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Pronounced regulatory T cell activity in human schistosomiasis: differences in T cell proliferation and cytokine responses before and after treatment with Praziquantel

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To the children in Africa

Content

1 Abbreviations

Ab	Antibody
Ag	Ag
APC	Allophycocyanin
AWA	Adult Worm Ag
BCG	Bacillus Calmette-Guerin
CD	Cluster of Differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CTLA-4	Cytotoxic T lymphocyte-associated Ag 4
DI	Division Index
EDTA	Ethylene Diamine Tetraacetic Acid
EU	European Union
FBC	Full blood count
FCS	Fetal calf serum
FACS	Fluorescence activated cell sorting
Figure	Figure
Foxp3	Forkhead / winged-helix transcription factor box P3
GITR	Glucocorticoid-induced tumor necrosis factor receptor family related gene
GM	Geometric Mean
Hb	Haemoglobin
HBSS	Hank's Buffered Salt Solution
Hp	Helicobacter pylori
ICH-GCP	International Conference on Harmonization of Good Clinical Practice
IFNγ	Interferon gamma
IL	Interleukin
Max	Maximum
MACS	Magnetic Activated Cell Separation

MED	Medium
Min	Minimum
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PFA	Paraformaldehyde
PHA	Phytohaemagglutinin
RPMI	Roswell Park Memorial Institute
SEA	Schistosome Egg Antigen
S.h.	Schistosomiasis haematobium
S.m.	Schistosomiasis mansoni
Spp.	Species (Pl.)
Th1	T-helper cell 1
Th2	T-helper cell 2
T-regs	T-regulatory cells
TNFα	Tumor necrosis factor alpha
TRANCHI	T cell Regulation and the Control of Helminth Infections
Tx	Treatment
WHO	World Health Organization

2 Figures and Tables

3 Introduction

3.1 Schistosomiasis

3.1.1 Bilharziosis: an Overview

Schistosomiasis, also known as Bilharziosis, is one of the most common helminth infections worldwide and after malaria the second greatest cause of parasitic disease. While globally an estimated 650 Million individuals are at risk of contracting this infection, Sub-Saharan Africa, Asia and small parts of Latin America bear the main burden (1) (Figure 1). According to the World Health Organization (WHO), at present there are annually approximately 230 Million individuals affected by the disease, of which an estimated 90% are based in Africa. Between 2006 and 2010 the number of individuals treated for Schistosomiasis rose by more than 20 million from 12.4 to 33.5 million / per year (2).

Figure 1: Global distribution of Schistosomiasis

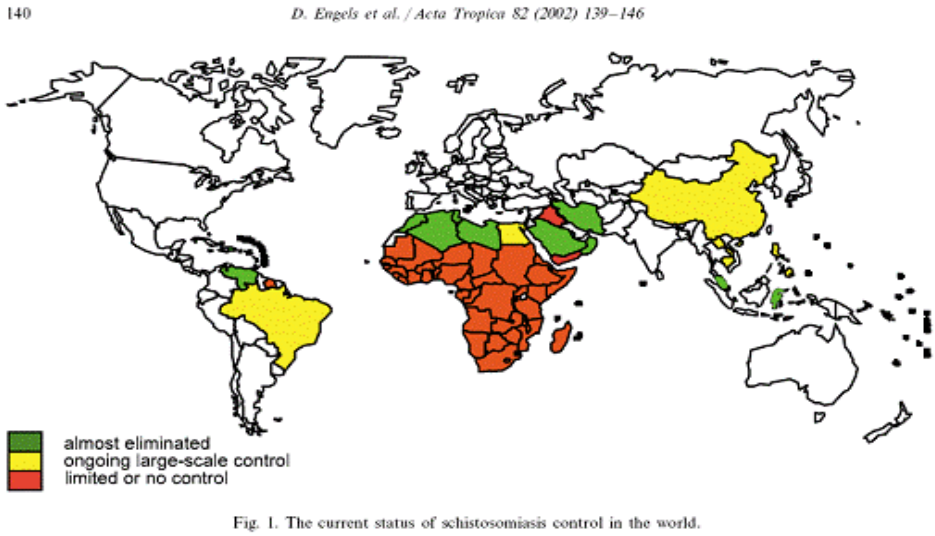


Figure 1: Global distribution of Human Schistosomiasis in 2002, showing that Sub-Saharan Africa is greatly affected with limited or no control of the disease (1)**.**

As is known, there are numerous Schistosome subspecies affecting different animals. The two major forms of Schistosomiasis, intestinal and the urogenital form including some subspecies, primarily target the human being. The four most common subspecies affecting the gastrointestinal tract system are *Schistosoma mansoni (S. mansoni), S. japonicum, S. mekongi* and *S. intercalatum,* while *S. haematobium* is the only one that has its final destination in the urinary tract system. These different types also show a variable prevalence worldwide (Figure 2). Yet, despite a certain variance regarding its global distribution, some species are found within the same area. Thus, in areas where different subspecies occupy the same habitat, concurrent infections may occur.

Figure 2: Global distribution of different Schistosomiasis subspecies

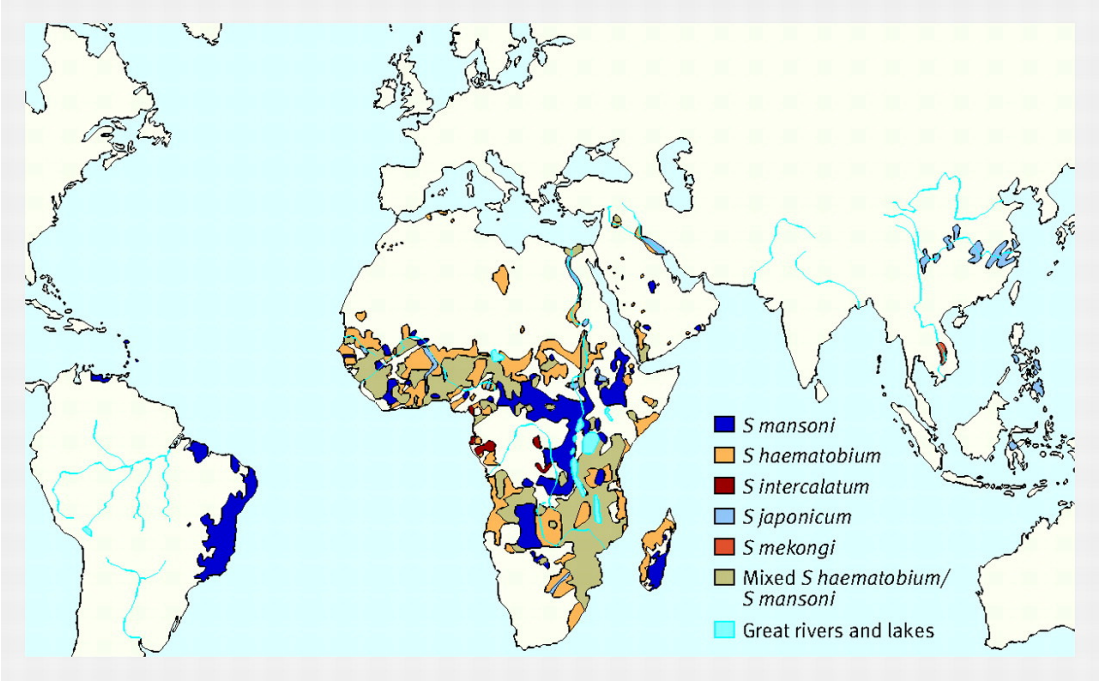


Figure 2: This map of displaying the global distribution of different Schistosomiasis subspecies shows a large prevalence of S. haematobium and S. mansoni in sub-Saharan Africa with mainly mixed occurrence of the two in the same area. S. mansoni shows further prevalence in Brazil. Small amounts of S. intercalatum are also found around the equator in central Africa, whereas S. japonicum and S. mekongi are primarily found in Asia and the Arabic peninsula (3).

3.1.2 The parasite life cycle

In order to complete its life cycle, the Schistosome parasite requires two different hosts (Figure 3). Schistosome eggs, that are being excreted in urine or faeces by the infected host infected, can remain viable for up to seven days. Yet, upon contact with preferably quiet waters, such as ponds or lakes, Schistosome eggs release miracidia, which aim to search for a fresh water snail as their intermediate host (3).

Figure 3: Schistosomiasis-life cycle

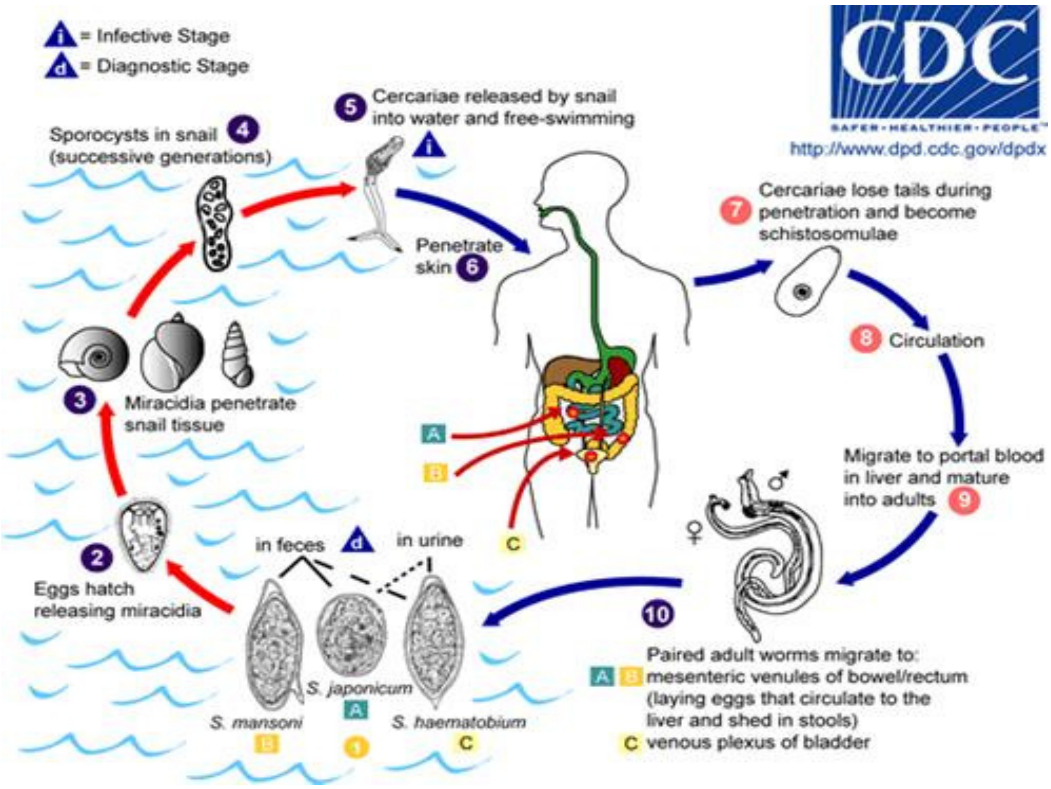


Figure 3: Schistosomiasis parasite lifecycle showing its 2-stage multiplication first as miracidia going through an asexual cycle within a water snail, turning into sporocytes and being released as cercariae-the stadium in which they penetrate human skin. Once they have entered the venous blood stream, they complete their sexual cycle in the hepatic veins and then aim for their final organ destination (4)**.**

After penetrating the snails parasites are able to perform asexual multiplication. Once inside the snail the miracidia undergo transformation and turn into sporocysts, which after the 2nd generation produce thousands of infective cercarial larvae. It then takes approximately 4-6 weeks until the larvae are ready to leave their temporary host. After excretion within the snails biotope the larvae have an estimated survival time of 48 to 72 hours before penetrating their definitive host through the skin (3). Having gained access to the venous circulation of a human being, migration continues through the systemic circulation via the host's heart and lung until the schistosomes reach the liver where sexual multiplication takes place in the portal vasculature. Depending on the subspecies the worm pair then migrates against portal blood flow to the vesical or mesenteric veins. Having reached its final organ destination the worm pair continues to migrate within the vessels of the organ. Here, they continuously dispose eggs preventing accumulated pathology in one particular segment, but instead causing mild pathology widely spread (5).

3.1.3 Epidemiology of Schistosomiasis in Gabon

It is well-known that tropical climate with high humidity levels in conjunction with little or no access to proper water sanitation serve as a favourable environment for the spread of parasitic infections such as S. *haematobium* (6). Rural Gabon, the site of study performance, serves as an example with conditions as described above. Looking at the global distribution of Schistosomiasis only provides a rough guide of its local spread. Besides the speculation that the disease may be underreported in some areas, there are great variances regarding its incidence within the same region. One of multiple reasons responsible is the parasite's complex life cycle and its dependence on specific water snails, showing limited occurrence, which restrict its successful survival to certain areas (7).

The WHO (8) estimates that in Gabon there are currently about 310.391 people (including 159.570 school aged children) at risk to acquire the disease. This makes up about approximately 25% of its total population requiring preventative chemotherapy in order to avoid an infection with Schistosomiasis. Exact figures on how many people actually suffer from the disease are being awaited.

As prevalence and incidence may greatly differ between the adjacent villages, predicting the potential to acquire the disease remains difficult. Yet, if no time is spent in-or one is not exposed to-any local conditions, where Schistosomes have their natural habitat, it is impossible to become infected. However, with current migration from rural towards urban areas trends as seen in Gabon and other developing countries plus uprising ecotourism, the disease is not only locally spread but also globally amongst those who travel (2.). Areas with high disease incidence also seem to have high infection intensity.

Most affected of the disease are children within the age group 5-15 years. While there is a raising trend over some years, the disease reaches its peak during adolescence and eventually shows a steady decline during adulthood (9),(10),(11). In particular limited access to running water encourages the use of local ponds for physical hygiene and playing, making children and pregnant women more susceptible to the disease (12).

3.1.4 General Pathology / Pathophysiology

The infection with Schistosomiasis can be divided into three phases: (1.) the pre-patent phase, (2.) the acute phase and (3.) the chronic phase. In the pre-patent phase, when the worm passes through the skin, most patients have little to no symptoms. Urticarial rashes have been reported at the site where cercariae penetrate the host's skin (3),(5). When the cercariae reach the vasculature, patients still remain mostly asymptomatic. A special tegument allows the worm to

hide from the immune system. Using the host's own major histocompatibility complex (MHC) antigens (Ag) and blood group glycolipids, the parasite cannot be recognized as foreign and is therefore protected from any host attack (13),(14). Other methods that successfully avoid effective parasite elimination include lysis of macrophages by local release of worm toxins, inactivation of complement-mediated lysis as well as secretion of immune-suppressive neuropeptides lowering T-cell activity. Regardless of the mechanisms applied, the worms effectiveness is expressed by its long survival within the host enduring up to 30 years (15).

The actual damage to the host occurs once the eggs have been released by the worm. The worm itself causes darkening of the parenchyma of the spleen and liver due to excretion of hematin from human red blood cells, of which the worms feed themselves (5). Acute infection coincides with egg release at the final organ destination, where eggs exude toxins. At this point immunological mechanisms become activated and the acute inflammatory response begins. Acute Schistosomiasis, also known as Katayama fever, varies with clinical symptoms from mild to severe. Besides fever, patients typically present with fatigue, myalgia, malaise, non-productive cough, eosinophilia, patchy infiltrates on the chest radiograph and possibly abdominal symptoms. Recovery from this phase usually occurs after 2–10 weeks as the infection moves on into its chronic phase (3),(5). Yet, at this point the disease may progress further with patients suffering from weight loss, dyspnea, diarrhea, diffuse abdominal pain, toxemia, hepatosplenomegaly and widespread rash.

The release of eggs is a continuous process lasting from 35 days up to several months after primary infection. While during the active phase the inflammatory response facilitates the eggs migration through the tissue, during the chronic phase the formation of granulomatous tissue protects other surrounding tissue areas of the host. The granulomatous tissue consist of a mixture of cells such as macrophages, lymphocytes, fibroblasts, mast cells, but first and foremost eosinophils accounting for about 50% of the cells (3),(5).

3.1.5 Urinary schistosomiasis

The eggs of *S. haematobium* provoke granulomatous inflammation, ulceration, and pseudopolyposis of the vesical and ureteral walls (16). Common early signs include dysuria, pollakisuria, proteinuria, and especially haematuria (17),(18). In endemic areas, the latter is known as a "red flag sign" of S. *haematobium* in children aged 5 –10 years, and may be confused with menstruation in girls or with a urinary tract infection (19). Typically, blood is first seen in the terminal urine, but in severe cases the whole urine sample can be dark colored. Bacterial super - infection and bladder stones can complicate this clinical picture. Those early clinical signs become less common after adolescence.

Yet, chronic lesions within the urinary tract can evolve into fibrosis or calcification resulting in hydronephrosis or hydroureter. Chronic compression may eventually lead to parenchymal damage and kidney failure. In non-treated populations exposed to *S. haematobium*, microhaematuria could be found in 41–100%, gross haematuria in 0% - 97% and radiologically visible lesions in the upper urinary tract in 2–62% of infected children (18). Kidney function was often well preserved and most lesions, including hydronephrosis, healed well either spontaneously or after antischistosomal treatment suggesting only temporary damage the renal parenchyma (18).

Several case-control studies have demonstrated a significant association between infection with *S. haematobium* and squamous cell cancer of the lower urinary tract (17),(16),(18). In Egypt, the incidence of bladder cancer has decreased in line with Schistosomiasis prevalence over the past few decades (20),(21). Nonetheless, epidemiological evidence showing an association between increased mortality rates and the occurrence of urinary Schistosomiasis is scanty. While autopsies and clinical observations leave no doubt that particularly elder patients die of Schistosomiasis-induced renal damage (22),(23),(24), contrasting surveys have not confirmed any specific mortality due to Schistosomiasis (18),(25),(26),(27).

Less controversial are the pathologies occurring due to the adult worms residing in-or the eggs deposited within the venous vessel system of the urinary tract resulting in urethritis, hematuria, and possible obstructive nephropathy. Hence, infection with S. *haematobium* is more often symptomatic than intestinal Schistosomiasis with schistosomes inhabiting the mesenteric veins. Alteration in host physiology and pathology can vary with the number and species of the infection causing schistosome (3).

3.2 Helminth infection and human immune defense

3.2.1 An overview

Once infected with helminths, the host's corresponding immune defense mechanisms evolve over time involving dynamic processes between host and parasite. From the acute phase of infection through to any chronic stages, both innate as well as acquired immune responses are involved in this process (28). In order to not only overcome parasite intrusion, but still react towards other pathogens effectively, a counterbalance between different immunological responses is necessary. This entails effector-as well as regulatory pathways.

As previously indicated parasites use a variety of mechanisms in order to effectively evade innate as well as acquired immunological responses. Besides interfering with Ag variation or subverting phagocytosis, it has been implicated that parasites make use of regulatory mechanism facilitating its maturation and multiplication which ultimately results in their long-term survival (29).

Comparing a number of pathogens including bacteria, viruses, parasites and other species, the immunological responses observed show different foci (28). While intracellular bacteria and protozoa lead towards a Th1- type response with activation of macrophages, extracellular bacteria seem to stimulate a Th17 led response with further activation of neutrophils (30). On the contrary, helminths-

equally to other parasites-show a pronounced Th2 response and eosinophil activation (31),(32),(33). While parasites search for mechanisms to overcome the host's response, the latter needs to propagate distinct pathways if a sustained self-defense including effective parasite expulsion is to be achieved. Once the host has become infected and eggs are disposed at their final organ destination, a Th2 type driven response takes over protecting the host from any further damage caused by parasite invasion (28).

Rodent models demonstrated a number of immune - modulatory effects helminth have on Ag-specific-as well as on non-specific immune responses. They suggest that once the adult worm has evolved from the schistosome during the initial phase of infection, immune recognition is hindered via a variety of mechanisms: this includes the induction of anti-inflammatory molecules (34), the incapacitation of lymphoid-(effector) cell function (35) and the coating of their surfaces with host Ags (36).

Any immune alterations may be variably pathogenic-yet with occasionally with possible lethal outcome to the host. Down-modulated responsiveness to specific Ags from the infecting parasite are well documented effects in helminth infection (37). Despite the evasion strategies the worms take in the initial phase, the immune system eventually reacts against the worms, although this unlikely ends in destroying them. In most cases though, it will offer protection against subsequent reinfection, which is a starting point to raise the idea of vaccine development (38). In case of ineffective immune responses due to failure of an adequate response of Th2 effector cells, exacerbated granulomatous tissue inflammation occurs. On the other hand, chronic infection is thought to be generally characterized by an ongoing Th2 type response causing small chronic lesions, which lead to tissue fibrosis and may also become pathological (39).

Interestingly though, besides the generally detrimental impact helminth infection can have on the host, there may also be secondary benefits due to the infection. For instance, it seems as though harmful inflammatory responses in

allergy are being reduced (40),(28). Diminished pathologies were also found in diseases caused by Plasmodium falciporum and Helicobacter pylori (H.p.) (41),(42), although on H.p. and S. *haematobium* co-infections the results are ambivalent (43).

Be that as it may, only cumulative exposure to the parasite anitgen (Ag), which most commonly occurs during childhood, allows the development of an Ag-specific immune responses (9),(44). As re-infection remains a major problem compromising current pharmacological treatment, it is pivotal to understand the immune - modulatory mechanisms evolving during infection. With children being the first and therefore most susceptible to acquire disease, discovering the evolvement of Ag-specific immune responses in this age-group is an essential step forward in establishing the disease immune epidemiology. Important findings in those few studies undertaken with children will be pointed out in the following sections.

3.2.2 Th1 / Th2 balance vs. T- reg cell activity

In chronic helminth infection immune responses are characterized by marked $CD4^+$ cell proliferation, by increased production of eosinophils, mast cells and IgE as well as by increased Interleukin (IL) 4, IL5, IL10 and IL13 levels. All of these are distinctive features of a typical Th2-type response comprising both innate and adaptive components (45),(46). In contrast to other pathogens, such as bacteria, which typically stimulates a pronounced Th1 response including $CD8^+$ activity as well as increases in IFNγ and TNFα (47),(30), in chronic helminth disease we observe a marked Th2 response (Figure 4) (39),(30).

Figure 4: Pathogen dependent T-cell pathways

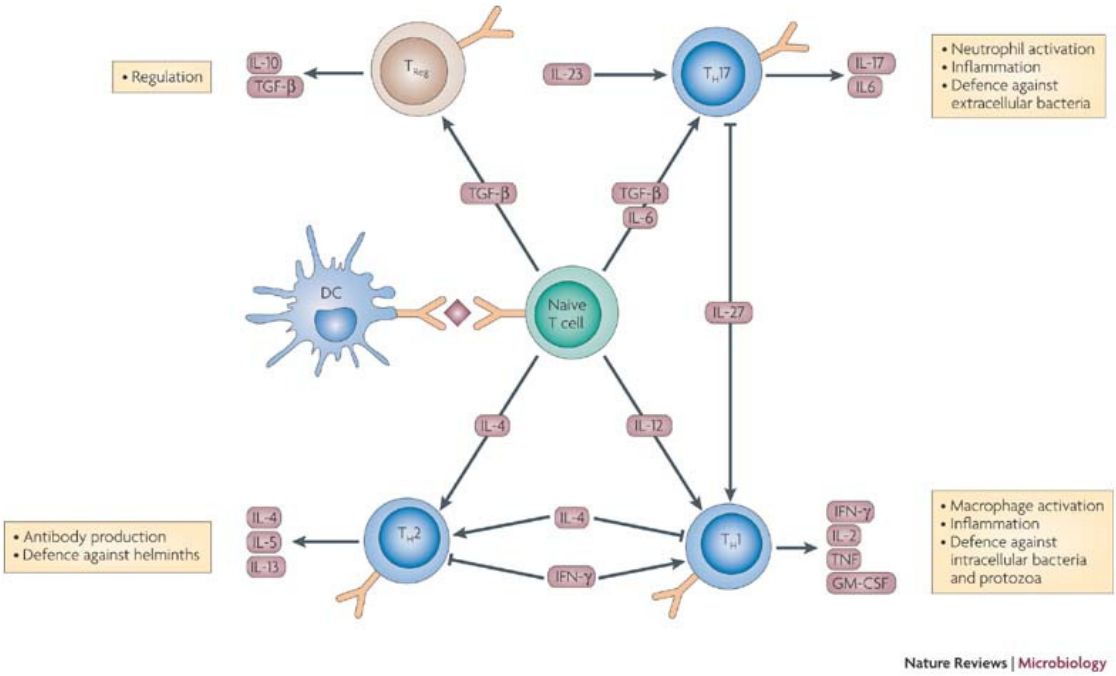


Figure 4: Showing that depending on the original stimulant, the naïve T-cell is driven towards a certain pathway. Helminths tend to trigger a Th2 driven reaction with its associated cytokines IL4, IL5 and IL13 (bottom left) (48)**.**

However, looking at the whole course of infection with Schistosomiasis-including the acute onset until its chronic state, it is important point out that human immune responses are made up of an interactive driven response comprising Th1 and Th2 cellular components. Precisely, while the acute infection-provoked by maturing worms-tends to stimulate a Th1 response, soon after egg release has taken place a transition to a Th2-type follows with the aid of co-stimulatory molecules as well as B-cells (28).

