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Immune regulation during parasitic infections: from bench to field

Proefschrift

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Chapter 1

General Introduction
Scope & aims of this thesis

Background

Helminth (a Greek word for 'worm') infections are a major – and neglected – public health problem. Chronic helminth infections are thought to induce a regulatory network in the host that prevents their elimination, on the one hand, while protecting the host against the pathological consequences of excessive inflammation, on the other[1]. Importantly, this downregulation is not only directed against helminth antigens, but can also extend to other antigens: so-called bystander suppression. This may have significant consequences for immunity to incoming infections, such as malaria[2], and for responses to vaccines administered to helminth-infected subjects[3]. At the same time, several studies have suggested a protective effect of chronic helminth infections on atopy and allergic diseases[4]. Moreover, there are indications that anti-inflammatory properties of helminths might be beneficial in dampening the excessive inflammation observed in chronic autoimmune and other inflammatory diseases[5]. To be able to translate these epidemiological findings into therapeutic strategies, it is essential to unravel the mechanisms underlying detrimental and beneficial effects of helminths. Since it is thought that immunological features of host parasite interaction during helminth infections could be responsible for these epidemiological findings, we set out to characterize the immune regulatory network associated with helminth infections.

Coevolution of humans and helminths

Currently, helminths affect millions of people worldwide, mostly inhabitants of rural areas in low- and middle-income countries[6]. The different species of helminths are classified into two major groups, nematodes and platyhelminths, or roundworms and flatworms. The nematodes include soil-transmitted helminths (STH), causing intestinal worm infections, and filarial worms, which lead to lymphatic (lymphatic filariasis, LF) or subcutaneous manifestations (onchocerciasis and loiasis). Platyhelminths are further divided into trematodes, such as schistosomes, and cestodes or tapeworms. Helminth-induced mortality is low, compared to other tropical diseases such as malaria, however the chronic presence of worms can have a major impact on health by affecting host nutrition, growth and cognitive development, which can be substantially impaired by chronic helminth infections[7]. In addition, although majority of infections with these parasites do not lead to noticeable immunopathologies, in a subset tissue pathology can cause significant disabilities, for example elephantiasis resulting from LF or liver granulomas formed around schistosome eggs.

Parasitic worm infections are likely to have been with us throughout evolution. In recent history, schistosome eggs were identified in Egyptian mummies that are approximately 3000 years old[8]. In many communities in endemic areas, people of all ages can be infected with helminths without much outward clinical signs of

infection. This has often been interpreted as a peaceful coexistence of worms with their human host. The coevolution of worms and humans has taught us not only that these parasites are endowed with immune evasion mechanisms, but also that helminths, more profoundly than other pathogens, have directly altered the host's genetic composition, as shown by research into the evolution of human interleukin (IL) genes[9]. Parasite richness in an environment has been shown to be correlated with various single nucleotide polymorphisms (SNP) in IL genes, indicating that helminths can act as a selective pressure to shape the immune system. These genetic alterations, while beneficial when humans are parasitized by helminths, might be detrimental when these parasites are eliminated.
Nowadays, parasitic infections have been largely eradicated in affluent countries due to improved sanitation, control of water bodies and housing. Although this can be regarded as a great accomplishment of the 20th century, the question has arisen as to whether the presence of helminth infections might have some beneficial aspects. The hygiene hypothesis was based on Strachan's publication on the negative association between hay fever incidence and the number of – especially older – siblings in a household, which was thought to reflect the burden of infection in childhood[10]. The hypothesis stated that the increase in atopic diseases in high-resource settings might be due to the improved hygiene and the reduction in childhood infections. The hygiene hypothesis has been extended to other diseases that stem from immune dysregulation such as inflammatory bowel disease, rheumatoid arthritis, multiple sclerosis, diabetes mellitus and cardiovascular diseases[11]. Whereas Strachan proposed that respiratory viral infections contribute to the observed protection against hay fever, it has become clear that other infectious agents, such as helminths, which are able to modulate the immune system and establish chronic infections in their human host, may also be associated with less allergies and other inflammatory diseases.

The immune regulatory network

It has been hypothesized that helminths are able to induce an immune regulatory network, which can suppress the host immune system in such a way that the parasite is not expelled and the host tissues are not damaged too extensively[12]. Unresponsiveness in lymphocyte proliferation to helminth antigens was already described in the 1970s for individuals with *Schistosoma mansoni* infection as well as for those with bancroftian filariasis[13,14]. Studies in lymphatic filariasis indicated that an adherent cell population within peripheral blood mononuclear cells (PBMC) could suppress anti-filarial immune responses[15]. Moreover, the T cell compartment was dominated by suppressor T cells, removal of which augmented lymphocyte proliferative responses[16].

At the same time, in the field of autoimmune diseases, it was recognized that diseased individuals were often affected by more than one autoimmune condition[17,18], leading to the idea that suppression of auto-reactive T cells could be mediated by a common mechanism. A landmark publication from Sakaguchi and colleagues showed that depletion of T cells expressing CD25, the α-chain of the IL-2 receptor, led to a range of autoimmune disorders in mice[19], whereupon several studies in humans indicated that $CD4^{+}CD25^{+}$ cells can also exert suppressive activities[20]. Another breakthrough reported by the same group was the discovery that transduction of T cells with the transcription factor Forkhead box protein 3 (Foxp3 for rodents, FOXP3 for humans) could prevent autoimmune gastritis and inflammatory bowel disease in a mouse model[21]. Mutations in the *Foxp3* gene had earlier been identified as the cause for scurfy mice and the human equivalent, immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), both characterized by multiple autoimmune and inflammatory processes[22,23]. TGF-β has been identified as the main cytokine capable of inducing Foxp3 expression in naïve T cells[24]. The subsequent generation of Foxp3-GFP reporter[25] and “depletion of regulatory T cell” (DEREG)[26] mice has enabled an exponential number of studies on the function of these regulatory T cells (Tregs) in autoimmune, allergic, infectious and malignant diseases as well as in transplantion tolerance.

Thus, cells coexpressing FOXP3 and high levels of CD25 were regarded as a novel T cell subset, termed Tregs. Soon thereafter these were called ‘natural’ Tregs, since these cells are thought to originate from the thymus and are naturally present in the periphery to maintain self-tolerance[27]. In addition, several other T cell subsets with regulatory activity have been described, which may or may not express CD25 or FOXP3. These subsets are jointly named adaptive or inducible Tregs, since these cells can be induced through specific antigenic stimulation[28]. For example, T cells in the periphery can be induced to express FOXP3 capable of suppression, T-regulatory-1 (Tr-1) cells secreting IL-10 and transforming growth factor (TGF)-β were demonstrated to have a regulatory role[29] as well as Th3 cells, which were originally identified as TGF-β secreting cells that could mediate oral tolerance to myelin peptides in MS patients[30]. Furthermore, some studies have described CD8-positive Tregs[31] and even $CD4^{-}CD8^{-}$ Tregs[32], however these subsets have not been characterized in much detail.

The mechanisms for Treg-mediated suppression are not fully understood in humans, but research in animals and *in vitro* models has made great progress in the last decade[33,34]. Since CD25 was the first surface molecule implicated in cells involved in self-tolerance[19], high consumption of IL-2 by Tregs (“cytokine sink”) was suggested as a way of depriving other T cells from this critical factor and thereby leading to apoptosis[35]. A well-characterized cell contact-dependent mechanism of suppression by Tregs is through the expression of inhibitory

molecules. CTLA-4 is the suppressory equivalent of CD28 expressed on T cells and is involved in costimulatory interactions with CD86 and CD80[36]. Importantly, agonistic CTLA-4 antibodies are now one of the options that can be used for therapeutic intervention to inhibit excessive T cell activation in rheumatoid arthritis[37]. Next to cell-contact mediated suppression, Tregs can also exert their regulatory functions by secreting immune modulatory molecules. IL-10 and TGF-β are the best-known examples of suppressory cytokines. Another cytokine lately added to this spectrum is IL-35, which was shown to contribute to the human regulatory network and possibly infectious tolerance[38]. Moreover, the expression of tumor necrosis factor (TNF) receptor II (TNFRII) has been observed in rheumatoid arthritis and malaria infections, with possible mode of actions being enhancement of Treg activity in TNF-rich environments and neutralization of TNF[39,40]. Recent work has further identified CD39 and CD73 co-expression on Tregs as part of their suppressory activity[41]. These ectoenzymes generate adenosine, which inhibits T cell proliferation and activation via the A2 adenosine receptor (A2AR) pathway.

Characterization of Tregs during helminth infections

Experiments in murine models have paved the way for studies that investigate the contribution of Tregs in human helminth infections. Although murine models, which provide homogenous genetic and environmental background, are expected to provide robust studies of infections, the results obtained have not always been clear-cut. For example regarding the role of IL-10 in murine schistosomiasis (*S. mansoni*), $CD4^{+}CD25^{+}$ cells expressing Foxp3 were able to inhibit Th1 cell proliferation through IL-10[42]. In another study IL-10 from Tregs was shown to be more important for host survival than IL-10 produced by other cell subsets[43]. However, other studies using the same models have shown that IL-10 is not essential for Treg-modulated suppression of Th1 or Th2 responses[44,45]. These discrepancies illustrate the degree of the complexity of the so-called “regulatory network” and the possible relation to parasites.
The group of Rick Maizels has contributed much to unraveling the cellular mechanisms of the immune regulatory network in murine models of filariasis (*Brugia malayi* or *Litomosoides sigmodontis*) and intestinal helminth infections (*Heligmosomoides polygyrus*). Although these models are very different, in all three an expansion of $Foxp3^{+}$ Tregs during early stages of infection was seen[46-48]. In successive studies, it has been shown that Tregs during *L. sigmodontis* infection suppress anti-parasite immunity through surface expression of CD25 and glucocorticoid-induced TNF receptor family related gene (GITR)[48], that Tregs in *H. polygyrus*-infected animals are more suppressive *in vitro* than those from uninfected mice[46], that adult parasite stages also induce Foxp3 expressing T cells

which have *in vitro* suppressive capacity which extends to bystander antigens[47] and finally, that *in vivo* CD25 depletion 7 days prior to infection could prevent the appearance of microfilaremia and reduce worm burden, indicating that Tregs suppress parasite killing *in vivo*[49]. *In vivo* and *in vitro* anti-CD25 treatment has been used in several other studies to deplete Tregs, leading to increased anti-parasite responses in murine infections with *Brugia pahangi*[50] and *Schistosoma mansoni*[43-45], but this same treatment did not enhance immune responses in other studies of *S. mansoni*[51] and *Trichinella spiralis*[52]. Another approach is depletion of Foxp3$^+$ Tregs by using DEREG mice. Expression of diphtheria toxin (DT) receptor fused with GFP under control of the *Foxp3* locus enables complete depletion of Foxp3$^+$ Tregs by administration of DT[26]. Interestingly, it was noted that only depletion of Tregs in the first days of infection is effective in improving resistance to worms. In a *Strongyloides ratti* mouse model, this method was applied and early depletion of Tregs increased type 2 responses and reduced worm burden significantly but did not change intestinal pathology, whereas depletion at a later time point (4 days after infection) had no effect on worm burden or pathology[53]. In a model of *H. polygyrus* infection however, the same Treg depletion applied at 4 days post-infection, while enhancing Th2 responses, did not affect the numbers of worms and exacerbated intestinal pathology[54]. Taken together, these data suggest that anti-CD25 treatment and Foxp3$^+$ Treg depletion show similar results; Tregs are rapidly increased upon helminth infection and are in particular important in inhibiting protective immunity at early stages of infection, whereas their effect on intestinal pathology is not consistent (summarized by Taylor et al.[55]).

In human infections, the characterization of Tregs has been more complicated (Figure 1). Up until recently, the presence of Tregs was shown indirectly by mRNA analysis of total peripheral blood mononuclear cells (PBMC)[56,57], but flow cytometry has now been established as the principal method for Treg identification during helminthic infections[58,59], however staining for FOXP3 was not available until recently. Since FOXP3 is an intracellular protein, expression can only be analyzed in fixed cells with a certain staining protocol[60]. Therefore, if isolated Tregs are needed, only surface molecules, which are less specific for Tregs, can be used for their identification and sorting[61]. Furthermore, large volumes of blood are needed for the purpose of isolating Tregs from peripheral blood, which may not always be feasible. Finally, the most state of the art methods for Treg characterization demand well-equipped laboratories with specialized operators that are more than often limited in low-resource settings. In the field of helminth research, we are therefore restricted to indirect methods of assessing Treg properties, such as phenotypic analysis of markers and depletion of Tregs in *in vitro* cell cultures (Figure 1).

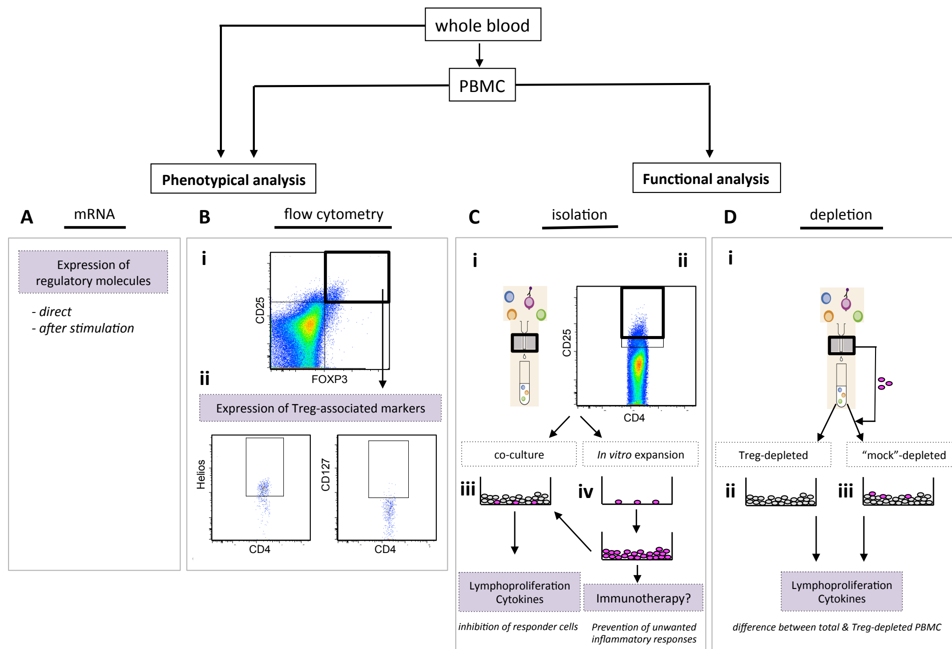

Figure 1. Different methods of characterizing human Tregs. Schematic representation of the different ways Tregs have been analyzed. In A and B, the methods of phenotypic characterization are summarized. (A) Whole blood as well as PBMC can be assessed for mRNA expression of regulatory molecules, such as IL-10 or FOXP3, directly *ex vivo* or after *in vitro* stimulation. (B) Flow cytometry can be used to assess expression of up to 17 different cell markers simultaneously. Cells from whole blood and PBMC can be stained and analyzed. The most common definition of Tregs is the population expressing CD4, FOXP3 and high levels of CD25; a representative example of Treg gating within $CD4^+$ T cells is shown (B-i). Furthermore, when selecting these cells, the expression of several other molecules can be analyzed as depicted in B-ii, where examples are shown of Helios and CD127 expression, two markers used for further characterization of Tregs. In C and D, options for functional characterization of Tregs are depicted. (C) Isolation of Tregs can be performed using magnetic beads (C-i) or fluorescent activated cell sorting (C-ii). The acquired cells can be directly co-cultured with other cell populations (C-iii) to determine the inhibitory capacity of Tregs, alternatively the cells can be expanded *in vitro* (C-iv) to obtain sufficient cell numbers for further applications, such as immunotherapy. (D) A more indirect, but more field applicable approach for the functional analysis of Tregs is the depletion method. By using magnetic bead isolation of CD25-positive or -highly positive cells (D-i), the flow-through can be regarded as Treg-depleted cells (D-ii), whereas the isolated Tregs can be added back to the flow-through to create a "mock"-depleted cell fraction (D-iii). Both cell populations can furthermore be cultured *in vitro* and the difference in immune responses can be analyzed.

– Representation adapted from Miltenyi Biotec –

Challenges in immunoepidemiological field studies

The immune regulatory network is thought to be essential for helminth-induced modulation of parasite-specific as well as bystander responses. Many research groups in affluent countries have addressed the question if and how helminths affect the immune system and whether, through modulation of bystander responses, helminths could influence the outcome of vaccinations or inflammatory diseases. Whereas the work has mainly been conducted in animal models, travellers or experimentally infected humans, only few groups have taken these questions to areas where helminth infections are highly endemic. Field studies in remote areas are complicated by the logistic challenges, lack of advanced technologies and, possibly, cultural obstacles. However, these studies analyzing human samples are of utmost importance for understanding the real-life situation, and moreover, for the opportunities of health education and bilateral knowledge transfer. Despite the difficulties, in the last decades several investigators have established collaborations with scientists in low-resource settings and these have generated important insight into the interaction of helminths with the immune system, vaccines, other infections and allergies.

It was established in various study sites that cellular immune responses to tetanus[62-64], cholera[65], BCG[66,67] and influenza[68] vaccines are impaired in helminth-infected individuals and some studies have shown increased responses to vaccines after anthelmintic treatment[62,67,69], although many were not placebo-controlled. The effect of helminths on coinfections has been addressed in a number of studies, but mostly in a cross-sectional manner. Helminth and malarial infections have overlapping distributions in tropical regions, raising the question what impact helminth infections may have on the plasmodial parasites that cause malaria. There is much controversy surrounding the effect of helminth infections on malarial parasitemia and clinical malaria episodes. Most studies have used cross-sectional designs and have variously reported detrimental[70,71] or beneficial[72,73] or no[74,75] effect of helminths on either burden of infection or clinical outcomes. Studies of anthelmintic treatment are expected to be more informative, but the trials that have been conducted so far have also shown detrimental[76] or beneficial[77] effects in small groups of children. The relationship between helminth infections and allergy has received much attention, also in terms of clinical trials conducted in areas endemic for helminth infections. Although the majority of cross-sectional studies have reported inverse associations between helminth infections and skin prick test (SPT) reactivity[78], a number show that certain helminths may increase the risk of atopy[79,80]. Two randomized trials with albendazole treatment have been carried out in cohorts of school children. A study in Ecuador showed no change in either SPT reactivity to allergens or allergic symptoms, but this study did not include a placebo group[81], while in a trial in

Vietnam, one year of albendazole treatment increased SPT reactivity but also did not change clinical allergy to any significant degree[82]. It has been suggested that longer anthelmintic treatment might be needed to reveal the modulatory effect of helminths[83].

So far, most field studies have not assessed immune alterations in parallel to clinical consequences of helminth elimination. The two albendazole trials assessing the effect on allergy in school children demonstrated lower IL-10 production in response to helminth antigens after anthelmintic treatment[82,84], suggesting a role for parasite-specific IL-10. The Ecuador study also showed an increase in helminth-specific Th2 responses[84] and enhanced Th2 responses were furthermore seen in a mebendazole trial in infants from Pemba[85] and after praziquantel treatment of pregnant women in Uganda[86], but two of these studies were not placebo-controlled and none of them involved a whole community but focused on specific age groups. There is therefore a need for randomized controlled interventional studies assessing the effect of deworming on the prevalence of coinfections and allergy, together with detailed assessment of immunological parameters, which might help us understand the causal pathways.

Scope and aims of this thesis

Since parasitic infections are still highly prevalent in tropical areas, there are opportunities to study the underlying immunological processes that might explain the possible beneficial effects of helminth infections. In particular, areas with few or no history of mass drug administration would be suited to analyze the 'natural' situation in which humans live with worms. By looking into our past, we may be able to find solutions for the current struggle worldwide with immune-associated diseases, not only in affluent countries but also in urban centers of the less affluent regions of the world. It is important to note that such studies are expected to help anticipate the consequences of the future epidemiological transition for low-to middle-income countries and thus prepare the health care systems for the challenge facing them. The dilemma between deworming and helminth immunotherapy is pressing and of major global public health impact.

The regulatory network, where Tregs play an important role, is thought to be central to the relationship of parasites with coinfections and inflammatory diseases. The characterization of this regulatory network, and helminth-induced Tregs in particular, forms the focus of this thesis. The specific aims are as follows:

i. To characterize T cell responses during parasitic infections

ii. To explore the mechanisms of immune modulation employed by parasites

  a. Proportions and phenotype of Tregs during parasitic infection

  b. Treg suppressive capacity measured by establishing a field-applicable assay

iii. To assess the immunological consequences of deworming

iv. To assess the clinical outcomes of deworming, in terms of malaria and allergy

References

1. Maizels, R.M. & Yazdanbakhsh, M. Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nature reviews. Immunology* 3, 733-744 (2003).
2. Druilhe, P., Tall, A. & Sokhna, C. Worms can worsen malaria: towards a new means to roll back malaria? *Trends in parasitology* 21, 359-362 (2005).
3. Borkow, G. & Bentwich, Z. Chronic parasite infections cause immune changes that could affect successful vaccination. *Trends in parasitology* 24, 243-245 (2008).
4. Flohr, C., Quinnell, R.J. & Britton, J. Do helminth parasites protect against atopy and allergic disease? *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 39, 20-32 (2009).
5. Weinstock, J.V., *et al.* The possible link between de-worming and the emergence of immunological disease. *The Journal of laboratory and clinical medicine* 139, 334-338 (2002).
6. Hotez, P.J., *et al.* Helminth infections: the great neglected tropical diseases. *The Journal of clinical investigation* 118, 1311-1321 (2008).
7. Bethony, J., *et al.* Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. *Lancet* 367, 1521-1532 (2006).
8. Ruffer, M.A. Note on the Presence of "Bilharzia Haematobia" in Egyptian Mummies of the Twentieth Dynasty [1250-1000 B.C.]. *British medical journal* 1, 16 (1910).
9. Fumagalli, M., *et al.* Parasites represent a major selective force for interleukin genes and shape the genetic predisposition to autoimmune conditions. *The Journal of experimental medicine* 206, 1395-1408 (2009).
10. Strachan, D.P. Hay fever, hygiene, and household size. *BMJ* 299, 1259-1260 (1989).
11. Rook, G.A. Review series on helminths, immune modulation and the hygiene hypothesis: the broader implications of the hygiene hypothesis. *Immunology* 126, 3-11 (2009).
12. Allen, J.E. & Maizels, R.M. Diversity and dialogue in immunity to helminths. *Nature reviews. Immunology* 11, 375-388 (2011).
13. Ottesen, E.A., Hiatt, R.A., Cheever, A.W., Sotomayor, Z.R. & Neva, F.A. The acquisition and loss of antigen-specific cellular immune responsiveness in acute and chronic schistosomiasis in man. *Clinical and experimental immunology* 33, 37-47 (1978).
14. Ottesen, E.A., Weller, P.F. & Heck, L. Specific cellular immune unresponsiveness in human filariasis. *Immunology* 33, 413-421 (1977).
15. Piessens, W.F., *et al.* Antigen-specific suppressor cells and suppressor factors in human filariasis with Brugia malayi. *The New England journal of medicine* 302, 833-837 (1980).
16. Piessens, W.F., *et al.* Antigen-specific suppressor T lymphocytes in human lymphatic filariasis. *The New England journal of medicine* 307, 144-148 (1982).
17. Irvine, W.J., Clarke, B.F., Scarth, L., Cullen, D.R. & Duncan, L.J. Thyroid and gastric autoimmunity in patients with diabetes mellitus. *Lancet* 2, 163-168 (1970).
18. Thomas, D.J., Young, A., Gorsuch, A.N., Bottazzo, G.F. & Cudworth, A.G. Evidence for an association between rheumatoid arthritis and autoimmune endocrine disease. *Annals of the rheumatic diseases* 42, 297-300 (1983).
19. Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. & Toda, M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155, 1151-1164 (1995).
20. Shevach, E.M. Certified professionals: CD4(+)CD25(+) suppressor T cells. *The Journal of experimental medicine* 193, F41-46 (2001).

21. Hori, S., Nomura, T. & Sakaguchi, S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299, 1057-1061 (2003).
22. Bennett, C.L., *et al.* The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nature genetics* 27, 20-21 (2001).
23. Brunkow, M.E., *et al.* Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nature genetics* 27, 68-73 (2001).
24. Chen, W., *et al.* Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *The Journal of experimental medicine* 198, 1875-1886 (2003).
25. Fontenot, J.D., *et al.* Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 22, 329-341 (2005).
26. Lahl, K., *et al.* Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. *The Journal of experimental medicine* 204, 57-63 (2007).
27. Gavin, M. & Rudensky, A. Control of immune homeostasis by naturally arising regulatory CD4+ T cells. *Current opinion in immunology* 15, 690-696 (2003).
28. Mills, K.H. & McGuirk, P. Antigen-specific regulatory T cells--their induction and role in infection. *Seminars in immunology* 16, 107-117 (2004).
29. Levings, M.K. & Roncarolo, M.G. T-regulatory 1 cells: a novel subset of CD4 T cells with immunoregulatory properties. *The Journal of allergy and clinical immunology* 106, S109-112 (2000).
30. Chen, Y., Kuchroo, V.K., Inobe, J., Hafler, D.A. & Weiner, H.L. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* 265, 1237-1240 (1994).
31. Cosmi, L., *et al.* Human CD8+CD25+ thymocytes share phenotypic and functional features with CD4+CD25+ regulatory thymocytes. *Blood* 102, 4107-4114 (2003).
32. Fischer, K., *et al.* Isolation and characterization of human antigen-specific TCR alpha beta+ CD4(-)CD8- double-negative regulatory T cells. *Blood* 105, 2828-2835 (2005).
33. Shevach, E.M. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* 30, 636-645 (2009).
34. Tang, Q. & Bluestone, J.A. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nature immunology* 9, 239-244 (2008).
35. Pandiyan, P., Zheng, L., Ishihara, S., Reed, J. & Lenardo, M.J. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nature immunology* 8, 1353-1362 (2007).
36. Krummel, M.F. & Allison, J.P. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *The Journal of experimental medicine* 182, 459-465 (1995).
37. Kremer, J.M., *et al.* Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig. *The New England journal of medicine* 349, 1907-1915 (2003).
38. Chaturvedi, V., Collison, L.W., Guy, C.S., Workman, C.J. & Vignali, D.A. Cutting edge: Human regulatory T cells require IL-35 to mediate suppression and infectious tolerance. *J Immunol* 186, 6661-6666 (2011).
39. Randall, L.M. & Engwerda, C.R. TNF family members and malaria: old observations, new insights and future directions. *Experimental parasitology* 126, 326-331 (2010).
40. van Mierlo, G.J., *et al.* Cutting edge: TNFR-shedding by CD4+CD25+ regulatory T cells inhibits the induction of inflammatory mediators. *J Immunol* 180, 2747-2751 (2008).