As the Th1 to Th2 switch normally occurs during the hepatic phase of the parasite life cycle, failures resulting in a non-switch from Th1 to Th2 lead to a Th1 and Th17-driven reaction. This causes detrimental tissue damage of the hepatic parenchyma and possibly even death (49). Although the Th2 response does not

clear the host from parasites, it prevents Th1 induced pathology leading to unrestricted inflammatory reactions. Instead, Th2 responses are associated with relatively mild granuloma formation made up of eosinophils, macrophages and lymphocytes.

While Taylor et al (2006) (50) found that Th2 lead responses, occurring chronic infection, had no major suppressing effect on Th1 responses, the exact mechanisms responsible for the Th1-Th2 switch remain unclear. It has been proposed that in the initial phase of helminth infection Th2 responses are being suppressed by non-egg Ags favoring a Th1 polarization (51). Yet, once egg release has taken place, egg Ag increases, triggering Th1 apoptosis. Hence, Th2 responses become more dominant resulting in an enhanced survival of both host and pathogen. Several studies show the involvement of other cells, such T-regulatory cells (T-reg cells), with it suppressive properties taking also effect on Th1 activity (50),(52).

3.2.3 T-regulatory cells: necessity and strain to the immune system

As the immune system is exposed to a variety of challenges, it has evolved several regulatory mechanisms essential to (1.) maintain homeostasis, (2.) prevent autoimmunity and (3) restrain inflammation induced by innocuous environmental triggers. Central to these mechanisms are T-regulatory cells (T-reg cells) that are now widely regarded as the primary mediators of peripheral tolerance. Many pathogens have developed mechanisms to manipulate the regulatory network of the host to their advantage, thereby generating conditions that ensure their survival for a prolonged period of time (53),(29),(54).

A number of reports on chronic viral and bacterial infections in humans have demonstrated a correlation between infection load and increased T-reg cell number or activity, either in the periphery or at the site of infection (55),(56),(57),(58). Prior

to this investigation, rodent based studies have also shown an association between increased T-reg cell activity and helminth infection. Human studies on this subject remain still scarce. Furthermore, there is a lack of understanding regarding any causative mechanisms leading to an increase in cells on one hand and the immunopathology leading to any unwanted effects to the host on the other (29).

In order to explore the versatile role of T-reg cells it is crucial to correctly identify T-reg cells. Yet, up to date there is not one single marker exclusive for T-reg cells. Instead, a great variety of surface markers have been attributed in trying to characterize T-reg cells. Among those, is the first and despite its low specificity still commonly used: highly expressed α-chain of IL2-equal to $CD25^{high}$. Later, the more specific intracellular Transcription Factor Forkhead Box Protein P3 (Foxp3) was introduced ahead of others such as $CD127^{-}$, CTLA-4, GITR, LAG-3. Despite numerous listings in the literature (59),(60), unfortunately they all remain non - exclusive in defining T-reg cells. Both: $CD25^{high}$ $Foxp3^{+}$, in particular as a combination are still commonly used, though they have shown to become up-regulated upon cell activation (29).

T-reg cells comprise a number of different subsets depending on their origin. Firstly, there are natural $CD4^{+}$ $CD25^{+}$ T-reg cells mainly originating from the thymus with the aim to control self-reacting, peripheral T- cells. Secondly, so called "inducible T-reg cells" have been described (61),(62),(63) emerging in the periphery from conventional $CD4^{+}$ cells upon stimuli caused by antimicrobial pathogens including viruses, such as HIV, cytomegalovirus and hepatitis, or bacteria, such as tuberculosis and listeria, but also parasites such as filariae and helminths. Subsequently, they are equally part of-and take effect on the system's adaptive immune response. Prior to this study, rodent models clearly showed a correlation between parasite infection, T-regs cells and prolonged disease (38),(64),(65).

The pathways used resulting in T-reg cell activation may be multifactorial. Inter alia, it has been suggested that T-reg cells alter the differentiation of various

cells lines including $CD8^+$ and $CD4^+$ T-effector cells. In the presence of other T-reg the latter were seen turning into IL10, TGFß adaptive T-regs (66),(67). However, the exact mechanism by which T-reg cell activation is finally initiated is not fully understood. However, there is sufficient evidence from what has been previously described, that T-reg cells interfere with both Th1 and Th2 cell line responses (Figure 5) and are therefore a main actor in the immune answer (68).

Figure 5: Interference of T-reg cells with the immune system

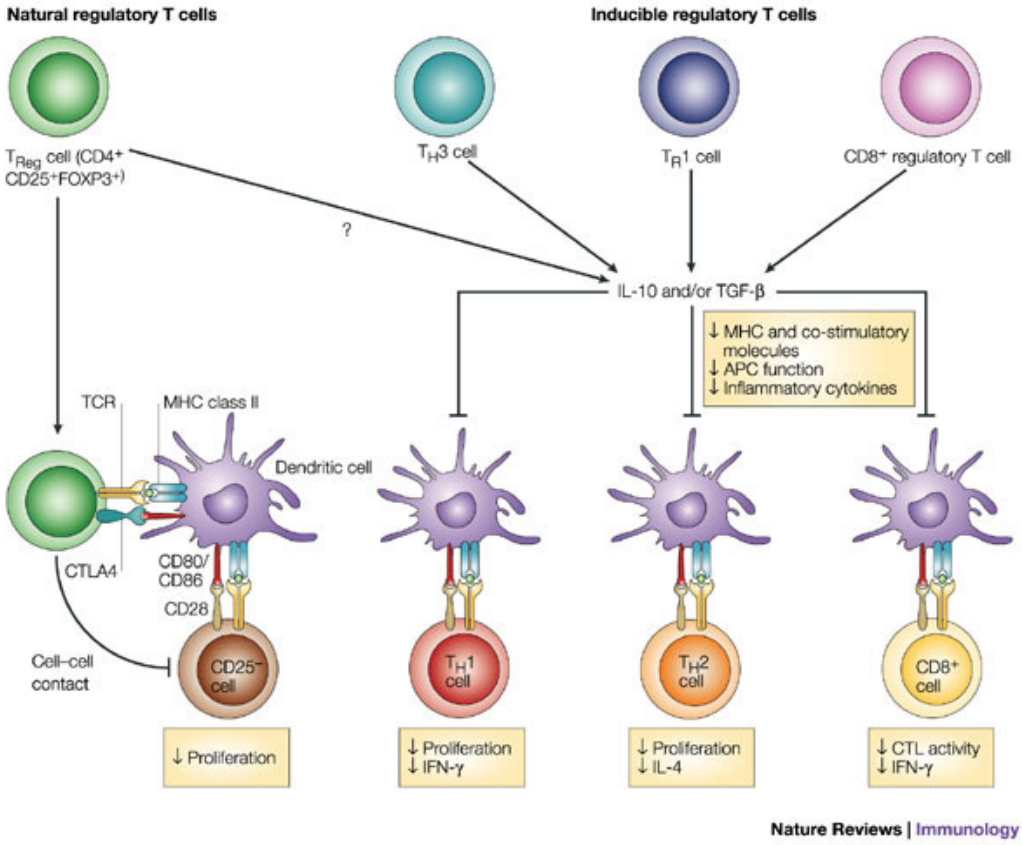


Figure 5: This graphs displays that both natural as well as inducible regulatory T-cells take an effect on different types of cells (Th1 and Th2) resulting in the attenuation of cell proliferation as well as the cell type specific cytokine release (IL4, IFNγ) (68).

Despite some contraindicating findings (69), there are strong indications that once T-reg cells have become activated they suppress in a non Ag-specific way (70),(71). This means, even T-reg cells with a single Ag-specificity are able to suppress effector cells with various other Ag specificities. This phenomenon has been considered as “bystander suppression”.

The role of T-reg cells is as variable as ambiguous – at times being beneficial and other times being harmful to host. For instance, one valuable capacity that T-reg cells had been assigned to is the control of local tissue damage and pathology. By controlling the expansion of self-reactive lymphocytes-hence preventing over-reactivity within the host (72),(53) naturally occurring T-reg cells were able to prevent autoimmune disease. Beyond, it was noted that during the cause of infection, but in the absence of T-reg cells both prolonged Th1 activity and general exertion of T-effector cells-would also lead to collateral tissue damage. In a study with S. mansoni infected individuals increased tissue damage was caused to the liver upon removal of T-reg cells (73). However, in the presence of T-reg cells these unwanted effects seem to be preventable. In consequence, it would be justified to say that that the control of immunopathology during infection seems a major component of regulatory T-cell effects. In fact both types of T-reg cells-inducible and natural T-reg cells-have shown to play a pivotal role in infection (29).

Although the occurrence of allergic or any other inflammatory diseases may coincide with helminthic disease (74),(75), it has been suggested that T-reg cells are responsible for the immune modulation happening in helminthic disease, which generally protects against inflammatory disease (76),(39),(28). This is in coherence with the finding of active helminth infection on one hand and asthma on the other, in which both (1.) the frequency of T-reg cells and (2.) inflammatory response worked in opposite directions (77). Another interesting finding was made in conjunction with tumorous disease, where the removal of T-reg cells improved the outcome of tumor growth (78),(79).

While the lack of T-regulatory cells may result in extensive inflammatory responses, there are strong indications that the accumulation of T-reg cells in parasitic disease may prevent efficient expulsion of the pathogen (80),(81) or may contribute to insufficient attenuation of viremia in other infectious diseases (82),(83). Although on one hand, their handling with inflammatory responses propagate the immune - protective role T-reg cells have, on the other hand their immune controlling effects seem to enhance pathogen survival with possibly detrimental consequences to the host.

Putting helminth infection back into focus, rodent as well as human models have shown that the parasites' presence results in both induction and expansion of naturally occurring regulatory T cells (84),(85). Interestingly, in a rodent model the parasite infection with *Litomosoides sigmodontis* was blocked following in vivo depletion of Treg cells and resulted in increased killing of the parasite (86). Further mice and human studies have proven an association between immunological hypo-responsiveness and the presence of helminth infection. It was suggested that this may be due to the increased mobility of T-regs cells produced via cytokinetic signaling upon stimulation in the periphery (87). Despite a number of hypotheses made on the possible mechanisms of T-reg cells suppression affecting T-effector cell proliferation and / or Cytokine production (88), including the involvement of enzyme release (i.e. activation of TGFβ on DC) (89), the exact mechanisms are far from being understood. All in all, it is justified to say that a very fine balance is required in order to find a healthy equilibrium not causing any damage to the host-neither due to the deficiency of T-reg cells nor due to its over-expression.

3.2.4 Cytokine activity

Following rodent models in the mid 90's, human studies looked at the release of different cytokines in infection with Schistosomiasis. As outlined earlier both Th1 as well as Th2 cytokines play an important role during the different stages of the disease. Taking their distinctive field of action into consideration, they seem to have mutual as well as cross-inhibitory effects on each other (90). Prior to this study little was known on the evolvement of cytokine responses especially in children infected with Schistosomiasis (91). Some recent studies give some hints on the cytokines interplay during this parasitic disease (92). Yet, as most first time contacts of this disease occur during childhood-when the development of Ag specific immune responses is at its most crucial point-it requires further attention.

Prior to any human studies, various rodent models with Schistosome infected mice have demonstrated a certain pattern in the release of cytokines. As indicated above, studies have shown that during the early stages of primary infection with *S. mansoni*, Th1 signals were in the limelight of immune responses (33),(46). Yet, once egg deposition had taken place, Th2 responses evolved with notable increases of Th2 cytokines including IL5 and IL13. Correlating findings were seen later in human studies with Th1 peaks during the acute phase and predominant Th2 cytokines IL4, 5 and 13 during the chronic phase of infection (90). The first peak in Th2 cytokines was seen at 2-5 weeks, while the second significant surge occurred at 8-12 weeks of infection. Inversely, Th1 responses-represented primarily by the release of IFNγ-showed an obvious decline at these time points (33),(46) with variations observed depending on the extent of disease (90).

Focusing more specifically at selected Th1 and Th2 cytokines shows a more ingenious system of interaction. Irrespective of the phase of the disease, alongside INFγ, the other pro - inflammatory cytokine, that has been mostly discussed to play a role in Schistosomiasis, is TNFα. In patients infected with *S. mansoni* high levels of both IFNγ and TNFα were found in those with signs of hepatosplenomegaly (93) as an expression of severe infection. An interesting Th1/Th2 interaction was found

in a rodent model with Trichuris muris infected mice, where the existence of TNFα seemed to be a prerequisite to the co-finding of Th2 cytokine IL13. A lack in TNFα consequently resulted in failed worm expulsion (94).

A further insight into different cytokines was gained investigating distinct profiles in either mild or severe disease with Schistosomiasis. For instance, studies with *S. mansoni* infected subjects revealed that individuals who developed severe disease had a rather low level of IL10, but high levels of INFγ and TNFα. Non-severe disease was associated with a pronounced Th2 response (95). The study of (96) showed that Th2 cytokine IL13 played a pivotal role in preventing severe granulomatous response. Yet, in general chronically infected individuals displayed a dominant Th2 response with high release of IL 4, 5 and 13. No relation was proven between infection intensity and specific levels of Th2 cytokine IL13 (92).

Somewhat particular seems to be the role of the “pro - inflammatory” cytokine IL17. Though prior to this investigation little was known on IL17, more recently first insights were gained. With both Th1 cytokine INFγ and certain Th2 cytokines found to exert suppressing effects on IL17, it was concluded that IL17 stemmed from a distinct cell lineage referred to as Th17 cells (97). These are now recognized as a distinct $CD4^+$ cell lineage next to Th1 and Th2 cells (98). Remarkably, in arthritis, an inflammatory disease, a reciprocal relationship was found between IL17 production and the development of T-reg cells (99). Similar to T- reg cells IL17 was induced via the transforming growth factor TGF-ß, though contrastingly to T-reg cells only in conjunction with IL6 (100). This suggests an efficient system between both cell types in order to balance tolerance and immunity. Although IL17 has shown to play an important role in clearing pathogens such as Candida albicans or extracellular bacteria during host defense, so far little is known about its role in parasitic disease. Yet, one study with children living in an epidemic region for S. *haematobium* (92) found that those with detectable IL17 levels in their plasma had little or no eggs.

Looking at the cytokine IL10 the findings are not only diverse in terms of its origin but also regarding its role. Studying the cytokines immune-regulatory function observations showed that exposure to Schistosome cercariae led to a particular increase of IL10 (101). Due to IL10's rather late secretion during the course of infection followed by a limited pro - inflammatory Th1 cytokines response highlight its down-regulatory role (102). The function is further supported by the observation that IL10 deficiency lead to an enhanced pathological Th1 response (103),(35) as seen by the increased inflammatory reaction on the skin of mice (104),(105). Further, IL10 supposedly contributes towards reduced morbidity in Schistosomiasis infected individuals (73). Some argue that the correlation between high infection intensity and high IL10 levels serves in order to prevent severe pathology (91),(33),(106). IL10 has also shown some inhibitory effects on Th2 cytokines IL4, IL5 (91). Although IL10 is released by a number of cells including T-reg cells, it does not seem to interfere with its immuno-regulatory function (65). Further studies may bring more lucidity into IL10's varied role description.

As the majority of these observations have been primarily made on rodents and human adults, it needs emphasizing that during childhood the immune response mechanism remain rather unclear and may well be diverse. During the various age-ranges in childhood distinct cytokine profiles could be highlighted. For instance, while IL10 had its peak during early childhood, IL5 had a rather slow raise with its peak at a later stage (91),(107).

In one study where children were repeatedly treated with Praziquantel (PZQ) the release of Th1 cytokine INFγ was reduced, while the production of Th2 cytokine IL5 became stimulated (108). Another investigation on specific Th1 and Th2 cytokine levels before and after the treatment of Schistsomiasis infected children showed that while during the infection both Th1 specific IFNγ and Th2 specific IL4 and IL5 were still raised, soon after treatment only Th2 cytokine levels demonstrated a further surge (109).

3.3 Aims and objectives

Helminth infections continue to have a detrimental effect on humans in particular during childhood. Although still effective, current pharmacological interventions are limited and compromised by limited compliance due to high re-infection rates. Understanding host and parasite interactions is crucial in order to move vaccine development forward. Previous studies indicated a decisive role of T-reg cells in the persistence of helminth disease (59),(73),(52). According to a recent model, T-reg cell induction is stimulated by the intruding parasite leading to a failed effector cell response and parasite persistence (110),(111).

The overall aim of this study was to investigate the role of T-reg cells during the infection with S. *haematobium*. The specific objectives of the study were: (1.) to determine the frequency of T-reg cells in S*. haematobium* infected children before and six weeks after successful treatment with PZQ and (2.) to evaluate the regulatory activity of T-reg cells in the infected subjects at both time points. For the evaluation of the latter, measurements of cell proliferation and cytokine activity were taken. Later, a small - scale auxiliary study was carried out (1.) to strengthen the correct identification of T-reg cells using a more sensitive marker and (2.) to carry out a comparison between infected vs. non-infected children.

3.4 Hypotheses

Based on previous works mentioned above, we hypothesized that (1.) infection with S. *haematobium* induces a higher frequency of T-reg cells compared to non-infected individuals. Subsequently, we put forward (2.) that an increase in T-reg cell frequency in S. *haematobium* infected individuals would suppress the functional activity of T-effector cells. Hence, we suggested that removing T-reg cell would lead to increased functional activity in *S. haematobium* infected children and that post-treatment this effect is minimized compared to pre-treatment.

3.5 Background

As worldwide helminth infections belong to the most neglected communicable diseases, this project was carried out in the sequence of EU financed projects called “TRANCHI” (T-cell Regulation and the Control of Helminth Infections) spread over certain institutes in Africa and Asia. The aim was to better understand T-reg cells in the context of immune responses gathering strategies for new treatments (Appendix 1). The study site for this investigation was within the vicinity of Lambaréné in Gabon, a high prevalence area for S. *haematobium*.

This study was carried out in collaboration between the “Eberhard Karls University” in Tübingen / Germany, the Medical Research Unit of the Albert Schweitzer hospital in Lambaréné / Gabon and the “Leiden Univeristy Medical Centre” (LUMC) in Leiden / the Netherlands. The Albert Schweitzer hospital in Lambaréné was founded in 1913 by Dr. Schweitzer, initially a practicing theologian from the Alsace Region, who took up his medical studies in his thirties to dedicate his life to the medical care of the people in Lambaréné. In 1981 the “Medical research Unit” was found in proximity to the hospital. Since 1992 the research unit is under the lead of Prof. Dr. Kremsner from the “Eberhard Karls University” in Tübingen.

With clinical studies on new anti-malarials and malaria vaccine in focus the research laboratory has further extended. Since 1996 there is a growing collaboration with the Parasitology Unit under the lead of Prof. Yazdanbakhsh. With experimental research being its central approach, so far, the unit’s main activities were on T-reg - , dentritic - cell and T-effector cell activity in helminth infection.

4 Material and Methods

4.1 Study area and population

The study subjects were schoolchildren from an area known as PK15 based at approximately 15km South of Lambaréné in the province of Moyen-Ogouée / Central Gabon (Figure 6). Lambaréné, since 1913 hometown of the “Hôpital du Dr. Albert Schweitzer” with its adjacent “Laboratoire de recherche”, is separated into 3 parts by Gabon’s largest river “Ogooue”. Further, Lambaréné with an estimated population of 35.000 of a total of 1.5 Million in Gabon is fifth biggest town of the country and situated approximately 80km south of the Equator (112). Being in the middle of Africa’s tropical climate zone, there are only minor fluctuations in temperature and humidity levels between its rainy and dry seasons.

Due to the strong current of the river Ogooue in Lambaréné itself, the prevalence of S. *haematobium* seems less widespread. However, at PK15, situated along the main road to Fougamou, children bathe and often play in standing water of nearby ponds with access to only one fresh water pump in the whole area extending as far as a couple of kilometres either way. Geographically and epidemiologically PK15 and its surroundings serve therefore as an ideal location for subject recruitment with high prevalence of S. *haematobium*, but also with close vicinity to the laboratory facilities. Previous studies have already described this area as endemic for *S. haematobium* (113),(40). The study conduction started during the great rainy season in the middle of April, when in fact transmission of disease is still before reaching its peak. The greatest risk of transmission is indeed at the beginning of the dry season when high waters are standing still (114).

Figure 6: Map of Gabon

Figure 6: Gabon is situated over the Equator in Central Africa with Northern borders to Equatorial Guinea and Cameroon, Eastern and Southern borders to the Republic of Congo and the Atlantic coastline to its west side (115)**.**

4.1.1 Recruitment/ Inclusion of children / diagnostics

For the recruitment of children in the chosen area at PK15 a provisional diagnosis of *S. haematobium* was made by questioning schoolchildren, whether they had observed blood in their urine. Once haematuria was affirmed and trial conduction was consented, an appointment was made for further investigations at the medical research unit in Lambaréné. Prior to inclusion of all study participants and in accordance with the International Conference on Harmonization of Good Clinical Practice (ICH-GCP), verbal explanation of how and why the study will be conducted was given to the child's parent or a legal guardian. Thereafter, signed consent (Appendix 2) was obtained from those before their child was included. In advance the "Comité d' Ethique Régional Indépendant de Lambaréné" had given ethical approval for conducting the study according to the GCP Guidelines (Appendix 3).

Upon arrival at the research laboratory a urine sample was collected from each child and immediately tested under optic microscope for *S. haematobium* eggs (Figure 7). If at least one egg was found, diagnostics and sample collection continued. Further procedure involved a brief physical examination including vital signs, height and weight measures, a brief medical history, blood sampling for a full blood count (FBC), analysis and for peripheral blood mononuclear cells (PBMC) as well as a random thick blood smear for Malaria screening. The results were recorded on a questionnaire (Appendix 4). Exclusion criteria were (1.) treatment with Praziquantel within the last 6 months prior to the study, (2.) the presence of fever (tympanic body temperature > 37.5C) and/or (3) other symptoms of acute illness.

A total number of 52 children were included over a period of three weeks with the sample collection taking place from Monday to Friday. The age of the children ranged from 5 to 15 years with a gender distribution of 25 females and 27 males at pre-treatment and 22 females and 26 males at post treatment. The mean of baseline characteristics at pre- and post-treatment are depicted in Table 1.

Table 1: Demographic Data of study participants at Pre- & at Post-Treatment

		Pre-Treatment (Baseline) (*N=52*)	Post-Treatment (*N=48*)
Age, years (yrs)	**Mean (SD)**	**10,2 (2,4)** **≤10 yrs: 27** **>10 yrs: 25**	
Gender	**Female/Male**	**25/27 (0.9)**	**22/26 (0.8)**
Height, cm	**Mean (SD)**	**134.4 (14,5)**	
Weight, kg	**Median (IQR)**	**28.4 (23.7-35.9)**	**28.4 (23.1-34.4)**
BMI	**Median (IQR)**	**16,09 (14.9-17.1)**	**16,2 (14.9-17.4)**
Temperature, ℃	**Median (IQR)**	**37,0 (36,7-37,3) *n=48**	**36,6 (36,3-36,8)*n=47**
Egg count, /10mL urine	**Median (IQR)** **Range (min-max)**	**34,5 (6,3-134,0)** **1-1812**	**0,0 (0-1,5)** **0-69 *n=49**
Haemoglobin, g/dL	**Mean (SD)**	**11,22 (0,12)**	**11,18 (0,13)**
WBC count, $x10^3/mm^3$	**Mean (SD)**	**8,52 (0,42)**	**9,47 (0.42)**
Eosinophils (%)	**Median (IQR)**	**1.03 (0.7-1.8)**	**1.5 (0.8-1.9)**

Table 1: The demographic data shows that study participants were very well balanced in terms of gender and age distribution. Differences between pre- and post- treatment measures were observed in the average temperature (the mean dropped by 0.4 Degree Celsius), in the Eosinophil count (Median rose by 69%) and evidently in the egg count (with the Median value dropping from 34.5 to 0). Haemoglobin levels instead remained consistent, while the WBC rose by almost 1 x103/mm3. Weight and BMI remained stable.

One year later a small scale auxiliary study was performed in order to strengthen T-reg cell analysis by adding $Foxp3^+$ as an additional marker (Appendix 6) Subject characteristics, external conditions as well as the study performance were equivalent to those in the main study. Furthermore, a small sample size of uninfected children was examined as a control group investigating any possible differences between infected and non-infected donors (Table 2). Gender balance

was not taken as a prerequisite as the analyses performed in the main study did not reveal any differences between male and female children.

Table 2: Demographic Data (auxiliary study): Uninfected vs. Infected children

Parameters	Unit	*S.haematobium* uninfected (N=7)	*S.haematobium* infected (N=10)
Age, years (yrs)	Mean (SD)	12.9 (2.6) ≤ 10yrs: 1 > 10yrs: 6	12.5 (1.5) ≤ 10yrs: 2 > 10yrs: 8
Gender	Female/Male	4/3	9/1
Height, cm	Mean (SD)	145.4 (9)	149.1 (13.9)
Weight, kg	Median (SD)	39.5 (9.2)	39.2 (10.5)
BMI	Median (SD)	16,04 (2,4)	16,13 (2,05)
Temperature, ℃	Median (IQR)	37.1 (36.7-37.5)	37.2 (36.9-37.4) *n=8
Egg count, /10mL urine	Median (IQR) Range (min-max)	0	19.5 (3.25-216.5) 1-532
Haemoglobin, g/dL	Mean (SD)	10.86 (0.72)	11.4 (0.31)
WBC count, $x10^3/mm^3$	Mean (SD)	6.33 (0.73)	8.82 (0.55)
Eosinophils (%)	Median (IQR)	0.43 (0.21) *n=5	1.25 (0.25) *n=6

Table 2: Within this small study sample there is good balance between both donor groups in age and constitution, yet a marked gender difference in the infected donor sample. Noticeable differences can be seen in WBC and Eosinophils with similar trends to the main study, while Hb and temperature levels of both groups remained within a similar range.

SD: Standard Deviation; IQR: Interquartile Range; SE: Standard Error of mean; WBC: White Blood Cell count. *n ≠ N because of missing data

4.2 Laboratory procedures in Lambaréné

4.2.1 Sample collection and processing

As a standardised and reliable measure to diagnose *S. haematobium* in the urine sample 10 mL of urine were taken with a 10 mL syringe and processed through a 12.0µm polyamide N filter (Millipore). The filter paper was then immediately applied to a slide and for Schistosome eggs examined under the microscope. This method has previously been used in *S. haematobium* studies (116). In order to coincide with the peak of *S. haematobium* egg excretion in urine (109), all samples of urine were collected and processed between 10 and 14h. A thorough egg count of S. haematobium showed a wide distribution between 1-1695 eggs / 10mL. However, it is necessary to mention that microscopically dead eggs cannot be differentiated from eggs alive.

After microscopic confirmation of the diagnosis "*S. haematobium*" (Figure 7), blood samples were taken and divided over two tubes. Between 3-5mL of blood were taken into an Ethylene Diamine Tetraacetic Acid (EDTA) prepared red topped vacutainer plastic tube for full blood count (FBC) analysis and another 7-10mL into a Heparin prepared orange topped vacutainer plastic tube for PBMC isolation.

Figure 7: Schistosomiasis haematobium egg

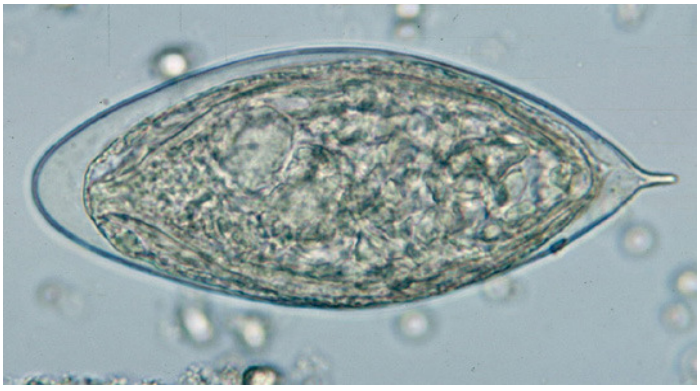


Figure 7: A typical S. haematobium egg under the microscope. They are considerably large (110-170 µm long by 40-70 µm wide), and with a conspicuous terminal spine. Eggs contain a mature miracidium when shed in urine (120).

Within one week of inclusion and execution of laboratory procedures all children were treated with a single dose of PZQ (PZQ) (40 mg/kg body weight). The same treatment was administered a second time three weeks later to maximize the clearance of parasites eggs. Six weeks after the first treatment, urine and blood samples were again taken a 2nd time under the exact same conditions as mentioned above for post-treatment analysis. Successful treatment was determined by the remaining egg load in urine at post treatment. At this point it is possible that, despite effective treatment, children still excrete Schistosome eggs. Although they are more likely to be dead-rather than viable eggs, microscopically they cannot be differentiated. Nonetheless, donors continuing to excrete Schistosome eggs after the 2nd treatment-disregarding their status of viability- were prophylactically given a third dose of PZQ and excluded from data analysis if their egg count was not reduced by at least 90% (N=5).

According to a pre-written protocol (Appendix 5) the following procedures were carried out in a standardized way: all freshly collected blood samples taken into heparinized vacutainer tubes were diluted (1:1) in Hank's Buffered Salt Solution (HBSS). With the addition of Ficoll (Amersham Biosciences, place, Netherlands), PBMC were purified through a Ficoll-Hypaque centrifugation for 25min. After finishing the centrifugation, at first plasma was removed and prophylactically stored at - 80°C for possible measures of antibodies at a later point of time. Then, the “white ring” comprising PBMC (Figure 8) was carefully extracted and for washing further processed with HBSS and Phosphate Buffered Saline (PBS). Re-suspended in Magnetic Activated Cell Separation (MACS) buffer, a counting sample was taken and cell solutions were separated over at least two tubes: one for whole-and another one for depleted PBMC. If more than 10 million cells/ ml were counted, a third tube was filled for possible phenotype measures at a later point of time.

Figure 8: PBMC post Centrifugation with FICOLL

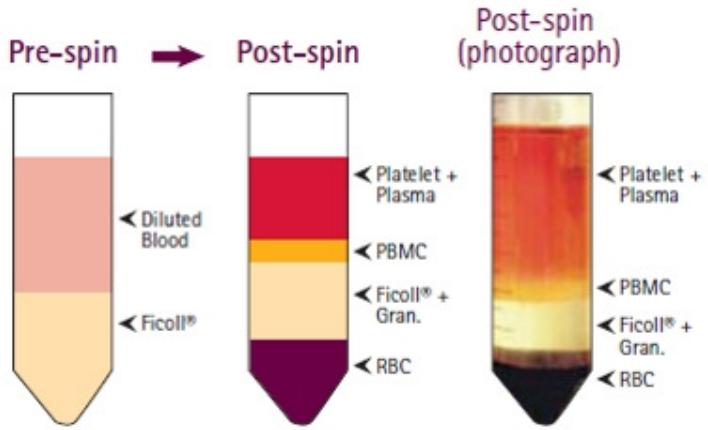


Figure 8: Typical separation of different blood products after centrifugation with the Polysaccharid FICOLL, which helps to separate products of different density and is commonly used for PBMC isolation in blood. Red blood cells sink to the base of the 50ml tube, with Ficoll on top, separating a white ring consistent of PBMC from it. Above the white ring accumulates white and red cell free plasma (117)**.**

4.2.2 Depletion of CD25hi T cells, Cell isolation, Extraction of T-regs

Depletion of $CD25^{high}$ T cells was performed using CD25 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. For this purpose $CD25^{high}$ T Cells were directly labelled with CD25 microbeads for 15 minutes at 4°C. The labelled cell suspension was then brought into a separator stand attached column, providing a magnetic field retaining $CD25^{high}$ cells and allowing $CD25^{high}$ depleted PBMC to pass through. While storing whole PBMC on ice in the meantime, this method allowed to produce T-reg cell depleted PBMC. Further, both samples were equally processed. Those cells remaining on the column, presumably only $CD25^{high}$ and therefore putative T-reg cells, were flushed out using a plunger in a MACS separator (Figure 9) and after put in lysis buffer solution stored at -80°C for possible RNA isolation. This method was performed in a slight modification to the protocol described by (118).

At pre- and post- treatment, identical procedures were carried out. In order to validate successful depletion, FACS analysis and the corresponding Flow Jo Software was used, confirming a reduction in $CD25^{high}$ cell count between the PBMC whole sample and the CD25high depleted sample.

Figure 9: OctoMACS™ Separator-for multiple sample processing

Figure 9: The Octo Macs was used to perform $CD25^{high}$ (T-reg cell) depletion of 4 to 8 patients maximum at the same time. Magnetic bead labelling allowed retention of $CD25^{high}$ cells in the separator's column, while $CD25^{high}$ depleted cells were collected in the Falcon tubes below the magnetic field (119)**.**

4.2.3 PBMC culture and CFSE cell staining

For cell proliferation analysis, $CD25^{hi}$ T cell-depleted-as well as total PBMC were stained with Carboxyfluoresceinsuccinimidyl ester (CFSE), a fluorescent cell staining dye, and stored in the dark for 15min at room temperature. Adding a 10% mix of fetal calf serum (FCS) in a buffer solution developed at **R**oswell **P**ark **M**emorial **I**nstitute 1640 (RPMI) (Invitrogen®) and supplemented with penicillin, streptomycin, L-glutamine and pyruvate to prevent additional bacterial growth, stopped the action of CFSE. RPMI was also used for the following washing process. After another washing with 20% FCS/ RPMI, a second counting sample was carried out. Thereafter cell suspensions were diluted to generate a

composition of 400.000 cells/ 100 µl. Providing cell numbers were sufficient, from each whole-and $CD25^{high}$ depleted PBMC sample five times (5x) 100 µl were brought into a round bottom plate and stimulated 1:1 with Medium (20%FCS/RPMI) as background "stimulant" (or negative control), Schistosome-specific Adult Worm Ag (AWA) (10 µg/ml), and Schistosome Egg Ag (SEA) (10 µg/ml) as well as non-specific Ag Bacillus Calmette-Guérin (BCG) (10 µg/ml) and as a positive control Phytohemagglutinin (PHA) (2 µg/ml). Being wrapped in cling film, the round bottom plates were stored in the incubator for four days at 37.5°C.

After this period each well was looked at under the microscope to look for proliferative effects of the different supplements. Supernatants were then removed and immediately frozen and stored at -80°C for cytokine measurement in Leiden. The remaining cells were washed out several times and fixed with 2% Paraformaldehyde (PFA) or with a fixation buffer provided by the FoxP3 staining kit (eBioscience, San Diego, CA 92121, USA) for the auxiliary study. For further analysis in Leiden using flow cytometry, all samples were cryopreserved at -80°C.

4.3 Laboratory procedures in Leiden

4.3.1 Flow cytometry analysis (FACS)

On arrival in Leiden all dry ice cooled FACS samples were stored in a -195°C Nitrogen Tower. For further processing of each sample a standardized protocol was followed (Appendix 7). Prior to FACS measurements samples were supplemented with Fetal FCS) and FACS buffer (= 3% FCS/ Dulbecco's Phosphate Buffered Saline (DPBS)) and centrifugated at 1600 rpm for 5 mins. Cells were then mixed and transferred into a V-bottom plate. Calibration controls were prepared as well as different label mixes. $CD4^+$ cells were labelled with Phycoerythrin (PE), $CD8^+$ cells with Peridinin Chlorophyll Protein (PerCP) and

CD25 cells with Allophycocyanin (APC) and to allow for absorption of the staining material, stored for 30min on ice in the dark. In advance a dilution series was carried out for each label. All labels were purchased from BD-Bioscience (San Diego, CA 9212, USA). After incubation FACS buffer was added to stop label activity. After centrifugation all cell suspensions were transferred into FACS tubes and further supplemented with FACS buffer.

Before individual samples were measured the FACS analyser (Calibur, Becton Dickinson) (Figure 10) was calibrated with the controls previously prepared. On the basis of APC labelled CD25 cells, depletion of T-reg cells was confirmed later via analysis with Flow Jo software. The extent of proliferation of $CD4^+$ as well as $CD8^+$ cells, which took place during the four days incubation period in Lambaréné, was measured via analysis of CFSE dilution (Figure 11). After individual sample measurements all FACS data were analysed using FlowJo Software (Tree Star, Ashland, Oregon, US).

The main component of FACS analysis expressing functional activity is proliferation. In this study it was determined through the division index (FlowJo Software, Tree Star, Ashland, Oregon, US) and represents the average number of responding cells undergoing cell divisions. Hence, it allowed a comparison of proliferation amongst all samples regardless of the original number of cells.

Figure 10: CD FACS Calibur

Figure 10: Becton Dickinson FACS analyser, 4 colour analyser used to measure cell proliferation and $CD25^{high}$ depletion effects. Cell Proliferation was visualized via CFSE staining using the Flow Jo software (Photo: by author).

Figure 11: Example of proliferation figure

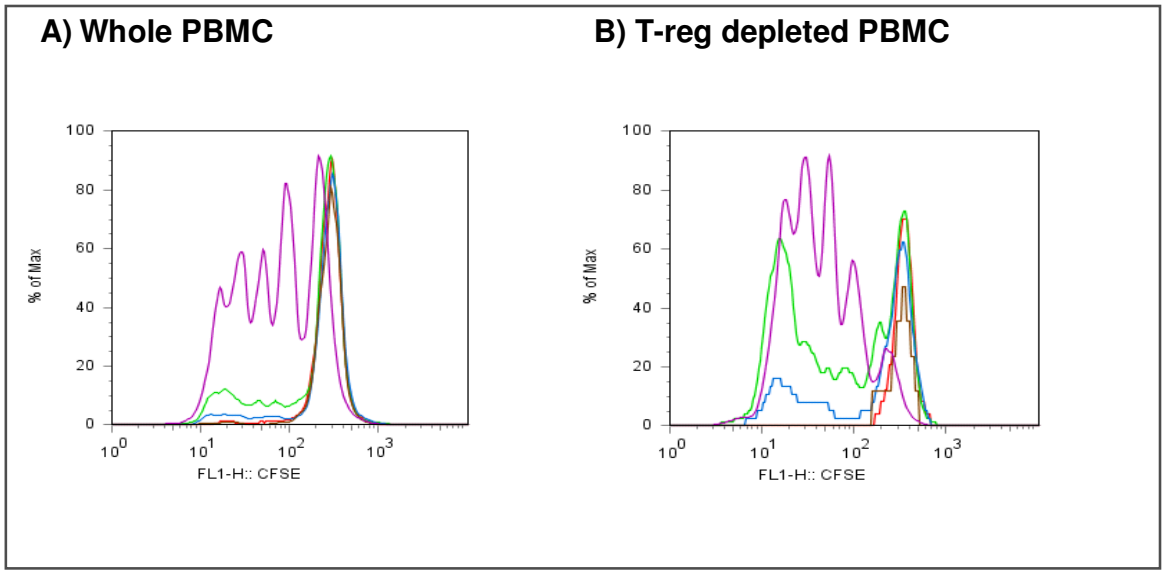


Figure 11: A) image of a donor's whole PBMC proliferation Figure at pre-treatment realised through CFSE staining with the different stimuli used (Medium, AWA, SEA, BCG, PHA) and B) the same donor's proliferation Figure showing the depleted sample at pre-treatment with the same stimuli applied.

4.3.2 Cytokine assays

Cytokines were measured from supernatants previously collected using Luminex 100 IS system (Figure 12) (Invitrogen) and commercially available beads and standards from BioSource (The Netherlands). Beads were titrated for optimal concentration before use according to the manufacturer's instructions. Once standard solutions were prepared and stored adequately (Appendix 8) beads and incubation buffer were mixed and centrifugated before samples were added into each well of a round bottom plate. Two hours of mixing in the dark allowed the beads to bind. Appropriate Antibody Solution was added in order to identify cytokines later on in the LS 100. Cytokines measured in this study included Interferon gamma (IFN-γ), Tumor Necrosis Factor alpha (TNF-α), Interleukin 17(IL-17), IL-5, IL-10 and IL-13.

Figure 12: Luminex LS 100

Figure 12: The multicolour Luminex LS 100 allows simultaneous measurement of 100 analytes in one single microplate well at the same time. Amongst the different assay formats available the one being used in this study was immunoassay (enzymatic assay) (Photo: by author).

4.4 Statistical analysis

Data collection and initial statistical analysis was performed using SPSS, version 14.0 (SAS Institute Inc., USA). In a second step Figure Pad Prism software, version 5, (Figure Pad Software, Inc.) was used independently-principally to make Figures and secondly to test for result conformity with data from SPSS. Since both analyses produced concurrent Figures, Figure Pad Prism was finally used for both Figures and statistics. After most data showed non-parametric distribution the two-tailed Wilcoxon test, making no distributional assumptions (120), seemed most suitable and was therefore applied to analyse the different variables comparing pre- versus post- as well as whole versus $CD25^{high}$ depleted PBMC. All data in our principal study were analysed in pairs, although distinct exclusion criteria were applied depending on the analysis.

General exclusion criteria for both cytokine and FACS data analysis were (1.) non-successful depletion, measured through $CD25^{high}$ percentage whole versus depleted PBMC (details see results section) (2.) non-successful treatment (N=5), defined by less than 90% egg clearance post vs. pre-treatment and (3) low cell counts (< 10.000) (N=2). Further reduction in numbers for paired analysis was caused by missing pre-or post-treatment samples (N=8) due either sample loss or due to drop out of donors (N=4) at post-treatment. Further exclusion criteria, applied to FACS analysis only, were (4) loss in CFSE staining (N=12) (possibly due to leakage) and (5) label errors (N=2).

In total 27 pairs were compared for cytokine analysis, while only 14 pairs were suitable to analyse FACS data. Where indicated, data are presented either as mean ± SEM or as geometric mean with 95%CI. *P* values of 0.05 or less are considered as statistically significant at 95%CI (Figures * $p<0.05$, ** $p<0.005$).

In the auxiliary study some non-paired analysis was performed due to low donor numbers using the Mann-Whitney-U test.

5 Results

5.1 Expression of T-regulatory cells in S. haematobium

5.1.1 Determination of T-reg cells via $CD25^{high}$ and $Foxp3^{+}$

In our principal study T-regulatory cells were defined as $CD4^{+}$ $CD25^{high}$ cells. During and after our study, more and more studies suggested (53),(121) that $Foxp3^{+}$ serves as a more precise marker for T-reg cells. Therefore, an auxiliary study was performed one year later adding the transcription factor $Foxp3^{+}$ in combination with $CD25^{high}$. Both studies analysed the effect of T-reg cells in children infected with *S. haematobium* in the same age group with otherwise comparable characteristics.

Hence, regardless of the markers used outlining regulatory T-cell, either $CD25^{high}$ only or both $CD25^{high}$ plus $Foxp3^{+}$, the first challenge was to determine a standardized gate applicable to all donors. Scrutinizing the individual dot plots of $CD4^{+}$ $CD25^{+}$ cells at high fluorescence levels, a subset of cells was distinguishable in most donors by a right shift (Figure 13). Yet as overall fluorescence levels varied amongst the different donors, so did the individual subsets of $CD25^{high}$ cells. In order to obtain a standardized gate-transferrable to all donors-distinct subsets were intercalated aiming to achieve one representable gate only.

Consequently, according to their most striking fluorescence levels, putative T-reg cells were gated in the principal study and in the auxiliary study at levels between 10^2 and 10^3 (Figure 13A) and at 10^2 (Figure 13B) respectively. Within each study, the same gate setting was strictly applied to every individual donor-assuring conformity in both studies. This method seemed reliable to draw conclusions about pre-and post T-reg cell levels. Another method (section 5.4.1) was used to measure depletion effects more effectively.

The average percentage of T-reg cells at pre-treatment measuring $CD25^{high}$ cells was 1.046% (GM) with a Minimum (Min) of 0.1% to a Maximum (Max) of 6.01%. In the auxiliary study the average percentage of $CD25^{high}$ $Foxp3^{+}$ was 0.65% ranging from a Min of 0.48% to a Max of 0.78%.

Figure 13: T-reg dot plots using CD25high and Foxp3 gates

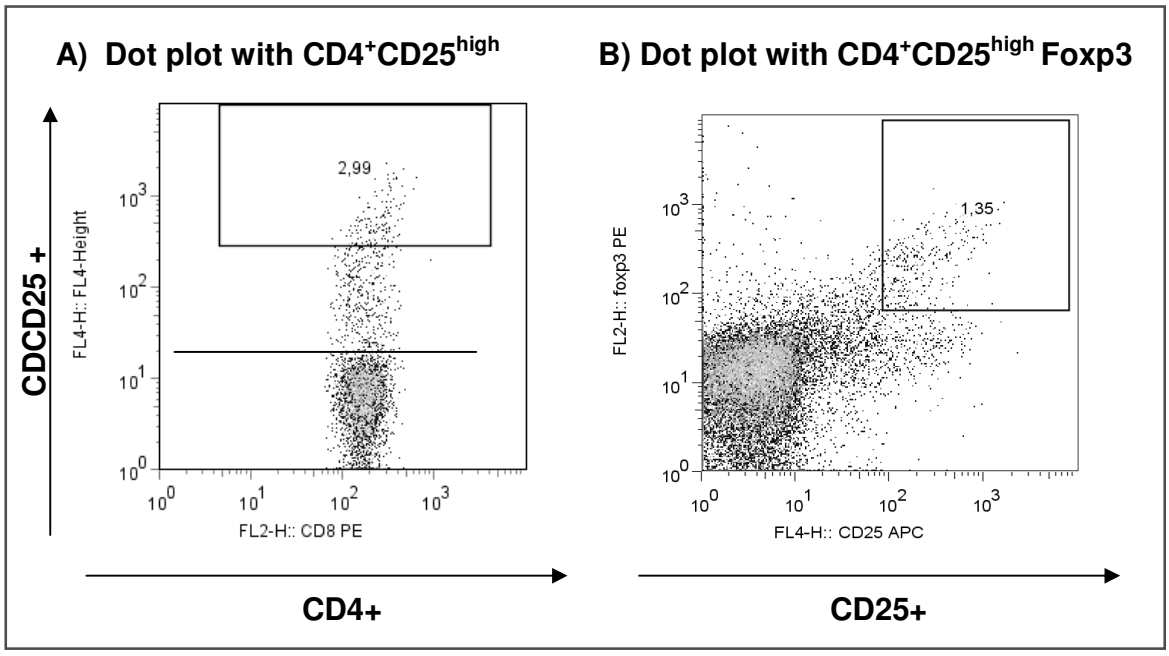


Figure 13: A) shows a typical dot plot used in the main study with the major cell population being $CD4^{+}$ only (on average between 40% and 50% with a Minimum of 30% and a Maximum of 68% of all PBMCs at pre - treatment) and $CD4^{+}$,$CD25^{+}$ cells (ranging from 4% to 16% in whole PBMC at pre - treatment) and on top with a slight right shift $CD4^{+}$,$CD25^{high}$ cells (with cell percentages between 0.07% to 6%). 1b) shows a dot plot with all $CD4^{+}$ cells, $CD25^{high}$ to the Y axis and $Foxp3^{+}$ cells on the X axis showing that the vast majority of $Foxp3^{+}$ cells were also $CD25^{+}$.

5.2 T-regulatory cells: Expression infected vs non-infected

5.2.1 Expression of Foxp3^{+}, CD25^{high} in S.haemtobium infected-vs. non-infected children at pre-and post-treatment

In addition to Foxp3^{+} measurements, we took the opportunity in the auxiliary study to measure and compare T-reg cell frequencies in *S. heamatobium* infected children and non-infected children allowing us to establish whether there was any association between T-reg cell activity and *S. heamatobium* infection.

Hence, samples were taken from both subgroups at the same time prior to administration of treatment to the infected individuals and analysed for expression of T-reg cell associated transcription factor Foxp3^{+} as well as CD25^{high} in CD4^{+} T cells. At this point a significant difference in the frequency of CD4^{+}, Foxp3^{+}, CD25^{high} within the infected group (n=10) expressing notably more T-reg cells than non-infected donors (n=7) ($p = 0.006$) was observed (Figure 14).

Figure 14: Foxp3+ CD25high T-reg cells: Infected and non-infected children

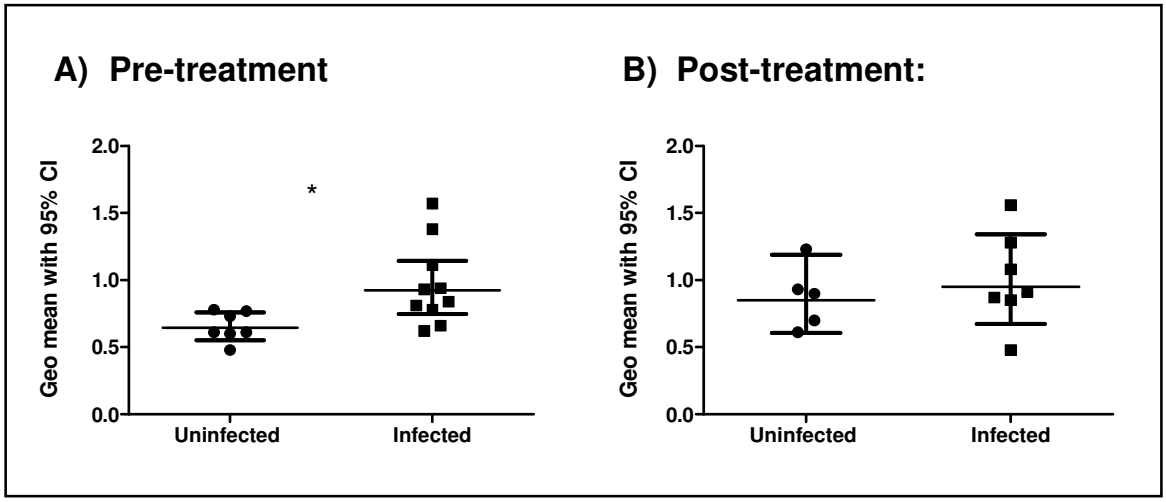


Figure 14: At pre-treatment there is a significant difference in the frequency of T-regs cells between infected and non-donors (*=p<0.05) (A), whereas at post-treatment the difference in T-reg cells has become insignificant between the treated infected group and the non-infected group (p=0.64) (B).