41. Deaglio, S., *et al.* Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *The Journal of experimental medicine* 204, 1257-1265 (2007).
42. McKee, A.S. & Pearce, E.J. CD25+CD4+ cells contribute to Th2 polarization during helminth infection by suppressing Th1 response development. *J Immunol* 173, 1224-1231 (2004).
43. Hesse, M., *et al.* The pathogenesis of schistosomiasis is controlled by cooperating IL-10-producing innate effector and regulatory T cells. *J Immunol* 172, 3157-3166 (2004).
44. Baumgart, M., Tompkins, F., Leng, J. & Hesse, M. Naturally occurring CD4+Foxp3+ regulatory T cells are an essential, IL-10-independent part of the immunoregulatory network in Schistosoma mansoni egg-induced inflammation. *J Immunol* 176, 5374-5387 (2006).
45. Taylor, J.J., Mohrs, M. & Pearce, E.J. Regulatory T cell responses develop in parallel to Th responses and control the magnitude and phenotype of the Th effector population. *J Immunol* 176, 5839-5847 (2006).
46. Finney, C.A., Taylor, M.D., Wilson, M.S. & Maizels, R.M. Expansion and activation of CD4(+)CD25(+) regulatory T cells in Heligmosomoides polygyrus infection. *European journal of immunology* 37, 1874-1886 (2007).
47. McSorley, H.J., Harcus, Y.M., Murray, J., Taylor, M.D. & Maizels, R.M. Expansion of Foxp3+ regulatory T cells in mice infected with the filarial parasite Brugia malayi. *J Immunol* 181, 6456-6466 (2008).
48. Taylor, M.D., *et al.* Removal of regulatory T cell activity reverses hyporesponsiveness and leads to filarial parasite clearance in vivo. *J Immunol* 174, 4924-4933 (2005).
49. Taylor, M.D., *et al.* Early recruitment of natural CD4+ Foxp3+ Treg cells by infective larvae determines the outcome of filarial infection. *European journal of immunology* 39, 192-206 (2009).
50. Gillan, V. & Devaney, E. Regulatory T cells modulate Th2 responses induced by Brugia pahangi third-stage larvae. *Infection and immunity* 73, 4034-4042 (2005).
51. Walsh, C.M., Smith, P. & Fallon, P.G. Role for CTLA-4 but not CD25+ T cells during Schistosoma mansoni infection of mice. *Parasite immunology* 29, 293-308 (2007).
52. Beiting, D.P., *et al.* Coordinated control of immunity to muscle stage Trichinella spiralis by IL-10, regulatory T cells, and TGF-beta. *J Immunol* 178, 1039-1047 (2007).
53. Blankenhaus, B., *et al.* Strongyloides ratti infection induces expansion of Foxp3+ regulatory T cells that interfere with immune response and parasite clearance in BALB/c mice. *J Immunol* 186, 4295-4305 (2011).
54. Rausch, S., *et al.* Establishment of nematode infection despite increased Th2 responses and immunopathology after selective depletion of Foxp3+ cells. *European journal of immunology* 39, 3066-3077 (2009).
55. Taylor, M.D., van der Werf, N. & Maizels, R.M. T cells in helminth infection: the regulators and the regulated. *Trends in immunology* 33, 181-189 (2012).
56. Babu, S., Blauvelt, C.P., Kumaraswami, V. & Nutman, T.B. Regulatory networks induced by live parasites impair both Th1 and Th2 pathways in patent lymphatic filariasis: implications for parasite persistence. *J Immunol* 176, 3248-3256 (2006).
57. King, C.L., *et al.* Cytokine control of parasite-specific anergy in human lymphatic filariasis. Preferential induction of a regulatory T helper type 2 lymphocyte subset. *The Journal of clinical investigation* 92, 1667-1673 (1993).
58. Garcia-Hernandez, M.H., *et al.* Regulatory T Cells in children with intestinal parasite infection. *Parasite immunology* 31, 597-603 (2009).

59. Watanabe, K., *et al.* T regulatory cell levels decrease in people infected with Schistosoma mansoni on effective treatment. *The American journal of tropical medicine and hygiene* 77, 676-682 (2007).
60. Law, J.P., *et al.* The importance of Foxp3 antibody and fixation/permeabilization buffer combinations in identifying CD4+CD25+Foxp3+ regulatory T cells. *Cytometry. Part A : the journal of the International Society for Analytical Cytology* 75, 1040-1050 (2009).
61. Gregori, S., Bacchetta, R., Passerini, L., Levings, M.K. & Roncarolo, M.G. Isolation, expansion, and characterization of human natural and adaptive regulatory T cells. *Methods Mol Biol* 380, 83-105 (2007).
62. Cooper, P.J., Espinel, I., Paredes, W., Guderian, R.H. & Nutman, T.B. Impaired tetanus-specific cellular and humoral responses following tetanus vaccination in human onchocerciasis: a possible role for interleukin-10. *The Journal of infectious diseases* 178, 1133-1138 (1998).
63. Nookala, S., Srinivasan, S., Kaliraj, P., Narayanan, R.B. & Nutman, T.B. Impairment of tetanus-specific cellular and humoral responses following tetanus vaccination in human lymphatic filariasis. *Infection and immunity* 72, 2598-2604 (2004).
64. Sabin, E.A., Araujo, M.I., Carvalho, E.M. & Pearce, E.J. Impairment of tetanus toxoid-specific Th1-like immune responses in humans infected with Schistosoma mansoni. *The Journal of infectious diseases* 173, 269-272 (1996).
65. Cooper, P.J., *et al.* Human infection with Ascaris lumbricoides is associated with suppression of the interleukin-2 response to recombinant cholera toxin B subunit following vaccination with the live oral cholera vaccine CVD 103-HgR. *Infection and immunity* 69, 1574-1580 (2001).
66. Elias, D., *et al.* Schistosoma mansoni infection reduces the protective efficacy of BCG vaccination against virulent Mycobacterium tuberculosis. *Vaccine* 23, 1326-1334 (2005).
67. Elias, D., *et al.* Effect of deworming on human T cell responses to mycobacterial antigens in helminth-exposed individuals before and after bacille Calmette-Guerin (BCG) vaccination. *Clinical and experimental immunology* 123, 219-225 (2001).
68. van Riet, E., *et al.* Cellular and humoral responses to influenza in gabonese children living in rural and semi-urban areas. *The Journal of infectious diseases* 196, 1671-1678 (2007).
69. Elias, D., Britton, S., Aseffa, A., Engers, H. & Akuffo, H. Poor immunogenicity of BCG in helminth infected population is associated with increased in vitro TGF-beta production. *Vaccine* 26, 3897-3902 (2008).
70. Nacher, M., *et al.* Intestinal helminth infections are associated with increased incidence of Plasmodium falciparum malaria in Thailand. *The Journal of parasitology* 88, 55-58 (2002).
71. Sokhna, C., *et al.* Increase of malaria attacks among children presenting concomitant infection by Schistosoma mansoni in Senegal. *Malaria journal* 3, 43 (2004).
72. Kung'u, J.K., *et al.* Early helminth infections are inversely related to anemia, malnutrition, and malaria and are not associated with inflammation in 6- to 23-month-old Zanzibari children. *The American journal of tropical medicine and hygiene* 81, 1062-1070 (2009).
73. Nacher, M., *et al.* Ascaris lumbricoides infection is associated with protection from cerebral malaria. *Parasite immunology* 22, 107-113 (2000).
74. Shapiro, A.E., *et al.* Epidemiology of helminth infections and their relationship to clinical malaria in southwest Uganda. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 99, 18-24 (2005).

75. Bejon, P., *et al.* Helminth infection and eosinophilia and the risk of Plasmodium falciparum malaria in 1- to 6-year-old children in a malaria endemic area. *PLoS neglected tropical diseases* 2, e164 (2008).
76. Brutus, L., Watier, L., Hanitrasoamampionona, V., Razanatsoarilala, H. & Cot, M. Confirmation of the protective effect of Ascaris lumbricoides on Plasmodium falciparum infection: results of a randomized trial in Madagascar. *The American journal of tropical medicine and hygiene* 77, 1091-1095 (2007).
77. Kirwan, P., *et al.* Impact of repeated four-monthly anthelmintic treatment on Plasmodium infection in preschool children: a double-blind placebo-controlled randomized trial. *BMC infectious diseases* 10, 277 (2010).
78. Feary, J., Britton, J. & Leonardi-Bee, J. Atopy and current intestinal parasite infection: a systematic review and meta-analysis. *Allergy* 66, 569-578 (2011).
79. Dagoye, D., *et al.* Wheezing, allergy, and parasite infection in children in urban and rural Ethiopia. *American journal of respiratory and critical care medicine* 167, 1369-1373 (2003).
80. Palmer, L.J., *et al.* Ascaris lumbricoides infection is associated with increased risk of childhood asthma and atopy in rural China. *American journal of respiratory and critical care medicine* 165, 1489-1493 (2002).
81. Cooper, P.J., *et al.* Effect of albendazole treatments on the prevalence of atopy in children living in communities endemic for geohelminth parasites: a cluster-randomised trial. *Lancet* 367, 1598-1603 (2006).
82. Flohr, C., *et al.* Reduced helminth burden increases allergen skin sensitization but not clinical allergy: a randomized, double-blind, placebo-controlled trial in Vietnam. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 40, 131-142 (2010).
83. Lau, S. & Matricardi, P.M. Worms, asthma, and the hygiene hypothesis. *Lancet* 367, 1556-1558 (2006).
84. Cooper, P.J., *et al.* Repeated treatments with albendazole enhance Th2 responses to Ascaris Lumbricoides, but not to aeroallergens, in children from rural communities in the Tropics. *The Journal of infectious diseases* 198, 1237-1242 (2008).
85. Wright, V.J., *et al.* Early exposure of infants to GI nematodes induces Th2 dominant immune responses which are unaffected by periodic anthelminthic treatment. *PLoS neglected tropical diseases* 3, e433 (2009).
86. Tweyongyere, R., *et al.* Effect of praziquantel treatment during pregnancy on cytokine responses to schistosome antigens: results of a randomized, placebo-controlled trial. *The Journal of infectious diseases* 198, 1870-1879 (2008).

Chapter 2

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

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– manuscript submitted –

Abstract

Background Filarial infections are associated with profound changes in the immune system. However, relatively little is known about *Loa loa* infection which causes subcutaneous filariasis and shows a spectrum of clinical manifestations.

Methods To characterize the effect of *Loa loa* infection on the immune system, the levels of IFN-γ, IL-5, IL-13, IL-10 and IL-17 produced by peripheral blood mononuclear cells in response to filarial antigen and mitogen were measured in infected subjects with and without microfilariae in blood as well as in endemic control subjects. Moreover the frequencies of CD4+ T cell subsets (regulatory T cells, Th1, Th2 and Th17 cells) were analyzed in the three groups using flow cytometry.

Results Levels of Th2-type cytokines were significantly higher in amicrofilaremic infected subjects whereas microfilaremics had lower levels of both Th1 and Th2 cytokines. The infected groups showed low levels of IL-17 and IL-17 producing cells while showing significantly higher regulatory T cells compared with endemic controls.

Conclusions These results suggest that *Loa loa* infection is associated with expansion of Th2 responses, however, when microfilaremic, the infection is associated with suppressed antigen specific Th2 as well as Th1 responses. *Loa loa* infections, irrespective of patent microfilaremia, lead to expansion of regulatory T cells and decrease in frequency of Th17 cells, which might prevent excessive inflammation in tissues affected by this parasite.

Introduction

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

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

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

immunological responses to filarial infection, particularly in animal models. Infection of mice with *Brugia malayi* resulted in the expansion of $CD25^{hi}Foxp3^{+}$ T cells within the $CD4^{+}$ T cell population, accompanied with raised CD103 and CTLA-4 expression[15]. Babu and co-workers have shown in humans that infection with *Wuchereria bancrofti*, results in impaired induction of Tbet and GATA3 mRNA while the expression of FOXP3 and regulatory effectors such as TGF-β, and CTLA-4 are enhanced[16]. A fourth lineage of $CD4^{+}$ cells, the Th17 cell, has been described that appears to play an important role in pathogenesis of inflammatory diseases mediated by the signature cytokine IL-17[17]. A recent study in an area endemic for lymphatic filariasis in India has shown that in subjects with chronic lymphedema but with no microfilaria or circulating antigens, antigen specific Th1 and Th17 responses are elevated compared to individuals who are infected but asymptomatic. Moreover, lymphedema was associated with impaired expression of FOXP3, GITR, CTLA-4 and TGF-β mRNA[18]. However, the latter study did not examine the responses in uninfected subjects living in the same endemic area. In a study on lymphatic filariasis in Indonesia, IL-17 production was compared between MF-positive and -negative subjects and found to be lower in microfilaremics, but the lower response did not seem to be mediated by Treg cells[19]. Furthermore, asymptomatic amicrofilaremic individuals have recently been assessed in a *W. bancrofti*-endemic area in Ghana showing higher IL-17 responses to *B. malayi* antigen compared to asymptomatic MF-positive subjects [20]. These data suggest that Th17 responses are suppressed in MF-positive lymphatic filariasis patients and that if IL-17 suppression is lost it may lead to pathology.
In loiasis, which has a different clinical presentation than lymphatic filariasis, there are very few cellular immunological studies and none regarding the expression of Treg cells or IL-17 producing cells. The current study examines the effect of *Loa loa* infection on the cellular immune responses of individuals residing in an endemic area in Gabon.

Methods

Study population and hematological analysis

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

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

Detection of *Loa loa* and categorization of infection status

Detection of microfilaria

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

Filaria IgG4 detection in serum by immunochromatography

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

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

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

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

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

Cytokine analysis in supernatants

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

Intracellular cytokines and circulating Tregs analyzed by flow cytometry

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

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

Statistical analysis

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

Results

Characteristics of the study population

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

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

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

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

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

Table 1. Characteristics of study population.

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

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

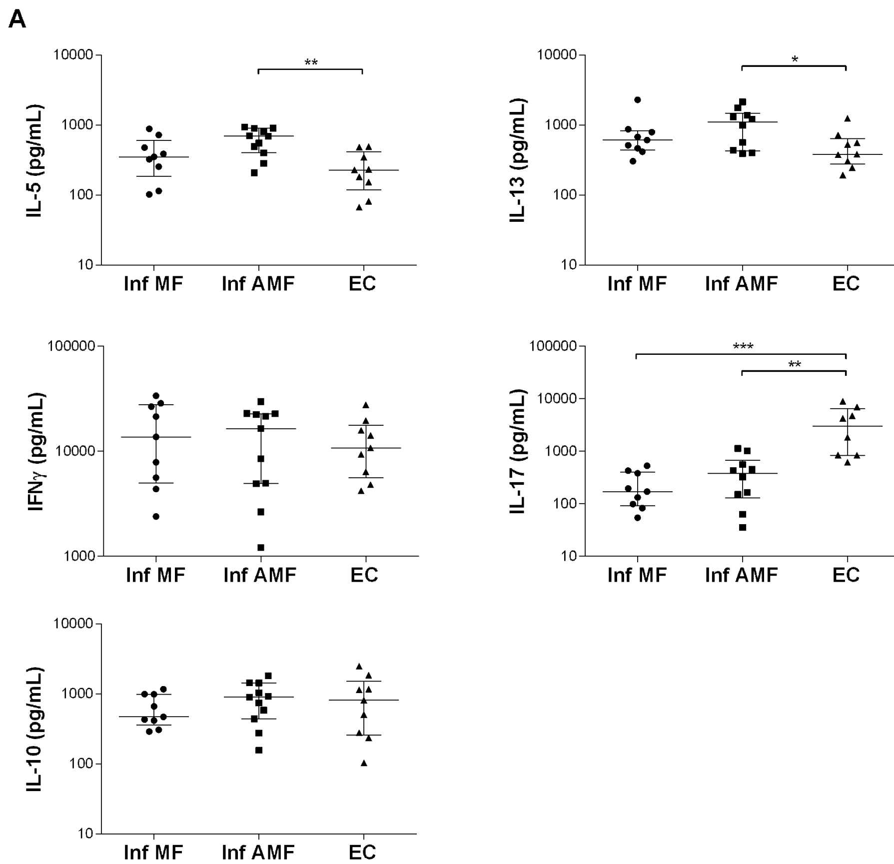
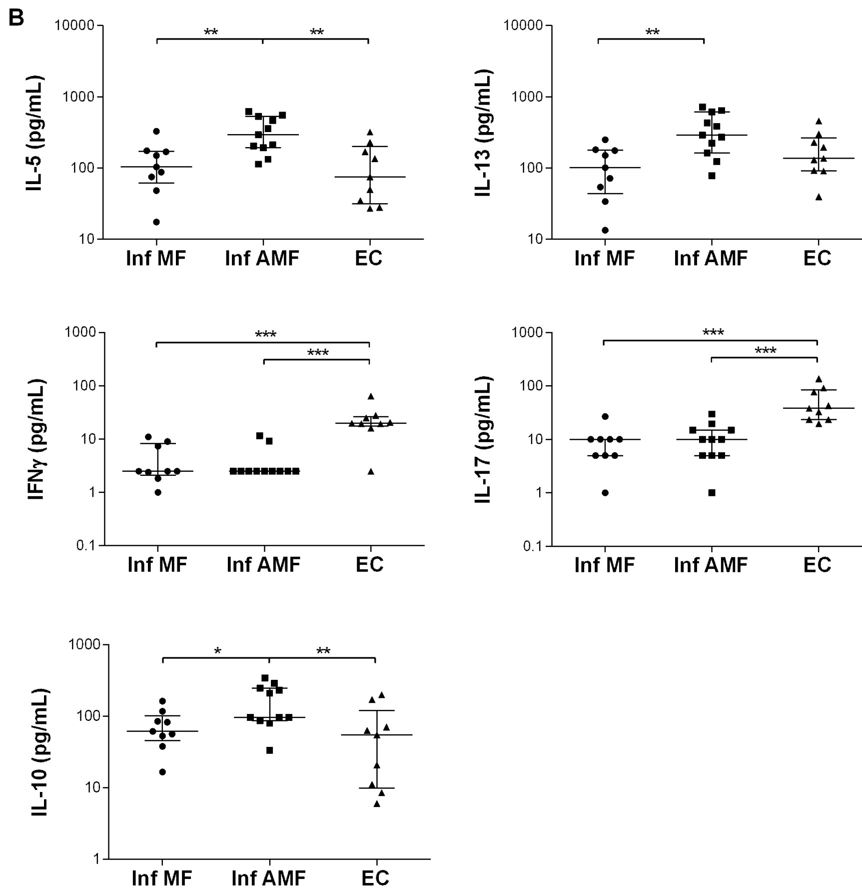


Figure 1. Altered filarial antigen specific and mitogen induced cytokine responses in microfilaremic and infected amicrofilaremic individuals. PBMC were stimulated for 72 hours with PHA (A; above) and BmA (B; right page) to assess cytokine production in culture supernatants. Cytokine levels were compared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



Circulating CD4⁺CD25ʰⁱFOXP3⁺ T cell frequencies are higher in *Loa loa* infected subjects

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

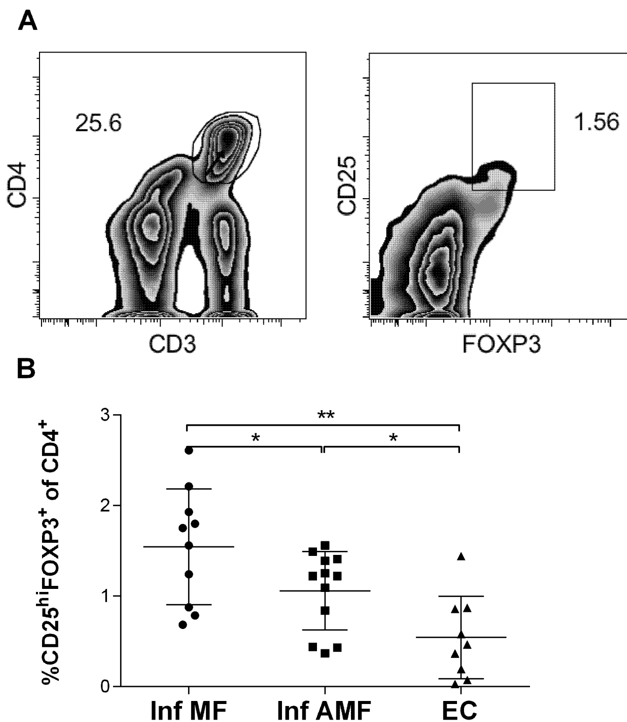


Figure 2. Higher Treg frequencies in loiasis. PBMC were fixed and stained for flow cytometry to analyse CD25 and FOXP3 expression. (A) A representative example is shown for the gating strategy of Tregs characterized as the $CD25^{high}FOXP3^{+}$ subset of $CD4^{+}$ T cells within PBMC (B) Percentage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

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

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

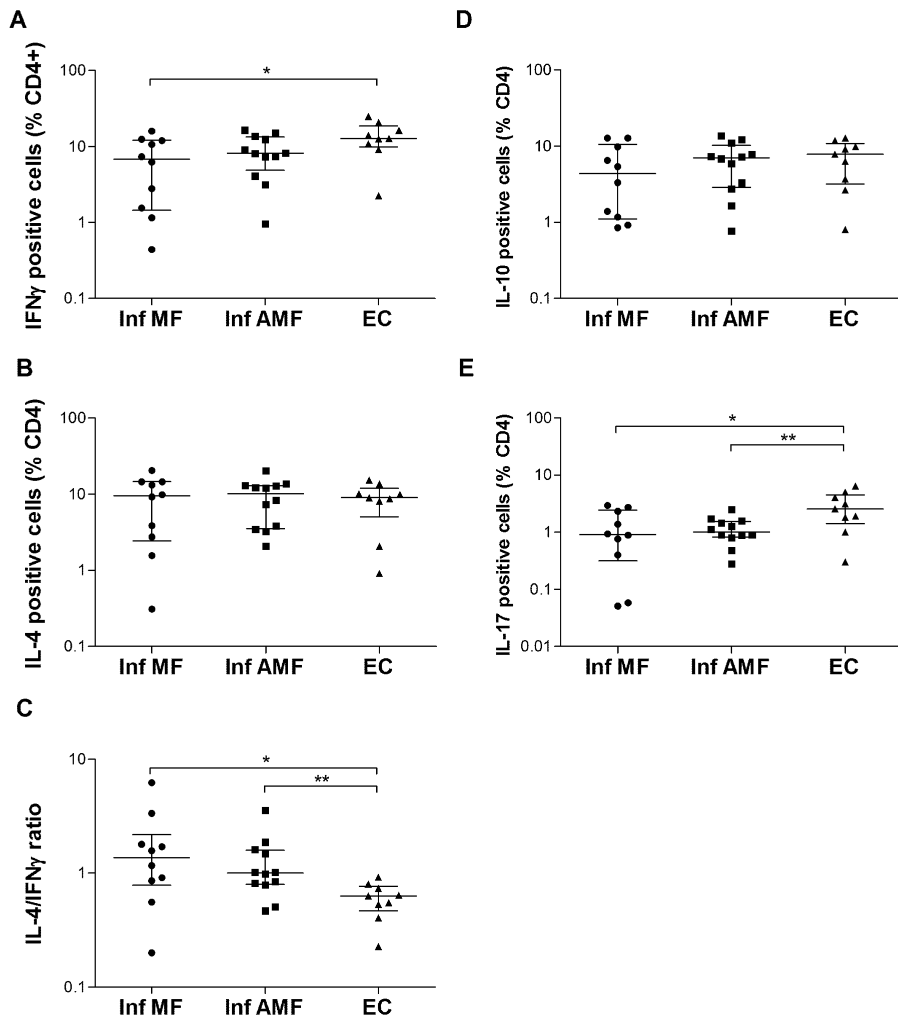


Figure 3. Intracellular cytokine production after PMA stimulation. PBMC were stimulated for 6 hours with PMA-ionomycin, and for 4 hours more in the presence of Brefeldin A, after which intracellular cytokines were detected by flow cytometry. MF+ (circles), MF– (squares) and EN (triangles) were compared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

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

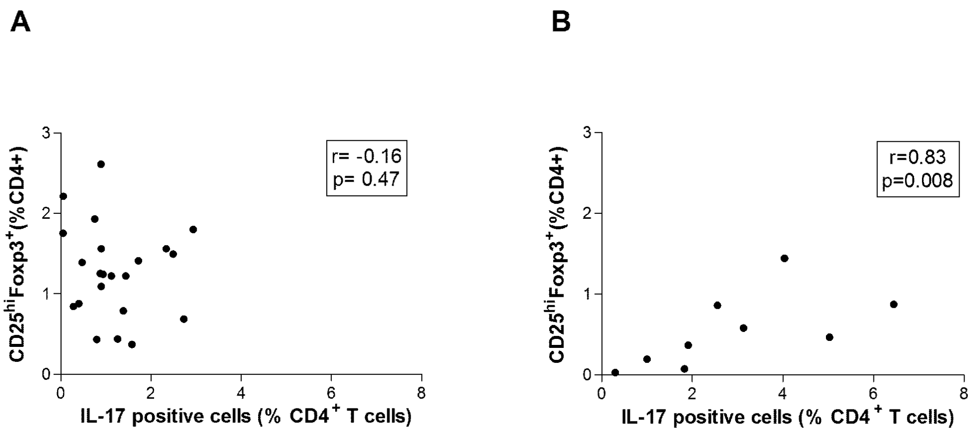


Figure 4. Correlation between Tregs and IL-17 producing CD4+ T cells. The correlation is shown between Treg frequencies (x-axis) and production of IL-17 by CD4+ T cells in response to PMA (y-axis). Spearman's Rank test was used to measure correlation, of which the coefficient r and p-values are depicted.

Discussion

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

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

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

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

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

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

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

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

References

1. Thomson, M.C., Obsomer, V., Dunne, M., Connor, S.J. & Molyneux, D.H. Satellite mapping of Loa loa prevalence in relation to ivermectin use in west and central Africa. *Lancet* 356, 1077-1078 (2000).
2. Zoure, H.G., *et al.* The geographic distribution of Loa loa in Africa: results of large-scale implementation of the Rapid Assessment Procedure for Loiasis (RAPLOA). *PLoS neglected tropical diseases* 5, e1210 (2011).
3. Klion, A.D., Massougbodji, A., Sadeler, B.C., Ottesen, E.A. & Nutman, T.B. Loiasis in endemic and nonendemic populations: immunologically mediated differences in clinical presentation. *The Journal of infectious diseases* 163, 1318-1325 (1991).
4. Nutman, T.B., Miller, K.D., Mulligan, M. & Ottesen, E.A. Loa loa infection in temporary residents of endemic regions: recognition of a hyperresponsive syndrome with characteristic clinical manifestations. *The Journal of infectious diseases* 154, 10-18 (1986).
5. Pion, D.S., *et al.* Structure of the microfilarial reservoir of Loa loa in the human host and its implications for monitoring the progr,ammes of Community-Directed Treatment with Ivermectin carried out in Africa. *Parasitology* 129, 613-626 (2004).
6. Van Hoegaerden, M., Chabaud, B., Akue, J.P. & Ivanoff, B. Filariasis due to Loa loa and Mansonella perstans: distribution in the region of Okondja, Haut-Ogooue Province, Gabon, with parasitological and serological follow-up over one year. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 81, 441-446 (1987).
7. Boussinesq, M. Loiasis. *Annals of tropical medicine and parasitology* 100, 715-731 (2006).
8. Akue, J.P., Egwang, T.G. & Devaney, E. High levels of parasite-specific IgG4 in the absence of microfilaremia in Loa loa infection. *Trop Med Parasitol* 45, 246-248 (1994).
9. Atmadja, A.K., *et al.* Differential decline in filaria-specific IgG1, IgG4, and IgE antibodies in Brugia malayi-infected patients after diethylcarbamazine chemotherapy. *The Journal of infectious diseases* 172, 1567-1572 (1995).
10. Egwang, T.G., Nguiri, C., Kombila, M., Duong, T.H. & Richard-Lenoble, D. Elevated antifilarial IgG4 antibody levels in microfilaremic and microfilaridermic Gabonese adults and children. *The American journal of tropical medicine and hygiene* 49, 135-142 (1993).
11. Winkler, S., *et al.* Increased frequency of Th2-type cytokine-producing T cells in microfilaremic loiasis. *The American journal of tropical medicine and hygiene* 60, 680-686 (1999).
12. Baize, S., Wahl, G., Soboslay, P.T., Egwang, T.G. & Georges, A.J. T helper responsiveness in human Loa loa infection; defective specific proliferation and cytokine production by CD4+ T cells from microfilaraemic subjects compared with amicrofilaraemics. *Clinical and experimental immunology* 108, 272-278 (1997).
13. Ungeheuer, M., *et al.* Cellular responses to Loa loa experimental infection in mandrills (Mandrillus sphinx) vaccinated with irradiated infective larvae. *Parasite immunology* 22, 173-183 (2000).
14. van Riet, E., Hartgers, F.C. & Yazdanbakhsh, M. Chronic helminth infections induce immunomodulation: consequences and mechanisms. *Immunobiology* 212, 475-490 (2007).
15. McSorley, H.J., Harcus, Y.M., Murray, J., Taylor, M.D. & Maizels, R.M. Expansion of Foxp3+ regulatory T cells in mice infected with the filarial parasite Brugia malayi. *J Immunol* 181, 6456-6466 (2008).

16. Babu, S., Blauvelt, C.P., Kumaraswami, V. & Nutman, T.B. Regulatory networks induced by live parasites impair both Th1 and Th2 pathways in patent lymphatic filariasis: implications for parasite persistence. *J Immunol* 176, 3248-3256 (2006).
17. Bettelli, E., *et al.* Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441, 235-238 (2006).
18. Babu, S., *et al.* Filarial lymphedema is characterized by antigen-specific Th1 and th17 proinflammatory responses and a lack of regulatory T cells. *PLoS neglected tropical diseases* 3, e420 (2009).
19. Wammes, L.J., *et al.* Regulatory T cells in human lymphatic filariasis: stronger functional activity in microfilaremics. *PLoS neglected tropical diseases* 6, e1655 (2012).
20. Arndts, K., *et al.* Elevated adaptive immune responses are associated with latent infections of Wuchereria bancrofti. *PLoS neglected tropical diseases* 6, e1611 (2012).
21. Noordin, R., *et al.* Multicentre evaluations of two new rapid IgG4 tests (WB rapid and panLF rapid) for detection of lymphatic filariasis. *Filaria journal* 6, 9 (2007).
22. WHO, U.W.B. Guidelines for rapid assessment of Loa loa. . in *Special Programme for Research & Training in Tropical Diseases* (2002).
23. WHO. Basic laboratory methods in medical parasitology. . 25-28 (1991).
24. Lal, R.B., Lynch, T.J. & Nutman, T.B. Brugia malayi antigens associated with lymphocyte activation in filariasis. *J Immunol* 139, 1652-1657 (1987).
25. Maizels, R.M., *et al.* Helminth parasites--masters of regulation. *Immunological reviews* 201, 89-116 (2004).
26. Shevach, E.M. CD4+ CD25+ suppressor T cells: more questions than answers. *Nature reviews. Immunology* 2, 389-400 (2002).
27. Finney, C.A., Taylor, M.D., Wilson, M.S. & Maizels, R.M. Expansion and activation of CD4(+)CD25(+) regulatory T cells in Heligmosomoides polygyrus infection. *European journal of immunology* 37, 1874-1886 (2007).
28. Hesse, M., *et al.* The pathogenesis of schistosomiasis is controlled by cooperating IL-10-producing innate effector and regulatory T cells. *J Immunol* 172, 3157-3166 (2004).
29. Taylor, M.D., *et al.* Removal of regulatory T cell activity reverses hyporesponsiveness and leads to filarial parasite clearance in vivo. *J Immunol* 174, 4924-4933 (2005).
30. Metenou, S., *et al.* At homeostasis filarial infections have expanded adaptive T regulatory but not classical Th2 cells. *J Immunol* 184, 5375-5382 (2010).
31. Watanabe, K., *et al.* T regulatory cell levels decrease in people infected with Schistosoma mansoni on effective treatment. *The American journal of tropical medicine and hygiene* 77, 676-682 (2007).
32. Nausch, N., Midzi, N., Mduluza, T., Maizels, R.M. & Mutapi, F. Regulatory and activated T cells in human Schistosoma haematobium infections. *PloS one* 6, e16860 (2011).
33. Babu, S., Ganley, L.M., Klei, T.R., Shultz, L.D. & Rajan, T.V. Role of gamma interferon and interleukin-4 in host defense against the human filarial parasite Brugia malayi. *Infection and immunity* 68, 3034-3035 (2000).
34. Winkler, S., *et al.* Microfilarial clearance in loiasis involves elevation of Th1 and Th2 products and emergence of a specific pattern of T-cell populations. *Parasite immunology* 18, 479-482 (1996).
35. Taylor, J.J., Krawczyk, C.M., Mohrs, M. & Pearce, E.J. Th2 cell hyporesponsiveness during chronic murine schistosomiasis is cell intrinsic and linked to GRAIL expression. *The Journal of clinical investigation* 119, 1019-1028 (2009).

36. Mangan, P.R., *et al.* Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* 441, 231-234 (2006).
37. Mbow, M., *et al.* T-helper 17 cells are associated with pathology in human schistosomiasis. *The Journal of infectious diseases* 207, 186-195 (2013).
38. Rutitzky, L.I., Smith, P.M. & Stadecker, M.J. T-bet protects against exacerbation of schistosome egg-induced immunopathology by regulating Th17-mediated inflammation. *European journal of immunology* 39, 2470-2481 (2009).
39. Walsh, K.P., Brady, M.T., Finlay, C.M., Boon, L. & Mills, K.H. Infection with a helminth parasite attenuates autoimmunity through TGF-beta-mediated suppression of Th17 and Th1 responses. *J Immunol* 183, 1577-1586 (2009).
40. Metenou, S., *et al.* Filarial infection suppresses malaria-specific multifunctional Th1 and Th17 responses in malaria and filarial coinfections. *J Immunol* 186, 4725-4733 (2011).
41. Hogewoning, A.A., *et al.* Prevalence and causative fungal species of tinea capitis among schoolchildren in Gabon. *Mycoses* 54, e354-359 (2011).
42. Chamilos, G., *et al.* Generation of IL-23 producing dendritic cells (DCs) by airborne fungi regulates fungal pathogenicity via the induction of T(H)-17 responses. *PloS one* 5, e12955 (2010).

CHAPTER 3

Asymptomatic plasmodial infection is associated with increased TNFRII-expressing Tregs and suppressed type 2 immune responses

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Abstract

Background In malaria-endemic areas, a proportion of the population becomes chronic carriers of parasites with few or no clinical signs. There is little information on cellular immune responses in asymptomatic parasite carriers.
Methods In 80 schoolchildren residing in a malaria-endemic area of Flores Island, Indonesia, T-helper subsets, regulatory T-cell (Treg) frequencies, TNFRII expression on Treg and *P. falciparum*-infected red blood cell (PfRBC)-induced cytokine responses were measured and asymptomatic infected subjects were compared to uninfected controls. To ascertain that alterations found was due to the presence of malaria parasites, the immune responses were analyzed in 16 children before and one month after anti-malarial treatment.
Results TNFRII expression, a marker of activation on Treg, was higher during infection, but decreased upon treatment. GATA3-positive cells as well as level of IL-13 secretion in response to PfRBC appeared to be suppressed by plasmodial infection as both increased after anti-malarial treatment. TNFRII expression on Treg correlated positively with TNF in response to PfRBC, but this association disappeared following treatment.
Conclusions Malaria parasites associated with asymptomatic infections seem to result in increased TNFRII expression on Tregs as well as suppressed Th2 cytokine responses, features that might be important for survival of the parasites in asymptomatic carriers.

Introduction

In malaria-endemic areas, immunity is gradually acquired, leading to lower malaria incidence and more frequent asymptomatic parasitemia with increasing age[1,2]. The presence of malarial parasites at subclinical levels is thought to be relevant for development and maintenance of protective immune responses associated with prevention of malaria attacks[3]. Studying immune responses during asymptomatic carriage of parasites, is expected to provide insight into mechanisms that allow parasite survival on the one hand and restrict the development of clinical symptoms on the other[4].

Immunological studies have focused mainly on the characterization of IFN-γ and TNF as these are considered to be important for destruction of the parasites[1]. Type 2 responses, which can interact with B-cells and induce antibody class switching, have not been characterized extensively during malaria infection. However, recently, attention has been given to the role of regulatory T-cells (Treg) in malaria as reviewed by Scholzen *et al.*[5]. Although definitions may vary, an expansion of CD4+ Treg is consistently reported in human experimental[6] and natural infection with *Plasmodium falciparum* (*P. falciparum*) as well as *P. vivax*[7-10]. The proportion of Treg has been reported to be positively correlated with parasite growth[7,10,11], which may suggest that either induction of Treg leads to parasite expansion, or blood-stage parasites recruit natural Treg and/or directly induce *de novo* Treg. In addition to their quantity, the quality of Treg in terms of their activation status might be an important determining factor in disease progression[5].

One of the activation markers of Treg that may be important during malarial infections is TNF-receptor type 2 (TNFRII). TNF(R) family members are implicated in parasite elimination as well as in the development of fever and other clinical symptoms[12]. Interestingly, TNFRII may have dual effects; while limiting TNF-induced fever and inflammation, it may also impair TNF bioactivity, which could favor parasite growth. A study in adults from Papua, Indonesia, concluded that TNFRII expression on Treg in peripheral blood and soluble TNFRII and TNF levels in plasma were higher in patients with severe versus uncomplicated malaria[10]. Furthermore, *P. falciparum*-parasitized (Pf)RBC-induced immune responses in malaria-naive donors were more strongly inhibited by CD25+TNFRII+ Tregs than by their TNFRII− counterparts[10]. In addition, in malaria-naive subjects, in vitro PfRBC stimulation of PBMC induces TNFRII expression on Treg[13].

To assess the immune regulatory network during asymptomatic parasitemia, we investigated the presence of TNFRII-expressing Treg and other T-cell subsets in a group of school children on Flores Island where malaria is endemic, by examining *ex vivo* T-cell subsets and *in vitro* cytokine responses to PfRBC. To determine whether observed differences were caused by malaria parasites, cells from a group of school children were analyzed before and after anti-malarial treatment.

Methods

Study population

In a cross-sectional study, *Plasmodium*-infected versus -uninfected children, and in a longitudinal study, infected children before and after treatment of plasmodial infection were compared. Participants resided in an area where *P. falciparum*, *P. vivax* and *P. malariae* are endemic on Flores island, Indonesia[14,15]. The cross-sectional study, aimed to recruit 100 children between 5 and 15 years, as the pilot study data from the area indicated that the prevalence of plasmodial infection by microscopy was 20%. Children were randomly selected from schools, of which 84 were willing to donate blood. A total of 80 subjects donated sufficient blood for peripheral blood mononuclear cell (PBMC) isolation as well as PCR and sufficient cells were available from 58 individuals for culture to assess cytokine responses. These 58 were similar to the total group in their baseline characteristics. For the treatment study, 20 *Plasmodium*-infected children with no clinical symptoms, in the same age group, were selected and were treated based on the blood slide result. Sixteen treated subjects donated sufficient blood for PBMC isolation after treatment. According to availability of cells, *ex vivo* phenotype and cytokine responses were measured.

The Committee of Medical Research Ethics of the University of Indonesia approved the study and the participant's parents or guardians gave informed consent.

Malaria diagnostics and treatment

Asymptomatic malaria was defined as a positive thick or thin blood smear, with no signs of fever or chills at consultation or in the last 48 hours and no other clinical complaint. Exclusion criteria were clinical symptoms in the last 48h or treatment for malaria in the preceding 7 days. Blood was drawn on the day of clinical examination. The 80 children who provided blood for the cross-sectional study were examined for malarial parasitemia by microscopy and later by real-time PCR for *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*[16] in the central laboratory. The infected children for the treatment study were selected by microscopy in the field with subsequent examination by PCR. Children in the longitudinal study were treated according to the current guidelines at the local health center, at the time comprising single-dose sulfadoxine-pyrimethamine (SP; 25 mg/kg bodyweight sulfadoxine and 1,25 mg/kg pyrimethamine) for *P. falciparum* and three days of chloroquine (total 25 mg/kg) combined with fourteen days of primaquine (0,25 mg/kg per day) for *P. vivax*. The WHO recommended artemisinin-based combination therapy (ACT) was not fully operational everywhere in Indonesia[17], including our study area. Treatment efficacy was assessed by microscopic detection of parasites at post-treatment blood sampling, 28–32 days post-treatment.

Hematological assessments
Blood was collected into sodium heparin-vacutainers (BD Biosciences, Franklin Lakes, USA) and complete blood counts were determined (Coulter® ACT Diff Hematology Blood Analyzer; Beckman Coulter, Brea, CA, USA). WHO reference values for anemia in school-age children were used (11.5 and 12 g/dl for hemoglobin in children <11 years and between 12 and 14 years of age respectively)[18].

Intestinal parasites
Geohelminth infections were determined by microscopic examination of formalin-preserved stool samples after applying the formol-ether-acetate concentration method[16].

Cell isolation and stimulation
PBMC were obtained by gradient centrifugation over Ficoll. After isolation, a small number of cells were fixed with the FOXP3 Staining set (eBioscience Inc., San Diego, USA) and cryopreserved until further analysis. Freshly isolated PBMC were cultured in RPMI 1640 (Gibco, Invitrogen, Carlsbad, USA) with 10% FCS (Greiner Bio-One GmbH, Frickenhausen, Germany). *P. falciparum*-infected and uninfected RBC (PfRBC, uRBC; kindly provided by the department of Microbiology, Radboud University Medical Centre Nijmegen) were used for stimulation. Information on preparation of PfRBC is provided in the supplementary material. After 96h supernatants were harvested and preserved at -20°C.

Flow cytometry
Fixed PBMC were thawed and permeabilized with FOXP3 Staining set (eBioscience). PBMC were stained with two panels of antibodies, details of which are shown in table S1. Extra information on our gating strategy is given in the supplementary material. PBMC from the two study groups were stained and acquired at different time points dictated by the study timelines and therefore the absolute values can not be compared between study groups; however the pre- and post-treatment samples were measured simultaneously within one experiment on the same day. Flow cytometry data were acquired on a FACSCanto machine (BD Biosciences) and analyzed with FlowJo software (Treestar Inc., Ashland, USA).

Cytokine multiplex analysis
Cytokines (IFN-γ, TNF, IL-10 and IL-13) were measured by Multiplex Bead Immunoassay, using Luminex 100™ xMAP (Luminex Corp., Austin, TX, USA), according to supplier's protocol (Biosource, Invitrogen). Half the detection limit indicated by manufacturer was used for values below detection limit and one

outlying data point was excluded. The background cytokine levels of cells stimulated with uRBC were not subtracted, but analyzed separately. Samples from the two study groups were measured at different times, precluding direct comparison of cytokine levels between the studies. Pre- and post-treatment samples were measured simultaneously within one experiment on the same day.

Data analysis

Analysis was performed in SPSS 18.0. Cross-sectional comparisons between groups were tested with Student's t-test or Mann-Whitney test for data not normally distributed. For data before and after treatment, paired analysis was done using paired t-test or Wilcoxon Signed Ranks Test. Correlations were analyzed using Spearman's test. In the multiplex cytokine analysis Bonferroni correction was applied by multiplying the p-values by the number of non-correlated measurements.

Results

Study population

In the cross-sectional study, T cell subsets and cytokine responses were compared in 80 schoolchildren (26 infected and 54 uninfected). To verify that differences found were due to malarial parasites, we designed a second study where we looked at the effect of malarial treatment on the same parameters. From a thick blood smear survey, 20 asymptomatic children infected with malarial parasites were identified and treated for their infection, 16 of whom also provided blood samples post-treatment. Characteristics of the children in the three groups, cross-sectional uninfected, cross-sectional infected and longitudinal infected before treatment are shown in Table 1.

Table 1. Demographic and infection characteristics of the study population. The study population is divided in three groups: cross-sectional uninfected, cross-sectional infected and longitudinal pre-treatment.

	cross-sectional study		**longitudinal study**
	uninfected	asymptomatic infected	asymptomatic infected
N	54	26	16
age (median; range)	8.8 (6 - 15)	9.3 (6 - 13)	8.7 (4 - 16)
sex (M / F)	25 / 29	12 / 14	5 / 11
BMI (mean ±SEM)	14.6 (±0.23)	14.7 (±0.33)	15.5 (±0.71)
%CD4+ (mean ±SEM)[a]	34.5 (±0.92)	32.3 (±2.19)	34.2 (±1.58)
***Plasmodium* species (n of Pf / Pv / Pm)[b]**	- / - / -	17 [c,d] / 9 [c] / 2 [d]	12 [c,d] / 5 [c] / 1 [d]
CT value of positive PCR (mean ±SEM)	N.A.	34.83 (±0.86)[e]	31.78 (±0.98)[e]
geohelminth prevalence[f]	80.4 % (37 of 46)	68.2 % (15 of 22)	58.3 % (7 of 12)

[a] *percentage of total lymphocytes*
[b] *assessed by PCR and if no PCR data by microscopy*
[c] *1 mixed Pf Pv infection*
[d] *1 mixed Pf Pm infection*
[e] *p=0.046 in t-test*
[f] *assessed by microscopy after formol-ether concentration*

In the cross-sectional group, microscopy identified 7 infected (8.8% of total) children; clearly much lower than the 18% prevalence found in pilot studies (data

not shown), however PCR revealed that 26 (32.5%) of the children were infected. The longitudinal study used microscopy, the method available in the field, for selection of study subjects. Examination of blood samples by PCR showed that 2 children in both groups (respectively 7.7% and 12.5% of infected) were infected with 2 *Plasmodium* spp., but excluding these children did not change the results, therefore they were retained in the analysis. Although children with microscopically detectable parasitemia in the longitudinal study did not report clinical symptoms or visit the health clinic in the week prior to inclusion, 41% had leukocytosis and 40% were anemic according to WHO guidelines[18]. There were no differences in immunological outcomes between children with or without either of these hematological alterations.

Frequency of and TNFRII expression by Treg decrease after anti-malarial treatment

To test the hypothesis that TNFRII-positive Tregs are present in asymptomatic parasitemia, we assessed the Treg compartment and its activation status by analyzing TNFRII expression on $CD25^{hi}FOXP3^{+}$ $CD4^{+}$ T-cells; the gating strategy is shown in Figure 1A. In the cross-sectional study, mean fluorescent intensity (MFI) of TNFRII expression on Treg as well as the proportion of $TNFRII^{+}$ Treg were significantly higher in the infected compared to the uninfected group (Figure 1B; mean MFI 702 in infected versus 610 in uninfected; p=0.008 and Figure 1C; 12.8% versus 8.5% respectively; p=0.007). This was not the case for whole $CD25^{hi}FOXP3^{+}$ Treg, which was lower in infected children, although the difference did not reach statistical significance (Figure 1D, mean 0.60% vs. 0.75% respectively; p=0.082). In the longitudinal study, TNFRII expression on Treg decreased significantly after treatment (Figure 1E, mean MFI of TNFRII 1526 to 1410; p=0.034), but $TNFRII^{+}$ Treg proportions did not change (Figure 1F, mean 10.3% to 8.9%; p=0.35). The $CD25^{hi}FOXP3^{+}$ Treg frequency also decreased, however this was not statistically significant (Figure 1G; mean 0.79% to 0.65% Treg of $CD4^{+}$ T-cells; p=0.091). The intensity of TNFRII expression on the total $CD4^{+}$ T-cell population was markedly lower compared to that of Treg and was similar in infected and uninfected individuals, but also decreased after anti-malarial treatment (mean MFI of TNFRII 911 to 808; p=0.0001, data not shown).

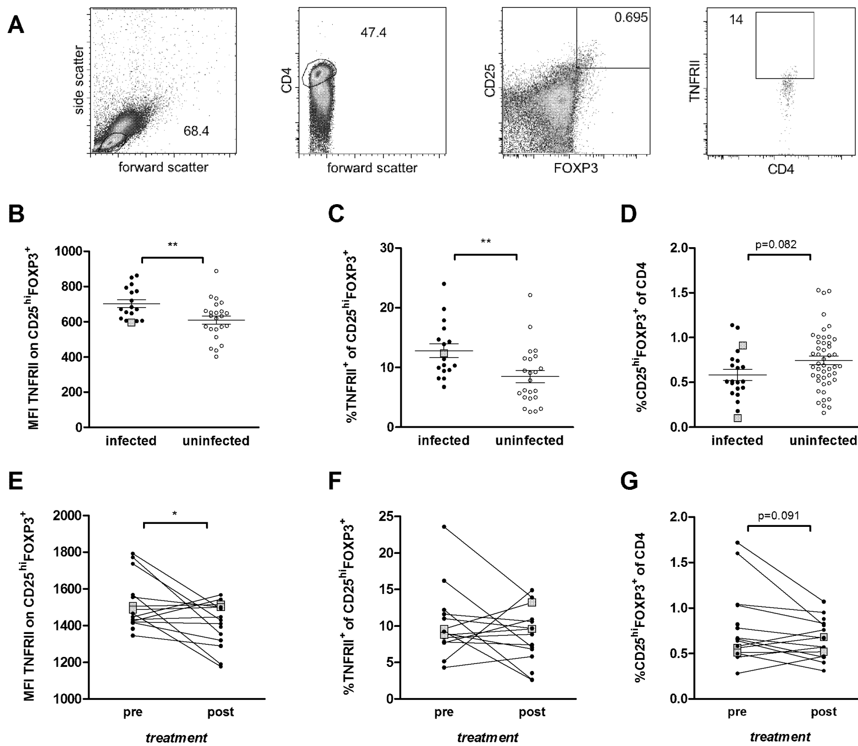


Figure 1. Increased TNFRII expression on Treg during malaria infection which decreases after treatment. PBMC were isolated and fixed and after preservation, cells were stained for flow cytometry to detect Treg and their TNFRII expression. For the cross-sectional study, TNFRII expression was measured on a subset of the samples. (A) A representative example illustrating the gating strategy for Treg as CD25^{hi}FOXP3^{+} subset of CD4^{+} T cells and TNFRII expression within the CD25^{hi}FOXP3^{+} subset. The gate for TNFRII expression on Treg was derived from a fluorescence-minus-one control (FMO). MFI of TNFRII expression on Treg (B, E), fraction of TNFRII^{+} Treg (C, F) and mean Treg frequencies (D, G) were compared between *Plasmodium*-infected (closed symbols) and -uninfected (open symbols) individuals (B-D), as well as before and after treatment (E-G). Squares represent individuals infected with 2 species of *Plasmodium*. Lines connect data points of the same individuals. Note that the fluorescent intensities and cell percentages are not comparable between the two study groups, as flow cytometric assays were performed on different days. *$p<0.05$, **$p<0.01$; p-values between 0.05 and 0.10 are indicated.

Th1 subset is not altered while Th2 cells increase after treatment

To assess whether circulating T-helper cells are polarized towards Th1 and/or Th2 during plasmodial infection, the transcription factors for Th1 (Tbet) and Th2 (GATA3) were analyzed in CD4^{+} T-cells. In the cross-sectional study, we found a higher percentage of Tbet^{+} cells in the infected group (Figure 2A; geometric means

infected 3.45% vs. uninfected 2.32%, p=0.046), while the GATA3+ subset was similar (Figure 2B; geomeans 0.92% in infected vs. 1.02% in uninfected). When we analyzed the subsets before and after treatment, the frequency of the Th1 cell subset was unaltered (Figure 2C; geomeans Tbet 3.08% pre- compared to 2.69% post-treatment), whereas elimination of parasites led to an increase in the frequency of Th2 (GATA3+) cells from 1.95% to 2.37% (p=0.021; Figure 2D).

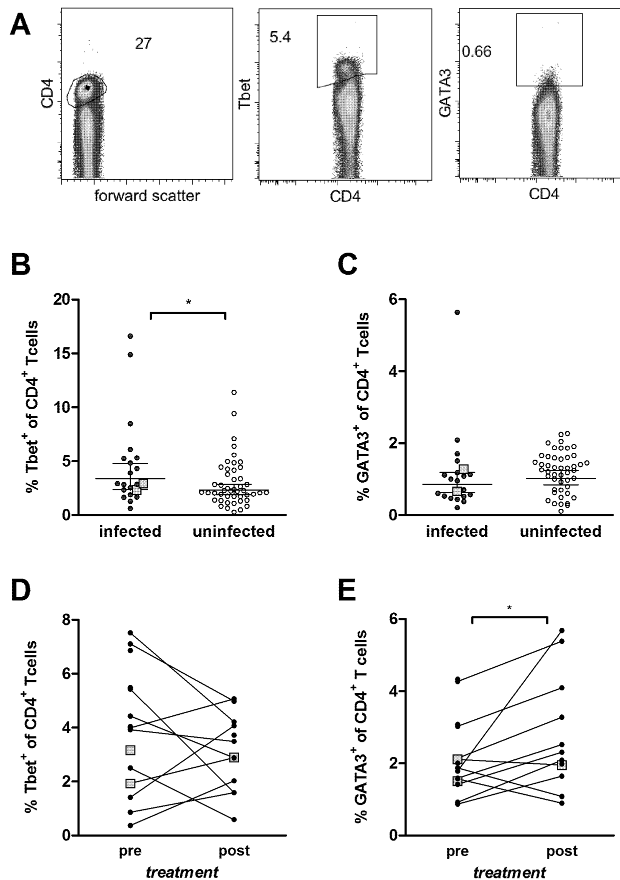


Figure 2. The proportion of Th2 cells in peripheral blood decreases after anti-malarial treatment. Isolated PBMC were stained and measured by flow cytometry and a representative example of the gating on CD4+ T cells is shown in (A). Percentages of Tbet+ (B, D) and GATA3+ (C, E) CD4+ T cells are shown for the cross-sectional subset (B-C; infected vs. uninfected n=22 vs. n=47) and the longitudinal study subjects (D-E; n=11). Closed symbols represent *Plasmodium*-infected individuals; open symbols indicate uninfected subjects, squares represent individuals infected with 2 species of *Plasmodium*. For the cross-sectional comparison, geometric means are depicted. Lines connect data points of the same individuals. *p<0.05

Parasite-specific cytokine responses before and after malarial treatment

We had sufficient cells in 58 children in the cross-sectional study and 15 subjects at both time points in the longitudinal study to be able to measure PfRBC-induced cytokine responses. Interestingly, we found no differences in TNF and IFN-γ production but lower IL-13 production in response to PfRBC in infected versus uninfected children (Figure 3A; geomeans of TNF 85 pg/ml vs. 98 pg/ml, IFN-γ 375 pg/ml vs. 398 pg/ml and IL-13 120 pg/ml vs. 349 pg/ml in infected and uninfected respectively; for IL-13 p=0.010). When we assessed the effect of anti-malarial treatment in the longitudinal study, IFN-γ production did not change while both TNF and IL-13 responses increased after treatment (Figure 3b; geomeans TNF from 364 to 745 pg/ml, p=0.041; IFN-γ from 1723 to 1960 pg/ml; IL-13 from 237 to 327 pg/ml, p=0.023). IL-10 responses to PfRBC did not differ between groups or after treatment (data not shown). After Bonferroni correction only the effects on IL-13 production remained significant. Cytokine responses to uninfected RBC did not differ in either the cross-sectional or the treatment study (data not shown).