Next, in order to prove that this finding was not only coincidental, but related to the children's infection status, six weeks later, after infected donors were given 2 sets of treatment regimen with PZQ, PBMC's of both groups were re-examined carrying out the same measurements as described above. Interestingly, at this point there was no statistical difference anymore in the frequency of $CD4^+$ $CD25^{high}$ $FoxP3^+$ T cells between previously infected (n=7; 3 missing) vs. non-infected children (n=5; 2 missing) (p=0.64) (Figure 14B). Noticeably, at post treatment the overall frequency of T-reg cells was increased in both groups compared to pre-treatment.

5.2.2 Expression of $Foxp3^+$, $CD25^{high}$ in S. *haematobium* infected children: Pre-vs. Post-Treatment

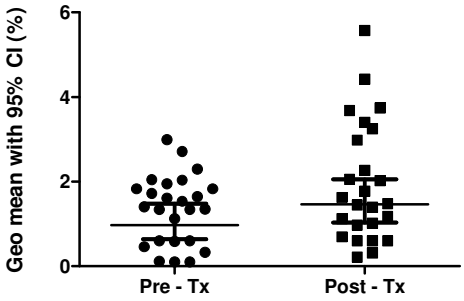
Looking at the dynamics of T-reg cells in *S. haematobium* infected children, the activity of $CD4^+$ $CD25^{high}$ $Foxp3^+$ cells was compared by looking at the frequency (%) and the Geometric Mean (GM) in whole PBMC samples at pre-and at post-treatment. The GM was useful as an indicator of cell activity while displaying the intensity of CD25 expression.

Firstly, measurement of the frequency of $CD4^+$ $CD25^{high}$ cells in our principal study showed only slim differences between pre-and post-treatment values. At post-treatment of previously S. *haematobium* infected subjects there was a small increase in frequency compared to pre-treatment (p=0.08) (Figure 15A). Similar results were also found in our auxiliary study, where $Foxp3^+$ was measured in addition to $CD4^+$ $CD25^{high}$ (p=0.74) (Figure 15B).

However, noticeably measurements of the GM in our principal donor study group showed significantly higher measurements at post-treatment (GM = 540) than at pre-treatment (GM = 490) (p=0.01) (Figure 15C).

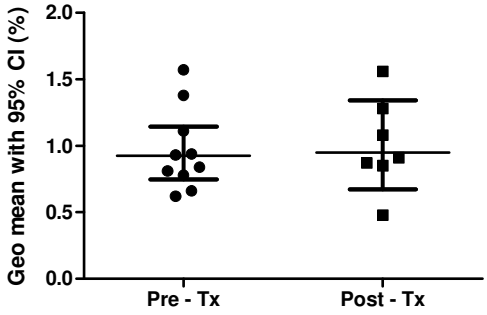
Figure 15: T-regs in whole PBMC at pre-vs. post-treatment

A) Frequency of CD25^{high}



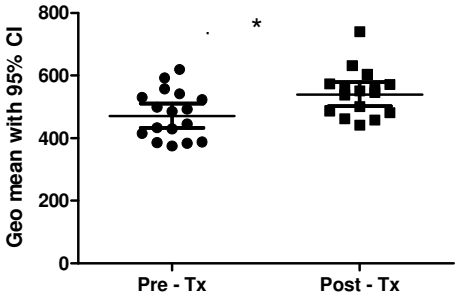
A) shows frequencies of CD4 + CD25^{high} cells in the principal study of whole PBMC (med) at pre-vs. post treatment showing no significant difference at 6 weeks later (p=0.08).

B) Frequency of CD25^{high} Foxp3^{+}



B) A similar trend to A) was observed in the auxiliary study measuring Foxp3 in addition to CD25^{high} with no remarkable difference in T-reg frequencies between pre-and post-treatment samples in whole PBMC (p =0.74).

C) Geometric mean of CD25^{high}



C) Yet, on comparison of the GM at pre-and post-treatment of CD25^{high} cells there is a significant difference between both measurements showing a considerable rise at the second measurement (* = p<0.05) *(SEE ALSO 6.4.)*

5.2.3 Treatment effect on $CD4^+$, $CD25^+$ T-effector cells (frequencies)

Neglecting the possible impact of T-reg cell depletion, treatment effects of PZQ on T-effector cells, defined as $CD4^+$ $CD25^+$ cells, were measured by comparing frequencies of whole $CD4^+$ $CD25^+$ PBMC samples in Medium at pre-vs. post-treatment. In addition, GMs were measured at both time points.

The analysis showed that frequencies of $CD4^+$ $CD25^+$ T-effector cells increased significantly at post-treatment vs. pre-treatment (p=0.038) (Figure 16A). Further, a visible increase was also seen in GM between pre-and post-treatment samples (p=0.0561) (Figure 16B).

Looking at $CD4^+$ cells only, there was no significant difference anymore between pre- and post-treatment samples neither in frequency nor in GM (data not shown).

Figure 16: Treatment effect on T- effector cells in Medium

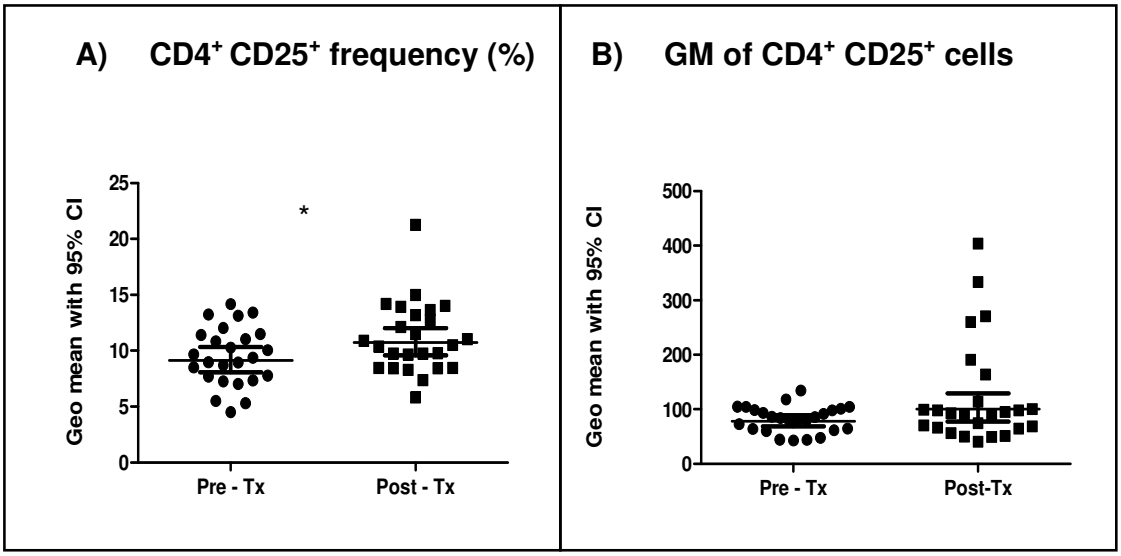


Figure 16: A) Frequencies of $CD4^+$ $CD25^{high}$ measured in Medium solution show a significant rise after treatment with PZQ (*=p<0.05). B) GM levels reveal a noticeable yet not significant difference at the second time point of measurement (p=0.06).

5.3 Specific and non-specific immune response upon anti-helminth treatment

5.3.1 Cell Proliferative responses

Comparing proliferative responses of Ag specific-and non-specific stimulated PBMC at pre-and at post-treatment allowed the evaluation of anti-helminthic treatment effects-not taking any cell suppressor activity into account. As mentioned above, samples were taken at time point zero and then again exactly 6 weeks later after having received the first of two courses of treatment with PZQ-following the initial sample taking.

Proliferative responses were determined by calculating the Division index (DI) (via Flow Jo Software) representing the average of cell divisions occurring and based on dilution of CFSE. Although the analyses showed no significant differences in proliferative responses between pre-and post-treatment samples, a visible trend was observed with increased responses at post-treatment to Ag-specific stimulated sample Anti Worm Ag (AWA). Overall, proliferative responses to the vaccine Ag BCG were low and at a comparable level to background proliferation represented by samples cultured in medium. Responses looked rather similar at both time points before as well as after treatment (Figure 17).

The DI values measured as stated above were as follows in medium at pre - treatment: 0,054 and at post - treatment 0,14 ($p = 0.21$), for AWA at pre-treatment: 0,2 and at post-treatment: 0,37 ($p = 0.09$), for SEA at pre - treatment: 0,09 and at post-treatment: 0,23 ($p = 0.33$), for BCG at pre-treatment: 0,06 and at post-treatment: 0,14 (0.19), for PHA at pre-treatment: 1,04 and at post-treatment 0,66 ($p = 0.05$) (PHA not shown in Figure as due to faulty dilution sample size very small).

Figure 17: Proliferative responses at pre-and post-treatment in specific and non-specific stimuli

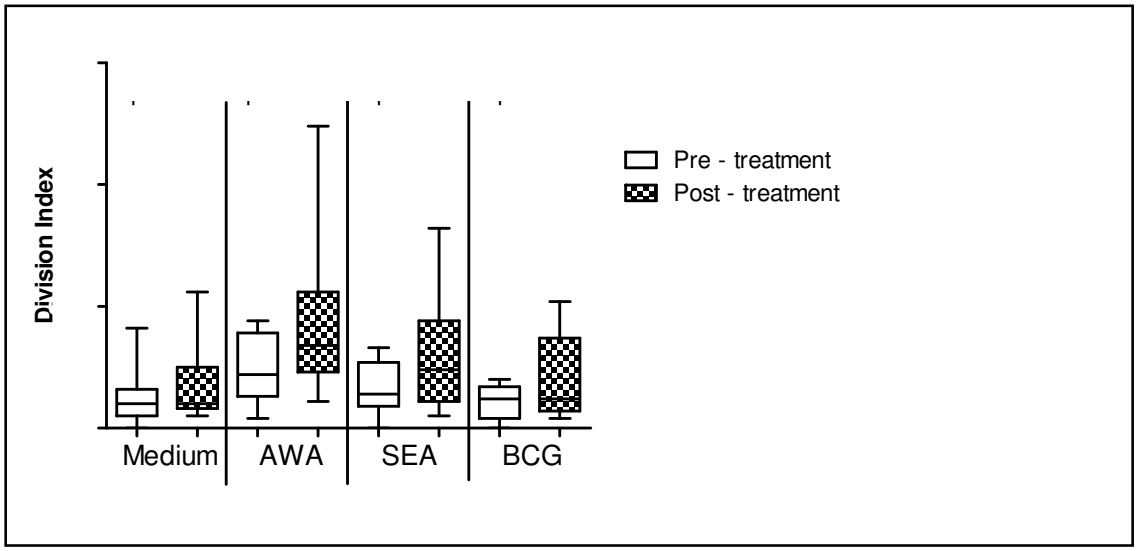


Figure 17: Cell proliferation: all stimuli including schistosome specific Ag AWA, SEA and non-specific Ag BCG show a raising tendency in cell proliferation after anti-helminth treatment with PZQ (Med: p=0.02, AWA: p=0.09, SEA: p=0.33, BCG: p=0.2). The raise is most obvious to Adult Worm Ag, though statistically not significant. Overall BCG shows very little response compared to background cultivation in Medium.

5.3.2 Cytokine responses

Anti-helminthic treatment effects were further established by comparing cytokine responses at pre-and post-treatment. In fact, cytokines from different cell lines were individually analysed to show their specific involvement during chronic infection.

Cytokines, supposedly stemming from different originator cells, including Th2 cytokines IL5 and IL13, Th1 cytokines INFγ and TNFα, Th17 cytokine IL17 as well as IL10 (122) were measured to evaluate Th1, Th2 and Th17 cell lines. Identically to the measurements of cell proliferation, all cytokine levels were analyzed prior- and at 6 weeks post administration of the first dose of PZQ.

At first glance, it was noticeable that overall Th2 cytokine responses were most expressive to specific Ags AWA and SEA at pre-as well as at post-treatment. Comparing the different time points, both Th2 cytokines IL5 and IL13 showed significantly higher responses to specific Schistosome Ags at post-treatment than at pre-treatment. While IL13 production was significantly increased to both AWA (0.004) and SEA (0.03), IL5 levels rose only remarkably to AWA (p=0.044). IL 5 showed a visible but non-significant tendency to increase to SEA (p=0.13) at post-treatment (Figure 18A, B). In general, IL13 showed slightly more activity than IL5. Th2 Cytokine responses to non-specific Ag BCG were generally very low and with no remarkable difference between pre-and post-treatment measures.

In contrast, at pre-and at post-treatment typical Th1 cytokines IFNγ and TNFα were produced at highest levels to non-specific Ag BCG amongst all the Ags utilised (Figure 18C, D). Further, IFNγ also showed significantly elevated responses to BCG at post-treatment (p=0.05), while there was only a rising trend in TNFα levels. Little activity was seen towards stimulation with specific Ags AWA and SEA in both cytokines. The overall activity of TNFα was marginally greater than the one of INFγ.

IL10 with its potentially diverse origin showed generally greater activity to Schistosome specific stimuli than to non-specific BCG. Although, there was an overall increased activity to all stimuli at post-vs. pre-treatment, a statistically significant difference was only seen to non-specific Ag BCG (Figure 18E).

IL17 coming from a distinct subset of cells (Th17 cells) showed no relevant changes after 6 weeks of treatment and overall activity of this cytokine to any of the stimulants was at rather low levels. In particular responses to AWA and SEA were only marginally greater than those to Medium. The greatest raise was observed to non-specific Ag BCG-similar to Th1 cytokines although to a far lower extent (Figure 18F).

Figure 18: Pre– and Post– treatment responses of Th1, Th2 and Th17 cytokines: IFNg, TNFa, IL5, IL13, IL10 and IL17

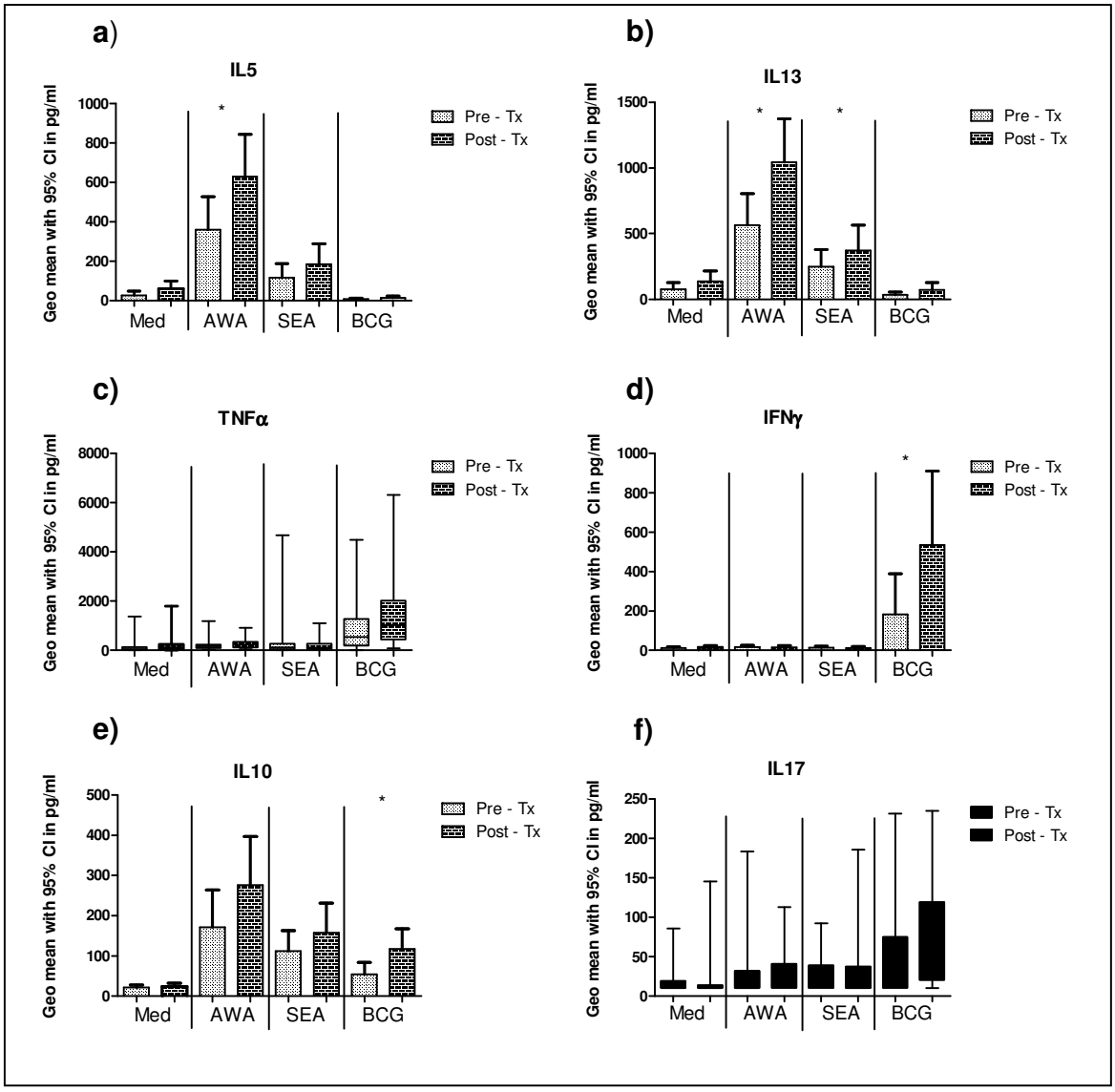


Figure 18: A-B) Different cytokine patterns in response to specific and non-specific Ags show that Th2-cytokines IL13 and IL5 not only have greatest responses to specific Ags AWA and SEA, but also that they increase post treatment with PZQ. C-D) Th1 cytokines IFNγ and TNFα show generally low responses to specific Ags, but high responses to non-specific Ag BCG. with significant increases post-treatment for this Ag. E) Although IL10 shows strong responses to AWA and SEA and less to non-specific Ag BCG, in response to treatment there is only significant increase to the latter one. IL-17 shows low responses to all stimuli with no or little difference post-treatment. *=p<0.05, **=p<0.005.

5.4 Depletion of $CD4^+CD25^{high}$: impact on effector T cell population

5.4.1 Gating the T-reg cell population

To study the assumptive suppressor effect of T-reg cells on proliferation and cytokine responses to helminth specific-and non-specific Ag stimulation, responses of effector PBMC needed analysing with and without T-reg cells. As described earlier (see Materials & Methods) $CD4^+CD25^{high}$ cells and thus putative T-reg cells were magnetically depleted from PBMC. Consequently, the difference in activity between (1.) whole vs. $CD4^+CD25^{high}$ depleted PBMC at pre-and at post-treatment as well as the difference between (2.) these results at pre-vs. post-treatment was compared. This allowed primarily the evaluation of (1.) any possible influence T-regs cells may have on immune responses during chronic infection and secondly (2.) whether and how any influence would be altered once the host was free of infection.

After the T-reg cell gate was set in the whole PBMC sample according to $CD25^{high}$ fluorescence levels as described in 5.1.2., it was transferred to its counterpart depleted sample at the same fluorescence level permitting a fair evaluation of the extent of depletion (Figure 19A, B). Any reduction of the proportion of T-reg cells in the depleted vs. the corresponding whole sample was considered as a successful depletion. However, in samples with a generally low cell counts, this effect was difficult to perceive.

Figure 19: FACS dot plot of $CD4^+$ $CD25^+$ $CD25^{high}$ cells before and after CD25high cell depletion

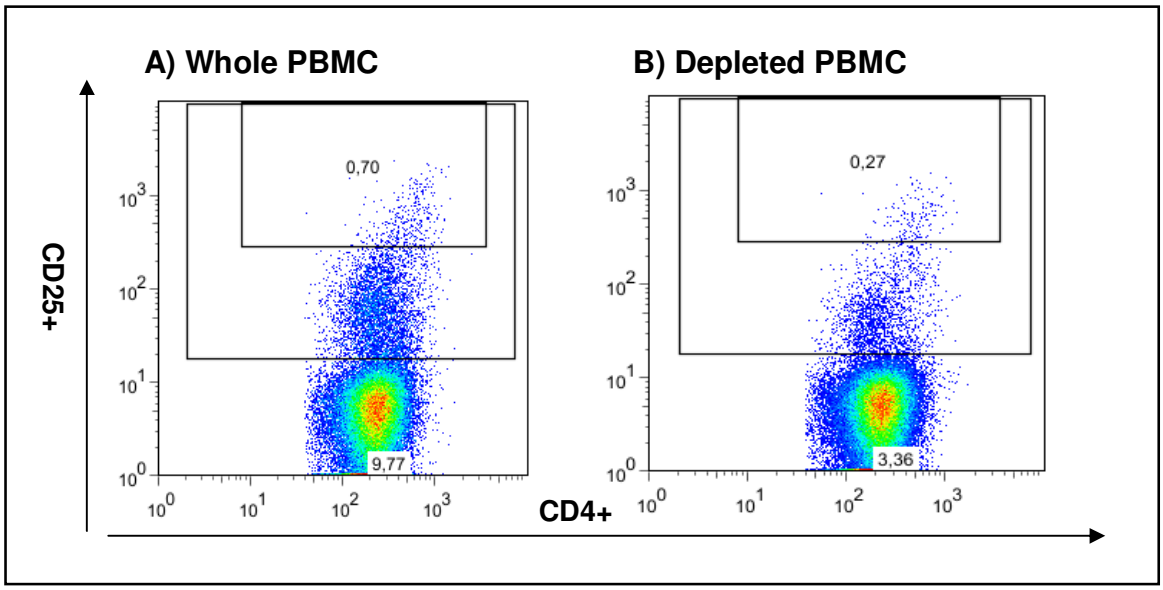


Figure 19: (donor 25 at Post - Tx) Dot plot of a donor sample at post treatment before (A) and after $CD25^{high}$cell depletion (B). All cells displayed are CD4+. In the intermediate square cells have $CD25^+$ expression. On top right shift is visible amongst the $CD25^+$ cells pointing towards a separate subset of cells. Being $CD25^{high}$ these cells were defined as putative T-reg cells. After $CD25^{high}$ cell depletion, there is also a generally reduced number of $CD25^+$ cells noticeable.

Therefore, we applied an additional method to analyse depletion effects in each individual sample. On the basis of previous study results stated in the literature, where T-reg cells had been assigned to constitute approximately 5% of all $CD4^+$,$CD25^+$ cells (66),(123),(124), we gated $CD25^{high}$ cells for each donor individually set at 5% within the whole PBMC set. Subsequently, the gate was transferred at the same fluorescence level to its depleted counterpart sample. By applying this method, each gate contained a greater number of $CD25^{high}$ cells allowing a more effective analysis of depletion effects. (Figure 20A, B). Successful depletion was defined as any reduced number in $CD25^{high}$ cells in the depleted vs. the whole sample.

Figure 20: Depletion of CD25high cells with a top 5% gate

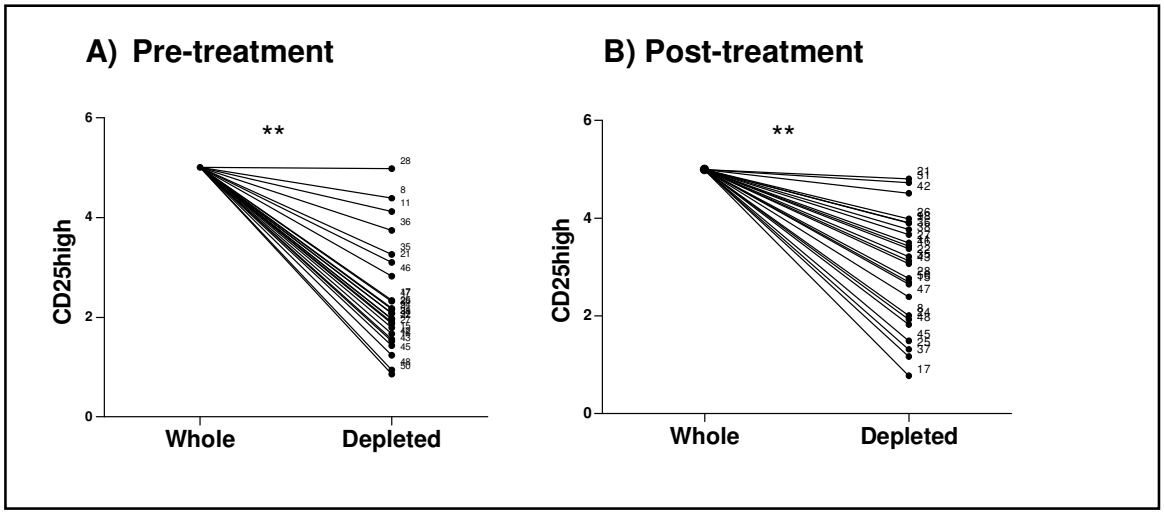


Figure 20: Displays the top 5% of CD25^{high} cells in whole PBMC at A) pre-and at B) post-treatment. Both gates, set at top 5% of total PBMC, were transferred at the same fluorescence level to the depleted sample and showed a significant decrease from 5% to an average of 2.42% at pre-treatment and to 3.45% at post-treatment. Lowest levels at pre-treatment were around 0.83% and at post –treatment around 0.78%. At both points in time the depletion effect highly significant. *=p<0.05, **=p<0.005.