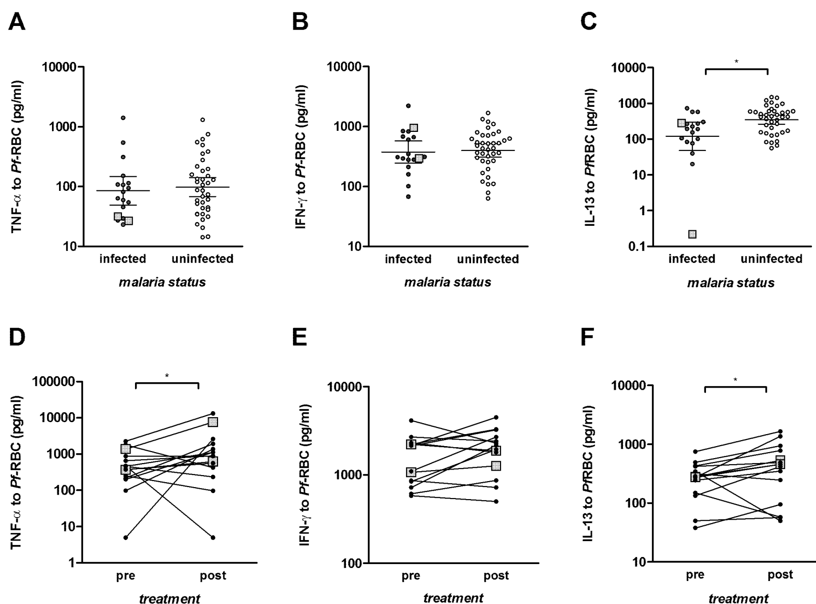


Figure 3. Suppression of TNF and IL-13 but not IFN-γ production to PfRBC. PBMC from Indonesian children were cultured with PfRBC for 4 days. Culture supernatants were analyzed for levels of TNF (A, D), IFN-γ (B, E) and IL-13 (C, F). Geometric means of cytokine production were compared between *Plasmodium*-infected and -uninfected children (A-C; n=19 vs. n=39 respectively). Squares represent individuals infected with 2 species of *Plasmodium*. Treatment effects on cytokine production were tested by paired analysis (D-F; n=15); lines connect data points of the same individuals. Cytokine levels are not comparable between the two study subsets, as multiplex assays were performed on different days. *p<0.05

Correlation of TNFRII expression on Treg and cytokine production to PfRBC
After observing higher TNFRII expression and lower cytokine production to PfRBC during asymptomatic parasitemia, we hypothesized that high TNFRII expression on Treg might be inversely correlated with PfRBC-specific cytokine production. We found a positive correlation between TNFRII expression on Treg and TNF production to PfRBC (Figure 4A; Spearman rho=0.66, p=0.002). A positive correlation was also observed between TNFRII expression on Treg and PfRBC-induced IL-13 (Figure 4B; rho=0.46, p=0.047), but the levels of TNF and IL-13 were themselves not correlated. After treatment these correlations were no longer evident (Figure 4C for TNF, rho=0.17, p=0.55; Figure 4D for IL-13, rho= -0.31, p=0.27).

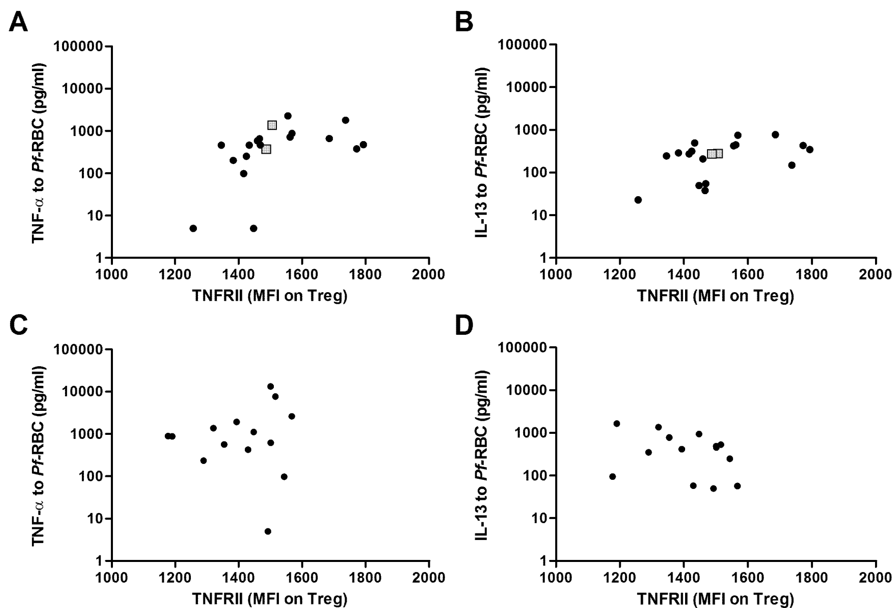


Figure 4. TNFRII expression on Treg is associated with cytokine production to PfRBC during plasmodial infection and stabilizes after treatment. PBMC were assessed for TNFRII expression on $CD25^{hi}FOXP3^{+}$ Treg and stimulated with PfRBC to detect cytokine production in culture supernatants. MFI of TNFRII expression on Treg is depicted on the x-axis and TNF (A, C) or IL-13 (B, D) production to PfRBC is depicted on the y-axis, at pre-treatment (A-B; n=19) and post-treatment (C-D; n=14) time points. Squares represent individuals infected with 2 species of *Plasmodium*. Spearman's correlation coefficients (rho) and p-values are indicated.

Discussion

We report increased TNFRII-expression on Treg during asymptomatic plasmodial infection, which decreases after anti-malarial treatment, suggesting that plasmodial parasites in children lead to activation of these cells even at a subclinical level. We also show that IL-13 responses to *P. falciparum* antigens (PfRBC) are downregulated during asymptomatic parasitemia, and restored after treatment, without changes in type 1 responses.

Very few studies have focused on asymptomatic infections, which from an immunological perspective are interesting, since in malaria-endemic areas large proportion of the population may harbor chronic, clinically silent infections[1]. We studied two groups of schoolchildren with plasmodial infections, confirmed by microscopy and/or PCR, but who were asymptomatic. Whether they were truly asymptomatic, cannot be concluded unequivocally, since we relied on symptoms assessed at the time of examination and on self-reported history of clinical symptoms in the previous 48h. However, we may conclude that in apparently healthy children, malaria parasites induce clear immunological changes.

An increased frequency of TNFRII-expressing Treg has been reported in adults with severe malaria in Papua, Indonesia, compared to uncomplicated malaria cases and asymptomatic controls[10]. Interestingly, Treg numbers and soluble TNFRII plasma concentrations decreased significantly in those with uncomplicated malaria when given artemisinin combination therapy. Another study in children in the Gambia, found that in contrast to the Papua study, both MFI and percentage of TNFRII expression by Treg and also FOXP3^{+}CD127$^{-/low}$ Treg frequency were not different between severe and uncomplicated acute *P. falciparum* malaria infections[11]. However, again after treatment lower TNFRII expression and TNFRII^{+} Treg were found. These two studies suggest that *P. falciparum* is associated with higher levels of TNFRII-expressing Treg. Our findings are in line, showing that even in school-age children with asymptomatic parasitemia, malarial parasites are associated with increased TNFRII expression on Treg.

When total Treg rather than TNFRII-expressing Treg were analyzed in the Gambian study, Treg frequencies were increased at convalescence, suggesting they were lost or sequestered during severe or uncomplicated clinical malaria[11]. We indeed found a tendency for lower frequencies of total Treg in peripheral blood in infected subjects in our cross-sectional study, yet after treatment, Treg numbers decreased, albeit non-significantly. Discrepancies found in studies of Treg, are likely to reflect differences in study population, presence of other co-infections and the Treg phenotypes studied. For example, in our study, malaria infection intensity was different in the groups participating in the cross sectional and the longitudinal studies. In the cross-sectional study, infection was detected by the more sensitive PCR, while in the longitudinal study, this was based on microscopy, which will only

detect higher intensity infections. It is possible that when assessing CD25 and FOXP3 expression, we look at a mixture of activated effector T cells and suppressive Tregs[19]. In higher intensity infections of our longitudinal study group, more activated T cells could have been included in the Treg gate. This is partly why we preferred to focus on the expression of TNFRII on Treg, as a more specific marker of suppressive function of Treg[10]. Moreover our study population consists of children exposed to both *P. falciparum* and *P. vivax*, which potentially lead to altered immunological outcomes compared with those exposed to *P. falciparum* alone[8,20].

Analysis of Th1 and Th2 cell subsets suggested a lower frequency of circulating Th2 cells and a lower in vitro IL-13 response to parasite antigens during asymptomatic plasmodial infection, which increased post-treatment. So far, few immunological studies of malaria have considered type 2 responses in any detail. In a study in Papua New Guinea, IL-4 responses to PfRBC did not differ between infected and uninfected children[21]. IL-4 production to *P. falciparum* schizont lysates was also unaffected by intermittent preventive treatment with SP of infants in Mozambique[22]. However, in the same study, IL-13, the Th2 cytokine examined in our study, was elevated in the plasma of children treated with sulfadoxine-pyrimethamine (SP)[22]. Interestingly, *P. falciparum*-lysate-specific IgE antibodies, dependent on Th2 cell activity, were associated with a reduced risk of malaria episodes regardless of age in a Tanzanian population[23]. Although not specifically addressed, this protective effect might have been due to the better control of malarial parasites. Moreover in the Fulani, an ethnic group in West Africa that is resistant to clinical malaria episodes and plasmodial parasitemia, GATA3 and IL-4 genes are increased, in parallel with the downregulation of FOXP3 and CTLA4 genes[24]. The Fulani have also been shown to have higher percentages of IL-4 producing cells in response to *P. falciparum* antigens compared to the sympatric malaria-susceptible Dogon tribe[25]. Taken together, it is tempting to speculate that, along with Th1-type IFN-γ and TNF responses, there may be a protective role for Th2-associated immune responses in plasmodial infections. Our observed lower frequency and cytokine responses of Th2 cells in subjects with plasmodial infection compared to uninfected and treated individuals may be in line with the notion that plasmodial parasites' survival is dependent on suppression of parasite-specific Th2 responses, which are known to be involved in promoting B cell survival and antibody switching.

Along with IL-13, TNF production induced by PfRBC in vitro was suppressed during asymptomatic infection. Although we hypothesized that TNFRII$^+$ Treg would suppress cytokine production to PfRBC, we found a positive association between TNFRII expression levels on Treg with both TNF and IL-13 levels stimulated by PfRBC. It is known that TNF induces TNFRII expression and expansion of Treg populations[12], presumably to control TNF-associated

inflammatory responses and tissue damage. Moreover, in rheumatoid arthritis (RA), some studies have suggested that shedding of TNFRII may be a mechanism whereby Treg can prevent TNF action[26]. The positive correlation between TNFRII expressing Treg and TNF as well as our observations that TNFRII expression decreased after parasite elimination and that the correlation of TNFRII-expression on Treg with PfRBC-induced cytokines waned supports the notion that there is a dynamic interaction between parasites, TNF and TNFRII expressing Tregs.

The positive association of TNFRII expressing Treg with IL-13 production during plasmodial infection might seem more difficult to reconcile with the hypothesis that Th2 responses may be suppressed by malaria parasites to enhance their survival. However, it is possible that IL-13 is correlated with TNF, which can induce TNFRII expression, and therefore an indirect causal relationship is found. The numbers in the separate studies were too small but when data from all malaria-infected individuals in the two study groups were combined, indeed TNF and IL-13 were significantly correlated (not shown). It is also known that Th2 cells can have a more pro-inflammatory character when co-expressing cytokines such as TNF[27], whilst modified Th2 cells co-expressing anti-inflammatory cytokines[28] could play a role in controlling pro-inflammatory responses. In addition, a recent paper has shown a tight co-regulation and cooperation of FOXP3 and GATA3 transcription[29]. Therefore, our data could suggest that the pro-inflammatory Th2 cells are suppressed during infection while the anti-inflammatory Th2 cells are correlated with TNFRII expression on Treg. Studies are needed to better characterize cytokine co-expression by single cells to determine the contribution of different cell subsets to malarial immunology.

In conclusion, since a considerable proportion of endemic populations may be asymptomatic parasite carriers, this group needs to be studied more intensively. Based on our data, we propose that in vivo malaria-induced TNF upregulates TNFRII on Treg, which might increase their activity and therefore more effectively prevent inflammation. Moreover we show that Th2 responses, which are often ignored, might be an important component of immunity to malaria parasites that are targeted by Tregs.

References

1. Langhorne, J., Ndungu, F.M., Sponaas, A.M. & Marsh, K. Immunity to malaria: more questions than answers. *Nature immunology* 9, 725-732 (2008).
2. Marsh, K. & Kinyanjui, S. Immune effector mechanisms in malaria. *Parasite immunology* 28, 51-60 (2006).
3. Males, S., Gaye, O. & Garcia, A. Long-term asymptomatic carriage of Plasmodium falciparum protects from malaria attacks: a prospective study among Senegalese children. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 46, 516-522 (2008).
4. Riley, E.M., Wahl, S., Perkins, D.J. & Schofield, L. Regulating immunity to malaria. *Parasite immunology* 28, 35-49 (2006).
5. Scholzen, A., Minigo, G. & Plebanski, M. Heroes or villains? T regulatory cells in malaria infection. *Trends in parasitology* 26, 16-25 (2010).
6. Walther, M., *et al.* Upregulation of TGF-beta, FOXP3, and CD4+CD25+ regulatory T cells correlates with more rapid parasite growth in human malaria infection. *Immunity* 23, 287-296 (2005).
7. Bueno, L.L., *et al.* Plasmodium vivax: induction of CD4+CD25+FoxP3+ regulatory T cells during infection are directly associated with level of circulating parasites. *PloS one* 5, e9623 (2010).
8. Goncalves, R.M., *et al.* CD4+ CD25+ Foxp3+ regulatory T cells, dendritic cells, and circulating cytokines in uncomplicated malaria: do different parasite species elicit similar host responses? *Infection and immunity* 78, 4763-4772 (2010).
9. Jangpatarapongsa, K., *et al.* Plasmodium vivax parasites alter the balance of myeloid and plasmacytoid dendritic cells and the induction of regulatory T cells. *European journal of immunology* 38, 2697-2705 (2008).
10. Minigo, G., *et al.* Parasite-dependent expansion of TNF receptor II-positive regulatory T cells with enhanced suppressive activity in adults with severe malaria. *PLoS pathogens* 5, e1000402 (2009).
11. Walther, M., *et al.* Distinct roles for FOXP3 and FOXP3 CD4 T cells in regulating cellular immunity to uncomplicated and severe Plasmodium falciparum malaria. *PLoS pathogens* 5, e1000364 (2009).
12. Randall, L.M. & Engwerda, C.R. TNF family members and malaria: old observations, new insights and future directions. *Experimental parasitology* 126, 326-331 (2010).
13. Scholzen, A., Mittag, D., Rogerson, S.J., Cooke, B.M. & Plebanski, M. Plasmodium falciparum-mediated induction of human CD25Foxp3 CD4 T cells is independent of direct TCR stimulation and requires IL-2, IL-10 and TGFbeta. *PLoS pathogens* 5, e1000543 (2009).
14. Elyazar, I.R., Hay, S.I. & Baird, J.K. Malaria distribution, prevalence, drug resistance and control in Indonesia. *Advances in parasitology* 74, 41-175 (2011).
15. Ende, H.D.A. Health profile of Ende District. (Ministry of Health Indonesia, 2009).
16. Wiria, A.E., *et al.* Does treatment of intestinal helminth infections influence malaria? Background and methodology of a longitudinal study of clinical, parasitological and immunological parameters in Nangapanda, Flores, Indonesia (ImmunoSPIN Study). *BMC infectious diseases* 10, 77 (2010).
17. Harijanto, P.N. Malaria treatment by using artemisinin in Indonesia. *Acta medica Indonesiana* 42, 51-56 (2010).
18. WHO. Nutritional anaemias. Report of a WHO scientific group. (1968).
19. Finney, O.C., Riley, E.M. & Walther, M. Phenotypic analysis of human peripheral blood regulatory T cells (CD4+FOXP3+CD127lo/-) ex vivo and after in vitro

restimulation with malaria antigens. *European journal of immunology* 40, 47-60 (2010).
20. Hemmer, C.J., *et al.* Stronger host response per parasitized erythrocyte in Plasmodium vivax or ovale than in Plasmodium falciparum malaria. *Tropical medicine & international health : TM & IH* 11, 817-823 (2006).
21. Robinson, L.J., *et al.* Cellular tumor necrosis factor, gamma interferon, and interleukin-6 responses as correlates of immunity and risk of clinical Plasmodium falciparum malaria in children from Papua New Guinea. *Infection and immunity* 77, 3033-3043 (2009).
22. Quelhas, D., *et al.* Intermittent preventive treatment with sulfadoxine-pyrimethamine does not modify plasma cytokines and chemokines or intracellular cytokine responses to Plasmodium falciparum in Mozambican children. *BMC immunology* 13, 5 (2012).
23. Bereczky, S., *et al.* Elevated anti-malarial IgE in asymptomatic individuals is associated with reduced risk for subsequent clinical malaria. *International journal for parasitology* 34, 935-942 (2004).
24. Torcia, M.G., *et al.* Functional deficit of T regulatory cells in Fulani, an ethnic group with low susceptibility to Plasmodium falciparum malaria. *Proceedings of the National Academy of Sciences of the United States of America* 105, 646-651 (2008).
25. Farouk, S.E., *et al.* Different antibody- and cytokine-mediated responses to Plasmodium falciparum parasite in two sympatric ethnic tribes living in Mali. *Microbes and infection / Institut Pasteur* 7, 110-117 (2005).
26. van Mierlo, G.J., *et al.* Cutting edge: TNFR-shedding by CD4+CD25+ regulatory T cells inhibits the induction of inflammatory mediators. *J Immunol* 180, 2747-2751 (2008).
27. Ito, T., *et al.* TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *The Journal of experimental medicine* 202, 1213-1223 (2005).
28. Maizels, R.M. & Yazdanbakhsh, M. Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nature reviews. Immunology* 3, 733-744 (2003).
29. Rudra, D., *et al.* Transcription factor Foxp3 and its protein partners form a complex regulatory network. *Nature immunology* 13, 1010-1019 (2012).

Supplementary methods

Specific gating of regulatory T cells by flow cytometry

Monocytes were excluded based on the forward scatter / side scatter plot, but since CD3 staining was not included, some monocytes could have been included in the CD4-positive T cell gate. However, by selecting CD25- and FOXP3-positive cells for assessing TNFRII expression on Treg, we have excluded all monocytes in the Treg analysis.
Since both CD25 and FOXP3 are also markers of T cell activation, we defined Treg cells as FOXP3-positive cells with high CD25 expression, to include more pure Treg cells with low contamination of activated (effector) T cells.

Preparation of *Plasmodium falciparum* -infected and uninfected RBC

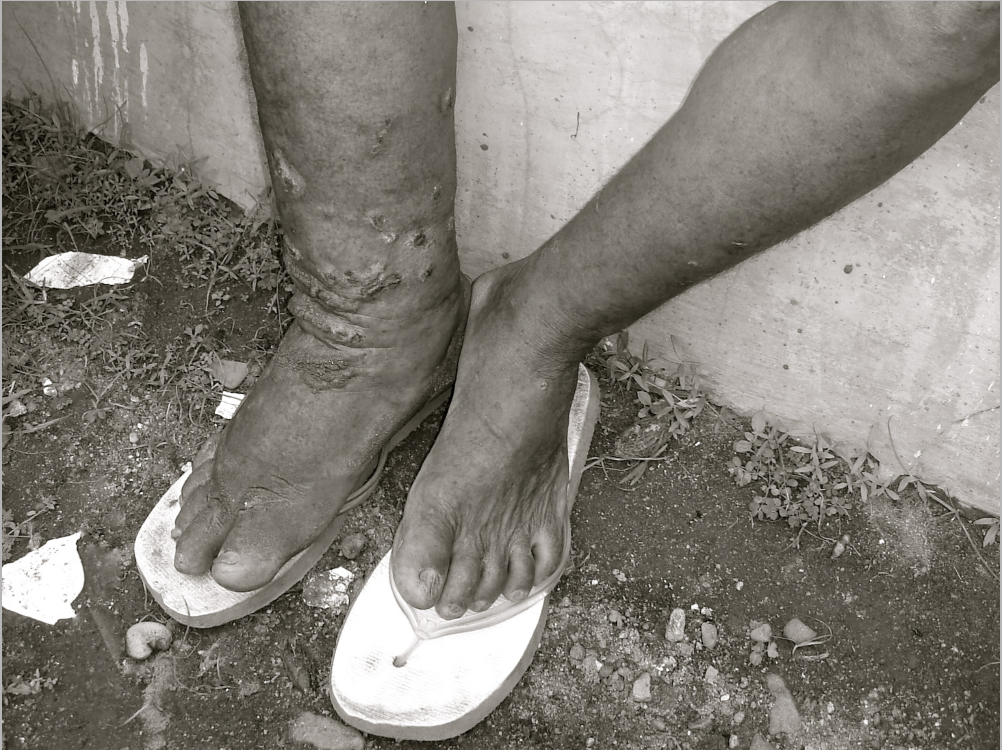
Cryopreserved *P. falciparum*-infected (PfRBC) and uninfected RBC (uRBC) were kindly provided by Professor Sauerwein at Radboud University Medical Centre Nijmegen). Mature asexual stages of the *P. falciparum* NF54 strain were purified by Percoll gradient centrifugation[1], resulting in 80-90% parasitemia in the obtained RBC, consisting of >95% schizonts / mature trophozoite[2,3]. RBC were used for stimulation in a PBMC:RBC ratio of 1:2.5; pilot experiments had indicated optimal dose and time for these experiments.

References

1. Rivadeneira, E.M., Wasserman, M. & Espinal, C.T. Separation and concentration of schizonts of Plasmodium falciparum by Percoll gradients. *The Journal of protozoology* **30**, 367-370 (1983).
2. Hartgers, F.C., *et al.* Responses to malarial antigens are altered in helminth-infected children. *The Journal of infectious diseases* **199**, 1528-1535 (2009).
3. McCall, M.B., *et al.* Plasmodium falciparum infection causes proinflammatory priming of human TLR responses. *J Immunol* **179**, 162-171 (2007).

Table S1. Details of the panel of antibodies used for flow cytometry.

Marker	Clone	Fluorochrome	Company information
CD4	SK3 RPA-T4	PE-Cy7 APC-eFluor780	BD Biosciences, Franklin Lakes, USA eBioscience Inc., San Diego, USA
CD25	2A3	PECy7	BD Biosciences
FOXP3	PCH101	APC or eFluor450	eBioscience
TNFRII	MR2-1	Biotin + streptavidin-Qdot525	Hycult Biotech Inc., Plymouth Meeting, USA Invitrogen, Carlsbad, USA
Tbet	4B10	PerCP-Cy5.5	eBioscience
GATA3	TWAJ	eFluor660	eBioscience



Chapter 4

Regulatory T cells in human lymphatic filariasis: stronger functional activity in microfilaremics

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Abstract

Background Infection with filarial parasites is associated with T cell hyporesponsiveness, which is thought to be partly mediated by their ability to induce regulatory T cells (Tregs) during human infections. This study investigates the functional capacity of Tregs from different groups of filarial patients to suppress filaria-specific immune responses during human filariasis.
Methods Microfilaremic (MF), chronic pathology (CP) and uninfected endemic normal (EN) individuals were selected in an area endemic for *Brugia timori* in Flores island, Indonesia. PBMC were isolated, $CD4CD25^{hi}$ cells were magnetically depleted and *in vitro* cytokine production and proliferation in response to *B. malayi* adult worm antigen (BmA) were determined in total and Treg-depleted PBMC.
Results In MF subjects BmA-specific T and B lymphocyte proliferation as well as IFN-γ, IL-13 and IL-17 responses were lower compared to EN and CP groups. Depletion of Tregs restored T cell as well as B cell proliferation in MF-positives, while proliferative responses in the other groups were not enhanced. BmA-induced IL-13 production was increased after Treg removal in MF-positives only.
Conclusions Thus, filaria-associated Tregs were demonstrated to be functional in suppressing proliferation and possibly Th2 cytokine responses to BmA. These suppressive effects were only observed in the MF group and not in EN or CP. These findings may be important when considering strategies for filarial treatment and the targeted prevention of filaria-induced lymphedema.

Introduction

Lymphatic filariasis (LF), caused by nematodes *Wuchereria bancrofti, Brugia malayi* and *B. timori*, affects around 120 million people worldwide and additionally 2 billion people are at risk in endemic areas[1]. Although not life-threatening, chronic manifestation of disease causes major disabilities and deformities, especially in areas with minimal access to health care facilities. Indonesia is one of the endemic countries in the South-East Asia region and accounts for the second highest burden of LF in the world. All three filarial parasites are prevalent in the archipelago and efforts are being made to control the disease in various areas (Global Programme to Eliminate Lymphatic Filariasis)[2,3].

Helminths such as filarial parasites have been shown to induce immune modulation, resulting in T cell hyporesponsiveness and failure to expel parasites[4]. Initially a phase of immune activation and pro-inflammatory cytokine responses is induced by the larval stages of filarial parasites[5]. However in patent infection, with circulating microfilariae (MF) and/or filarial antigens, decreased proliferative responses and increased anti-inflammatory cytokines, such as IL-10 and TGF-β, reflect a state of immune hyporesponsiveness[6]. At the transcriptional level, it has been shown that in infected subjects both Th1 and Th2 pathways are downmodulated by the enhanced expression of molecules such as FOXP3, CTLA-4 and TGF-β involved in regulatory networks[7]. In patients with chronic pathology this seems to be reversed; in PBMC from these patients enhanced inflammatory Th1 and Th17 responses as well as decreased levels of mRNA for different Treg markers were observed as compared to asymptomatic infected individuals[8].

The suppressive capacities of Tregs have been implicated in many infectious diseases, including filariasis. Induction of Tregs by pathogens is regarded as one of the mechanisms to evade the human immune system[9]. A recent report demonstrated that in animal models, early recruitment of Tregs affects the course of the immune response that leads to the development of chronic filariasis, indicating that Tregs are important regulators of the overall immune response to filarial nematodes in mice[10]. In human filariasis, different Treg subsets have been the focus of recent studies in different age groups and different clinical categories. While in India, higher frequencies of regulatory T cell markers were found in asymptomatic microfilaremics compared with chronic pathology patients[8], a recent study in Mali reported higher frequencies of Tregs ($CD4^{+}CD25^{+}FOXP3^{+}CD127^{-}$) in MF or circulating filaria antigen-positive versus uninfected adolescents, but also suggested a more prominent regulatory role for IL-10 producing, so-called adaptive Tregs ($CD4^{+}CD25^{-}$) cells[11].

Altogether, in previous studies of regulatory networks in human filariasis phenotypes of participating lymphocyte subsets and key regulatory molecules have been investigated, whereas the functional capacity of Tregs remained largely

unknown. In this study we aimed to explore the immune regulatory activity in different disease states of microfilaremia (MF), chronic pathology (CP) presented as elephantiasis and uninfected endemic normals (EN) as controls in a population living in an area endemic for *B. timori* in Indonesia. By *in vitro* depletion assays we determined the effect of Tregs on filaria-specific T and B cell proliferation and cytokine production.

Methods

Study population and parasitological diagnostics

In Sikka district, Flores, east Indonesia, an area endemic for *B. timori* was identified. Study participants were recruited from surrounding villages, written informed consent was obtained and night blood samples were collected to determine microfilaremia. Morning venous blood samples were collected from 24 MF-negative asymptomatic endemic normals (EN), 24 MF-positive asymptomatic individuals (MF) and 26 MF-negative chronic pathology (uni- or bilateral elephantiasis) patients (CP). 1 ml of blood was used for filtration to quantify mf load and thick blood smears were screened for the presence of malaria parasites. The study was approved by the Committee of Medical Research Ethics of the University of Indonesia.

Cell isolation and Treg depletion

Peripheral blood mononuclear cells (PBMC) were obtained by gradient centrifugation of heparinized venous blood over Ficoll. Based on sufficient numbers of PBMC, of 69 individuals (23 in each group) $CD4^{+}CD25^{hi}$ T cells were isolated by magnetic cell sorting (MACS) using the $CD4^{+}CD25^{+}$ Regulatory T Cell Isolation Kit (Miltenyi Biotec GmBH, Bergisch Gladbach, Germany); details have been described previously[12]. The $CD4^{+}CD25^{hi}$ -depleted PBMC were compared with PBMC, which were treated in an identical manner, however to which the eluted $CD4^{+}CD25^{hi}$ cells were added back to (this is referred to as “mock-depleted”).