Based on those criteria, a total of 25 donors could be included, while at pre-treatment 8 donors and at post-treatment 11 samples were excluded because of either equal or more cells in the depleted vs. the whole PBMC samples. Other criteria to exclude samples were (1.) if cell counts were too low to detect depletion (2.) if pre-or post-donor samples were missing.

5.4.2 Effect of T-reg depletion on CD25^{high} percentage and GM

In order to discriminate not only whether treatment had any effects on whole PBMC, but also whether it had any effect on T-reg depletion, we applied the initial T-reg gating analysis (according to their fluorescence levels) to quantify these effects. At both times, pre-and post-treatment, analysing the proportional percentage of CD25^{high} in CD4^{+} CD25^{+} PBMC showed that there was a significant reduction in the depleted vs. the whole samples. At both times the reduction in T-reg cells was highly significant: from 0.9753 (%) to 0.5578 (%) (p=0.0054) at pre - treatment compared to 1.461 (%) to 1.143 (%) at post treatment (p=0.0098) (Figure 21A). At post-treatment the percentage of CD4^{+}CD25^{high} was recognizably greater than at pre-treatment.

Intensified expression of CD25^{+} - measured via CD25^{+} GM levels-indicated the cells activity levels. While at pre-treatment GM levels were significantly higher in the depleted (GM = 490) vs. the whole samples (GM = 583) (p = 0.0002), at post-treatment there was no difference between the samples (Fig. 21B).

Figure 21: Proportional % and GM of CD4+ CD25^{high}

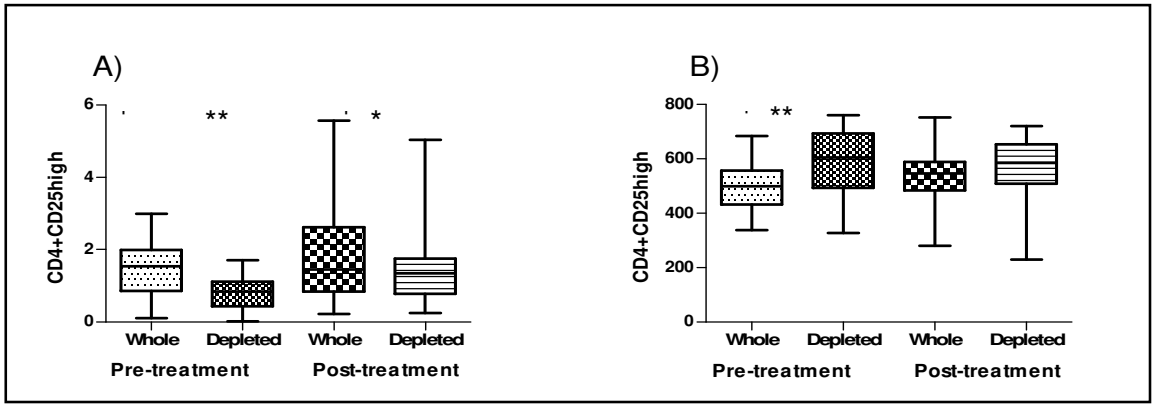


Figure 21: The proportional % of CD25^{high} cells amongst CD4^{+}CD25^{+} cells at pre-and post-treatment before and after depletion with a significant reduction in CD25^{high} percentage at both times (A). The Geometric mean of CD25^{high} cells measured in the same samples with a significant increase of GM after depletion at pre-treatment, but only a marginal difference a post-treatment (B). *=p<0.05, **=p<0.005.

5.4.3 Depletion of $CD4^+CD25^{high}$ $FoxP3^+$ T cells

In the auxiliary study, where $Foxp3^+$ was used as an additional marker to $CD25^{high}$ to define T-reg cells, similar results were observed as described in the principal study. Within the group of $CD4^+$ cells, a subgroup of $Foxp3^+$ and $CD25^{high}$ cells were "encircled" forming the subset of T-reg cells. Transferring this gate from whole to depleted samples the FACS dot plot analysis showed a successful reduction within this defined cell group (Figure 22 A, B). This intensified the assumption that by magnetic depletion of $CD4^+CD25^{high}$ cells, putative T-reg cells were effectively removed.

Figure 22: Foxp3+ cell gating before and after depletion of CD25high cells

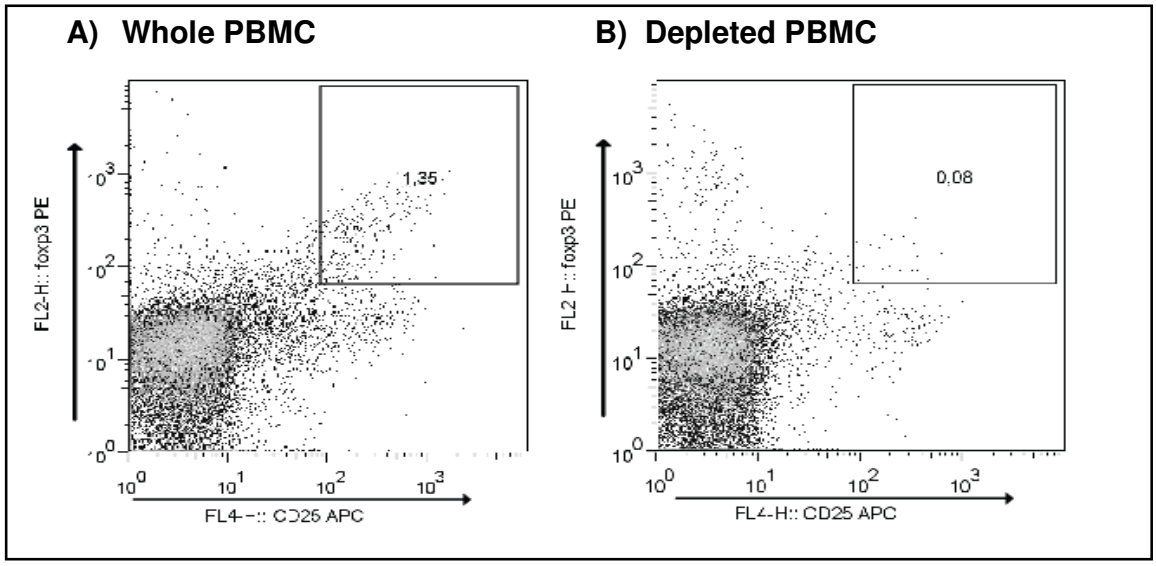


Figure 22: As a subgroup amongst CD4 cells in the right upper corner the gating encloses $Foxp3^+$, $CD25^{high}$ cells (A and B). After depletion (B) the number of Foxp3+, $CD25^{high}$ cells is clearly diminished compared to whole PBMC (A).

As in the principal study successful depletion was confirmed by a diminished count in Foxp3^{+} CD25^{high} cells. Analyzing the subsets individually, CD25^{high} cells with and without Foxp3^{+} cells, showed good depletion effects. In the whole samples the average cell percentage was 0.86% compared to 0.32% after depletion ($p<0.0001$) (Figure 23A). Yet, a significant reduction was also found measuring CD4^{+} CD25^{+} whole (14.76%) vs. depleted (10.89%) cells only ($p=0.0003$) (Figure 23B).

Figure 23: Auxiliary study: measurement of Foxp3+ CD25high and CD4+ CD25+ cells before and after depletion at pre-treatment

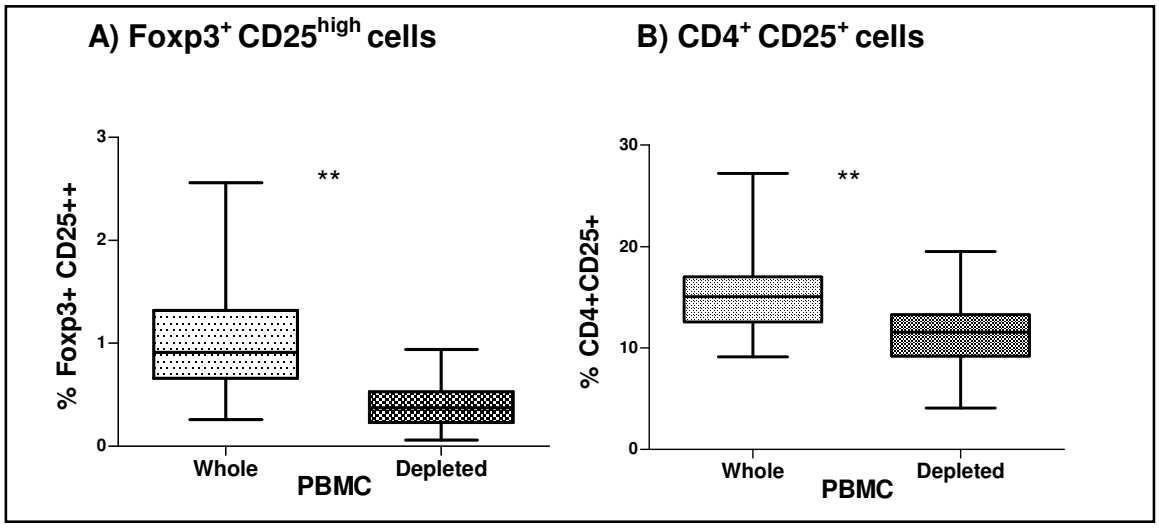


Figure 23: The above Figure shows the content of T-reg cells amongst CD4^{+} cells measuring Foxp3^{+} and CD25^{high} cells before and after magnetic cell depletion of CD25^{high} cells at pre-treatment (A). It reveals that not only CD25^{high} cells (putative T-reg cells), but also T-effector cells (B) were depleted. *=p<0.05, **=p<0.005.

5.4.4 Depletion effect on T-effector cells

As indicated above, in aiming to deplete $CD25^{high}$ cells only, it was remarkable to observe the side effect in both studies displaying a significant decrease in the total number of $CD4^+CD25^+$ cells taking place at pre-as well as at post-treatment (Figure 24A). Interestingly though, as discussed later, this did not seem to affect the activation ability of the effector cells. Analysing the GM of the $CD4^+$ $CD25^+$ effector cells discloses similar values throughout all whole and depleted samples at pre-as well as at post-treatment (Figure 24B).

Figure 24: Proportional % and GM of CD4+ CD25+ T-effector cells

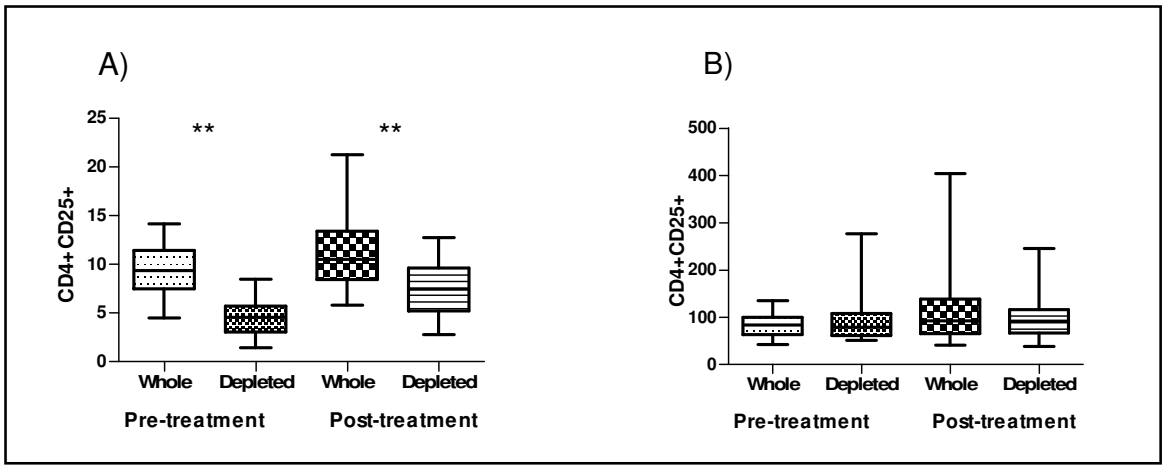


Figure 24: $CD4^+CD25^+$ cells have significantly decreased amongst the $CD4^+$ PBMC after magnetic depletion of $CD25^{high}$ cells. At pre-and at post-treatment $CD25^+$ cells were proportionally equally depleted (A). Throughout all samples at pre-and at post-treatment there is no significant difference in GM (B). *=p<0.05, **=p<0.005.

5.5 Depletion effects on Proliferation and Cytokines at pre- and at post- treatment

5.5.1 Enhanced Ag-specific proliferation in S. *haematobium* infected PBMC after removal of $CD4^{+}CD25^{high}$ T cells at pre-treatment.

In order to investigate the influence of T-reg cells on the functional activity of T-effector cells in S. *haematobium* infected individuals, firstly this study measured and compared each donor's proliferation levels (based on DI) of whole PBMC-with $CD4^{+}CD25^{high}$ depleted PBMC at pre-treatment and at post-treatment. A typical plot showing proliferation changes is illustrated in Figure 25. Looking at pre-and post analyses individually allowed to judge the immune processes happening during each phase-(1.) while disease was in progress and (2.) early after successful treatment. Secondly, a comparative analysis between pre-and post-treatment changes gave room for conclusions how immune responses had altered over time and with treatment.

Figure 25: Representative plot of CFSE staining illustrating proliferation of CD4+CD25+ T cells to different stimuli before and after T-reg depletion

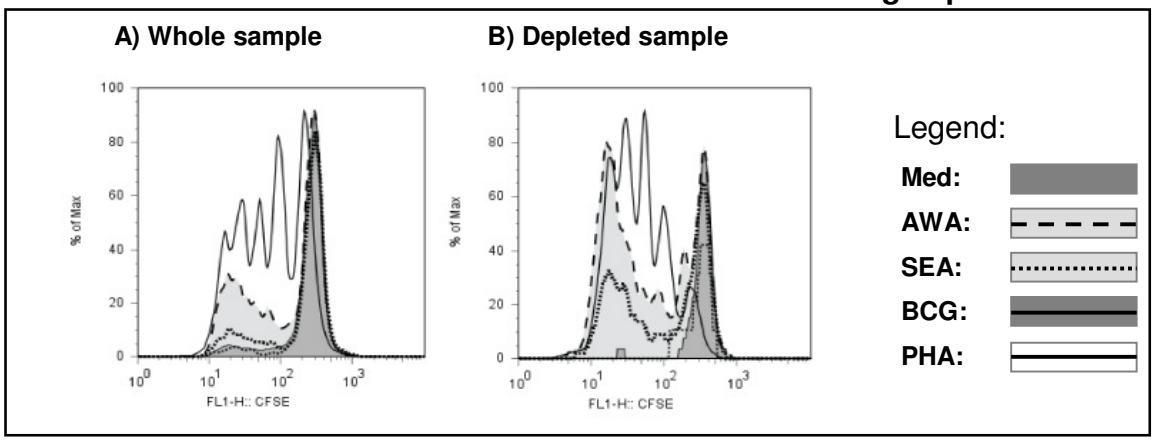


Figure 25: On the left: a typical pattern displaying cell proliferation of whole $CD4^{+}$ $CD25^{+}$ cells to specific and non-specific stimuli (A). Post T-reg depletion: PBMC proliferation is visibly increased to all stimuli (B).

At pre-treatment, proliferation of T-reg depleted PBMC vs. whole PBMC resulted in spontaneous enhancement first and foremost in response to Schistosome specific Ags AWA (GM: pre-Tx whole = 0.198, pre depleted: 0.393; p = 0.0039) and SEA (GM pre-Tx whole = 0.086, pre depleted: 0.397; p = 0.0021), but also to non-specific vaccine Ag BCG (GM pre-Tx whole = 0.056, pre depleted: 0.191; p = 0.0029), although overall responses to BCG were hardly more than those to background proliferation based in Medium GM: pre-Tx whole = 0.054, pre-Tx depleted = 0.177; p = 0.0051) (Figure 26).

Figure 26: Proliferation to specific and non-specific Ags measured via cell DI

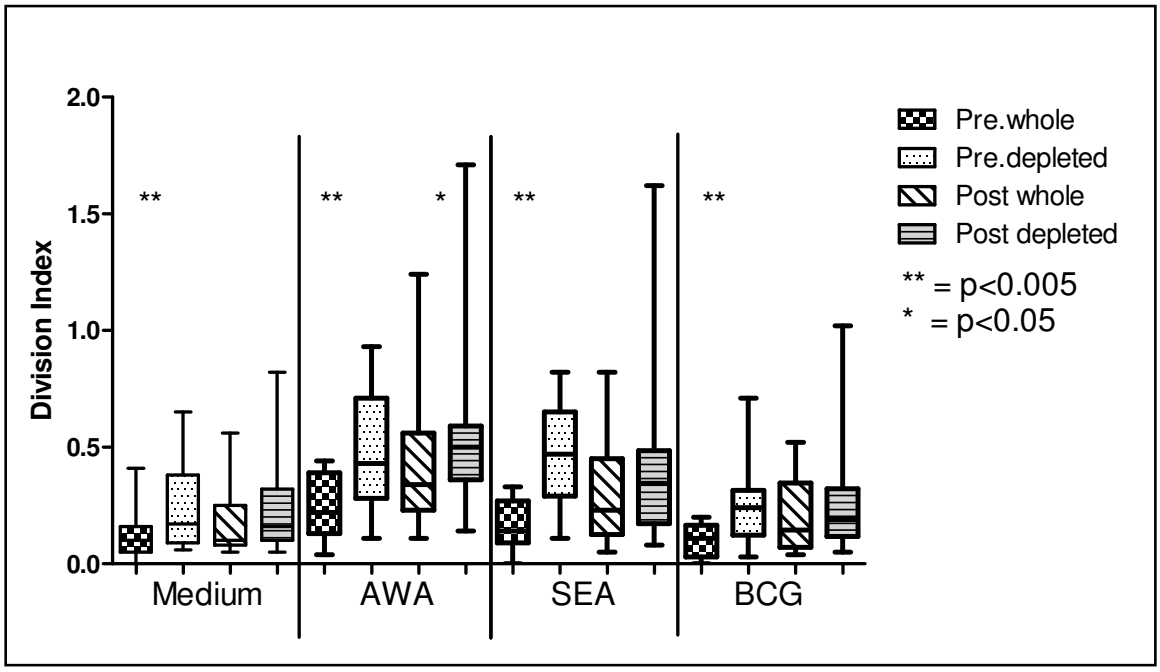


Figure 26: shows that T-reg depletion in the Pre-treatment group caused significant increase in Cell Proliferation to specific Ag AWA and SEA, but also to non-specific Ag BCG. At post treatment after depletion this increase in proliferation becomes insignificant for Ags SEA and BCG and less significant for AWA. (Legend: AWA = Adult Worm Ag; SEA = Schistosome Egg Ag; BCG = Bacille Calmette-Guerin).

5.5.2 Diminished raise of cell proliferation after T-reg depletion at post-treatment

Interestingly, as illustrated in the Figure above (Figure 26): upon successful anti-helminth treatment, this striking effect of enhanced proliferation due to depletion of T-reg cells was diminished in all stimuli including the background stimulus (GM: Medium post total = 0.14, post depleted = 0.16; AWA post total = 0.27, post depleted = 0.38 ($p = 0.02$); SEA post total = 0.23, post depleted = 0.32 ($p = 0.06$); BCG post total = 0.14, post depleted = 0.18 ($p = 0.31$). A significant raise remained only to AWA, although at this point less pronounced than at pre-treatment.

5.5.3 Specific-and non-specific cytokine responses in whole and depleted PBMC from S. *haematobium*-infected children at pre-treatment

In a next step cytokine responses were measured and compared in the same way as cell proliferation. As indicated earlier, Th2 cytokines IL-5 and IL13 showed highest responses to specific Ag such as AWA and secondly to SEA. Cellular stimulation with BCG resulted in even lower responses than those cultured in Medium. Yet, upon depletion of $CD4^{+}CD25^{high}$ T cells at pre-treatment strong increases were observed in both Th2 cytokines to Ag specific SEA (Figure 27A, B). In contrast, depletion had no stimulating effect to AWA.

Next, IL-10 on one hand, showed a typical Th2 reaction with generally strong responses to AWA and SEA and also some visible increases to BCG-yet, no significant differences were observed between whole and depleted samples (Figure 27C).

As opposed to anti-inflammatory driven Th2 cytokines, the pro-inflammatory Th-1 cytokines IFN-γ, TNF-α and Th17 cytokine IL-17 reacted most strongly in response to BCG. Upon depletion at pre-treatment, convincing increases were observed in all of these three cytokines to this non-specific Ag (IFN-γ < TNF-α < IL-17) (Figure 27D, E and F).

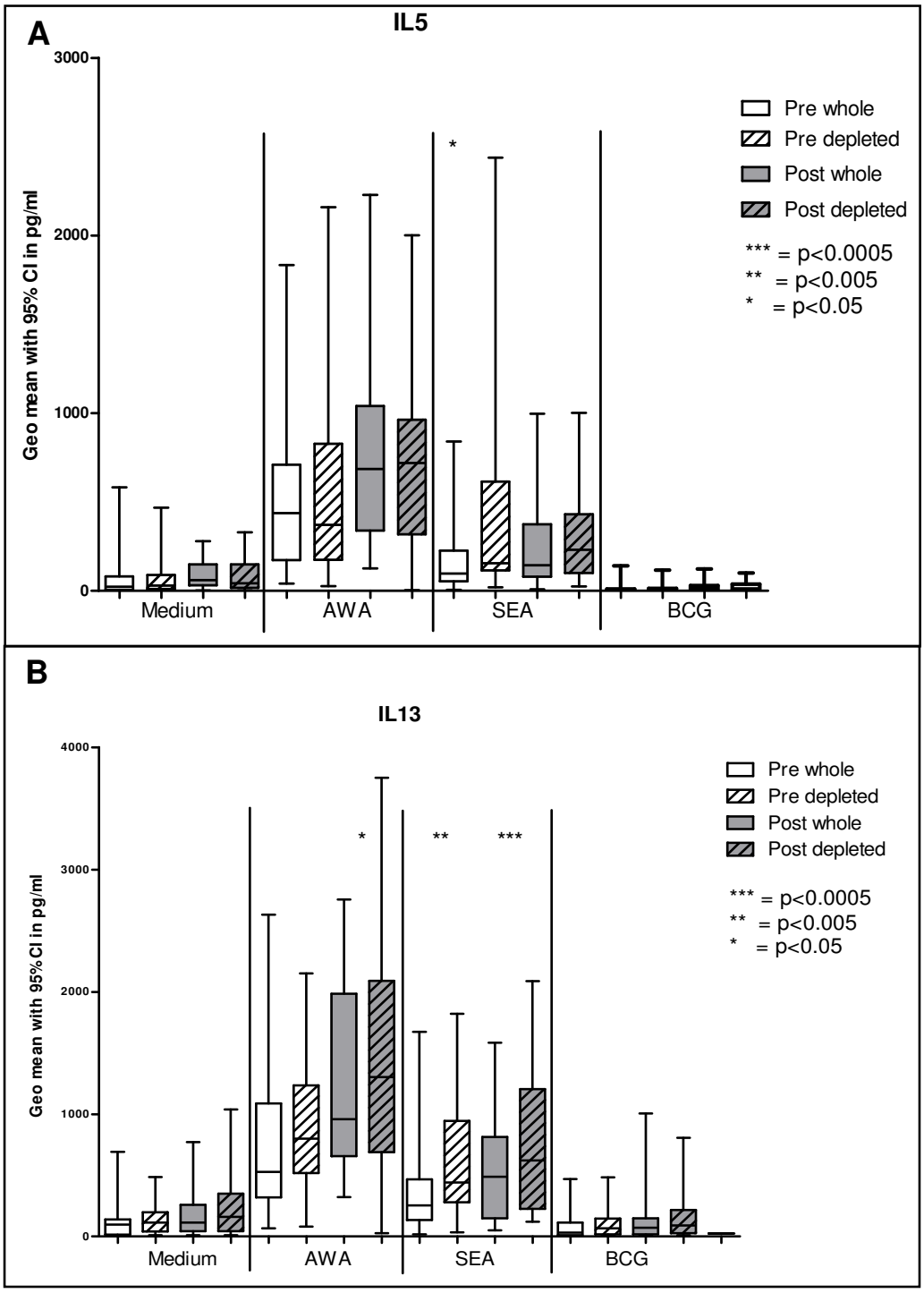
As opposed to what has been observed with Th2 cytokines in response to BCG, Th1 cytokines IFN-γ and TNF-α had lowest-comparable with background stimulation Medium responses to AWA and SEA. Despite the low level stimulation to both Ags, there was still a significant increase upon depletion in IFN-γ to AWA and SEA and in TNF-α to AWA only. IL17 responses to AWA and SEA were visibly greater than to background stimulation, though depletion had no impact and overall stimulation was relatively low compared to IFN-γ and TNF-α.