PBMC stimulation assay for proliferation and cytokine production

The green-fluorescent dye carboxyfluorescein succinimidyl ester (CFSE; Sigma-Aldrich, CA, USA) was used to monitor proliferation. CFSE is divided over daughter cells upon cell division and this can subsequently be tracked by decreasing fluorescence intensity. After labeling with 2 μM CFSE, mock- and $CD4^{+}CD25^{hi}$ -depleted PBMC were cultured in RPMI 1640 (Gibco, Invitrogen, Carlsbad, CA, U SA) supplemented with 10% FCS (Greiner Bio-One GmbH, Frickenhausen, Germany) with or without *B. malayi* adult worm antigen (BmA, 10 μg/ml). After 96h cell supernatants were collected and cells were fixed in 2% formaldehyde (Sigma-Aldrich), after which all samples were preserved at -20°C first, then at -80°C.

Flow cytometry

After thawing, the CFSE-positive cells were labeled with fluorochrome-conjugated anti-CD3, anti-CD4, anti-CD25 (BD Biosciences, Franklin Lakes, NJ, USA) and anti-CD19 (biotinylated antibody from eBioscience Inc., San Diego, CA, USA; streptavidin-Qdot525 from Invitrogen) antibodies, acquired on a FACSCanto II machine (BD Biosciences) and analyzed with FlowJo software (Treestar Inc.,

Ashland, OR, USA). Proliferation of effector T cells was determined in a FlowJo Proliferation application by calculation of the fraction of cells from the starting population that had divided, within the $CD3^+CD4^+CD25^+$ T cell and $CD3^-CD19^+$ B cell subsets. Since background levels of cell proliferation were high, spontaneous proliferation was subtracted from BmA-stimulated values to compare proliferative responses in the three study groups.

Cytokine multiplex analysis

Cytokine production was assessed using the Multiplex Bead Immunoassay for interferon-gamma (IFN-γ), interleukin (IL)-13, IL-17 and IL-10 according to the protocol supplied by the manufacturer (Biosource, Invitrogen, Carlsbad, CA, USA). Samples were acquired with Luminex 100™ xMAP technology (Luminex Corp., Austin, TX, USA). Half the detection limit supplied by the manufacturer was used for values below detection limit and the values above upper detection limit were given the upper limit value. The cytokine data were not normally distributed and therefore are presented as raw unmanipulated data. Thus, there was no subtraction of or division over unstimulated samples, but data are shown separately as medium-stimulated or antigen-stimulated cytokines.

Data analysis

Statistical analysis was performed in SPSS 16.0. Not-normally distributed values (cytokine levels in supernatants) were log-transformed. Both age and sex were incorporated into univariate analysis to compare different infection and clinical groups. Resulting adjusted means were anti-log-transformed when needed. For mock- versus Treg-depleted samples, paired analysis was done using paired t-test or Wilcoxon Signed Ranks Test. In the multiplex cytokine analysis Bonferroni correction was taken into account where applicable, by multiplying the p-values by the number of non-correlated measurements.

Results

Study population and parasitological examination

Individuals from an area endemic for lymphatic filariasis in the north of Flores, Indonesia, were recruited for a night blood survey. Based on the microfilaremic status and sufficient number of PBMC, 23 MF-negative asymptomatic endemic normals (EN), 23 MF-positives (MF) and 23 chronic pathology (CP) patients were included for immunological studies. Microscopic *Plasmodium* spp. parasitemia was found in 2 CP patients, but had no effect on the analyses shown here. The characteristics of the study population are summarized in Table 1. Age was significantly higher in the CP group (medians 42, 46 and 54 years for EN, MF and CP respectively; p=0.038), while male to female ratio was lower in the CP group (percentage male 48 %, 65 % and 22 % in EN, MF and CP respectively; p=0.012). Because of these differences, comparisons between groups were adjusted for age and sex. The lymphocyte count (PBMC/ml blood) was similar in the three groups (medians 1.09, 0.97 and 0.92 for EN, MF and CP respectively), as well as the frequencies of T and B cells (data not shown).

Table 1. Study population characteristics.

	endemic uninfected (EN)	***microfilaremic (MF)***	***chronic pathology (CP)***	*p*	***total***
N	23	23	23		69
age (median [range])	42 [19 – 67]	46 [14 – 72]	54 [20 – 76]	0.038	46 [14 – 76]
sex (M / F)	11 / 12	15 / 8	5 / 18	0.012	31 / 38
malaria parasitemia (n of *Pf, Pv*)*	0	0	1,1	ns	2
PBMC/ml ($\cdot 10^6$) (median [range])	1.09 [0.6 – 2.3]	0.97 [0.7 – 5.6]	0.92 [0.7 – 1.6]	ns	0.98 [0.6 – 5.6]

** Pf Plasmodium falciparum; Pv Plasmodium vivax*

Filaria-specific proliferative responses of T and B cells are suppressed in microfilaremics

To analyze suppression of lymphocyte proliferation during filarial infection, cell proliferation to filarial antigen was determined by CFSE dilution in PBMC. Divided cell subsets were measured in activated T ($CD4^+CD25^+$) and in B ($CD19^+$) cell populations. Net T cell proliferation was lower in the MF group, which was mainly caused by high background proliferation in unstimulated condition (response to

medium, Figure 1A; age- and sex-adjusted means 2.66 %, -0.236 %, 4.02 % divided in EN, MF and CP respectively; p=0.043 for EN vs. MF, p=0.010 for MF vs. CP). Also B cell proliferation was lower in MF, shown in figure 1B (adjusted means 1.13 %, -1.11 %, 2.07 % divided for EN, MF and CP respectively; p=0.002 for EN vs. MF and p=0.0002 for MF vs. CP).

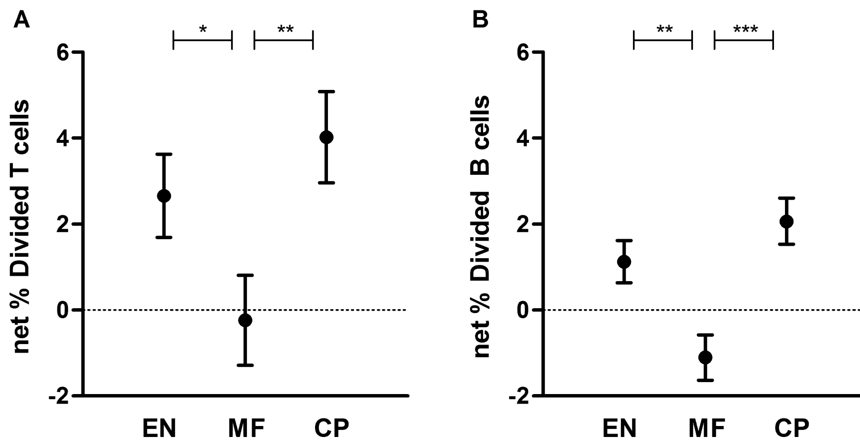

Figure 1. Suppressed T cell and B cell proliferative response to filaria antigen in microfilaremics. CFSE-labeled PBMC from uninfected endemic normals (EN), microfilaremic (MF) and chronic pathology (CP) subjects were stimulated with *Brugia malayi* adult worm antigen (BmA). After 4 days of culture cells were fixed, cryopreserved and after thawing CFSE division was analyzed by flow cytometry. Depicted are means and standard errors of net % divided subsets of $CD4^{+}CD25^{+}$ T cells (A) and $CD19^{+}$ B cells (B), adjusted for age and sex. * $p \leq 0.05$ **$p \leq .01$ ***$p \leq .001$

Lower filarial-specific Th1, Th2 and Th17 cytokine responses in MF

To assess the modulation of differentiated T helper cell subsets by filarial infection, hallmark cytokines for Th1 (IFN-γ), Th2 (IL-13), Th17 (IL-17) and regulatory (IL-10) responses were assessed in culture supernatants from cells stimulated with BmA (Figure 2). IFN-γ production was lower in the MF group than in EN or CP (adjusted means 573, 146 and 1318 pg/ml in EN, MF and CP respectively; p=0.004 for EN vs. MF, p=0.00007 for MF vs. CP). Both IL-17 and IL-13 levels were decreased in the MF group compared to EN, however were not different from CP (IL-17 adjusted means 130, 48 and 100 pg/ml; p=0.037 for EN vs. MF; IL-13 adjusted means 1472, 895 and 1276 pg/ml in EN, MF and CP respectively; p=0.029 for EN vs. MF). IL-10 production was similar in all three groups (adjusted means 333, 427 and 355 pg/ml in EN, MF and CP respectively). Spontaneous production of these cytokines was not significantly different between the groups and it was

noted that IFN-γ and IL-17 levels in BmA-stimulated PBMC supernatants were hardly above spontaneous (unstimulated) production, particularly in the MF group (Figure S1). After applying correction for multiple analyses, only IFN-γ levels were significantly lower in microfilaremic individuals.

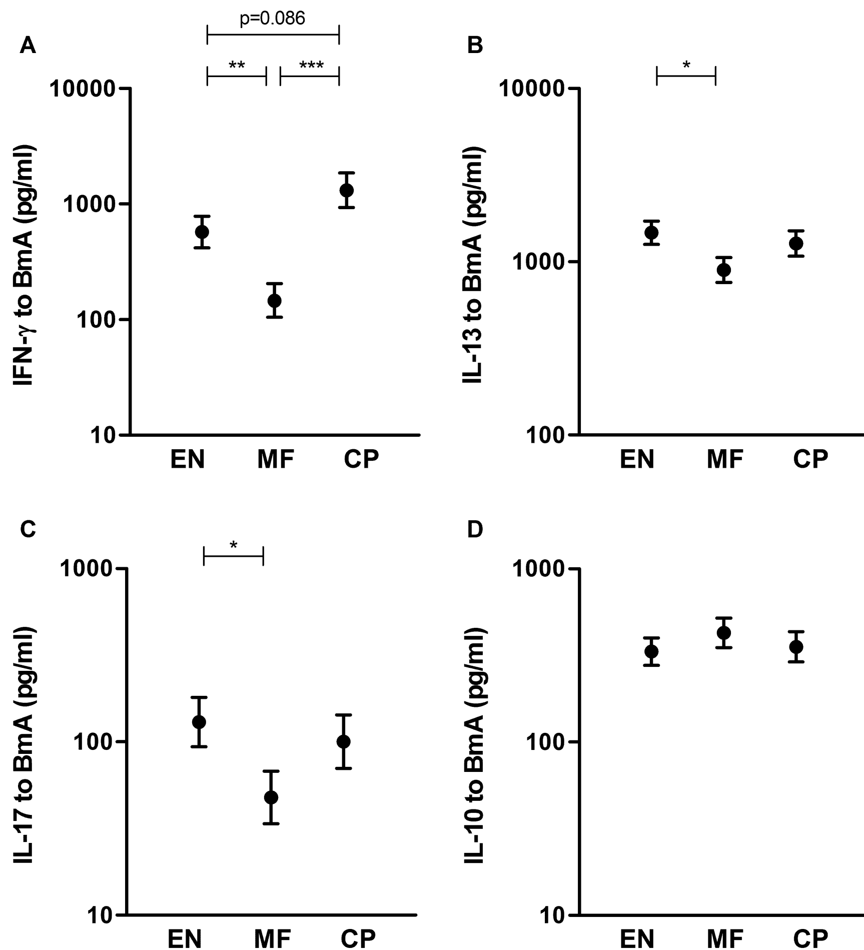

Figure 2. Altered filaria-specific cytokine production in different study groups. PBMC from uninfected endemic normals (EN), microfilaremic (MF) and chronic pathology (CP) subjects were stimulated with BmA. After 4 days of culture supernatants were harvested and assessed for IFN-γ (A), IL-13 (B), IL-17 (C) and IL-10 (D) production. Plotted values are age- and sex-adjusted means and standard errors; *p≤.05 **p≤.01 ***p ≤.001, p-values between 0.05 and 0.10 are indicated.

Similar Treg depletion in EN, MF and CP groups

To assess the functional contribution of Tregs to *in vitro* immune responses, we performed magnetic depletion of $CD4^{+}CD25^{hi}$ cells. By flow cytometry mock- and Treg-depleted PBMC were assessed for expression of CD25 and FOXP3 on CD4 T cells, of which a representative example is shown in Figure 3A. Treg frequencies decreased in most cases (Figure 3B), which was highly significant and similar in all three clinical groups (Figure 3C; $p=1.7 \cdot 10^{-4}$ for EN, $p=2.7 \cdot 10^{-5}$ for MF, $p=3.1 \cdot 10^{-5}$ for CP). Geometric mean of $CD25^{hi}FOXP3^{+}$ cell percentages of CD4 cells decreased from 1.69 % to 0.83 % after depletion (mean extent of depletion was 46.5 %). For 5 donors, 4 in EN and 1 in CP group, Treg frequency either could not be assessed or did not decrease after depletion, therefore these patients were excluded for further analysis.

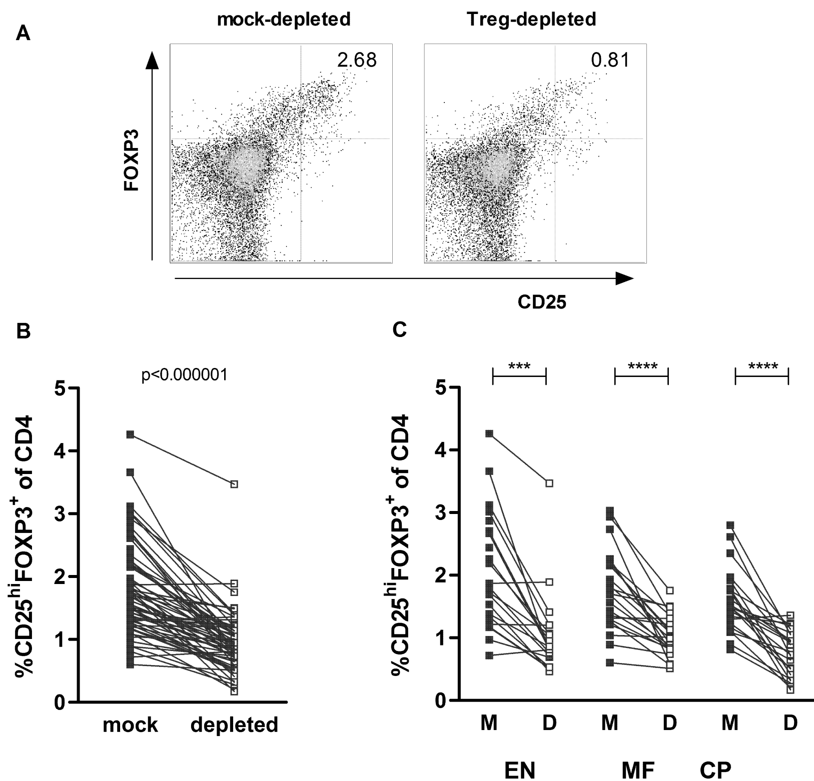

Figure 3. Efficient Treg depletion for all infection groups. $CD4^{+}CD25^{hi}$ T cells were isolated by magnetic bead separation. Treg frequencies were defined as percentages of $CD25^{hi}FOXP3^{+}$ cells from total $CD4^{+}$ fractions for mock- and $CD4^{+}CD25^{hi}$ cell -depleted PBMC that were cultured for 4 days in medium (representative example in A). Treg frequency of mock (M) and depleted (D) PBMC is shown for all donors (B) as well as for the different infection groups (C). Lines represent data points from one individual, data were analyzed by non-parametric paired tests; ***$p \leq 0.001$ ****$p \leq 0.0001$ or p-value as indicated.

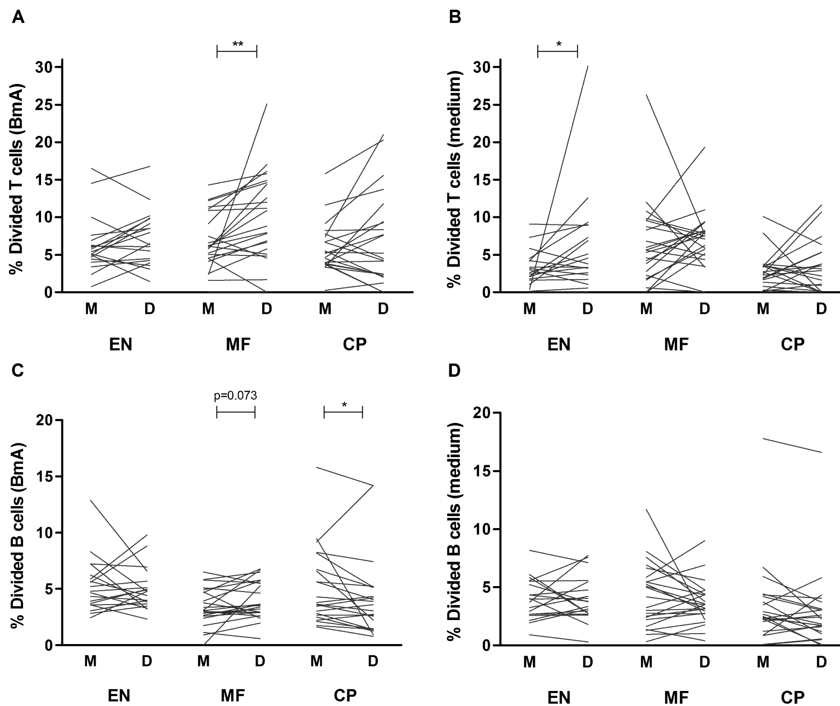

Figure 4. Suppressed lymphocyte proliferation is restored after Treg depletion in microfilaremics. Divided cell populations were assessed in mock- (M) and Treg-depleted (D) PBMC from EN, MF and CP subjects. CFSE dilution was analyzed for $CD4^+CD25^+$ T cells [26] and $CD19^+$ B cells (C-D). Cultures were stimulated with BmA (left panel; A&C) or left unstimulated (cultured with medium) (right panel; B&D). Plotted lines represent data points from single individuals; tested by Wilcoxon signed rank test, *p≤.05 **p≤.01, p-values between 0.05 and 0.10 are indicated.

Depletion of Tregs enhances filaria-specific lymphocyte proliferation

To evaluate the influence of Tregs on BmA-specific T and B cell proliferation, we analyzed CFSE dilution in $CD4^+CD25^+$ and $CD19^+$ cell subsets before and after Treg depletion. Here we present unadjusted proliferative responses to BmA and medium separately. For $CD4^+CD25^+$ effector T cells we observed an increase in proliferation to BmA in the MF group after removal of Tregs, whereas proliferative responses did not change significantly in EN or CP groups (Figure 4A; p=0.004 for MF). Treg depletion did not enhance spontaneous proliferation (medium condition) in MF-positives, however spontaneous responses were increased in uninfected individuals (Figure 4B, p=0.04 for EN). Interestingly, B cell proliferative responses in response to BmA were also enhanced in Treg-depleted conditions for MF patients, although this fell short of statistical significance (Figure 4C; p=0.07 for

MF). In contrast, after Treg depletion B cells proliferated to a lesser extent in CP patients (Figure 4C; p=0.01 for CP). Unstimulated B cell proliferative responses were not influenced by Treg removal (Figure 4D). To check whether Treg depletion completely restored lymphocyte proliferative responses in the MF group to levels seen in the other groups, we compared age- and sex-adjusted net proliferative responses of T and B cells to BmA in Treg-depleted conditions. Although responses in the CP group remained high for both T and B cells, T and B cell proliferation in the MF group was no longer different from EN individuals (Figure S2).

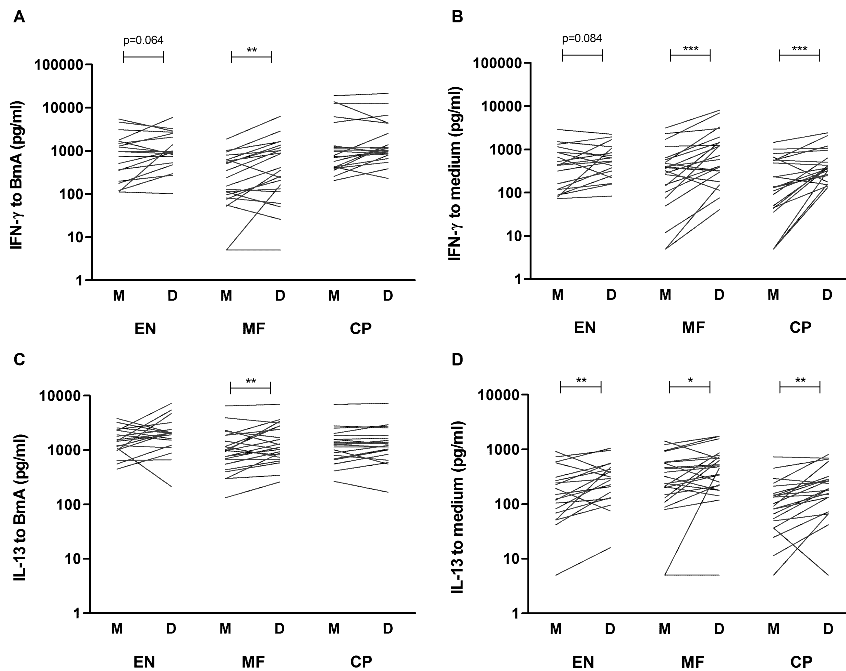

Figure 5. Removal of Tregs enhances filaria-specific Th1 and Th2 responses. Cytokine secretion was assessed in mock- (M) and Treg-depleted (D) PBMC cultures from EN, MF and CP individuals. IFN-γ [26] and IL-13 (C-D) secretion is depicted for BmA- (left panel) and unstimulated (right panel) conditions. Connecting lines represent data points of one individual, for mock- and Treg-depleted cultures; tested by paired t-test *p≤.05 **p≤.01***p≤.001, p-values between 0.05 and 0.10 are indicated.

Depletion of Tregs enhances filaria-specific Th2 responses

Next, we investigated the capacity of Tregs to suppress the filaria-specific cytokines by measuring IFN-γ, IL-13, IL-17 and IL-10 in response to BmA in culture supernatants of mock- and $CD4^+CD25^{hi}$ - depleted PBMC. In Figure 5, unmanipulated cytokine responses to BmA and medium are shown separately. Filaria-specific IFN-γ production was significantly upregulated after removal of Tregs in the MF group only (Figure 5A; p=0.064, p=0.004 for EN and MF respectively). However, the IFN-γ response to BmA was weak and similar in magnitude to responses seen in medium-stimulated PBMC, which also increased after depletion of Treg in MF as well as CP (Figure 5B; p=0.084, p=0.0002, p=0.001 for EN, MF and CP). With respect to IL-13, the response to BmA increased after depletion of Tregs in MF-positive individuals only (Figure 5C; p=0.41, p=0.008, p=0.20 for EN, MF and CP respectively). Spontaneous IL-13 production was low compared to BmA-stimulated conditions and also increased significantly upon removal of Treg, but this was still negligible compared to levels induced by BmA (Figure 5D; p=0.007 for EN, p=0.038 for MF and p=0.002 for CP). IL-17 and IL-10 responses before and after Treg depletion were comparable and unchanged in all three groups (data not shown).

Discussion

To investigate the function of Tregs in different infection and clinical groups of human filariasis, we studied the effect of Treg depletion on *in vitro* responses to BmA using human PBMC from individuals in an area endemic for *B. timori* lymphatic filariasis in Flores, Indonesia. Our main findings were diminished T and B cell proliferation as well as lower IFN-γ, IL-17 and IL-13 production in MF-positives, but similar IL-10 secretion compared to CP and EN groups. Treg depletion resulted in antigen-specific increase of lymphocyte proliferation and IL-13 responses in the MF group only.

Since our study population was not optimally age- and sex- matched, it was necessary to adjust for age and sex in the comparisons made between the infection groups. In studies on human filariasis it is often difficult to obtain comparable patient groups. One reason for this is the pathophysiology of this disease; microfilaremia can be present in all ages but particularly in young adults, while CP is an end stage disease that develops in older age. Importantly, a recent paper demonstrated a relevant effect of age on infection-induced regulatory immune responses; intensity of infection with *Schistosoma haematobium* was positively correlated with Treg frequency in the age group 8 – 13 years, while the opposite was observed for the group older than 14 years[13]. Age and sex should thus be taken into account carefully when interpreting cellular immunological data.

Lymphocyte proliferation in filariasis has been studied since the 1970s and is consistently shown to be diminished in microfilaremic patients, including previous population studies by our group in Sulawesi, Indonesia[14-19]. We have now established that the well-described T cell hyporesponsiveness can be measured by CFSE dilution assays in PBMC stimulated with BmA, and also show that in addition to T cells, B cell proliferation is considerably lower in MF-positives. Previously, it has been shown that the functional capacity of B cells, in terms of specific IgE and IgG production, was lower in MF versus CP patients[20,21]. Here, we extend this to B cell proliferation, showing for the first time to our knowledge B cell proliferative hyporesponsiveness in microfilaremics. Interestingly, despite lower IgG found in earlier studies, the number of positive individuals for filaria-specific IgG4, an isotype shown to be associated with elevated plasma IL-10[22], was higher in microfilaremics (data not shown). It is tempting to speculate that B cells in MF subjects that are hyporesponsive are also contributing to immune regulation by producing IL-10 and IgG4, as is suggested for venom-specific B cells from beekeepers (reviewed in).[23]

The suppressed cytokine responses in MF-positive individuals here correspond with a recent study, showing higher IFN-γ and IL-17 responses to BmA in chronic pathology patients compared to MF-positive individuals[8]. In microfilaremics IFN-γ and IL-17 production were not induced above background levels; this is also in line

with the findings by Babu *et al.*, who analyzed the production of IFN-γ and the expression of IL-17 mRNA in 24h BmA-stimulated PBMC[8]. However, Treg removal did not affect the Th1 and Th17 cytokines, which may suggest that these cytokines are not regulated by Tregs. IL-13 production in response to BmA was increased after Treg depletion, however this result must be considered with caution, since medium responses were also changed. Since IL-10 levels were high in MF before as well as after Treg depletion, IL-10 derived from $CD4^{+}CD25^{-}$ T cells could be responsible for the observed decreased cytokine responses in microfilaremics, supported by two studies which showed the majority of IL-10 during filarial infection was produced by effector T cells, despite higher Tregs in the MF group[11,24].

Contrary to our expectations, Treg depletion had little or no effect on BmA responses in the other groups, although these individuals live in a filaria-endemic area and do have filaria-specific proliferative and cytokine responses. One explanation might be that active Tregs in MF are filaria- or BmA-specific, which are only actively induced and/or expanded during patent microfilaremia. Since there are very few studies on the function of Tregs in human helminth infections, it would be interesting for future studies to determine antigen specificity and functional characteristics of the Tregs in the different study groups. Furthermore, due to limited number of available cells we were unable to determine the mechanisms by which this $CD4^{+}CD25^{hi}$ subset affects immune responses; an area that should be investigated in the future. A previous study concluded that *in vitro* blockade of CTLA-4 and PD-1 reverted suppression of *M.tuberculosis*-specific immune responses, suggesting cell-contact mediated mechanisms of suppression during microfilaremia[25].

Regarding the limitations of the current study, we were not able to evaluate whether the Treg depletion procedure has led to depletion of any other cell subsets, as a possible explanation for the reduced B cell proliferation in CP. In addition, our plan to confirm previous studies that show higher FOXP3 in *ex vivo* PBMC of MF patients failed due to a technical problem with FACS staining of FOXP3. We only had 4-days cultured PBMC that we could stain for FOXP3 and thereby we were able to show the depletion of $CD25^{hi}FOXP3^{+}$ cells. However the level of CD25 and FOXP3 in medium-cultured cells may not be fully representative for the circulating levels of Tregs. Nevertheless, although important to gather data on Treg frequencies, the primary objective of our study was to assess their functional capacity in different infection and clinical groups. It should also be mentioned that our previous study of geohelminth infection in Indonesia indicated that it was not the number but the suppressive capacity of Tregs which was altered in infected children[12].

In conclusion, we report active contribution of Tregs to modulation of T and B cell proliferation and polarized cytokine production by effector T cells in MF-positive

individuals in Flores, Indonesia. Since chronic lymphedema appears to be concurrent with lack of Treg-associated suppressive capacity, further research on targeted activation of specific Tregs would be important to be able to decrease the morbidity and disabilities induced by LF.

References

1. WHO. Health Topics: Filariasis. (2010).
2. Chu, B.K., Hooper, P.J., Bradley, M.H., McFarland, D.A. & Ottesen, E.A. The economic benefits resulting from the first 8 years of the Global Programme to Eliminate Lymphatic Filariasis (2000-2007). *PLoS neglected tropical diseases* 4, e708 (2010).
3. Oqueka, T.*, et al.* Impact of two rounds of mass drug administration using diethylcarbamazine combined with albendazole on the prevalence of Brugia timori and of intestinal helminths on Alor Island, Indonesia. *Filaria journal* 4, 5 (2005).
4. Maizels, R.M. & Yazdanbakhsh, M. Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nature reviews. Immunology* 3, 733-744 (2003).
5. Babu, S. & Nutman, T.B. Proinflammatory cytokines dominate the early immune response to filarial parasites. *J Immunol* 171, 6723-6732 (2003).
6. O'Connor, R.A., Jenson, J.S., Osborne, J. & Devaney, E. An enduring association? Microfilariae and immunosuppression [correction of immunosupression] in lymphatic filariasis. *Trends in parasitology* 19, 565-570 (2003).
7. Babu, S., Blauvelt, C.P., Kumaraswami, V. & Nutman, T.B. Regulatory networks induced by live parasites impair both Th1 and Th2 pathways in patent lymphatic filariasis: implications for parasite persistence. *J Immunol* 176, 3248-3256 (2006).
8. Babu, S.*, et al.* Filarial lymphedema is characterized by antigen-specific Th1 and th17 proinflammatory responses and a lack of regulatory T cells. *PLoS neglected tropical diseases* 3, e420 (2009).
9. Belkaid, Y. Regulatory T cells and infection: a dangerous necessity. *Nature reviews. Immunology* 7, 875-888 (2007).
10. Taylor, M.D.*, et al.* Early recruitment of natural CD4+ Foxp3+ Treg cells by infective larvae determines the outcome of filarial infection. *European journal of immunology* 39, 192-206 (2009).
11. Metenou, S.*, et al.* At homeostasis filarial infections have expanded adaptive T regulatory but not classical Th2 cells. *J Immunol* 184, 5375-5382 (2010).
12. Wammes, L.J.*, et al.* Regulatory T cells in human geohelminth infection suppress immune responses to BCG and Plasmodium falciparum. *European journal of immunology* 40, 437-442 (2010).
13. Nausch, N., Midzi, N., Mduluza, T., Maizels, R.M. & Mutapi, F. Regulatory and activated T cells in human Schistosoma haematobium infections. *PloS one* 6, e16860 (2011).
14. Lammie, P.J.*, et al.* Bancroftian filariasis in Haiti: preliminary characterization of the immunological responsiveness of microfilaremic individuals. *The American journal of tropical medicine and hygiene* 38, 125-129 (1988).
15. Nutman, T.B. & Kumaraswami, V. Regulation of the immune response in lymphatic filariasis: perspectives on acute and chronic infection with Wuchereria bancrofti in South India. *Parasite immunology* 23, 389-399 (2001).
16. Ottesen, E.A., Weller, P.F. & Heck, L. Specific cellular immune unresponsiveness in human filariasis. *Immunology* 33, 413-421 (1977).
17. Piessens, W.F.*, et al.* Immune responses in human infections with Brugia malayi: specific cellular unresponsiveness to filarial antigens. *The Journal of clinical investigation* 65, 172-179 (1980).
18. Sartono, E.*, et al.* Reversal in microfilarial density and T cell responses in human lymphatic filariasis. *Parasite immunology* 21, 565-571 (1999).
19. Yazdanbakhsh, M.*, et al.* T cell responsiveness correlates differentially with antibody isotype levels in clinical and asymptomatic filariasis. *The Journal of infectious diseases* 167, 925-931 (1993).

20. King, C.L., *et al.* Immunologic tolerance in lymphatic filariasis. Diminished parasite-specific T and B lymphocyte precursor frequency in the microfilaremic state. *The Journal of clinical investigation* 89, 1403-1410 (1992).
21. Nutman, T.B., Kumaraswami, V., Pao, L., Narayanan, P.R. & Ottesen, E.A. An analysis of in vitro B cell immune responsiveness in human lymphatic filariasis. *J Immunol* 138, 3954-3959 (1987).
22. Adjobimey, T. & Hoerauf, A. Induction of immunoglobulin G4 in human filariasis: an indicator of immunoregulation. *Annals of tropical medicine and parasitology* 104, 455-464 (2010).
23. Hussaarts, L., van der Vlugt, L.E., Yazdanbakhsh, M. & Smits, H.H. Regulatory B-cell induction by helminths: implications for allergic disease. *The Journal of allergy and clinical immunology* 128, 733-739 (2011).
24. Mitre, E., Chien, D. & Nutman, T.B. CD4(+) (and not CD25+) T cells are the predominant interleukin-10-producing cells in the circulation of filaria-infected patients. *The Journal of infectious diseases* 197, 94-101 (2008).
25. Babu, S., *et al.* Human type 1 and 17 responses in latent tuberculosis are modulated by coincident filarial infection through cytotoxic T lymphocyte antigen-4 and programmed death-1. *The Journal of infectious diseases* 200, 288-298 (2009).
26. Peisong, G., *et al.* An asthma-associated genetic variant of STAT6 predicts low burden of ascaris worm infestation. *Genes and immunity* 5, 58-62 (2004).

Supplementary material

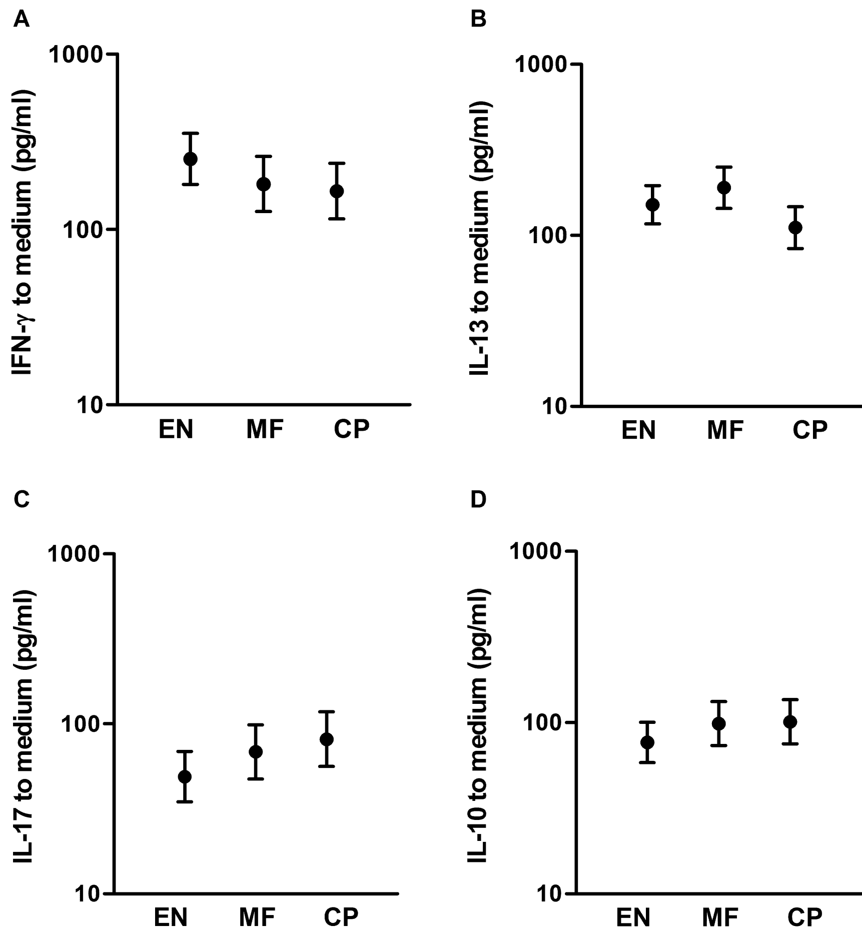

Figure S1. Similar spontaneous cytokine production in different disease stages. Culture supernatants of unstimulated PBMC from EN, MF and CP subjects were assessed for IFN-γ (A), IL-17 (B), IL-13 (C) and IL-10 (D) production. Plotted values are age- and sex-adjusted means and standard errors. Mean values are not different between the three groups for all cytokines.

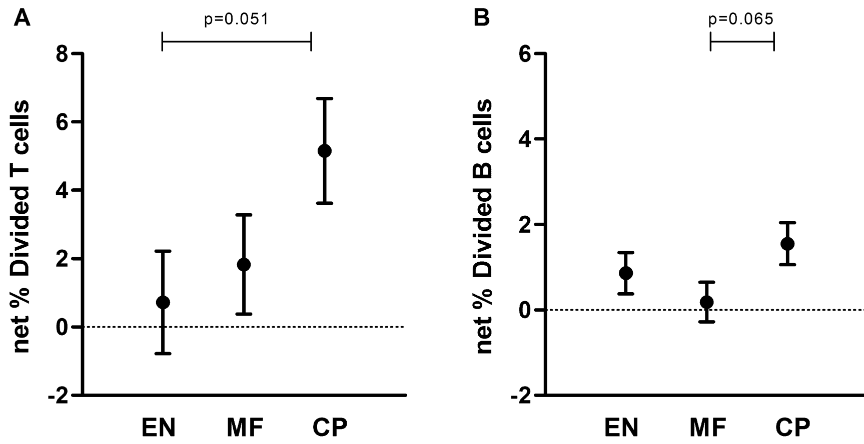

Figure S2. Similar lymphocyte proliferative responses to filaria antigen in Treg-depleted conditions. Divided cell populations were assessed in Treg-depleted PBMC from EN, MF and CP subjects using CFSE dilution analysis. Depicted are means and standard errors of net % divided subsets of $CD4^{+}CD25^{+}$ T cells (A) and $CD19^{+}$ B cells (B), adjusted for age and sex. p-values between 0.05 and 0.10 are indicated.

Chapter 5

Regulatory T cells in human geohelminth infection suppress immune responses to BCG and *Plasmodium falciparum*

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Abstract

Background Chronic helminth infections induce T cell hyporesponsiveness, which may affect immune responses to other pathogens or to vaccines.
Methods This study investigates the influence of regulatory T cell (Treg) activity on proliferation and cytokine responses to BCG and *P. falciparum*-parasitized red blood cells (PfRBC) in Indonesian schoolchildren.
Results Geohelminth-infected children's *in vitro* T cell proliferation to either BCG or PfRBC was reduced compared to that of uninfected children. Although the frequency of $CD4^{+}CD25^{hi}FOXP3^{+}$ T cells was similar regardless of infection status, the suppressive activity differed between geohelminth-infected and -uninfected groups: antigen-specific proliferative responses increased upon $CD4^{+}CD25^{hi}$ T cell depletion in geohelminth-infected subjects only. In addition, IFN-γ production in response to both BCG and PfRBC was increased after removal of $CD4^{+}CD25^{hi}$ T cells.
Conclusions These data demonstrate that geohelminth-associated Treg influence immune responses to bystander antigens of mycobacteria and plasmodia. Geohelminth-induced immune modulation may have important consequences for co-endemic infections and vaccine trials.

Introduction

Rural parts of Indonesia, particularly on islands further away from the more developed areas of Java, are characterized by a traditional lifestyle and by high burdens of parasitic infections such as geohelminths and malaria. One of the hallmarks of chronic helminth infections is induction of T cell hyporesponsiveness[1]. While the mechanisms involved may be multiple, several studies have pointed towards the possible involvement of natural and inducible T regulatory (Treg) cells in downregulating effector T cell responses upon chronic infection[2]. A limited number of studies have been performed on Treg dynamics in human helminth infection. *Schistosoma mansoni* infected subjects in Kenya had higher $CD4^{+}CD25^{hi}$ T cell levels compared to uninfected individuals and the numbers decreased after treatment[3]. In lymphatic filariasis, patients show decreased Th1 and Th2 cell frequencies, which might in part be explained by the upregulation of expression of Treg associated FOXP3, TGF-β and CTLA-4 in response to live *B. malayi* parasites[4].
Interestingly, it has also been shown that helminth infections can affect responses to unrelated antigens, such as those expressed in vaccines or by other pathogens[5]. Geohelminth infections have, for example, been associated with reduced immune responses to BCG vaccination[6] and to the cholera vaccine[7]. With respect to co-infections, epidemiological studies in areas where helminths and *Plasmodium spp.* are co-endemic, have so far not clarified whether there is a detrimental or beneficial interaction (reviewed in [5] and [8]). At the immunological level, a recent study has shown higher IL-10 responses to malaria antigens in children infected with *S. haematobium* and/or geohelminths such as *Ascaris lumbricoides*, *Trichuris trichiura* and hookworm[9]. These results would support the recently proposed hypothesis that helminth infections might facilitate the establishment of malaria infection through compromising immune responses, while simultaneously may prevent severe malaria-related pathology through counteracting strong inflammation[10].
While numerous studies in experimental models have provided evidence for increased $FOXP3^{+}$ Treg function during different helminth infections, only a few studies have addressed the functional capacity of these human Treg. To investigate Treg activity in geohelminth infections, we have analyzed Treg frequencies and immune responses to BCG and *P. falciparum*-parasitized red blood cells (PfRBC) in geohelminth-infected and -uninfected subjects from a rural area of Flores island, Indonesia. Proliferative responses to BCG and PfRBC were lower in helminth-infected compared to -uninfected children. After $CD4^{+}CD25^{hi}$ T cell depletion, proliferation and IFN-γ production were increased in response to both stimuli, but only in infected children, suggesting differential Treg activity as a consequence of geohelminth infections.

Methods

Study population and parasitological diagnostics

The study was approved by the Committee of the Medical Research Ethics of the University of Indonesia. Study participants were recruited from a primary school in Welamosa village on Flores Island, Indonesia, where preliminary surveys showed 65% prevalence of geohelminth infections. Informed consent was obtained from either parents or guardians and single stool samples were collected. Fresh stool samples were processed according to the Harada Mori method to detect hookworm larvae and formalin preserved stool was prepared using the formol-ether acetate concentration and microscopically assessed for eggs of the soil transmitted helminths (STH) *Ascaris lumbricoides*, *Trichuris trichiura* and hookworm species. Children were considered geohelminth-positive if either Harada Mori or microscopy results were positive. Blood slides were screened for the presence of malaria parasites and quantitative PCR analysis was used to detect *Plasmodium spp.* in whole blood. Heparinized venous blood was drawn from 20 children, 10 helminth-positive and 10 helminth-negative.

Cell isolation, depletion and phenotyping

Peripheral blood mononuclear cells (PBMC) were obtained by gradient centrifugation of heparinized venous blood over Ficoll. $CD4^+CD25^{hi}$ T cells were isolated by magnetic cell sorting (MACS) using the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec GmBH, Bergisch Gladbach, Germany). According to the protocol recommended by the manufacturer a two-step isolation was performed, firstly isolating $CD4^+$ cells and secondly enriching for $CD25^{hi}$ T cells using a (suboptimal) concentration of CD25 MicroBeads. $CD4^+CD25^{-/low}$ T cells and $CD4^-$ cells together were considered as Treg-depleted PBMC. For the total PBMC populations the obtained cells were added back (mock depletion). For 3 donors the depletion was not successful (no decrease in Treg frequency after depletion) and these donors were excluded for analysis of depletion effects. Mean depletion was 62.9% (range 20.9 – 100%).

To analyze Treg phenotype, PBMC were fixed and permeabilized with a FOXP3 Staining set (eBioscience Inc., San Diego, CA, USA) and stained with fluorochrome labeled anti-CD3, anti-CD4, anti-CD25, anti-CTLA-4 (BD Biosciences, Franklin Lakes, NJ, USA), anti-FOXP3 (Miltenyi) and anti-GITR (R&D Systems, Minneapolis, MN, USA) antibodies.

BrdU proliferation assay

To monitor proliferation BrdU incorporation was assessed using the BrdU Flow Kit (BD). Total and $CD4^+CD25^{hi}$ depleted PBMC were cultured in RPMI 1640 (Gibco, Invitrogen, Carlsbad, CA, USA) supplied with 10% FCS (Greiner Bio-One GmbH,

Frickenhausen, Germany) and 10 μM BrdU. BCG (Bio Farma, Bandung, Indonesia, 0.5 μg/ml), $1x10^6$ *P. falciparum* parasitized red blood cells (PfRBC) or $1x10^6$ uninfected RBC (uRBC) were used for stimulation. After 96h cells were fixed in 2% formaldehyde (Sigma-Aldrich, CA, USA) and preserved at -20°C. After thawing, cells were permeabilized and incubated with DNase (Sigma-Aldrich), labeled with anti-BrdU, anti-CD4 and anti-CD25 antibodies (BD), acquired and analyzed. Proliferation of effector T cells was defined as the percentage of BrdU-positive cells within the $CD4^+CD25^+$ T cell population.

Cytokine multiplex analysis

Cytokine production was assessed using the Multiplex Bead Immunoassay for interferon-gamma (IFN-γ), interleukins (IL)-5, and -13 according to the supplied protocol (Biosource, Invitrogen, Carlsbad, CA, USA). Samples acquired with Luminex 100™ xMAP technology (Luminex Corp., Austin, TX, USA). Half the detection limit supplied by the manufacturer was used, relevant background values (control medium for BCG, uRBC for PfRBC) were subtracted and zero or negative values were set at 1 pg/ml.

Data analysis

Statistical analysis was performed in SPSS 14.0. Comparisons of basic phenotypes and responses were tested with Mann-Whitney test for data not normally distributed. For total versus depleted samples paired analysis was done using Wilcoxon Signed Ranks Test. In the multiplex cytokine analysis Bonferroni correction was applied by multiplying the p-values by the number of non-correlated measurements.

Results

Study population

School-age children were recruited from Welamosa primary school. Stools were microscopically examined for soil-transmitted helminth (STH) eggs and two groups of 10 children, either geohelminth-infected or -uninfected were included for immunological studies. Within the geohelminth-infected children 4 had *Ascaris lumbricoides*, 4 had hookworms, 1 had both *A. lumbricoides* and hookworm and 1 had both *Trichuris trichiura* and hookworm infections. *Plasmodium* spp. infections were absent as determined both by microscopy and by quantitative PCR analysis of donor blood. The median age (11 years) and gender ratio was identical in geohelminth-infected and -uninfected groups of children.

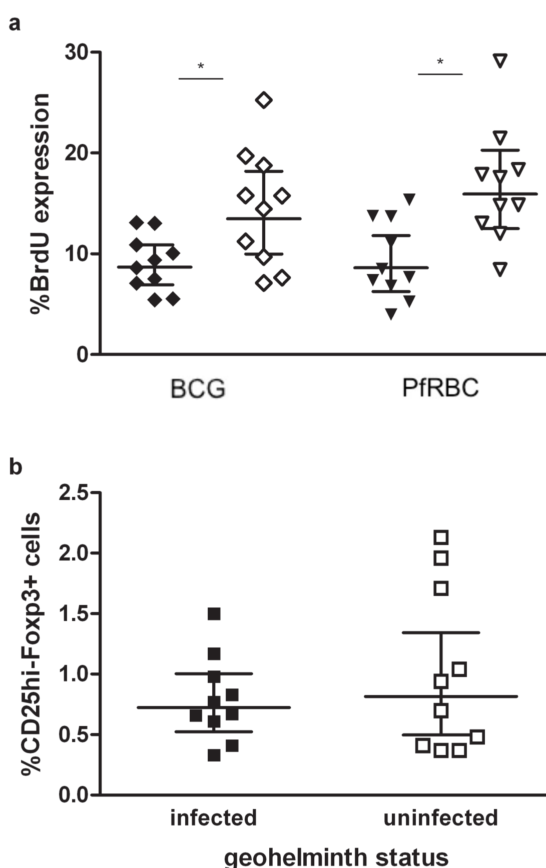

Figure 1. Helminth-infected children show lower T cell proliferation responses but similar Treg frequency. Donors were grouped by infection status; geohelminth-infected and -uninfected (both n=10) groups are shown by filled and open symbols respectively. (a) Proliferation was analyzed by flow cytometric analysis of BrdU incorporation by $CD4^{+}CD25^{+}$ T cells. Helminth-positive donors showed lower proliferative responses to both BCG (◆; p=0.021) and PfRBC (▼; p=0.005) stimulation. (b) Donor-derived PBMC were analyzed for CD25 and FOXP3 expression by flow cytometry. CD25 and FOXP3 co-expression in $CD4^{+}$ T cells was compared in the two groups and revealed no significant differences (p=0.68). Analysis of infected versus uninfected groups was performed using the Mann-Whitney test; lines represent geometric mean with 95% confidence intervals; *p≤0.05 **p≤0.01.

Lower BCG or PfRBC-induced proliferation in geohelminth-infected children

To determine the immunological reactivity of geohelminth-infected versus -uninfected children, we analyzed antigen-specific T cell responses to BCG vaccine, *P. falciparum*-parasitized RBC (PfRBC) or uninfected (u)RBC. BrdU incorporation by $CD4^+CD25^+$ cells was assessed to measure effector T cell proliferation. T cell proliferation to BCG and PfRBC was lower in helminth-infected children (Figure 1a) compared to uninfected children (geomeans 8.7% vs.13.5% and 8.6% vs.15.9%; p-values 0.021 and 0.005 respectively), whereas proliferation in medium only or in response to uRBC did not differ between the groups (data not shown).

Similar Treg frequency in geohelminth-infected and -uninfected children

As the observed helminth-dependent differences in immune responses could be the result of helminth-induced Treg, $CD25^{hi}$-$FOXP3^+$ T cell numbers and costimulatory molecules were compared in helminth-infected and -uninfected individuals. Similar proportions of $CD4^+$ T cells from the two groups expressed CD25 (20% vs. 25%; p=0.85), and there were similar populations of $CD25^{hi}$ T expressing cells (5.4% vs. 4.7%; p=0.57) as well as of $CD25^{hi}$-FOXP3 co-expressing T cells (0.7% vs. 0.8%; p=0.68; Figure 1b) in the $CD4^+$ population. In a subset of the donors the expression of the activation markers CTLA-4 and GITR was assessed. Within these small sub-groups (4 infected and 7 uninfected), no significant differences were observed in expression of these two markers on either $CD4^+FOXP3^+$ or $CD4^+CD25^{hi}FOXP3^+$ T cells (data not shown).

5

Higher suppressive Treg activity in geohelminth-infected children

To examine the functional capacity of Treg, $CD4^+CD25^{hi}$ T cells were depleted from PBMC by magnetic beads. Following $CD4^+CD25^{hi}$ T cell depletion, $CD4^+CD25^{hi}$ T cell populations decreased from 1.74% to 0.67% and in parallel the $CD4^+CD25^{hi}$-$FOXP3^+$ population diminished from 0.90% to 0.33% (p<0.001 for both, Figure 2a) in total $CD4^+$ T cells. In 3 donors with very low numbers of $CD4^+CD25^{hi}$ T cells, depletion failed and they were excluded from further analysis.

Proliferation in response to different stimuli was measured in $CD4^+CD25^{hi}$ T cell-depleted and mock-depleted populations. Segregation according to geohelminth infection status revealed a significant increase in the proliferative response to BCG in samples from geohelminth-infected children following depletion of $CD4^+CD25^{hi}$ T cells (geomeans 9.1% to 12.8%; p=0.008), an effect that was not observed in the equivalent samples from geohelminth-uninfected children (geomeans 15.0% and 12.8%, p=0.83; Figure 2b). Significantly enhanced proliferation in response to PfRBC after Treg depletion was also seen in samples from helminth-infected

(geomeans 8.8% to 12.7%; p=0.038) but not in those from -uninfected children (geomeans 17.9% and 18.7%, p=0.87; Figure 2b). No such differences were seen in response to uRBC (Figure 2b). In geohelminth-infected subjects, proliferative responses to BCG and PfRBC in depleted PBMC were equivalent to levels found in uninfected children. Interestingly, enhanced IFN-γ production in response to either BCG- or PfRBC-stimulation after depletion was also only observed in samples from the geohelminth-infected children (geomeans for BCG 46.7 to 66.8 pg/ml and for PfRBC 313.8 to 574.3 pg/ml; Figure 2c), while IL-5 or IL-13 production was unchanged (data not shown).

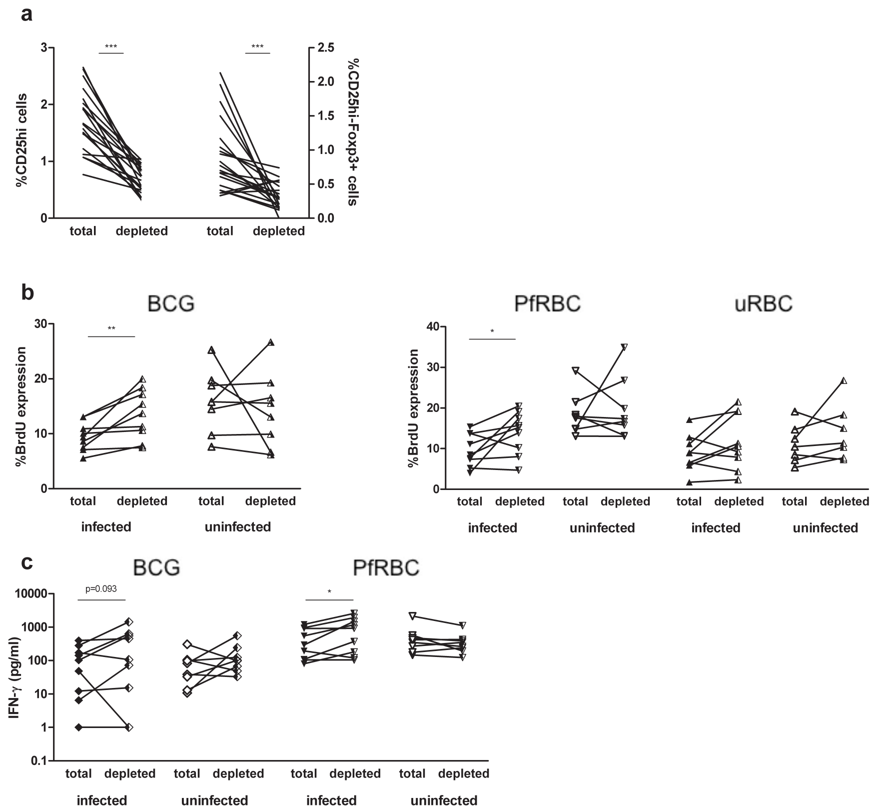

Figure 2. Treg depletion restores BCG and PfRBC-induced proliferation and enhances antigen-specific IFN-γ production in geohelminth infected children. (a) $CD4^{+}CD25^{hi}$ T cells were isolated by magnetic bead separation. The 'total' and 'depleted' data points and the connecting line represent paired data within one individual, for whole PBMC and for PBMC depleted of $CD4^{+}CD25^{hi}$ T cells, respectively. Depletion was effective in terms of $CD25^{hi}$ (left panel) and CD25-FOXP3 co-expressing (right panel) cell percentages within $CD4^{+}$ T cells (both $p<0.001$). Only donors with decreasing Foxp3 and or $CD25^{hi}$ expression were taken into further analysis (9 infected and 8 uninfected donors). (b) Effect of Treg depletion is shown for proliferation in response to BCG, PfRBC and uRBC. Only in the helminth-infected groups cell proliferation increased significantly after depletion, in response to both BCG- (infected p=0.008 vs. uninfected p=0.83) and PfRBC- (p=0.038 vs. p=0.87) stimulation. For uRBC no differences were found (p=0.17 vs. p=0.16). (c) Before and after Treg depletion, IFN-γ production was measured in day 4 cell culture supernatants. Treg depletion upregulated IFN-γ production in response to BCG or PfRBC in helminth-infected children only (p=0.093 and p=0.025; after Bonferroni correction p=0.186 and p=0.05 respectively). *p≤0.05 **p≤0.01 ***p≤0.001; analysis by Wilcoxon Signed Rank Test and Bonferroni correction for the multiplexed cytokines.