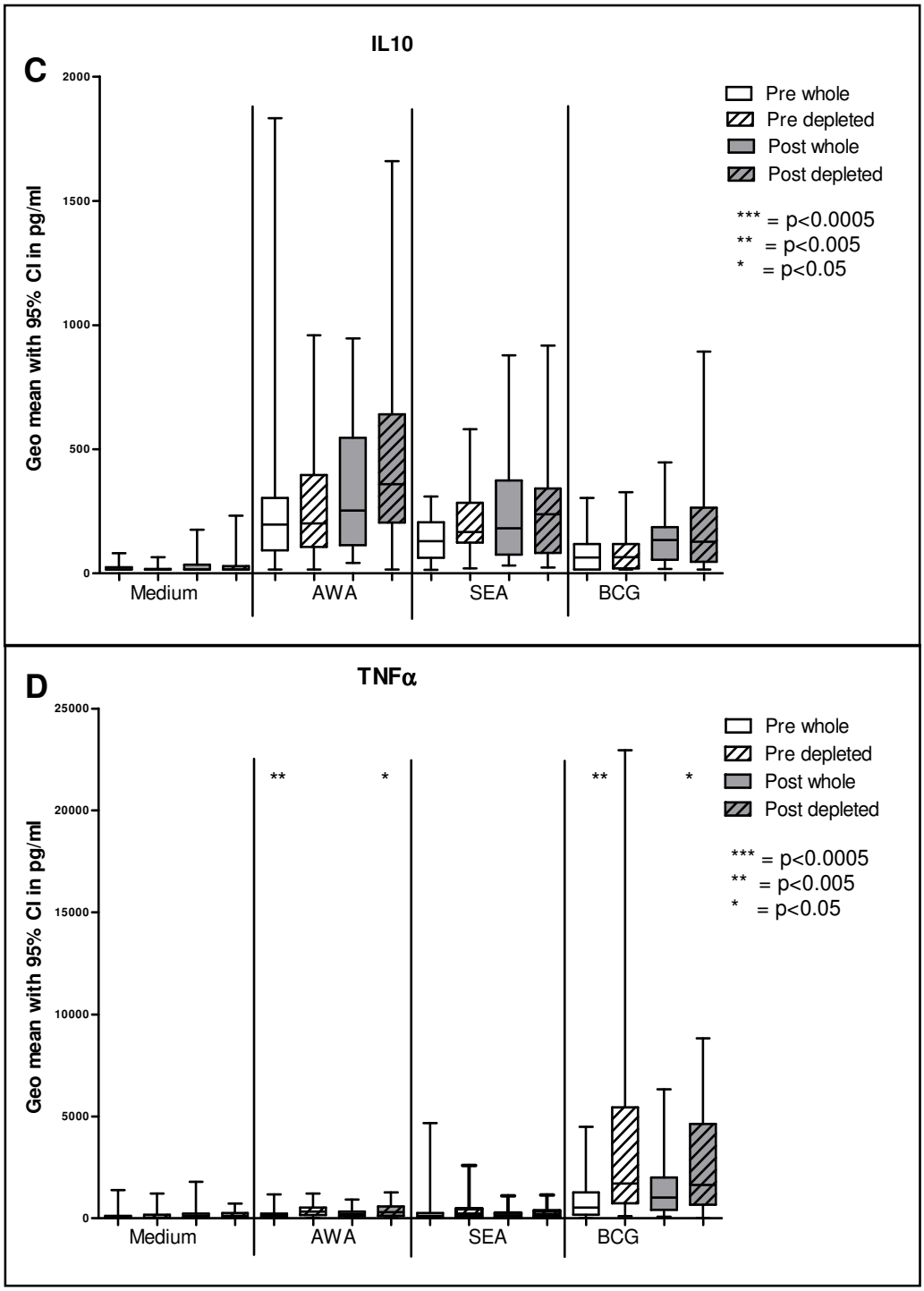
5.5.4 Specific and non-specific cytokine responses in whole and depleted PBMC from previously S. *haematobium* infected children upon anti-helminthic treatment.

At post-treatment upon depletion of $CD4^{+}CD25^{high}$ Th2 cytokines varied in their response. While no significant changes were seen in IL5 to any of the stimuli, IL13 showed significantly increased responses to SEA and to AWA after depletion (Figure 27A, B). IL10's responses post-treatment were similar to those seen at pre-treatment with no remarkable effects after T-reg depletion (Figure 27C).

For Th1 cytokines IFN-γ, TNF-α, and IL-17 depletion of $CD4^{+}CD25^{high}$ cells resulted overall in similar effects as described at pre-treatment. Yet, for Th1 cytokines IFN-γ and TNF-α the depletion effect was diminished compared to pre-treatment in particular to Th1 principal stimulus BCG. Most surprisingly, IL17 reacted most sensitively to all stimuli at post - treatment after depletion despite its overall lowest activity amongst all cytokines (Figure 27 D, E, F).

Figure 27 A-F: Th1 and Th2 cytokine responses to specific and non-specific Ag at pre-and at post-treatment after T-reg cell depletion




C
IL10
Geo mean with 95% CI in pg/ml
2000
1500
1000
500
0
Medium
AWA
SEA
BCG
Pre whole
Pre depleted
Post whole
Post depleted
*** = p<0.0005
** = p<0.005
* = p<0.05
D
TNFα
Geo mean with 95% CI in pg/ml
25000
20000
15000
10000
5000
0
**
*
**
*
Medium
AWA
SEA
BCG
Pre whole
Pre depleted
Post whole
Post depleted
*** = p<0.0005
** = p<0.005
* = p<0.05

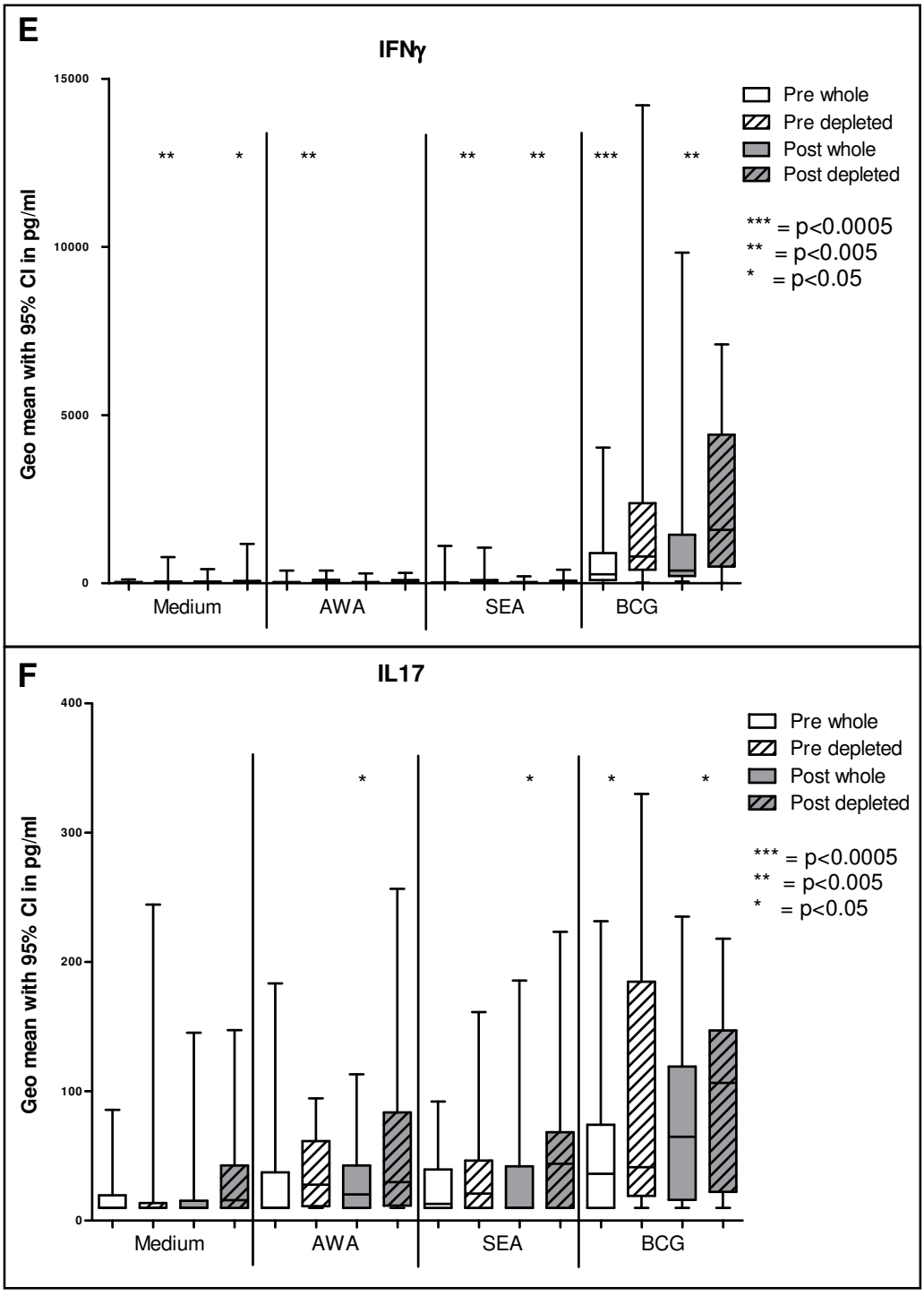

E
IFNγ
Geo mean with 95% CI in pg/ml
15000
10000
5000
0
Pre whole
Pre depleted
Post whole
Post depleted
*** = p<0.0005
** = p<0.005
* = p<0.05
Medium
AWA
SEA
BCG
F
IL17
Geo mean with 95% CI in pg/ml
400
300
200
100
0
Pre whole
Pre depleted
Post whole
Post depleted
*** = p<0.0005
** = p<0.005
* = p<0.05
Medium
AWA
SEA
BCG

Figure 27: Th2 driven cytokines IL5 and IL13 show strongest overall responses to AWA and secondly to SEA. Only to SEA, there is a significant rise in response after depletion at pre-treatment, whereas at post-treatment in IL13 there is a significant increase to AWA and SEA (A, B). IL10's profile for its overall responses are similar to IL13, yet upon depletion neither at pre-nor at post-treatment there is a significant differences to any of the stimuli (C). Th1 driven cytokines TNFα and INFγ show in general very little reaction to Ag-specific stimuli, but enhance visibly to non-specific Ag BCG. At pre-and post-treatment responses are significantly enhanced upon depletion, though at pre-treatment to a greater extent than at post-treatment. Increased responses upon depletion to AWA and SEA are negligible at pre-and at post-treatment as they are at the same level as background stimulation with Medium (D, E). IL17 displays Th1-type responses with overall somewhat higher responses to AWA and SEA than to Medium, but overall low responses. Most stimulation is seen to non-specific Ag BCG. Significant depletion effect is seen to all stimuli post-treatment and only to BCG at pre-treatment (F).

5.6 Correlations between Cytokines and Proliferation vs. infection activity

In order to evaluate whether infection levels, measured by the number of Schistosome eggs/ 10ml, were associated with immune activity, correlation statistics compared cytokine and proliferation levels with infection intensity. Out of all cytokines only one negative correlation could be made between IL10 and the number of eggs counted. At pre-treatment a significant association was made between low IL10 levels and high infection activity-reflected by an increased number of eggs ($p = 0.04$) – in other words higher IL10 levels were seen in those with lower egg count.

For all other cytokines to none of the stimuli correlations could be made between activity levels and infection activity-neither at pre-nor at post - treatment. However, looking at the extent of proliferation and egg count, at pre-treatment a positive correlation could be made between high proliferative responses to AWA and increased egg count (whole PBMC). No correlations could be made between all other stimuli such as SEA or BCG and infection load.

6 Discussion

6.1 T-reg cells in S. haematobium infection

An acute infection with Bilharziosis, which cannot be fought off neither by the innate nor by the acquired immune system, will turn into a chronic infection, that without any treatment appears difficult to be overcome. The concept of innate and acquired immunity is still being studied, but it is evident that susceptibility to this disease is higher in children than in adults (3). Rodent models, as well as human studies indicated that parasites are capable to use immunogenic mechanisms evading the host's active defence responses prolonging their lifetime within the host (110),(125). Having been subject to other infectious diseases T-reg cells have already shown their dampening effect on the immune system (126),(127). Looking at the impact T-reg cells have on children's immune response, this study aimed to investigate as to whether there is an association between T-reg cells and altered immune function in *S. haematobium* infected children.

As they form the central part of this study, the identification of T-reg cells was of primary importance in order to draw useful conclusions from any further functional studies. Therefore, in addition to the principal study, where the IL2 receptor α-chain $CD25^{high}$ was used to characterise T-reg cells, an auxiliary study was performed using the intracellular transcription factor $Foxp3^{+}$ plus $CD25^{high}$. Although, alike other markers, $Foxp3^{+}$ is not an exclusive marker for T-reg cells, so far it may qualify best to outline this particular subset (29). The FACS analysis revealed a convincing overlap between both gates displaying a distinct subset of cells identified as T-reg cells. Hence, using both - $CD25^{high}$ and $Foxp3^{+}$ - increased the validity of this study's results. The method applied within each study was consistent and therefore considered as robust.

Comparing T-reg cells of *S.haematobium* infected- with non-infected children, this report provides clear evidence that those infected with the parasitic worm had a considerably higher frequency of $CD4^{+}$ $CD25^{high}$ $Foxp3^{+}$ cells in their peripheral

blood than their healthy controls. This observation indicated that the infection with *S. haematobium* induced an increased number of T-reg cells. This finding is also in agreement with previous studies where schistosome infection had prompted an expansion of Treg cell number and / or activity (106),(113),(128).

Interestingly, when analysing of $CD4^+$ $CD25^{high}$ cell frequencies amongst S. *haematobium* infected children at pre-and at six weeks post clearance of infection, there was little difference. This observation was made in both studies (1.) the principal and (2.) the auxiliary study where $Foxp3^+$ was used in addition to $CD25_{high}$ as defining marker for T-reg cells.

The principal argument explaining the discrepancy between these results of (1.) infected vs. non-infected and (2.) infected pre-vs. post-treatment is likely to be related to cell kinetics: with the second time point of measurement having taken place at only 6 weeks post-treatment, T-reg cell reduction was likely to be incomplete. This argument is supported by a study describing a decrease in circulating Treg cells at only 8 to 14 months post anti-helminth treatment in *S. mansoni*-infected Kenian sand harvesters (128).

Another approach trying to explain this finding suggests, that at 6 weeks post administration of PZQ, T-effector cell activation is flourishing, resulting in increased expression of the surface marker CD25. Hence, both cell types, T-reg-cells as well as activated T-effector cells, would express high levels of CD25 making them indistinguishable (29). Observations made in this study support this idea by showing a remarkable increase in the percentage of $CD4^+CD25^+$ T cells as well as a raised GM of $CD25^{high}$ at post-treatment. Signs of increased cell activity are more likely due to increased T-effector rater than T-reg cell acitivity. This is in accordance with findings made by Watanbe (2007).

Lastly, seasonal differences may also exert a certain effect on immune responsiveness. Shiff et al (1979) found, that especially at the beginning of the dry season, when second measurements of this study were performed, the transmission of the disease was at its peak due to lots of standing waters in place.

On the contrary, in the middle of the rainy season, when this study was started, transmission would be normally at its lowest point (129). Assuming a certain impact of seasonal changes on immune activity, this would add to the effects mentioned above. Such seasonal influences may also explain the phenomenon found in the auxiliary study, where infected, but also non-infected children have a greater $CD25^{high}$ count at the post-treatment time point-6 weeks later than pre-treatment and right at the beginning of the dry season.

6.2 Treatment effects on immune response in S. haematobium

Assessing the effects of anti-helminth treatment on immune responsiveness-irrespective the influence of suppressor cells undepleted PBMC were compared at pre- vs. post-treatment. Only donors with at least 90% egg clearance were included in the paired data analysis. Although this precautionary measure can be argued as PZQ is known to be highly effective and the likelihood of reinfection with S. *haematobium* within such a short amount of time is small (130) both circumstances cannot be considered as irrevocable.

The diverse picture observed comparing basic immune responses vs. post-treatment responses, leaves room for speculation. At first, this study showed a significant increase in frequency of $CD4^{+}CD25^{+}$ T- effector cells at post-treatment. Yet, looking at cell functionality: cell proliferation levels remained surprisingly static at post-treatment regardless of the Ag's origin. Yet, assessing functional activity via cytokine responses, there was a clear uptake in activity to specific- as well as non-specific Ags. The latter observation led to the assumption that the apparent rise in $CD4^{+}CD25^{high}$ cells at post-treatment was due to T-effector cell activation expressing increased levels of CD25 as a marker of activity but not regulation. Both: early post-treatment measurements and activated T-effector cells expressing the same marker as T-reg cells make it difficult to draw definitive conclusions.

The vivid responses observed amongst various cytokine profiles were applicable to both Th1 and Th2 cytokines and were brought forward by specific as well as non-specific stimuli. Expectably both helminth-specific stimuli, AWA and SEA, though principally the former one, induced significant increases in the Th2 cytokines: IL-5 (AWA) and IL-13 (AWA, SEA). Such findings were in agreement with other studies demonstrating increased Th2 responses to schistosomal Ags post treatment with PZQ (131),(108),(113). The principal effect on AWA may be explained by the drugs main action on the adult worm causing an increased Ag release by dead worms in vivo resulting in a strong stimulating Th2 response. Non-Ag specific BCG resulted primarily in remarkable increases in Type 1 cytokine IFNγ and in non-cell type specific IL10 after treatment. Even those responses were observed in previous studies (108).

The heterogeneity in cell proliferation and cytokine release was unexpected and stands in contrast to a study showing at 6 weeks post-treatment concordant increases in both cell proliferation and cytokine responses (132). Analysing these discrepancies a variety of potentially responsible factors can be put forward. Yet, taking Mduluza et al's (2009) results into account technical issues appear quite plausible. It has been shown that CFSE can have a toxic effect on cells destroying their cell membrane (133) which may result in lowered sensitivity measuring proliferative T-effector cell activity, while cytokine measurements are little and not at all affected.

Secondly, despite the speculation about the role of T-reg cells during the infection, at post-treatment their role may be modified possibly involving immune response modification. Arguments showing a change in T-reg cell function at post-treatment have become evident in previous studies, i.e. an increase in T-reg cell activity was found in hepatitis C-infected patients after viral clearance. It was suggested that this is a mechanism to limit collateral damage (134). The same or similar mechanisms may be applicable to their role post anti-helminth treatment, in particular during the early phase when a high load of antigenic material is released.

As the latter effect may potentially induce active tissue-damaging effector cell responses, mechanisms for prevention would be required.

Despite the lack of increased cell proliferation leaving room for further speculation, the significant rise in cytokine release at post-treatment in particular to Ag specific stimuli showed certainly a convincing degree of immune response reactivation as a result of the applied treatment.

6.3 Effect of T-reg cell depletion on immune responses

To test whether schistosome-induced T-reg cells had an effect on the immune hyporesponsiveness of effector T-cells in *S. haematobium* infected individuals, immune responses were measured and compared before and after T-reg cell depletion (1.) at the time of chronic infection and (2.) six weeks later after having undergone two treatment regimen with PZQ. As earlier described in more detail this was done by measuring cellular responses including T-cell proliferation and distinct Th1 and Th2 cytokine profiles. While at pre-treatment this study looked mostly at the immune status during chronic infection, measures at 6 weeks post - treatment assessed rather early treatment responses on the immune system.

Depleting T-reg cells from PBMC of infected donors before treatment resulted clearly in significantly enhanced proliferative responses to both helminth-specific Ags AWA and SEA but also to non-helminth specific Ag BCG. Overall, at post-treatment T-reg cell depletion had clearly reduced effects on Ag-specific proliferation demonstrating a clear impetus of T-reg cells on immune reactivity during the chronic state of S. *haematobium* infection.

More diverse-and hence consequently somewhat more complex to interpret- were this study's results of cytokine production before and after treatment. As expected, at pre-treatment T-reg cell depletion resulted in a considerable increase of Th2 as well as Th1 cytokine production – highlighting their varying Ag specificity.

Generally, Th2 cytokines as well as IL10 responded predominantly to AWA and SEA with BCG responses being similar to background stimulation. In contrast, Th1 cytokines TNFα and INFγ as well as Th17 cytokine IL17 (IFN-γ < TNF-α < IL-17) were first and foremost raised to non-specific Ag BCG. Responses to Ag-specific AWA and SEA particularly those of TNFα and INFγ were negligible with marginal differences to Medium.

The declining prominence of Th1 cytokines in chronic S. haematobium infection underlines observations made in previous studies (90),(91). Yet, despite this repeatedly observed trend, Th1 cytokines responses to non-Ag specific BCG before, but even more so after T-reg depletion were outstanding. This raises the question on extent of T-reg suppression possibly going far beyond the disease specific immune suppression.

A clear indication of chronic infection was that at pre-treatment post depletion there was a significant increase in both Th2 cytokines to SEA (IL13 > IL5), but not to AWA. This was unsurprising as adult worms play the main role during the acute phase of infection, while chronic disease-as seen here-schistosome eggs are the main actors (38),(39),(135). Hence, during the chronic phase of S. *haematobium* infection, SEA is likely to be more affected by T-reg suppression than AWA.

At post-treatment a varied picture was observed between IL5 and IL13. Expectably, IL5 secretion showed no significant increase anymore upon depletion showing the diminishing influence of T-reg cells. In contrast, IL13 showed greater activity upon depletion than at pre-treatment to both stimuli SEA and AWA. With IL13 known to have an important role in the down-modulation of Granuloma formation during chronic infection (96), one attempt to explain this effect may be that at this early stage post-treatment IL13 is still actively involved in the phase of egg-expulsion – a process that can take up to several months. A rather sophisticated interaction of control mechanisms may continue to take place at this point-with T-regs still down-modulating IL13.

In general, Th1 cytokines displayed a similar picture in activity at pre-and post-treatment-with a slight decline at the latter point in time. Contrastingly, measures for IL17 showed an equally (BCG) or significantly higher increase (AWA, SEA) at post-treatment. Although the patterns observed reflected a certain Th1 polarization, the responsiveness to AWA and SEA was clearly more pronounced than by Th1 cytokines INFγ and TNFα. Yet, the enhanced activity upon T-reg depletion at post-treatment was unusual and reveals further queries regarding the role of IL17. In fact, the question may be raised, whether IL17's main activity may be in the immediate phase post parasite expulsion rather than at pre - treatment. IL10, which appears to have different originator cells (73), seemed to be least affected by T-reg depletion at pre-as well as at post-treatment.

In an attempt to explain some of these incongruent responses between T-effector cell proliferation-and cytokine responses several arguments, including some of the technical issues earlier mentioned, can be put forward. Looking at the extensive impact of T-reg cells, they may cause a shift in the host's cellular composition post treatment exerting different effects on cytokine release and cell proliferation. As matter of fact it is known that T-reg cells also influence other cells lineages such as Ag presenting cells, as dendritic cells, monocytes and B cells whilst reducing their immunogenicity (70). An indirect influence of T-reg cells has also been observed on NK (T) cell activity (136) mast cells, basophils and eosinophils (110). Lastly, depletion itself may exhibit stimulatory effects potentiated by helminthic treatment and therefore possibly leading to increased cytokine release post treatment and post depletion.

6.4 Limitations, strengths and weaknesses

Having all donors included from the same area within the age group (7-14) in which risk of infection is at its peak (91),(92) allowed a fair comparison amongst different individuals with similar exposures to their immunity. As no gender difference was observed in the main study, the gender difference in the auxiliary study was not considered as relevant.

One of the pitfalls in this study is the coexistence of other parasitic infections, which were measured at random and therefore not taken into account. Although Schistosomiasis in humans is still scarcely studied, other investigations have undoubtedly shown some evidence of interactions between different parasitic infections (138),(139). As many common parasitic diseases occur in the same area, i.e. Malaria, Schistosomiasis, Filariasis, children in this study are likely to have suffered from more than one parasitic illness prior or at the conduction of this study. Random checks for Malaria and Filaria as well as S. mansoni showed the coexistence of these infections despite the absence of any clinical signs or symptoms. However, making specific exclusions as a result of coexisting infections was not as a feasible option since only random checks were performed. Hence, the diagnosis of other parasitic diseases was considered as coincidental findings only-leaving any questions open as to whether they may have influenced immune responses.

Another important issue that needs consideration when drawing conclusions is the short post-treatment interval looking at immediate rather than any long-term effects on the immune system. Both are likely to differ, as at short-term T-reg cells as well as effector T cells are likely to remain at a certain activity level, while several months post-treatment immune responses may have not only settled, but be modulated. All in all, it seems justified to say that, at 6 weeks post-treatment immune responses have not yet returned to a status of complete immunological homeostasis.

A general shortage, also affecting this study, is the lack of a definitive marker to mark out T-reg cells (29). Although $CD25^{high}$, the surface marker used in our principal study, has been widely used, Foxp3 – in the meantime increasingly used – seems to be more sensitive. This lead to the conduction of an auxiliary study exactly one year later – using both: $CD25^{high}$ and Foxp3. A clear overlap between both gates to mark out T-reg cells not only confirmed the method used in the main study underlining its validity, but also strengthened any following results.

Another difficulty that became apparent during the analysis is related to the possible toxicity of CFSE toxicity. In some samples a so called “leakage” phenomenon occurred and may be due to cell rupture as a result of toxic effects. Apart from partially low cell numbers, which had made it impossible to analyze T-reg depletion reliably, this was considered a causal factor why in some samples there was no successful depletion observed.