Discussion

Geohelminth infections are usually found in areas co-endemic for multiple infectious agents and may increase susceptibility to other important tropical diseases such as malaria, HIV and tuberculosis[5]. Furthermore the presence of geohelminths may impair responses to vaccines[11]. These issues have recently lead to priority recommendations for the research agenda in Europe[12]. To explore cellular immune mechanisms underlying helminth-induced hyporesponsiveness, we have performed *in vitro* Treg depletion experiments with PBMC isolated from groups of geohelminth-infected and -uninfected school children living in a rural area of Flores Island, Indonesia. The data presented here show lower proliferative responses to BCG and to parasitized RBC in geohelminth-infected compared to uninfected children. These effects were not associated with a concomitant higher number of FOXP3$^+$ Treg in those infected; however, T cell proliferative responses to both BCG and PfRBC were restored after Treg depletion. Depletion also enhanced IFN-γ responses to both stimuli, demonstrating a generalized suppression of Th1 cells by geohelminth-induced Treg.

Although the observed suppression of immune responses in helminth infection was not associated with higher Treg numbers, our data do indicate increased functional Treg activity as a result of geohelminth infection. CD4$^+$CD25^{hi} T cell depletion significantly enhanced specific immune responses to BCG and Plasmodium-infected RBC in infected individuals only, implying a specific immunomodulatory effect during persistent geohelminth infections. Proliferative and IFN-γ responses were not correlated, which indicates that increased cytokine production is not associated with higher cell numbers. This observation would suggest that Treg are indeed able to influence the capacity of individual cells to produce effector cytokines. Despite the fact that some effector T cells in the CD25$^+$ T cell compartment may be removed along with depletion of Treg, we still see clear upregulation in T cell proliferation and IFN-γ production to BCG and PfRBC. Moreover, since Th2 cytokines were not affected, the enhancement of Th1 responses was not attributable to the removal of counteracting Th2 cells.

One of the few studies performed on Treg in human helminth infection showed expansion of Treg in schistosomiasis[3]. In our limited group of subjects, no differences in FOXP3, GITR or CTLA-4 expressing T cells were seen. This is in line with a number of studies that show no differences in Treg frequencies, but do in Treg activity, consistent with our data. For example, in lymphatic filarial patients from India expression of the Treg activation markers CTLA-4 and PD-1 was only different in infected versus uninfected individuals once cells had been stimulated *in vitro*[4]. In addition, studies with cells from patients with autoimmune diseases have reported comparable results: patients with either diabetes or multiple

sclerosis displayed Treg numbers characteristic of healthy controls, but Treg suppressive capacity was changed in diseased subjects[13,14].

In this study FOXP3$^+$ Treg appeared to be more active in helminth-infected children. Geohelminth-induced Treg activity might be able to control and divert selective proliferative and cytokine responses to third party antigens such as vaccine antigens or other pathogens. Helminths are usually found in areas where multiple tropical infections are endemic and where prevention of mortality through vaccination is of crucial importance. Therefore, the immunological background of target populations and their geohelminth infection status should be taken into careful consideration when designing mass vaccination strategies. Further studies are needed to assess the effect of helminths on the development of protective immunity to other infections.

References

1. Maizels, R.M., Bundy, D.A., Selkirk, M.E., Smith, D.F. & Anderson, R.M. Immunological modulation and evasion by helminth parasites in human populations. *Nature* 365, 797-805 (1993).
2. Belkaid, Y. Role of Foxp3-positive regulatory T cells during infection. *European journal of immunology* 38, 918-921 (2008).
3. Watanabe, K., *et al.* T regulatory cell levels decrease in people infected with Schistosoma mansoni on effective treatment. *The American journal of tropical medicine and hygiene* 77, 676-682 (2007).
4. Babu, S., Blauvelt, C.P., Kumaraswami, V. & Nutman, T.B. Regulatory networks induced by live parasites impair both Th1 and Th2 pathways in patent lymphatic filariasis: implications for parasite persistence. *J Immunol* 176, 3248-3256 (2006).
5. van Riet, E., Hartgers, F.C. & Yazdanbakhsh, M. Chronic helminth infections induce immunomodulation: consequences and mechanisms. *Immunobiology* 212, 475-490 (2007).
6. Elias, D., Britton, S., Aseffa, A., Engers, H. & Akuffo, H. Poor immunogenicity of BCG in helminth infected population is associated with increased in vitro TGF-beta production. *Vaccine* 26, 3897-3902 (2008).
7. Cooper, P.J., *et al.* Human infection with Ascaris lumbricoides is associated with suppression of the interleukin-2 response to recombinant cholera toxin B subunit following vaccination with the live oral cholera vaccine CVD 103-HgR. *Infection and immunity* 69, 1574-1580 (2001).
8. Druilhe, P., Tall, A. & Sokhna, C. Worms can worsen malaria: towards a new means to roll back malaria? *Trends in parasitology* 21, 359-362 (2005).
9. Hartgers, F.C., *et al.* Responses to malarial antigens are altered in helminth-infected children. *The Journal of infectious diseases* 199, 1528-1535 (2009).
10. Specht, S. & Hoerauf, A. Does helminth elimination promote or prevent malaria? *Lancet* 369, 446-447 (2007).
11. Borkow, G. & Bentwich, Z. Chronic parasite infections cause immune changes that could affect successful vaccination. *Trends in parasitology* 24, 243-245 (2008).
12. Boraschi, D., *et al.* Immunity against HIV/AIDS, malaria, and tuberculosis during co-infections with neglected infectious diseases: recommendations for the European Union research priorities. *PLoS neglected tropical diseases* 2, e255 (2008).
13. Lindley, S., *et al.* Defective suppressor function in CD4(+)CD25(+) T-cells from patients with type 1 diabetes. *Diabetes* 54, 92-99 (2005).
14. Viglietta, V., Baecher-Allan, C., Weiner, H.L. & Hafler, D.A. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *The Journal of experimental medicine* 199, 971-979 (2004).

CHAPTER 6

Three-monthly albendazole treatment alleviates geohelminth-induced immune hyporesponsiveness; results of a double blind placebo-controlled household-randomized trial

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– Manuscript in preparation –

Abstract

Background Chronic helminth infections are proposed to induce cellular immune hyporesponsiveness, which secures their long-term survival in their host, but which also may affect immune responses to unrelated antigens. As there are several other causes of immunosuppression, we conducted a household-clustered RCT to evaluate the specific effect of deworming on cellular immune responses in a helminth-endemic area in Indonesia.

Methods Cytokine (IL-2, IL-5, IL-10, IFN-γ and TNF) responses to antigens and mitogens were assessed in 1059 subjects at baseline, 9 and 21 months after three-monthly treatment with either albendazole or placebo.

Results This intensive treatment resulted in significant increase in malaria-specific and mitogen-induced TNF and IFN-γ responses. This effect was not associated with changes in cell counts or BMI.

Conclusions These findings establish unequivocally that helminth infections suppress pro-inflammatory responses, which may help to understand the possible protective effect of helminths on inflammatory diseases in rural areas of the world.

Introduction

Infection with soil-transmitted helminths (STH) is the most common infectious disease worldwide and affects mostly inhabitants of rural areas in low- and middle-income countries[1]. In addition to causing direct worm-associated morbidities, chronic STH infections may magnify poor health conditions common in communities remote from health care facilities, such as anemia, poor nutritional status, stunting and possibly poor cognitive development[1,2].

An important hallmark of chronic helminth infections is cellular hyporesponsiveness, which is thought to allow the long-term survival of these parasites within their host[3,4]. Although unresponsiveness in lymphocyte proliferation was already described in the 1970s for individuals with *Schistosoma mansoni* infection or bancroftian filariasis[5,6], the evidence for this has not moved beyond animal models and cross-sectional studies in humans (reviewed by Danilowicz-Luebert et al.[7]). An important drawback to the cross-sectional nature of these studies is that other factors, which are also associated with immune suppression, could bias the results. Individuals infected with helminths may be in a poor nutritional state and shortage of proteins or amino acids can interfere with expression of immune effector molecules. Malnutrition has been specifically associated with decreased cell-mediated immunity, exemplified by atrophy of the thymus and other lymphoid tissues leading to lower T-cell numbers and reactivity[8]. It is also known that other microorganisms and parasites can be associated with immune suppression or T-cell exhaustion[9] and therefore coinfections could act as confounders[10].

The consequences of immunosuppression are manifold and could be of major public health importance. Immune hyporesponsiveness, in the presence of helminth infections, can affect responses to unrelated antigens, it could curtail the development of effective immune responses to incoming protozoan, bacterial or viral infections, thereby increasing susceptibility to these pathogens. Similarly, vaccination studies have shown suboptimal responses to childhood vaccinations in subjects infected with STH[11,12]. On the other hand, the dampened immune responses associated with helminths might help to prevent immune-induced pathology during coinfections and, possibly, overt reactivity to self- or environmental antigens[13].

A few longitudinal studies have been undertaken to assess the effect of anthelmintic treatment on cellular immune responsiveness, however these were in small number of subjects or specifically targeted children[14-16]. Conversely, clinical trials that have experimentally infected humans have mostly not evaluated cellular immune responses and have all been conducted in adults[17,18]. Moreover, therapeutic infections are often not long enough to establish a chronic infection, which could be important for the gradual onset of hyporesponsiveness. So far

there are no large-scale community-based intervention studies that show helminth infections lead to immune hyporesponsiveness in man.

To disentangle the impact of helminths on the immune system from other influences, we conducted a randomized double blind placebo-controlled trial of three-monthly single dose albendazole treatment in an area where STH are highly endemic. Here we present the results of our trial; the effect of anthelmintic treatment on the peripheral blood cytokine responses of a community in Flores island, Indonesia.

Methods

Study design

This report describes a nested study within the ImmunoSPIN trial[19,20]. The trial was conducted in two villages in Ende district, Flores island, Indonesia. The coastal village Nangapanda is located around the main road of Flores and can be characterized as semi-urban, based on the location and the presence of a primary healthcare centre. Anaranda village is located 80 km north of Nangapanda and is more remote from roads, health centres and other facilities. In 2008 the double blind placebo-controlled trial of two year duration was initiated by randomizing all households in the two villages to receive either a single dose of 400 mg albendazole or a matching placebo every three-months over a two year study period (tablets from PT Indofarma Pharmaceutical, Bandung, Indonesia). Treatment allocation was based on household to minimise the risk cross-contamination and therefore reinfection of treated individuals. Treatment was provided to all household members older than two years of age, except for pregnant women (according to Indonesian national guidelines), and intake was observed by field workers. The study was approved by the Ethical Committee of the Medical Faculty, University of Indonesia, Jakarta (ref: 194/PT02.FK/Etik/2006) and has been filed by the ethics committee of the Leiden University Medical Center, the Netherlands. The trial was registered as clinical trial (ISRCTN83830814). Informed consent or parental consent was obtained from all participants.

Study population

The randomization for the total study was based on 954 households in the two villages, comprising of 4004 individuals, resulting in 2022 (481 houses) and 1982 (473 houses) subjects in placebo and albendazole group, respectively. For the immunological component of this study in Nangapanda, aiming at 1000 participants, 250 households were randomly selected and individuals older than 4 years of age were invited for morning venous blood sampling and assessment of anthropometric parameters. This resulted in the inclusion of 882 individuals from the semi-urban area, of which 858 provided sufficient blood samples for whole blood cultures. In the rural area Anaranda, only children were included since this area was included for our allergy studies[19]. 250 children were randomly selected from the total population and children from the same households were also included, leading to a total number of 295 children with whole blood cultures. After exclusion of cytokine data from wells that were suspected of being infected (see below), the number of subjects included at baseline was 839 and 220 for the two respective areas, corresponding to 572 placebo- and 487 albendazole-treated individuals.

Whole blood culture and cytokine measurements

Heparinized blood was diluted 1:4 with RPMI 1640 medium (Invitrogen, Breda, the Netherlands) and cultured in 96-well round-bottomed plates. Cultures were stimulated for 24h to assess innate responses (to lipopolysaccharide (LPS) from *E. coli*, Sigma-Aldrich, Zwijndrecht, the Netherlands), and for 72h to detect adaptive responses (to *Ascaris lumbricoides* antigen, *Plasmodium falciparum*-parasitized red blood cells (PfRBC), uninfected RBC (uRBC) and phytohaemagglutinin (PHA, Wellcome Diagnostics, Darford, UK)) and at each time point unstimulated control wells were included. PfRBC and uRBC were kindly provided by professor Sauerwein from Radboud University Medical Center Nijmegen, the Netherlands and were only used in the semi-urban area, since the rural area was not endemic for malaria. The cultures were carried out in the field laboratory in Nangapanda and the supernatants were kept at -20°C and transported to Jakarta. There cytokine responses were quantified using Luminex cytokine kits (Biosource, Camarillo, CA, USA) and run on a Liquichip 200® Workstation (Qiagen, Venlo, The Netherlands) equipped with Liquichip analyzer software (Qiagen, Venlo, The Netherlands). TNF and IL-10 were assessed in 24h supernatants and TNF, IFN-γ, IL-2, IL-5 and IL-10 in 72h supernatants. Samples with TNF levels ≥250 pg/mL in unstimulated blood were excluded from the analyses as they are considered unreliable with respect to possible infection in the culture. This value was derived from the data distribution, which indicated outliers to be identified with this cut-off. Cytokine levels that fell below the assay's detectable range were replaced by half of the detection limit.

Stool examination by microscopy and PCR

In order to examine the effect of treatment on helminth prevalence, yearly stool samples were collected. *T. trichiura* was detected by microscopy after formol-ether concentration and 18S-based multiplex real-time PCR was used for the specific amplification and detection of hookworm (*Ancylostoma duodenale*, *Necator americanus)*, *A. lumbricoides*, and *Strongyloides stercoralis* DNA, as described previously[20].

Complete blood counts

Complete blood counts (CBC) and differential counts before and one year after treatment were determined using heparinized blood on a routine cell counter (Coulter® Ac-T™ diff Analyzer, Beckman Coulter Inc., Fullerton, CA, USA), while CBC 2 years after treatment were determined using heparinized and EDTA blood on Sysmex KX-21N hematology analyzer (PT Sysmex Indonesia, Jakarta, Indonesia). Since both heparinized and EDTA blood samples were used at the last time point, 325 samples were tested in parallel analysis. All outcomes were highly

comparable except for thrombocyte counts, thus the data of all parameters but thrombocyte counts were pooled.

Statistical analysis

The cytokine data were log transformed (log10(concentration+1)) to obtain normally distributed variables. For children ≤19 years, BMI age-standardized z-scores were calculated according to WHO references[21]. To assess treatment effects, generalized linear mixed models were used with addition of three random effects, namely a random household-specific intercept to model clustering within households and a random subject-specific intercept and slope to model correlation within subjects. Linear or logistic mixed-effects models[22] were applied for continuous and binary outcomes, respectively. Parameter estimates for treatment effects at 9 and 21 months and 95% confidence intervals are reported. The reported p-values are obtained using likelihood ratio tests by comparing the model with and without the treatment effect. Unless stated otherwise, all outcomes were adjusted for area by using area as covariate in the model. All models were fitted using the lme4 package[23].

Results

Study population

The baseline characteristics of the study participants are shown in table 1. At baseline 88.7% of the individuals were infected with one or more helminth species, with hookworm infection being the most prevalent (77.1% of total). The consort diagram of the trial is shown in figure 1; follow-up after 9 months was 88% for both groups and 76% for placebo- and 75% for albendazole-treated groups after 21 months, corresponding to a total loss of 138 and 123 individuals respectively. Six subjects died during the study period, which were all above the age of 35, suggesting non-infectious causes of death. The analysis was intention-to-treat, and involved all participants as assigned randomly at the start of the trial. No significant change in BMI was observed over the 2-year study period in children (analyzed by zBMI, p=0.70) or in adults (BMI, p=0.45; data not shown).

Effect of albendazole treatment on helminth prevalence

Treatment with albendazole resulted in a reduction in the prevalence of geohelminths both after 9 (51.9% vs. 84.1% for placebo) and after 21 months (39.2% vs. 80% for placebo) (Table S1). Albendazole had the most effect on hookworm (from 78.4% at baseline to 32.6% at 9 months and 21.6% at 21 months post-treatment) compared to placebo (from 76.1% to 70.5% and 67.0% respectively), followed by on *Ascaris* (albendazole from 32.7%, to 13.3% and 12.2%; placebo from 31.3% to 33.5% and 24.2%), while the effect on *Trichuris* was less pronounced (from 20.0% at baseline to 21.0% at 9 months and 16.2% at 21 months post-treatment, compared to placebo from 25.5% to 31.7% and 28.8%). When assessing the intensity of infection in categories based on cycle threshold values of PCR, it was in particular the high-load infections that were greatly diminished in the treatment group[24].

Table 1. Baseline characteristics of the study population

	N	Placebo	N	Albendazole
Age (mean in years, SD)	572	25.7 (18.5)	487	24.9 (18.4)
Sex (female, n, % of total)	572	328 (57.3)	487	279 (57.3)
Area (rural, n, % of total)	572	114 (19.9)	487	106 (21.8)
BMI > 19 years old (mean, SD)	264	22.1 (4.1)	220	22.1 (3.8)
Z score of BMI ≤ 19 years old (mean, SD)	194	-1.15 (1.11)	386	-1.14(1.15)
Parasite infection (n, %)*				
Helminth *(any spp)*	322	286 (88.8)	237	210 (88.6)
- Hookworm[1]	335	255 (76.1)	245	192 (78.4)
N. americanus[1]	335	252 (75.2)	245	188 (76.7)
A. duodenale[1]	335	25 (7.5)	245	17 (6.9)
- *A. lumbricoides*[1]	335	105 (31.3)	245	80 (32.7)
- *S. stercoralis*[1]	335	3 (0.9)	245	14 (5.7)
- *T. trichiura*[2]	415	106 (25.5)	310	62 (20.0)
Malarial parasitemia *(any spp)*[2]	567	24 (4.2)	483	24 (5.0)
- *P. falciparum*	567	16 (2.8)	483	11 (2.3)
- *P. vivax*	567	8 (1.4)	483	10 (2.1)
- *P. malariae*	567	0 (0.0)	483	4 (0.8)
Cytokine production (pg/mL [median, IQR])				
LPS				
TNF	554	743 [368-1293]	468	769 [339-1318]
IL-10	554	271 [163-441]	468	256 [158-406]
PHA				
TNF	516	100 [50-222]	435	103 [50-214]
IL-10	515	76 [41-129]	435	70 [37-116]
IFN-γ	516	1625 [584-3983]	435	1270 [538-4340]
IL-2	516	23 [0-101]	432	23 [0-92]
IL-5	516	563 [309-840]	435	520 [317-829]
PfRBC				
TNF	299	18 [4-42]	237	14 [3-38]
IL-10	300	10 [5-19]	238	10 [5-20]
IFN-γ	300	163 [75-388]	239	176 [70-376]
IL-2	300	50 [5-125]	239	40 [5-112]
IL-5	300	14 [5-26]	239	12 [4-23]
Ascaris				
TNF	517	5 [0-15]	438	6 [0-14]
IL-10	516	7 [2-15]	438	7 [1-14]
IFN-γ	516	19 [6-47]	441	21 [7-47]
IL-2	497	38 [4-114]	426	36 [0-107]
IL-5	515	24 [9-68]	440	24 [9-63]

[1]*diagnosed by PCR;* [2]*diagnosed by microscopy.*
**The number of positives (n) of the total population examined (N)*

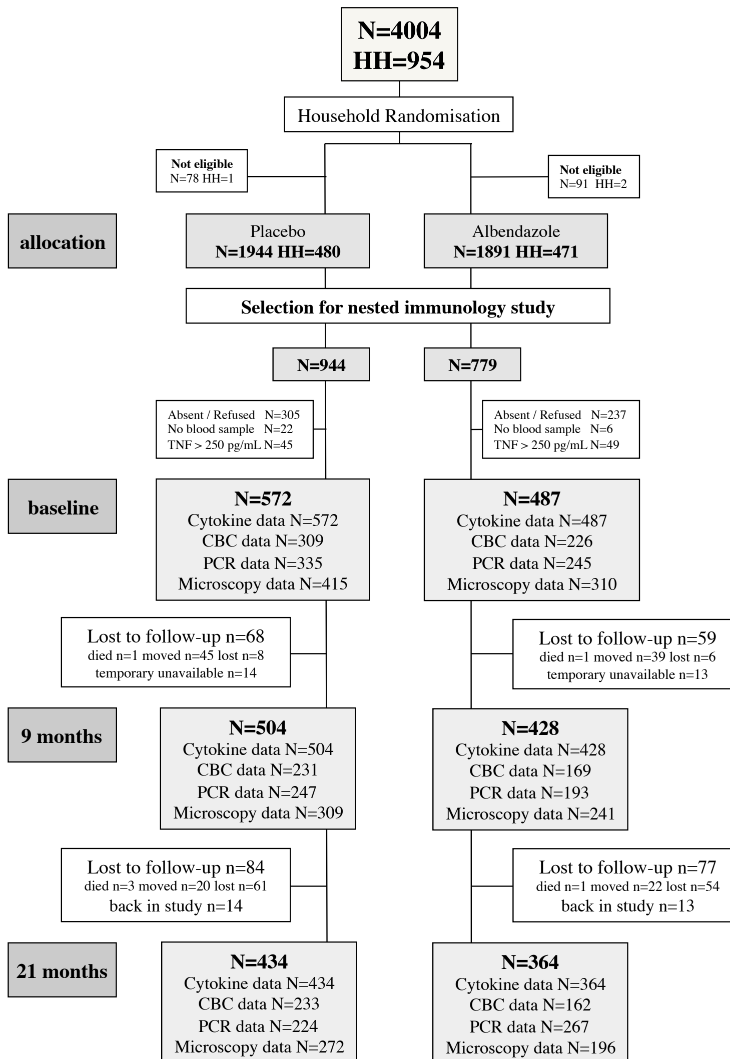

Figure 1. Consort diagram. The current study is nested within the ImmunoSPIN trial, with a total of 4004 individuals in two participating villages. Allocation of placebo and albendazole treatment resulted in 480 and 471 households including 1944 and 1891 in the two groups, respectively. For the immunological studies, a random selection was made and 1723 individuals were invited to participate (n=944 and n=779 respectively). Cytokines were assessed for 1059 subjects, of which 572 in the placebo and 487 in the albendazole arm. After 9 months 504 and 428 and after 21 months 434 and 364 individuals were analyzed, in placebo and albendazole group, respectively. Availability of complete blood counts (CBC) and parasitological data is indicated at the different time points for both groups.

Effect of albendazole treatment on whole blood cytokine responses

In figure 2, we present the effect of treatment on cytokine responses to *Ascaris* antigens, PfRBC and PHA. The model-estimated treatment effects on cytokines at 9 months (figure 2A) and 21 months (figure 2B) are shown. Regarding helminth (*Ascaris*) antigen-specific cytokine responses, IL-2 responses were significantly enhanced by treatment over the study period (p_{time}=0.018), with significantly higher IL2 in the treated group at 9 months (estimate [95% CI]: 0.17 [0.05–0.28], figure 2A). Neither Th1, nor Th2 responses changed with treatment. In response to *P. falciparum* antigens, there was an increase over time in pro inflammatory cytokine TNF, which was highly significant (p_{time}<0.0001), and IFN-γ (p_{time}=0.036), in the albendazole-treated group. As shown in figure 2A, both TNF and IFN-γ were significantly higher than in the placebo treated group at the 9 months time point (0.37 [0.21-0.53] for TNF and 0.14 [0.03-0.24] for IFN-γ). Moreover, the general adaptive response (cytokine production stimulated by PHA), albendazole treatment significantly increased TNF and IL-10 secretion (p_{time}=0.011 and p_{time}=0.003 respectively) over the trial period. Interestingly, for TNF, albendazole treatment resulted in elevated response at 9 months whereas for IL-10 the response was significantly higher at the later 21 months time point (for TNF 0.14 [0.05–0.24], figure 2A; for IL-10 0.12 [0.05–0.19], figure 2B). At 9 months post-treatment, IFN-γ (0.10 [0.01-0.19]) and IL-2 (0.12 [0.01-0.23]) responses were transiently increased (figure 2A) and at 21 months a significant enhancement of IL-5 production (0.10 [0.01-0.19]) was observed (figure 2B), however these alterations were not significant over the whole trial time period (IFN-γ p_{time}=0.076, IL-2 p_{time}=0.11, IL-5 p_{time}=0.068). Albendazole had no effect on immune responses to LPS (overall p-value for TNF p=0.77, for IL-10 p=0.12, data not shown). Analysis of cytokines in unstimulated whole blood cultures revealed no treatment-related differences (data not shown). When assessing responses to uRBC as control for PfRBC, we found that IFN-γ levels were not different between the treatment arms (p=0.91), however TNF production was increased post-treatment in the albendazole arm, although to a lesser extent than what was seen in response to PfRBC (p=0.018; figure S1).

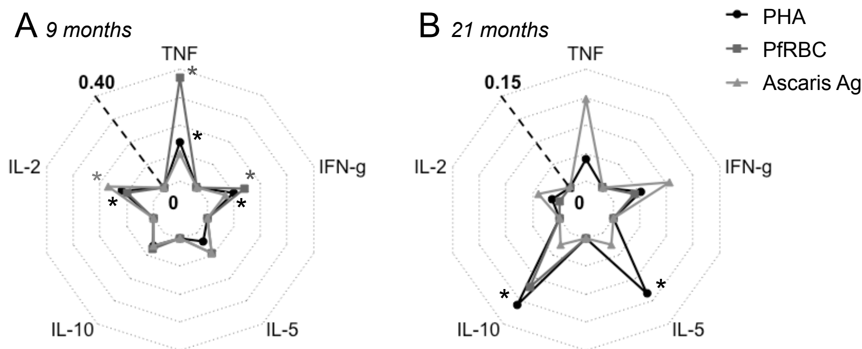

Figure 2. Effect of deworming on cytokine responses to *Ascaris*, PfRBC and PHA. TNF, IFN-γ, IL-2, IL-5 and IL-10 concentrations were assessed in supernatants of 72h-stimulated whole blood cultures. The effect of albendazole treatment on cytokine responses to PHA (black circles), PfRBC (dark grey squares) and *Ascaris* (light grey triangles) is shown. The estimates of the treatment effect in the whole study population after 9 (A) and 21 (B) months of albendazole treatment were obtained by general linear mixed models; asterisks with corresponding colors (black for PHA, dark grey for PfRBC, light grey for *Ascaris*) indicate a significant effect.

Increase in pro-inflammatory responses after treatment in helminth-infected individuals

To determine whether the enhanced cytokine responses could be due to a direct effect of albendazole, we stratified the analysis based on STH infection status at baseline. The enhancement of mitogen- as well as malaria-induced TNF by albendazole treatment was observed in helminth-infected individuals (overall p-values for PHA and PfRBC were p_{time}=0.098 and p_{time}=0.0004 respectively, figure 3A and 3B), but not in uninfected ones (data not shown). Importantly, uRBC-induced TNF responses were not increased in either helminth-infected or uninfected subjects (data not shown). Also in the response to *Ascaris* antigen, enhancement of TNF was observed in the stratified analysis of helminth-positives at baseline (p_{time}=0.044, figure 3C) but not in helminth negatives (data not shown). Moreover, elevated IFN-γ and IL-2 responses to Ascaris were only observed after treatment of the helminth-infected (p_{time}=0.028 and p_{time}=0.006 respectively; not shown).