6.5 Conclusion

This study strongly suggests that S. *haematobium* infection is associated with increased T-reg cell frequency. Evidence for this stems from a small-scale auxiliary study, which should be followed by an investigation on a larger scale. Yet, as other findings deduced from this study were comparable to those from the principal study, this result seems equally plausible and credible. In contrast, in neither of the two studies performed a reduction of T-reg cell frequency could be proven at 6 weeks post anti-helminthic treatment. Due to the short interval post-treatment, this may be due to elevated levels of CD25 by activated T-effector cells, which cannot be differentiated from $CD25^{high}$ expressed by T-reg cells. Besides, a raised CD25 GM as well as increased $CD25^{high}$ levels at post-treatment are likely to represent a boost of T-effector cells rather than raised T-reg cell levels.

At the same time, it is possible that T-reg cell levels would only be noticeably reduced at a later stage. It has been observed that the decline of T-reg cells post infection may take up to several months (128). Continously high T-reg cell frequencies may be associated with the ongoing post-disease process such as “dead” egg excretion. Yet, the uncertainty in correctly interpreting this finding shows the need for an adequate marker uniquely identifying T-reg cells, which would allow its differentiation from any other cell type. Currently, only prolonged measurements of T-reg cells at post-treatment might help to gain more clarity and establish the duration of altered immune responses.

The increase in Th1 and Th2 cytokines at post-treatment reflect a boosting effect of anti-helminth therapy on the immune system. As discussed in detail a number of causes including technical weaknesses may be responsible for the discrepancy found between cell proliferative and Th1/Th2 cytokine responses. The plausibility of causes would have established in further studies: while a comparison between CFSE and other staining materials might establish any toxic effects of CFSE, a longitudinal study with follow-ups over several months would allow to determine whether proliferative responses recover at a later stage and if at any stage they may correlate with cytokine responses.

According to this study’s hypothesis, T-reg cell depletion effects at pre-treatment were conclusive for both cell proliferative – as well as Th1 and Th2 cytokine responses. While a clear Th1 / Th2 polarization was noticeable with respect to the Ag responses, both Th1 (IFNγ, TNFα) as well as Th2 (IL5, IL13) cytokine responses were enhanced after T-reg cell depletion to BCG or AWA and SEA, respectively. This showed the T-reg cells suppressive effects on Ag – specific as well as Ag non – specific responses.

Post-treatment depletion effects were more diverse and this study’s findings leave room for speculation on one hand, but require further inquiries on the other. Looking at the Th2 cytokines, IL5’s activity had expectably settled upon depletion, yet IL13 still showed strong reactivity. This mismatch may indicate that IL13 is

possibly more involved in the early post-disease process compared to IL5. Only repeated measurements over a prolonged period at post treatment may help to gain a clearer picture of IL13's role. Due to generally very low Th1 cytokine responses to AWA and SEA – at pre- and at post-treatment, any interpretation of depletion effects is not very expressive. Depletion effects to non-specific Ag BCG were reduced, but still visible showing an ongoing suppression by T-reg cells on Th1 cellular responses. Predominant depletion effects on IL17 – as seen in this study – remain unclear and need further investigations.

Overall, the extent of suppression on Ag-specific as well as non-specific responses showed the ubiquitary effects T-reg cells have on the immune response in S. *haematobium* infection. This indicates that T-reg cells suppressive effects on the immune system go beyond the affecting disease resulting in a general state of immune hyporesponsiveness. Hence, children infected with S. *haematobium* may be more susceptible to acquire other diseases or have more difficulties in fighting them off. Further, this results also underlines Hu et al's (2010) hypothesis that T-reg cells induction, but not their field of action is Ag-specific (140).

Concluding, this study revealed not only the increased T-reg frequency in S. *haematobium* infection, but also that T-reg cells functionally impinge on children's immune responsiveness. The inhibitory effects of T-reg cells make it difficult for the immune system to unfold to its full capacity. While the presence of T-reg cells seems to be beneficial to the parasite's survival within the host, in consequence the latter seems to be unable to expel the parasite. Yet, there are many outstanding questions that need to be answered. Some of them include the mechanisms by which T-reg cells are induced, the specific role of certain cytokines (IL13, IL17) as well as long-term observations of proliferative cell responses and T-reg cell frequencies.

7 Summary

Schistosomiasis is a parasitic disease, which occurs predominantly in Sub - Saharan Africa. While annually an estimated 230 Million individuals become affected, mainly children are at risk of contracting this disease (9),(10),(11). S. *haematobium,* one of the 5 main types of Schistosomiasis, is the only one affecting the urinary tract system. With its hot and humid climate as well as often poor water sanitation facilities Gabon is widely affected by S. *haematobium.* Currently the disease is still effectively treated with the anti-helminth medication Praziquantel, though rapid re-infection compromises long-term effectiveness of this therapy. Hence, life-long immunity via an effective vaccine would be desirable.

A number of studies involving various infectious diseases showed a strong correlation between T-reg cells and prolonged disease (38),(64),(82),(83). This led to the assumption that T-reg cells could play a key role in the immune process once the host becomes infected with the parasite.

This study was dedicated to investigate the role of T-reg cells in children infected with S. *haematobium* in a high prevalence area. Measuring and comparing cell proliferative- and cytokine responses prior and after depletion of T-reg cells allowed to evaluate the cells' effects. Measurements were carried out before and 6 weeks after treatment allowing to establish any treatment effect on T-reg cells.

After identifying T-reg cells as $CD4^+$ $CD25^{high}$ $Foxp3^+$ cells, a comparison of their frequency in different subgroups showed that children infected with S. *haematobium* had significantly more T-reg cells than non-infected children. Less striking were the results when comparing T-reg cell frequencies in infected children at pre- vs. 6 weeks post-treatment. Possible explanations include the activation of T-effector cells expression increased CD25 – making them undistinguishable from $CD25^{high}$ T-reg cells. Alternatively, the reduction of Treg cell may only occur at a later stage post - infection as previously observed in other studies (128).

Increased T-effector cell activity at post-treatment was also reflected by elevated Th2 cytokines IL5 and IL13 to Schistosome specific Ags AWA and SEA as well as by a remarkable increase of Th1 cytokine IFNγ to non-specific Ag BCG. However, no decisive treatment effect was visible in cell proliferation which may be related to early measurements or low sensitivity of material.

Overall, depletion of T-reg cells resulted in greatest effects at pre-treatment, with generally diminished effects at post-treatment. Varying responses were seen for cytokines IL13 and IL17. Possible explanations may include early measurements post-treatment with still some continued T-reg activity or active post-infectious or post-inflammatory cytokine responses. The observed cytokine responses to specific or non-specific antigens showed a clear Th1 /Th2 polarization. While Th2 cytokines responded primarily to specific helminth antigens AWA and SEA, Th1 cytokines INFγ and TNFα were most responsive to non-specific BCG.

In summary, the data presented in this study indicate that human Schistosomiasis is associated with the expansion and increased activity of $CD4^{+}CD25^{high}$ $Foxp3^{+}$ Treg cells, suppressing both Th1 and Th2 cell proliferation and cytokine release-in particular during the chronic stage of infection. This may contribute to a long-term down-modulation of the host's immune response while infection is in place. Unclear remains the role of T-reg cells in the immediate phase post-treatment as well as the involvement of certain cytokines (IL13, IL17) re-establishing homeostatis. Besides the need for some long-term observations of T-reg cell and T-effector cell levels, further studies should be directed at identifying the mechanisms of T-reg cell induction and expansion as well as the precise role of specific cytokines, such as IL13 and IL17, post-infection.

8 Zusammenfassung *(deutsch)*

Schistosomiasis ist eine parasitäre Erkrankung, die vorwiegend in den Sub-Sahara Staaten Afrikas auftritt. Jährlich sind geschätzte 230 Millionen Menschen von der Krankheit betroffen, wobei Kinder die Hauptrisikogruppe darstellen (9),(10),(11). S. *haematobium,* eine der 5 Subspezies, ist die einzige Art, welche die Blase und ableitende Harnwege angreift. Tropisches Klima und oft mangelnder Zugang zu fließend Wasser begünstigen das weit verbreitete Vorkommen von S. *haemtobium* in Gabun. Aktuell erfolgt eine effektive Therapie durch das Medikament Praziquantel. Doch rasch auftretende Neuinfektionen kompromittieren die Langzeiteffektivität der Therapie, so dass eine lebenslange Immunität mittels eines effektiven Impfstoffs wünschenswert wäre.

Verschiedene Infektionsstudien zeigten eine deutliche Korrelation zwischen T-reg Zellen und anhaltendem Krankheitsverlauf (38),(64),(84),(85). Dies führte zu der Annahme, dass T-reg Zellen eine Schlüsselrolle im Immunprozess spielen könnten, sobald sich der Parasit im Wirt eingenistet hat.

Das Ziel dieser Studie war es, die Rolle von T-reg Zellen in S. *haematobium* infizierten Kindern in einem Hochprävalenzgebiet näher zu erforschen. Hierfür wurden jeweils vor und nach T-reg-Depletion Ag-spezifische und -unspezifische Zellproliferationsraten sowie verschiedene Th1 und Th2 Zytokin-Antworten gemessen und miteinander verglichen. Des Weiteren wurde ein Vergleich der Messungen vor und 6 Wochen nach der Therapie mit Praziquantel durchgeführt.

T-reg Zellen wurden zunächst mittels des Oberflächenmarker $CD4^+$ $CD25^{high}$ und des Transkriptionsfaktors $Foxp3^+$ identifiziert. Während sich S. *haematobium* positive Kindern deutlich mehr T-reg Zellen im Blut hatten als S. *haemtobium* negativen Kinder, bestand bei den gleichen Kindern vor- und 6 Wochen nach der Behandlung nur ein geringer Unterschied. Mögliche Erklärungen hierfür könnten zum einen aktivierte T-Effektorzellen mit vermehrter Expression von CD25 und dadurch nicht unterscheidbar von $CD25^{high}$ T-reg Zellen oder zum anderen eine erst später auftretende Abnahme von T-reg Zellen (128) sein.

Bestätigt wird diese Annahme durch einen signifikanten Anstieg von T-Effektor Zellen nach der Therapie, der sich in einem deutlichen Schub der Th2 Zytokine IL5 und IL13 auf die spezifischen Antigene AWA und SEA und des Th1 Zytokins IFNγ auf nicht-spezifisches Antigen BCG wiedergespiegelte. Ein Anstieg der Zell Proliferation konnte zu diesem Zeitpunkt nicht festgemacht werden, was möglicherweise auf den frühen Messzeitpunkt oder ggf. auf mangelnde Sensitivität der verwendeten Materialien zurückzuführen sein könnte.

Im Allgemeinen waren die Depletionseffekte von T-reg Zellen vor der Behandlung deutlich ausgeprägter als 6 Wochen danach. Abweichungen hiervon zeigten sich bei den Zytokinen IL3 und IL17 und könnten möglicherweise auf den frühen Messzeitpunkt nach der Therapie zurückzuführen sein. Bezüglich der Antigen Reaktionen war eine deutliche Th1 / Th2 Polarisierung zu erkennen. Nach Depletion der T-reg Zellen lösten die spezifische Antigene AWA und SEA v.a. eine Stimulation der Th2-Zytokine IL5 und IL13 aus, während Th1-Zytokine INFγg und TNFα primär auf nicht -spezifisches Antigen BCG reagierten.

Zusammengefasst deuten oben genannte Ergebnisse darauf hin, dass Schistosomiasis mit der Expansion und vermehrter Aktivität von $CD4^{+}CD25^{high}$ $Foxp3^{+}$ T-reg Zellen einhergeht. Während einer chronischen Infektion mit Schistosomiasis werden dabei sowohl Th1- als auch Th2-Zellproliferation mit ihren entsprechenden Zytokin-Antworten unterdrückt. Solange die Infektion aktiv ist, könnte dies langfristig zur Herunterregulierung der Immunantwort des Wirts führen. Unklar bleibt die Rolle von T-reg Zellen sowie von vereinzelten Zytokinen (IL13, IL17) in der unmittelbaren Phase nach der Therapie in der Wiederherstellung eines immunologischen Gleichgewichts. In weiteren Studien sollten sowohl T-reg Zellen sowie auch T-Effektorzellen postinfektiöse längerfristig beobachtet werden. Des Weiteren sollten sich zukünftige Fragen mit den Mechanismen der T-reg Zellen Induktion und Expansion sowie der präzisen Rolle vereinzelter Zytokine postinfektiös, wie z.B. von IL13 und IL17, beschäftigen.

9 Appendices

9.1 TRANCHI - Gabon

Project full title: T cell regulation and the control of parasitic infections

Specific sub project: Regulatory T cells in Schistosome infections

Project summary

Schistosoma haematobium infections are continuing to be a major problem in foci around Lambarene. Pharmacological treatments are compromised by rapid re-infection and variable compliance. Immunological intervention by vaccination has not yet succeeded in evoking strong resistance. The critical barrier in control of *S. haematobium* infection remains the failure of the immune system to clear parasites despite antigen recognition and stimulation of immune cells. By addressing the fundamental question of why the immune system is restrained from killing parasites, we hope to offer a novel route for intervention to achieve an immunological cure.

Recent developments in immunology now offer a conceptual framework to understand the failure of immunity and how medical science may intervene to restore the full potential of our defense mechanisms. In this new model, parasites induce “regulatory T cell” populations which suppress effector cells and dampen anti-parasite activity, as part of the parasites’ own strategy for survival in the human host. Because the regulatory T cells are stimulated by parasite antigens, repeated infection or vaccination will induce a measureable immune response in terms of antibodies and cytokines, but fall short at the critical effector cell level required for parasite killing. Only by inactivating or removing the regulatory T cells can the immune response display its full capability to eliminate the parasites.

The project is therefore of major significance to provide specific pathways to novel treatments of human schistosomiasis by ablating parasite-specific regulatory T cells in schistosomiasis patients.

Overall Objective
To determine the role of regulatory T cells in schistosome infections in Lambarene region.

Specific Objectives
1. determine the frequency of regulatory T cells in schistosome infected subjects before and one month after treatment with praziquantel
2. determine the degree of regulatory T cell activity in schistosome infected subjects before and six weeks after treatment with Praziquantel

Study Population
S. haematobium infected subjects (20) at the age range from 5 to 16 yrs old will be identified and recruited into the study from ongoing population surveys. These subjects will be asked to donate blood (5 mls) and will be given treatment (PZQ). One month following treatment they will be visited and blood (8 mls) will be examined again.

Immunological studies
The heparin anti coagulated blood will be assessed for differential cell counts and hematological parameters using 100 ul in haematology machine. The blood will be separated into PBMC and 2 million cells will be fixed and frozen for cytometric analaysis of the regulatory T cells. The rest of the PBMC will be divided into two, one total PBMC and another will be passed over an anti-CD25 column to remove CD25+ cells. The protocol will use suboptimal concentrations of the antibodies to ensure that only CD25++ are removed. These cells along with the total PBMC will be cultured with medium, schistosome soluble egg and adult worm antigen from and depending on cell numbers with BCG, PHA and TLR ligands (TLR2 and TLR4).
Proliferation: the proliferation assay will use CSFE and BRDU to assess what the effect of removing CD25++ is on schistosome specific responses.
Cytokine: the levels of IFN-g, TGF-b, IL-1b, IL-5, IL-6, IL-13, IL-10, IL-17, IL-21, IL-23, IL-32 and TNF-a will be assessed and the effect of removing CD25++ will be assessed.
FACS: the expression of CD4, CD25, Foxp3, CTLA-4, CD127 (if possible surfact TGFb) will be assessed as surface markers to identify regulatory T cells. The intracellular cytokine expression will be assessed using antibodies to IFN-g, IL-4, IL-10 and TGF-b.

9.2 Consent form / Consentement écrit éclaire

Titre du protocole:

Étude de la réponse immunitaire des cellules T régulatrices (T-reg) chez les enfants infectés par le paludisme (compliqué et non compliqué) ou/et la bilharzie au Gabon

Consentement éclairé:

Chaque participant ou parent légal doit absolument recevoir un exemplaire du consentement éclairé.

Numéro du participant : **Initiales du participant :**

Nous venons demander l'autorisation pour que vous (votre enfant) participiez à une étude de recherche immunologique. L'objectif de ce formulaire est de vous expliquer cette étude et de recueillir votre consentement (accord) pour que votre enfant participe à cette étude

Objectif/bénéfices de l`étude

Madame, (monsieur) nous invitons votre enfant à participer à cette étude dont le but est de déterminer la réponse immunitaire des cellules T régulatrices (T-reg) chez les enfants infectés par le paludisme (compliqué et non compliqué) ou/et la bilharzie au Gabon. En effet les infections parasitaires telles que le paludisme, et/ou les schistosome entraînent des modifications immunitaires énormes chez les enfants affectés comparativement aux enfants non affectés. En effet les études ont déjà montré une modification des réponses immunitaires lors des infections parasitaires aigues ou chroniques. Toutefois le mécanisme de médiation de ces réactions immunitaires n'est pas encore total connu. Ainsi la compréhension de ce mécanisme pourra contribuer à l'amélioration de leur prise en charge et surtout á la mise au point de vaccins appropriés. Pour atteindre ce but la participation de votre enfant à cette étude est très importante.

Méthode

Si vous acceptez la participation de votre enfant à cette étude, le médecin de l'étude vous posera certaines questions, relatives à sa santé, puis il procédera à un bref examen clinique de son état général plus particulièrement de l'état de, suivi de prise de sang dont la quantité dépendra de l'âge.

- Pour les essais immunologiques, 5 à 10 mL de sang chez les jeunes enfants de six (6) mois à quatorze (14) ans et 72 mL de sang chez les adultes seront prélevés dans le tube á héparine avant le traitement et après la guérison qui interviendra dans les deux mois avenir.

- Pour la réalisation de l'hémogramme, la goutte épaisse et la recherche du paludisme et des filarioses (parasites du paludisme et des filaires) et ultérieurement pour les tests de génotypage, 1mL de sang dans le tube à EDTA sera prélevé en même temps que le précédent. Outre ces prélèvements de sang, il vous sera demandé d'une part des urines pour la recherche des schistosomes, et d'autre part des selles pour la recherche des vers intestinaux.

Les bénéfices et avantages

Votre participation à cette étude, vous garantit le traitement gratuit des affections cliniques et biologiques que le médecin aura retrouvées. Aussi les résultats de l'étude peuvent apporter des informations nouvelles sur les mécanismes de défense de votre organisme.

Risques et désagréments

Les prise de sang constituent une contrainte, et peuvent entraîner des douleurs et voir parfois des hématomes. Aussi certaines sensations de vertiges ou de faiblesses peuvent survenir.

Toute fois, vous êtes libre d`interrompre votre participation à l`étude sans aucune conséquence négative pour votre enfant.

Confidentialité

Tous les documents de l`étude seront disponibles à l'unité de recherches médicale de l'Hôpital Albert Schweitzer, mais de façon anonyme. Un numéro sera utilisé pour identifier votre enfant. Les noms des participants ne figureront nulle part sur les documents tels que les publications ou les rapports découlant de l`étude. L´accès direct à votre dossier médical ou celui de votre enfant est possible à l'Unité de Recherches Médicale de Lambaréné, ainsi qu'aux autorités administratives.

Vos droits et ceux de votre enfant

Si pendant l`étude, vous avez des questions concernant la nature de l`étude, ou si vous pensez que vous ou votre enfant aviez souffert physiquement de l`étude, contactez le médecin de garde de l'unité de recherches. Si vous aviez des questions concernant vos droits et ceux de votre enfant dans le cadre de l`étude, vous pouvez contacter le médecin responsable de l'Unité de Recherches Médicale de l`hôpital Albert Schweitzer, Lambaréné en la personne du **Dr Saadou Issifou au 58 10 99**

Liberté de participation

Votre décision ou celle permettant à votre enfant de participer à l`étude est totalement libre et un refus de votre part est sans conséquence. De plus, vous pouvez à tout moment décider de quitter l`étude sans préjudice.

Consentement écrit

J'ai soigneusement lu les informations contenues dans le document qui m'a été remis et je les ai bien compris. On a répondu à toutes mes questions. On m'a donné un exemplaire du formulaire de consentement. J'ai compris que je suis libre à tout moment de me (mon enfant) retirer de l'étude, sans que cela présente pour mon enfant d`inconvénient dans l'avenir. J'accepte ma participation (la participation de l'enfant) à cette étude:

NOM ET NUMERO DU PARTICIPANT

SIGNATURE PARTICIPANT / DATE / HEURE

-

----------/----------/-------- :----------

NOM et SIGNATURE du PARENT si mineur / DATE / HEURE

----------/----------/------- :-----

NOM DU MEDECIN INVESTIGATEUR

SIGNATURE DU MEDECIN INVESTIGATEUR / DATE / HEURE

Consentement oral avec témoin

J'ai expliqué en détail les modalités de l'étude au participant (parent de l`enfant) et il/elle a compris le contenu. Le médecin a répondu à toutes les questions du participant (parent de l'enfant). Le médecin a donné au participant (parent de l'enfant) un exemplaire du formulaire de consentement. Le participant (parent de l'enfant) a compris qu'à tout moment, il/elle (son enfant) peut se retirer de l'étude sans que cela ne présente pour lui/elle ou l`enfant aucun inconvénient dans l'avenir.

NOM ET NUMERO du PARTICIPANT

.----------

NOM DU TEMOIN

SIGNATURE DU TEMOIN DATE / HEURE

----------/----------/-------

NOM DU MEDECIN INVESTIGATEUR

SIGNATURE DU MEDECIN INVESTIGATEUR / DATE / HEURE

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9.3 Ethics Approval

Comité d'Ethique Régional Indépendant de Lambaréné

CERIL

Président:
Dr. Bintou Kouyaté Amara
B.P. 216
Lambaréné, Gabon
Tél: (00241) [illegible]
comite_ethique.ceril@yahoo.fr

ATTESTATION D'AVIS FAVORABLE

Date de réunion: 07-02-2008
N° du protocole (CERIL):
Numéro de l'étude (Investigateur) :
Nombre de membres présents : 02
Numéro d'amendement:
Promoteur de l'étude: **Université de Leiden, Pays-Bas**
Titre de l'étude : **Innate Immune Responses and Immune regulation in Schistosomiasis: Mechanisms in the control of infection and disease (TRANCHI)**

Je soussignée, Dr Bintou KOUYATE, Présidente du **CERIL,** atteste que le protocole de recherche sus nommé, a reçu un avis favorable après délibération à la majorité des membres du **CERIL**. Les documents suivants ont été examinés et approuvés :

Protocole de la recherche proposée
Résumé en français du protocole de recherche/synopsis
Note d'information aux participants à l'étude
Formulaire de consentement éclairé

Date 07-02-2008 Signature

Comité d'Ethique Régional Indépendant de Lambaréné
CERIL
GABON

9.4 Questionnaire

Date (baseline)	**Date (follow up)**	
Name	**Initials**	**Patient No**
Gender	**Village**	
Age	**Height**	
Physical exam, comment (including pain, swollen lymphnodes, nausea):	**Medical Hx (anti - helminth within the last 6 months):**	
CLINICAL DATA at Baseline:	**Follow-up:**	
Weight	**Weight**	
Body Temperature	**Body Temperature**	
Blood Pressure	**Blood Pressure**	
Heart Rate	**Heart Rate**	
Schisto egg count	**Schisto egg count**	
Malaria	**Malaria**	
Filariasis	**Filariasis**	
LAB DATA at Baseline:	**Follow-up:**	
Hb	**Hb**	
HCT	**HCT**	
WBC	**WBC**	
Lymphozyten	**Lymphozyten**	
Neutrophile	**Neutrophile**	
Monozyten	**Monozyten**	
Basophile	**Basophile**	
Eosinophile	**Eosinophile**	
Platelets	**Platelets**	

9.5 T-reg (Tranchi) Study protocol

Updated 19.07.07 by Yvonne Schmiedel

FINAL…… Protocol 'malaria' and 'schisto' study.

For both studies the procedures are the same, only the stimulations are different.

Solutions:

Penicillin:
Dry stock of penicillin is stored by room temperature. Dilute the powder stock in 5 ml of distilled water. Store the diluted penicillin stock by 4 C.
(Stock of dry penicillin vials + syringe for drawing up are in cupboard under the sink)

Streptomycin: (also an antibiotic)
1 gram in 5 ml distilled water. Filter sterilized! Only 250 ul per medium flask, so fill out and store by -20 C (-20 C in Judith's lab).

L-Glutamin & Pyruvate: (amino acids)
1,46 gram glutamin and 0,55 gram Pyruvate diluted in 50 ml distilled water. Dissolve by putting in 37 C water bad, and filter sterilize. Only 5 ml needed per medium flask, fill out 5 ml and store by -20 C-filled up in 15ml tubes with blue lid (in Judith's lab).

*HBSS: (wash medium *Keep sterile**
Use the bottles with HBSS in the fridge. When using it between one week, you don't have to add antibiotics. After a week add antibiotics, because of perhaps contamination. Then add 250 ul penicillin per 500 ml media (2000 times diluted).

*RPMI: (culture medium) *Keep sterile**
Use the bottles RPMI in the fridge. When opening add 250 ul of penicillin, 250 ul of streptomycin, 5 ml of glutamin/pyruvate.

*EDTA: (0,5 M) *Already sterilized**
Stock is in fridge, in total 4 x 50 ml tubes.
(How it was made, weight of 3,7 grams of EDTA. Dilute this 20 ml distilled water, this is the 5 M stock. This stock have to be diluted 1:10 in distilled water of PBS, to get a 0,5 M stock. Then after filter sterilization store in fridge 4C).

*6,25% BSA: *Already sterilized**
Stock in fridge, in total 4 x 50 ml tubes.

(How it was made, weight of 3,125 grams of BSA (dry stock also in fridge-2 white bottles), dilute this in 50 ml room temperature PBS otherwise it does not dissolve. Don't shake-let dissolve slowly (After that filter sterilize and store in the fridge).

*MACS buffer: (for Macs isolation) *Keep sterile**
DURING PROCEDURE BUFFER NEEDS TO BE COLD, SO LEAVE ON ICE!
* 0,5 % BSA (so add 40 ml of 6,25% stock)
* 2 ml of 0,5 M EDTA stock (final concentration = 2mM)
* 500 ml PBS

Turk stain: (colours nucleus cells)
* 10 mg gentian violet
* 500 ul acetic acid
* in 50 ml distilled water

Trypan blue: (colours the death cells)
0,20 gram trypan blue in 50 PBS.

*Ficoll: (separation of the PBMC) *Keep sterile**
Store at room temperature (in silver suitcase, storeroom). .After opening store at 4 C.

2% Formaldehyde 'PFA' (for fixation):
37% flask is in cupboard under the sink, stored by room temperature. To get a 2% dilute 1:20 times in cold PBS(don't need to be sterile). During procedure keep cold on ice.

20%FCS / 20%DMSO / 60%RPMI (freezing medium):
DMSO (dimethyl sulphoxide) is stored by room temperature in cupboard under the sink. For 50 ml tube, add 10 ml of FCS then 30 ml of RPMI and add 10 ml DMSO at last (don't pipette this to the side of the tube because it reacts with plastic). Put solution afterward soon on ice because DMSO makes the solution warm.

Stimulations:

Medium: Just RPMI without FCS.

iRBC (red blood cells infected with malaria) (round bottom)**:** Shipped from Leiden, and stored at -80C. Per vial there's 20 ul antigen, you have to add 200 ul medium. And then you have enough for 2 wells to stimulate. Dilute iRBC just before use in RPMI, so before leave vials on plenty of ice. When defrosted the red blood cells lysate, so this doesn't have to take to long before culturing (same for uRBC).

uRBC (control red blood cells) (star bottom)**:** Same as RBC, stored at -80C and per vial add 200 ul medium, then also enough for 2 wells.

There are 4 bags with RBC's in the -80C:
Bag 1) uRBC cells batch 1
Bag 2) uRBC cells batch 2
Bag 3) iRBC cells batch 1
Bag 4) iRBC cells batch 2

PHA: Powder stock was already diluted by Yvonne in 5 ml sterile PBS. Now concentration is 2 mg/5ml = 4 mg/10ml. For study we need 4ug/ml, so dilute stock 1000x in RPMI. This can be done by dilute 25 ul of the stock in 25 ml RPMI. Then fill out 1,5 per cryovial with screw on lid. Store the 4 ug/ml stocks in -80C box with all stimuli's. After adding PHA stimuli added to the wells 1:1, concentration of PHA is 2 ug/ml.

AWA (adult worm antigen Hematobium): Stock we brought from Leiden was 1200 ug/ml. For study we need 20 ug/ml, so it have to be diluted 60 times. We made 20 ml, 0,33 ml (=330 ul) stock + 19,67 ml RPMI. This is also filled out and stored by -80C. After adding AWA to the wells the concentration is 10 ug/ml.

SEA (schisto egg antigen): Stock brought from Leiden is 309 ug/ml, for the study we need a concentration of 20 ug/ml so dilute 15 times. We made 15 ml, that's 1 ml of the stock with 14 ml RPMI. Also filled out and stored by -80C. After adding SEA to the wells the concentration is 10 ug/ml.

BCG (Baccillus Calmette Guerin, TBC antigen): Powder stock is diluted in 1 ml sterile water (vials in fridge). Concentration is 0,5 mg/ml. For study we need 20 ug/ml so 25 times diluted. One vial of 1 ml 0,5 mg/ml is diluted in 25 ml RPMI. Filled out and stored by -80C. After adding BCG to the wells concentration is 10 ug/ml. (there are still 2 powder stocks in the -20C on Judith's lab)

Procedure:

DAY 1:

Make sure that the media (HBSS) and the ficoll are at room temperature.

→ Fill all the information of the patients in on the forms.

Start with the PBMC isolation:

- Put all collected blood in a sterile 50 ml tube (not more then 20 ml).
- Dilute 1:1 with HBSS, mix well but gently.
- Add carefully 13 ml ficoll under the blood/HBSS-suspension

(let the Ficoll flow out off the pipette by itself, until the level of blood in the tube is reached, put your finger on top of the pipette and point the pipette tip towards the Ficoll-blood separation level and let some more Ficoll flow (very slowly), put your finger on top of the pipette again and take the pipette out of the tube, about 2.5ml Ficoll will be left in the pipette)

- spin 25 minutes at 1500 rpm, room temp (15C), acceleration 6, **no brake**
- Put 11 ml of plasma in a 15 ml tube. Discard the rest of the plasma carefully with pipette until ± 1cm above the white ring.
- Keep the 15ml tube (with patient no, study name, 1:1 Plasma and date), don't need to be sterile, and store at-80°C.
- Take the white ring carefully with a 3ml plastic-Pasteur pipette and put it into a new sterile 50ml tube
- Add HBSS until 40ml
- Spin 15 minutes at 1500 rpm, brake 6, acceleration 6, 15C.
- Pour off the fluid and re-suspend the pellet in 10ml PBS.
- Spin 15 minutes at 1500 rpm, brake 6, acceleration 6.
- Re-suspend the pellet in 1ml MACS buffer → usually there's still 200 ul in the tube, so **add 800 ul**.
- Bring all: 1 ml over to a 5 ml falcon tube.
- Take a 20 ul counting sample.
- Wash rest of the cells with 2 ml of MACS buffer (so add still 1ml) and spin down for 5 minutes at 1470 rpm (=300 *g), brake 6, acceleration 6, Temp. 15C. (new lab centrifuge/ this adjustments will remain the same for the rest of the procedure).
- During spinning count the cells with a counting-chamber (180 µl Türk-stain + 20 µl couting sample/ cell suspension).
- Count the amount of cells in 25 big squares, divide that number by 10 (dilution factor türk stain) gives you the number of …. x 10^6 cells/ml.
- If you have more than 10 million cells/ml, then separate cells over 3 falcon tubes or while FACS is to be carried out in Leiden, 2 Falcon and 1 Eppendorf tube). When less, don't leave cells for the FACS, so separate cells only over 2 tubes (whole and depleted PBMC). So after spinning, decant supernatant and dry on tissue and resuspend pellet with 870 ul (for 3 tubes) or 770 ul (for two tubes) of MACS buffer.
- Now separate cells in 3 portions (300 ul/tube) or 2 portions (400ul/tube). Put cells for MACS and for whole PBMC in a falcon tube and cells for the FACS in a 1,8 ml eppendorf vial.
- Wash/ Centrifugatie cells for the MACS with 2 ml of MACS buffer, leave whole PBMC on ice and process Eppendorf tube with FACS according to day 4 procedure (see below).
- After washing, resuspend cells, dry on tissue and take pellet up in 90 ul MACS buffer → if dried on a tissue, then there's 30 ul left in the tube, so only **add 60 ul** of MACS buffer.

Continue with the MACS separation:

- Add 10 µl of CD25 Micro-beads (10 µl/maximum10^7 cells).
- Mix well and incubate for 15 minutes at 4-8 degrees.
- Wash cells with 2 ml with MACS buffer and centrifuge at 1470 rpm for 5 minutes.
- Prepare everything for the MACS. Place column in the magnetic separator. And prepare column by rinsing with 500 µl buffer.
- Resuspend up to 10^8 cells in 500 µl of buffer.
- Apply cell suspension onto the column.
- Collect unlabeled cells (these are the depleted PBMC) that pass through and wash column three times with 500 µl buffer. Only add new buffer when the column reservoir is empty.
- Remove column from separator and place on suitable collection tube (falcon tube)- these are CD25++ cells.
- Pipette 1 ml of buffer onto the column and immediately flush out by using the plunger (these are the CD25 ++ cells).

Procedure for the CD25 ++ cells:

- Wash cells with cold PBS. Spin cells down. Dry pellet out on tissue till there's no liquid there anymore.
- Take cell-pellet up in 100 ul lysis buffer, resuspend and put all of it over into a 1,5 ml eppendorf. Store cells, make sure of name and at -80 C. Keep this for RNA isolation.

For the other cells, go further with the CFSE labelling:

- Wash the whole PBMC and the depleted PBMC cells twice with 2-4 ml PBS. Centrifuge 5 minutes by 1470 rpm and decant supernatant.
- Resuspend cells at 2 x 10^7 cells/ml in PBS. (Example: when you have 6 x 10^6 cells/ml and you want 2 x 10^7 cells/ml (same as divide 6 by 20) = 0,305 ml, so resuspend cells in 305 µl PBS to get the concentration).
- Make the 50 µM CFSE stock. Stock in freezer is 5 mM, so make a 100x dilution in PBS (10 µl CFSE in 1 ml PBS). Now you have a 50 µM stock. Then from 50 µM stock → 2 µM is 25x diluted.
- To get a 2 µM CFSE final concentration in your sample, you have to dilute 25 times. That's why you have to divide the amount of cells by 25.
  (Example: Your cell sample is 305 µl from above. Then 305/25 = 12,2 µl 2µM CFSE-stock)
- Add the amount to your cells, mix but do NOT vortex.
- Incubate sample for 15 minutes (exactly) at room temperature in dark. Mix now and then.

- Make a 10% FCS/RPMI solution.
- After 15 minutes add 4 ml of 10% FCS/RPMI (that blocks the CFSE action), incubate 1 minute (time to walk to the centrifuge) and spin down for 5 minutes 1470 rpm.
- Decant supernatant and wash again with 2 ml 10% FCS/RPMI.
- Make a 20% FCS/RPMI solution.
- After spinning, take pellet up in total 1 ml 20% FCS/RPMI medium. Take again a 20 µl counting sample and count with 180 µl türk stain.
- Wash the rest of the cells again with 2 ml medium (so add 1 ml to the tubes, total 2 ml) 20% FCS/RPMI.

Go further with the cytokine assay:

- Take the whole PBMC and depleted PBMC cells up to 4 * 10^6 cells/ml (is number of million cells divided by 4) in 20% FCS/RPMI. Because when you add in wells, you get now 400.000 cells/well.

There are 2 different protocols for the stimuli used for the 'malaria' and the 'schisto' study.

Malaria: Stimulate with medium, uRBC, iRBC, PHA, AWA and BCG
Schisto: Stimulate with medium, PHA, AWA, SEA and BCG.

For the stimulations, the way they are written above is how important they are for the study. We assume that we have enough cells after all procedures (you need at least for the malaria study 2,4 million cells and for the schisto 2 million cells) otherwise you don't stimulate the cells with BCG (last one in the row) etc.

- Stimulate the whole PBMC fraction as the CD25 depleted fraction. Put first 100 ul of the cells in a round bottom plate. Then add 100 ul of the stimulations.
- Rap plate in with plastic foil, and incubate for 4 days by 37 C.

Go further with the cells for the FACS (don't have to be sterile anymore):
Because FACS doesn't work well here, we bring these cells home.

- Wash the eppendorfs with the cells twice with 1 ml PBS and spin down at 2500 rpm for 5 minutes.
- Make the fixation buffer (2% PFA) and make sure it's cold (so leave on ice).
- After the second wash step, discharge supernatant and dry pellet on tissue.
- Add 500 ul of the 2% PFA to the cells, close eppendorf and vortex for 3 sec.
- Incubate the cells for 15 minutes by room temperature (just leave on bench).
- Spin cells down and dry pellet on tissue.
- Wash cells with 1 ml RPMI (don't need to be sterile) and spin cells down.
- Make freezing medium and store it on ice.

- Discharge supernatant, dry on tissue - assuming there is still pellet + 100 ul RPMI, add further 400 ul RPMI.
- Put total of 500 ul over to a 1,8 ml cryovial (write number patients etc on the vials with pencil because marker fades out by the alcohol). Add 500 ul of the freezing media, close vials, vortex 3 sec and quick put the cells on ice.
- When finished get the freezing boxes out of the fridge (filled with 96% Ethanol and cold). Put cells into the box and put box in -80 C freezer o/n.
- Next day get a plastic bag and put in the eppendorf tubes for longer storage also in the -80 C. Put the freezing box again in the fridge. After 5 times using the box, replace the 96% Ethanol for fresh on.

DAY 4:

Go further with the stimulated cells:

- Begin with writing tubes for the supernatant of the cells (Use RED for the whole PBMC cells and Green for the depleted PBMC cells). It's better do this equally.
- Get cells out of the incubator and place on bench, it doesn't have to be sterile anymore.
- Make sure there's enough fixation buffer and freezing media and put on ice.
- Take of 180 ul of the supernatant without getting the pellet of cells. Put the supernatant in small bags, labelled for each patient one bag and store in bigger bag in -80 C.
- Add 180 ul PBS to the wells with cells and resuspend the cells. Bring al the cells over to different 1,5 eppendorf tubes.
- Wash cells with 1 ml PBS and spin down for 5 minutes at 2500 rpm (small centrifuge). Pour of supernatant and dry on tissue, resuspend by vortex the tubes. There's still about 100 ul of liquid still in the eppendorf tube.
- Add 500 ul of fixation buffer, close lid and vortex for 1 sec.
- Incubate for 15 min by room temperature.
- Spin cells down and dry on tissue, resuspend-vortex for 1s.
- Wash cells with 1 ml RPMI and dry pellet on tissue, resuspend-vortex 1s. Now there's still like 100 ul left in the eppendorf.
- Add 400 ul of RPMI to the vials, bring all 500 ul to 1,8 ml cryovials (cryovials have to be written on with a pencil, name patient, stimuli, whole or depleted and date).

Setup for the vials for the malaria study (x is patient number):
x.1 = whole PBMC simulated with MED.
x.2 = whole PBMC stimulated with uRBC.
x.3 = whole PBMC simulated with iRBC.
x.4 = whole PBMC stimulated with AWA.
x.5 = whole PBMC stimulated with BCG.
x.6 = whole PBMC stimulated with PHA.

x.7 = depleted PBMC simulated with MED.
x.8 = depleted PBMC stimulated with uRBC.
x.9 = depleted PBMC simulated with iRBC.
x.10 = depleted PBMC stimulated with AWA.
x.11 = depleted PBMC stimulated with BCG.
x.12 = depleted PBMC stimulated with PHA.

Setup for the vials for the schisto study (x is patient number):
x.1 = whole PBMC simulated with MED.
x.2 = whole PBMC stimulated with AWA.
x.3 = whole PBMC simulated with SEA.
x.4 = whole PBMC stimulated with BCG.
x.5 = whole PBMC stimulated with PHA.
x.6 = depleted PBMC simulated with MED.
x.7 = depleted PBMC stimulated with AWA.
x.8 = depleted PBMC simulated with SEA.
x.9 = depleted PBMC stimulated with BCG.
x.10 = depleted PBMC stimulated with PHA.

- Add 500 ul freezing media to the cells, close lid, vortex for 3 sec.
- And put vials immediately on ice.
- Get freezing box out of the fridge and store cells overnight by -80 C.
- Next day place cells into bags that are labelled (each patient in one little bag, and all bags again in a bigger bag) and store again for longer time by -80 C. Put freezing box back in de fridge.

For shipment to Leiden:

You have to order dry ice.
When the follow-up study of the children is finished, send everything to Leiden.
List of things:

- Plasma of the patients (per patient 15 ml tube) **BAG 1**
- CD25 pellet in lysisbuffer of the patients (small 1,5 eppendorf vials) **BAG 2**
- Supernatants of the patient (small bags with vials) **BAG 4**
- FACS cells fixated (each patient one cryotube) **BAG 3**
- Stimulation cells fixated (10-12 cryotubes per patient) **BAG 5**

9.6 Auxiliary study protocol

Followed the same protocol as the main study including Foxp3 staining:

Fixing cells by eBioscience fixation (FOXP3 staining)

- After PBMC isolation, transfer cells to 5 ml Falcon tube
  Fix at least 0.5 million, maximally 2 million cells (see remarks)
- Wash cells with 2 ml (room temp) PBS; 5 min 1600 rpm 20°C
- Make the fixation buffer (eBioscience): *1 part fixation/permeabilisation* ***concentrate*** *plus 3 parts fixation/permeabilisation* ***diluent***
- Discard supernatant and dry cell pellet on tissue
- Add 250 µl of diluted eBioscience fixation-buffer while gently pipetting up and down
- incubate **60** min in the dark, at rT or at 4°C
- Add 2 ml PBS and spin at 1600 rpm for 6 min, rT
- Flick off supernatant and repeat washing with 1 ml RPMI-10% FCS, spin at 1600 rpm for 5 min, rT

9.7 Luminex assay for cytokine measurement

Preparing beads

Vortex the tubes with the beads for 30 seconds and sonicate them for 20 seconds before use.
For 1 plate (100 wells): mix 60 µl of each bead solution and add PBS-Tween until the total volume is 2500 µl. (If your plate is not full, recalculate these volumes for the appropriate number of wells)

Preparing the standard curve

Resuspend the lyophilized standard(s) in assay diluent (red cap) (end volume: 1 ml, independent on the number of lyophilized standards used)
Aliquot in PCR tubes (70 µl/tube) and store at –20°C. One tube is needed for each plate.

Make assay diluent/RPMI (800 µl needed for 1 plate)
Mix 400 µl assay diluent (red cap) and 400 µl RPMI in an eppendorf tube

Take 10 eppendorf tubes to prepare the standard (2x standard 1 + standard 2-9, standard 10 is the undiluted stock standard in PCR tube). Add 70 µl assay diluent/RPMI to each tube.
Standard 10: resuspended standard (undiluted stock)
Standard 9: add 35 µl from standard 10
Standard 8: add 35 µl from standard 9 etc.
Standard 1 (2x): only 70 µl assay diluent/RPMI (no cytokines)

Assay procedure

- Put 25 µl prepared beads in each well of a round bottom plate
- Add 125 µl PBS-Tween to each well
- Spin plate for 4 minutes, RT, 4000 rpm
- Remove the supernatant (gently!)
- Add 25 µl incubation buffer (transparent cap) to each well
- Centrifuge the samples for 10 minutes, 4000 rpm before use
- Put 25 µl of the samples or standard in each well
- Shake the plate for 2 hours at RT/dark
- Add 100 µl PBS-Tween to each well
- Spin plate for 4 minutes, RT, 4000 rpm
- Remove the supernatant (gently!)
- Prepare Biotinylated Antibodies (Biot-Ab) 10 minutes before using. For 1 plate (100 wells): mix 30 µl of each Biot-Ab solution and add Biotin diluent (blue cap) until

the total volume is 2500 µl. (If your plate is not full, recalculate these volumes for the appropriate number of wells)

- Add 25 µl Biot-Ab to each well
- Shake the plate for 1 hour at RT/dark
- Add 125 µl PBS-Tween to each well
- Spin plate for 4 minutes, RT, 4000 rpm
- Remove the supernatant (gently!)
- Prepare SA-PE just before use! For 1 plate, mix 250 µl streptavidin (light sensitive!) and add until 2500 µl SA-PE diluent (green cap)
- Add 25 µl SA-PE to each well
- Shake the plate for 30 minutes at RT/dark
- Add 125 µl PBS-Tween to each well
- Spin plate for 4 minutes, RT, 4000 rpm
- Remove supernatant (gently!)
- Add 80 µl PBS-Tween to each well (at this point, the plate can be stored O/N at 4^{o}C/dark)
- Shake plate for 2 minutes at RT/dark
- Measure on Luminex

Wash buffer: PBS 0.05% Tween

9.8 Facs Tranchi Protcoll

- Take 20 samples out of the N2, tower 12,draw 11.
- Add 150 ul pure FCS to the half frozen samples.
- Transfer cells to FACS tubes.
- Add 1 ml FACS buffer (3% FCS/DPBS).
- Centrifugate 5' 1600 rpm.
- Take off sup.
- Mix cells.
- Transfer cells to 96-wells V-bottom plate.
- Make 5 calibration controls : transfer from 4 samples 3 ul cells to one well
    - (cal.1, cal2, cal 3, cal 4 and cal.5)
- Add 100 ul FACS buffer.
- Centrifugate 3' 1200 rpm.
- Throw out sup.
- Dry plate on filter paper.
- Mix plate gently.
- Make a mix of labels, per 20 samples:
  23 ul CD4/PE (box 1)

  46 ul CD8/PerCP (box 1)

  115 ul CD25/APC (box 2)

  276 ul FACS buffer.

- Add 20 ul per sample, only to the samples.
- For the calibration wells, add:
    - Cal.1: nothing
    - Cal 2: 1 ul CD3/FITC
    - Cal3: 1 ul CD3/PE
    - Cal4: 1 ul CD4/PerCP
    - Cal5: 1 ul CD3/APC
- Incubate half an hour on ice in the dark.
- Add 150 ul FACS buffer.
- Centr. 3'1200 rpm.
- Throw out sup.
- Mix plate gently.
- Add 100 ul FACS buffer.
- Transfer samples and cal. controls
- Do FACS (template YF (namen labels kloppen niet, even aanpassen als je kan), instr.settings BrdU 4 kleuren 051206.
- Succes!

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11 Accreditations

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12 Curriculum Vitae

Personal information

Name:	Yvonne Schmiedel
Date & Place of Birth:	19.04.1977, Neu-Ulm
Nationality:	German

Pre-and Postgraduate Education

Currently:	
09/2012-on going	*MSc Epidemiology, London School of Hygiene and Tropical Medicine, London, UK*
10/ 2002-04/2009	Medical Training
26/05/2009	Registration as a Medical Doctor in Germany
28/04/2009	Final Medical State Exam
2000-2001	BSc Nursing (1st class), "St. George's Hospital Medical School/ Kingston University", Tooting/ Kingston, London, UK.
1997-2000	Diploma Nursing (Distinction), "St. George's Hospital Medical School/ Kingston University", Tooting/ Kingston, London, UK.

Research

09/2007-12/2007	Scientific co-worker: continuation of the "T-reg project" , "Leiden University Medical Centre" (LUMC), Leiden, Netherlands.
10/2006-09/2007	Scientific co-worker in clinical and experiemental trials including the T-reg project at the "Laboratoire de Recherche du Dr. Albert Schweitzer", Lambaréné, Gabon.

Employment

12/2011-09/2012	Senior House Officer ("Assistenzärztin"), "Medical Research Centre Borstel", Borstel, Germany, Department of Respiratory & Infectious Diseases
09/2010-10/2011	Senior House Officer ("Assistenzärztin"), "Academic Teaching Hospital, Asklepios Wandsbek", Hamburg, Germany. Dept: Gastroenterology

08/2009 - 08/2010	FY 2, “St. Mary’s Hospital”, Newport, Isle of Wight, UK. Dept.: T&O, A&E, ICU
2001-2002	Staff Nurse, Rotation in Acute Medicine (MAU, ICU, A&E), “Central Middlesex Hospital”, London, UK.

Internship

12/ 2008	Gynaecology, Victoria Teaching Hospital“, Banjul, Gambia
10-12/ 2008	Anaesthesiology, Academic Teaching Hospital „Asklepios St. Georg”, Hamburg, Germany
08-09/ 2008	Trauma, Plastic Surgery and Orthopaedics, “Berufs-genossenschaftliches Krankenhaus”, Hamburg Boberg, Germany
06-07/ 2008	General Surgery, “Bethesda Krankenhaus”, Bergedorf , Hamburg, Germany
02-06/ 2008	Internal Medicine, “Spital Zimmerberg”, Horgen, Switzerland

Electives

05-06/2007	Surgery & Gynaecology, “Hopital du Dr. Albert Schweitzer”, Lambaréné, Gabon
08/2006	Radiology, “Röntgenpraxis”, Schäferkampsallee, Hamburg
07/2006	Paediatric Orthopaedics, “Altonaer Kinderkrankenhaus”, Hamburg
09/2005	Neurology, “Unitverstätsklinikum Eppendorf“, Hamburg
07/2005	Accident and Emergency (“ER”), “Swedish Medical Centre”, Seattle, Washington, USA

Volontary Work / Work experience

05/1996- present	Sailing instructor / Co- Skipper, “Hanseatische Yachtschule”, Glücksburg, Germany
05/2002-06/2002	Work experience at the „Hopital de Infectologia“, Guayaquil, Ecuador.
11/1996-06/1997	Voluntary “Care Assistant, “Independent Living Alternatives” (ILA), Edgware, London, UK