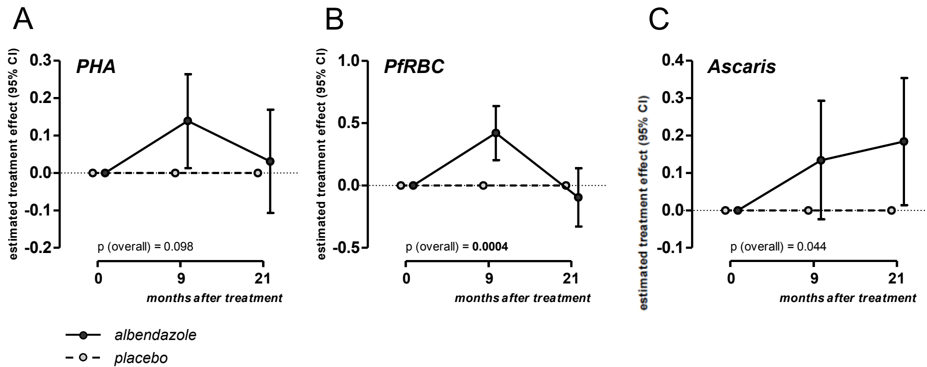

Figure 3. Effect of deworming on TNF responses in helminth-infected. TNF secretion was measured in supernatants from whole blood stimulated with (A) PHA, (B) PfRBC and (C) *Ascaris* antigen for 72 hours. The estimated effect of albendazole treatment on TNF responses in helminth-infected subjects is displayed for the 9 and 21 months time points, with corresponding 95% confidence intervals. The estimates of the treatment effect were obtained by general linear mixed model and overall p-values over time are indicated.

Increased cytokine responses are not associated with changes in cell counts

The total leukocyte count increased in the albendazole group at 9 months post-treatment and was similar in both groups at 21 months (p=0.035 and p=0.14 respectively; data not shown). However, we observed a negative association of leukocyte counts and both TNF and IFN-γ responses to PfRBC, indicating that increased leukocyte numbers could not be responsible for the enhanced cytokine responses after treatment. When analyzing differential cell counts and proportions, the lymphocytes and granulocytes did not change after treatment, whereas monocyte proportions and numbers were higher in the albendazole group. No association was found between monocyte numbers and cytokine production in response to any of the stimuli (data not shown). No treatment effect was noted on thrombocyte or erythrocyte counts, hemoglobin levels or hematocrit.

Discussion

This is the first time that helminth-specific and -unrelated cytokine responses have been analyzed in a whole community before and after repeated long-term placebo-controlled anthelmintic treatment. We show that treatment of STH infections increases cytokine responses, with profound effects on helminth-specific and other adaptive immune responses, providing conclusive evidence for helminth-induced immune hyporesponsiveness in humans.

Most pronounced were elevated pro-inflammatory, TNF and IFN-γ, cytokine responses after stimulation with mitogen and malarial antigens. Albendazole is a drug, which might induce production of inflammatory cytokines in a human monocytic cell line[25]. Stratifying the analysis for helminth infection status at baseline revealed stronger effects in the helminth-infected group, indicating that the suppression of pro-inflammatory cytokine responses is unlikely to be due to direct effects of albendazole, but can be regarded as a true helminth-induced phenomenon.

Subsequent to the rise in pro-inflammatory responses at 9 months, an interesting finding was the enhancement of IL-5 and IL-10 responses at 21 months post-treatment. Although helminth infections skew the immune system towards type 2 responses, suppression of these responses during helminth infections has been reported before in studies comparing helminth infected and uninfected subjects[26,27]. IL-10 upregulation appears particularly surprising; as it has been postulated that helminth-associated inhibition of pro-inflammatory responses is mediated by this suppressory cytokine[28]. Increased IL-10 responses after anthelmintic treatment have previously been observed in schistosomiasis[29] and STH infection[16]. Whether the increased pro-inflammatory responses in the first year leads to higher IL-10 in the second year to prevent overt inflammation, is not clear from these data. Moreover, it is known that IL-10 can be part of the Th2 response and therefore the increased IL-10 might be a component of the enhanced Th2 response following deworming, leaving the question whether IL-10 originating from other cells is involved in cellular hyporesponsiveness caused by helminth infections. However, the fact that different cytokines appear to all increase in response to antigens and mitogens after anthelmintic treatment seems to indicate that all adaptive immune responses are enhanced after deworming. This would predict that there is a general helminth-mediated hyporesponsiveness which is neither restricted to a particular pro- or anti-inflammatory nor to a Th1 or Th2 response, but might stem from a common general effect such as alternation in cell counts, changes in nutrients essential to functioning of the immune system or suppressory cells and factors which do not involve IL-10.

Importantly, cell counts were affected by reduction in helminths, but did not show any correlation with cytokine responses, excluding the possibility that the general

increase in responsiveness is due to higher numbers of cytokine-secreting cells. As immune responses can be enhanced by improved energy resources, we assessed BMI, and fasting glucose level (not shown), as proxies for nutritional status but these parameters were not affected by deworming.

The three-monthly albendazole treatment over a two-year period was not effective in eliminating all helminth infections. Treatment efficacy was particularly low for *Trichuris*, shown in earlier studies that used single or double doses of albendazole and / or mebendazole[30,31]. Here we show that even 7 doses of albendazole over a 21 month period is not sufficiently effective against *Trichuris* infection. By using a household-clustered design for randomization, repeated treatments and observed intake, we had expected a more effective reduction in transmission of STH. For better deworming results, more intensive treatment or inclusion of environmental control would be needed. However, it is clear that even a reduction in helminth infections in the community can lead to alleviation of immune hyporesponsiveness and that a more effective deworming, might result in even more pronounced immunological effects.

There is an increasing awareness that helminths might play an essential role in the development and homeostasis of the human immune system[32,33]. In areas where chronic helminth infections are persistent, the immune system may have evolved to operate optimally in the face of helminth-induced downmodulation; any disturbance of the long evolutionary coexistence between humans and helminths might be associated with the emergence of pathological conditions[34]. The question of what the clinical consequences of the enhanced adaptive immune responses are following deworming will need to be addressed next. So far, our trial of three-monthly albendazole treatment over a 21-month period did not show clear clinical changes[24]. Although we found a transient increase in malarial parasitemia at 6 months post treatment, the time point after the rainy season, this could not be confirmed during the further follow-up period, as the prevalence of malaria decreased drastically in the study area. With respect to allergy, skin prick test (SPT) reactivity was assessed in school children in our study cohort. This revealed a specific increase in SPT reactivity to cockroach allergens after two years of anthelmintic treatment, but no effect on allergy symptoms[24]. The effects of anthelmintic treatment on other infectious or inflammatory diseases should probably be investigated over a longer period, since the immunomodulatory effects of helminths are likely to have developed over several years of chronic infection. In the case of allergic diseases this is illustrated by a recent publication, showing an increase in allergen SPT reactivity and possibly eczema symptoms after more than 15 years of ivermectin treatment[35], whereas studies with a shorter treatment course failed to show increased SPT reactivity or symptoms[14,15].

Several clinical trials are underway to evaluate the possible beneficial effect of helminth infection or their excretory products on the symptoms and prevalence of

inflammatory diseases[36,37], while at the same time efforts are made to control STH by implementing regular treatment programs in developing countries[38]. These studies, if conducted within appropriate time frames, should be able to answer the question what clinical consequences of human helminth infections are. Given our results that there are major effects on immune responses following deworming, it will be important to include immunological measurements in future deworming programs in order to understand causation and predict clinical outcomes.

References

1. Hotez, P.J., *et al.* Helminth infections: the great neglected tropical diseases. *The Journal of clinical investigation* **118**, 1311-1321 (2008).
2. Bethony, J., *et al.* Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. *Lancet* **367**, 1521-1532 (2006).
3. Allen, J.E. & Maizels, R.M. Diversity and dialogue in immunity to helminths. *Nature reviews. Immunology* **11**, 375-388 (2011).
4. van Riet, E., Hartgers, F.C. & Yazdanbakhsh, M. Chronic helminth infections induce immunomodulation: consequences and mechanisms. *Immunobiology* **212**, 475-490 (2007).
5. Ottesen, E.A., Hiatt, R.A., Cheever, A.W., Sotomayor, Z.R. & Neva, F.A. The acquisition and loss of antigen-specific cellular immune responsiveness in acute and chronic schistosomiasis in man. *Clinical and experimental immunology* **33**, 37-47 (1978).
6. Ottesen, E.A., Weller, P.F. & Heck, L. Specific cellular immune unresponsiveness in human filariasis. *Immunology* **33**, 413-421 (1977).
7. Danilowicz-Luebert, E., O'Regan, N.L., Steinfelder, S. & Hartmann, S. Modulation of specific and allergy-related immune responses by helminths. *Journal of biomedicine & biotechnology* **2011**, 821578 (2011).
8. Chandra, R.K. Nutrition and the immune system from birth to old age. *European journal of clinical nutrition* **56 Suppl 3**, S73-76 (2002).
9. Wherry, E.J. T cell exhaustion. *Nature immunology* **12**, 492-499 (2011).
10. Stelekati, E. & Wherry, E.J. Chronic bystander infections and immunity to unrelated antigens. *Cell host & microbe* **12**, 458-469 (2012).
11. Cooper, P.J., *et al.* Human infection with Ascaris lumbricoides is associated with suppression of the interleukin-2 response to recombinant cholera toxin B subunit following vaccination with the live oral cholera vaccine CVD 103-HgR. *Infection and immunity* **69**, 1574-1580 (2001).
12. Elias, D., *et al.* Effect of deworming on human T cell responses to mycobacterial antigens in helminth-exposed individuals before and after bacille Calmette-Guerin (BCG) vaccination. *Clinical and experimental immunology* **123**, 219-225 (2001).
13. Rook, G.A. Review series on helminths, immune modulation and the hygiene hypothesis: the broader implications of the hygiene hypothesis. *Immunology* **126**, 3-11 (2009).
14. Cooper, P.J., *et al.* Repeated treatments with albendazole enhance Th2 responses to Ascaris Lumbricoides, but not to aeroallergens, in children from rural communities in the Tropics. *The Journal of infectious diseases* **198**, 1237-1242 (2008).
15. Flohr, C., *et al.* Reduced helminth burden increases allergen skin sensitization but not clinical allergy: a randomized, double-blind, placebo-controlled trial in Vietnam. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **40**, 131-142 (2010).
16. Wright, V.J., *et al.* Early exposure of infants to GI nematodes induces Th2 dominant immune responses which are unaffected by periodic anthelminthic treatment. *PLoS neglected tropical diseases* **3**, e433 (2009).
17. Bourke, C.D., *et al.* Trichuris suis ova therapy for allergic rhinitis does not affect allergen-specific cytokine responses despite a parasite-specific cytokine response. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **42**, 1582-1595 (2012).
18. Gaze, S., *et al.* Characterising the mucosal and systemic immune responses to experimental human hookworm infection. *PLoS pathogens* **8**, e1002520 (2012).

19. Hamid, F., *et al.* A longitudinal study of allergy and intestinal helminth infections in semi urban and rural areas of Flores, Indonesia (ImmunoSPIN Study). *BMC infectious diseases* **11**, 83 (2011).
20. Wiria, A.E., *et al.* Does treatment of intestinal helminth infections influence malaria? Background and methodology of a longitudinal study of clinical, parasitological and immunological parameters in Nangapanda, Flores, Indonesia (ImmunoSPIN Study). *BMC infectious diseases* **10**, 77 (2010).
21. WHO. WHO Child Growth Standards: Lenght/height-for-age, weight-for-age, weight-for-length, weight-for-height and body mass index-for-age: Method and development. *Geneva: World Health Organization*, 312 (2006).
22. Laird, N.M. & Ware, J.H. Random-effects models for longitudinal data. *Biometrics* **38**, 963-974 (1982).
23. R-Forge. lme4 - Mixed-effects models project. (2011).
24. Wiria, A.E., *et al.* The effect of three-monthly albendazole treatment on malarial parasitemia and allergy: a household-based cluster-randomized, double-blind, placebo-controlled trial. *PloS one* **8**, e57899 (2013).
25. Mizuno, K., Toyoda, Y., Fukami, T., Nakajima, M. & Yokoi, T. Stimulation of pro-inflammatory responses by mebendazole in human monocytic THP-1 cells through an ERK signaling pathway. *Archives of toxicology* **85**, 199-207 (2011).
26. Grogan, J.L., Kremsner, P.G., Deelder, A.M. & Yazdanbakhsh, M. Antigen-specific proliferation and interferon-gamma and interleukin-5 production are down-regulated during Schistosoma haematobium infection. *The Journal of infectious diseases* **177**, 1433-1437 (1998).
27. Wammes, L.J., *et al.* Regulatory T cells in human lymphatic filariasis: stronger functional activity in microfilaremics. *PLoS neglected tropical diseases* **6**, e1655 (2012).
28. Couper, K.N., Blount, D.G. & Riley, E.M. IL-10: the master regulator of immunity to infection. *J Immunol* **180**, 5771-5777 (2008).
29. van den Biggelaar, A.H., Borrmann, S., Kremsner, P. & Yazdanbakhsh, M. Immune responses induced by repeated treatment do not result in protective immunity to Schistosoma haematobium: interleukin (IL)-5 and IL-10 responses. *The Journal of infectious diseases* **186**, 1474-1482 (2002).
30. Namwanje, H., Kabatereine, N.B. & Olsen, A. Efficacy of single and double doses of albendazole and mebendazole alone and in combination in the treatment of Trichuris trichiura in school-age children in Uganda. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **105**, 586-590 (2011).
31. Speich, B., *et al.* Efficacy and safety of nitazoxanide, albendazole, and nitazoxanide-albendazole against Trichuris trichiura infection: a randomized controlled trial. *PLoS neglected tropical diseases* **6**, e1685 (2012).
32. Elliott, D.E. & Weinstock, J.V. Helminth-host immunological interactions: prevention and control of immune-mediated diseases. *Annals of the New York Academy of Sciences* **1247**, 83-96 (2012).
33. Hoerauf, A. Microflora, helminths, and the immune system-who controls whom? *The New England journal of medicine* **363**, 1476-1478 (2010).
34. Maizels, R.M. & Yazdanbakhsh, M. Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nature reviews. Immunology* **3**, 733-744 (2003).
35. Endara, P., *et al.* Long-term periodic anthelmintic treatments are associated with increased allergen skin reactivity. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **40**, 1669-1677 (2010).
36. Falcone, F.H. & Pritchard, D.I. Parasite role reversal: worms on trial. *Trends in parasitology* **21**, 157-160 (2005).

37. Harnett, W. & Harnett, M.M. Helminth-derived immunomodulators: can understanding the worm produce the pill? *Nature reviews. Immunology* **10**, 278-284 (2010).
38. Utzinger, J. A research and development agenda for the control and elimination of human helminthiases. *PLoS neglected tropical diseases* **6**, e1646 (2012).

Supplementary material

Table S1. Prevalence of helminth infections at post-treatment time points.

		N	any spp	*Hookworm*[1]	*A. lumbricoides*[1]	*T.trichiuria*[2]
			n (%)	n (%)	n (%)	n (%)
9 months	Placebo	227	191 (84.1%)	160 (70.5%)	76 (33.5%)	72 (31.7%)
	Albendazole	181	94 (51.9%)	59 (32.6%)	24 (13.3%)	38 (21.0%)
21 months	Placebo	215	171 (80.0%)	144 (67.0%)	52 (24.2%)	62 (28.8%)
	Albendazole	148	58 (39.2%)	32 (21.6%)	18 (12.2%)	24 (16.2%)

*[*1]diagnosed by PCR. [2]diagnosed by microscopy.*

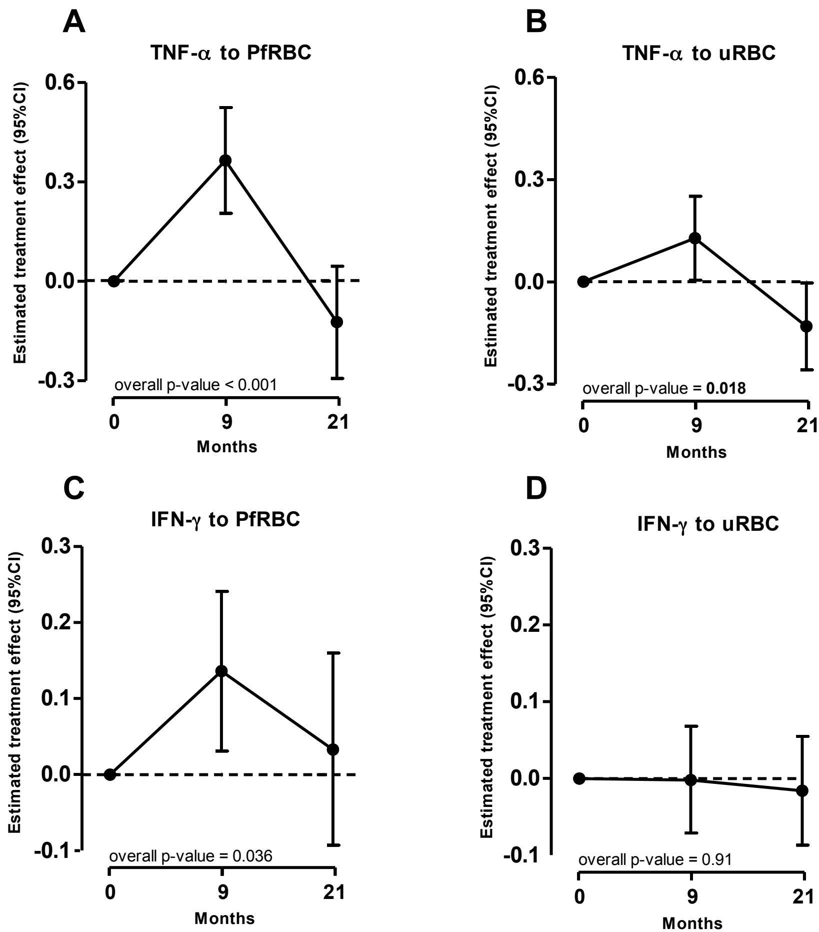

Figure S1. Effect of deworming on TNF and IFN-γ responses to PfRBC and uRBC. TNF (A, B) and IFN-γ (C, D) responses were measured after 72h of stimulation with *Plasmodium falciparum*-infected and -uninfected RBC (PfRBC (A, C) and uRBC (B, D), respectively). The estimated effect of albendazole treatment on TNF responses in helminth-infected subjects is displayed for the 9 and 21 months time points, with corresponding 95% confidence intervals. The estimates of the treatment effect were obtained by general linear mixed model and overall p-values over time are indicated.

Chapter 7

The effect of three-monthly albendazole treatment on malarial parasitemia and allergy: a household-based cluster-randomized, double-blind, placebo-controlled trial

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Abstract

Background Helminth infections are proposed to have immunomodulatory activities affecting health outcomes either detrimentally or beneficially. We evaluated the effects of albendazole treatment, every three months for 21 months, on STH, malarial parasitemia and allergy.
Methods A household-based cluster-randomized, double-blind, placebo-controlled trial was conducted in an area in Indonesia endemic for STH. Using computer-aided block randomization, 481 households (2022 subjects) and 473 households (1982 subjects) were assigned to receive placebo and albendazole, respectively, every three months. The treatment code was concealed from trial investigators and participants. Malarial parasitemia and malaria-like symptoms were assessed in participants older than four years of age while skin prick test (SPT) to allergens as well as reported symptoms of allergy in children aged 5-15 years. The general impact of treatment on STH prevalence and body mass index (BMI) was evaluated. Primary outcomes were prevalence of malarial parasitemia and SPT to any allergen. Analysis was by intention to treat.
Results At 9 and 21 months post-treatment 80.8% and 80.1% of the study subjects were retained, respectively. The intensive treatment regiment resulted in a reduction in the prevalence of STH by 48% in albendazole and 9% in placebo group. Albendazole treatment led to a transient increase in malarial parasitemia at 6 months post treatment (OR 4.16 [1.35-12.80]) and no statistically significant increase in SPT reactivity (OR 1.18 [0.74-1.86] at 9 months or 1.37 [0.93-2.01] 21 months). No effect of anthelminthic treatment was found on BMI, reported malaria-like and allergy symptoms. No adverse effects were reported.
Conclusions The study indicates that intensive community treatment of 3 monthly albendazole administration for 21 months over two years leads to a reduction in STH. This degree of reduction appears safe without any increased risk of malaria or allergies.

Introduction

Soil transmitted helminths (STH) (hookworms, *Ascaris lumbricoides* and *Trichuris trichiura*) establish chronic infections in a large proportion of the world population[1]. Major intervention programs using mass drug administration (MDA) to control STH have been launched[2]. However, STH infections seem to persist in the targeted populations raising concern over the development of drug resistance[3]. It is therefore important to conduct well-designed studies that allow evidence-based decisions to be made to maximize effective STH control toward elimination.

While there is no doubt that STH are associated with morbidities in billions of people worldwide, there is also increasing awareness that helminth infections might, like bacterial commensals, play an important role in shaping human health[4]. Helminths may contribute to immunologic and physiologic homeostasis. The immune system is thought to have evolved to operate optimally in the face of helminth-induced immune regulation, and that any disturbance of this long evolutionary co-existence between humans and helminth parasites might be associated with the emergence of pathological conditions[5] possibly involving outcomes of exposure to other pathogens or the development of inflammatory diseases.

In many parts of the world helminths and malarial parasites are co-endemic raising the question of what impact helminth infections may have on the plasmodial parasites that cause malaria. The results have been conflicting in this regard. In some studies a positive association has been reported between helminths and malarial parasitemia while in others, this has been refuted or in yet others a negative association has been shown between helminths and the severity of the clinical outcomes of malaria (reviewed by Nacher)[6] .

An increase in the prevalence of allergies has been reported worldwide, in particular in the urban areas of low- to middle-income countries[7]. Although majority of cross-sectional studies have reported inverse associations between helminth infections and allergies[8,9], two randomized trials with albendazole, have shown conflicting results. One in Ecuador, based on school randomization, reported no change in either SPT reactivity to allergens or allergic symptoms after one year of albendazole treatment[10] while another in Vietnam, in which the randomization unit was individual schoolchildren, showed increased SPT reactivity after one year of albendazole treatment, but consistent with the Ecuadorean study, clinical allergy did not change significantly[11]. It has been suggested that anthelminthic treatment of longer duration might be needed to reveal the modulatory effect of helminths[12,13].

In the light of global deworming initiatives, it is important to assess the effectiveness of and to monitor the risks associated with anthelminthic treatment regiments. There is as yet no report of a household-based cluster-randomized

double-blind placebo-controlled trial of repeated anthelminthic administration in a community that would be expected to more effectively reduce transmission of STH by decreasing household cross-contamination.
In an area where STH and malaria are co-endemic on Flores Island, Indonesia, we conducted a household cluster-randomized trial of three-monthly albendazole treatment over a two-year study period in a whole community to assess benefits and risks associated with this anthelmintic treatment. Specifically we assessed its impact on STH, malarial parasitemia and allergy.

Methods

Study population and design

This trial was conducted in two villages in the Ende District of Flores Island, Indonesia (supplementary appendix, p2) as described in detail elsewhere[14,15]. The treatment was based on household and given to all household members except those less than two years old or pregnant (the Indonesian national program guideline). Directly observed treatment was given three monthly during the trial period (June 2008 to July 2010, with treatment starting in Sept 2008). The primary outcomes were prevalence of malarial parasitemia and SPT reactivity to allergens. Additional outcomes were treatment effect on STH and BMI as well as malaria-like and allergy symptoms.

We measured malaria outcomes in Nangapanda only. Malaria was not endemic in Anaranda. Artemisinin-combination therapy (ACT) treatment and treated bed net distribution were not implemented during our study period[16,17].

Allergy outcomes were measured, in both villages, in school-age children (5-15 years old) as this group is particularly at risk of developing allergy and asthma[18] and is the target population of global deworming programs.

The study was approved by the Ethical Committee of the Medical Faculty, University of Indonesia (ethical clearance ref: 194/PT02.FK/Etik/2006) and filed by the Committee of Medical Ethics of the Leiden University Medical Center. The trial was registered as clinical trial (Ref: ISRCTN83830814). Prior to the study, written informed consent was obtained from participants or from parents/guardians of children. The study is reported in accordance with the CONSORT guidelines for cluster-randomized studies.

Randomization and masking

The population was randomized by IA using computer aided block randomization at household level utilising Random Allocation software to receive albendazole (single dose of 400 mg) or a matching placebo (both tablets from PT Indofarma Pharmaceutical, Bandung, Indonesia). The treatment code was concealed from trial investigators and participants. The un-blinding of treatment codes occurred after all laboratory results had been entered into the database (August 2011).

Procedures

Trained community workers measured fever, administered monthly malaria-like symptoms questionnaire which was based on WHO definitions[19] and took finger-prick blood for the three-monthly malarial parasitemia survey. Subjects with fever (≥37.5^{o}C) or additional malaria-like symptoms (headache, fatigue and nausea) at the time of visits were referred to the local primary health centre (puskesmas). Thick and thin Giemsa-stained blood smears were read at University of Indonesia.

At baseline, 9 months and 21 months after the first round of treatment blood was collected for PCR-based detection of *Plasmodium spp.* (supplementary appendix, p2), a method that is more sensitive than microscopy[20].

Regarding allergy outcomes, skin prick tests (SPT) with allergens were performed on school-age children in Nangapanda and Anaranda and clinical symptoms of allergy were recorded. House dust mite (*Dermatophagoides pteronyssinus* and *D. farinae*; kindly provided by Paul van Rijn from HAL Allergy Laboratories, Leiden, The Netherlands) and cockroach (*Blatella germanica*; Lofarma, Milan, Italy) were used for SPT, which was considered positive with 3 mm cut off.(Hamid et al. 83) The SPT was performed by one investigator. IgE with specificity for aeroallergens (*D. pteronyssinus* and *B. germanica*) was measured in plasma using an ImmunoCAP 250 system (Phadia, Uppsala, Sweden) following the manufacturer's instructions. All measurements were conducted in one laboratory in the Netherlands. Symptoms of asthma and atopic dermatitis were recorded using a modified visually-assisted version of the International Study of Asthma and Allergy in Childhood (ISAAC) questionnaire as reported before[14].

Yearly stool samples were collected on a voluntary basis. *Trichuris* was detected by microscopy and a multiplex real-time PCR was used for detection of hookworms (*Ancylostoma duodenale*, *Necator americanus)*, *Ascaris lumbricoides*, and *Strongyloides stercoralis* DNA as detailed before[15] (supplementary appendix, p2). Very few subjects were infected with *S. stercoralis* and therefore this infection was not included in analyses.

Body weight and height were measured using the National Heart Lung and Blood Institute practical guidelines (scale and microtoise from SECA GmBH & Co, Hamburg, Germany).

Power calculation

Sample size estimation was based on the expected change in primary outcomes taking into account a power of 90% and a significance level of <0.05 with a loss to follow-up of 20%. Based on previous observations we expected to find a decrease in malarial parasitemia prevalence and an increase in SPT reactivity after anthelminthic treatment. Based on a prevalence of about 10% and a risk ratio (RR) of 0.60 we aimed to include 2412 people in the malaria assessments. In a pilot study we found SPT to *D. pteronyssinus* to be around 15%, and expected that due to treatment the prevalence would increase. In order to find a RR of 1.5 we aimed to include at least 1418 children.

Statistical analyses

For children ≤19 years, BMI age-standardized z-scores were calculated according to WHO references[21]. The IgE data were log-transformed to obtain normally distributed variable. To assess treatment effects generalized linear mixed models

were used which provide a flexible and powerful tool to derive valid inference while capturing the data correlations induced by clustering within households and repeated evaluations in time of the same subject. Parameter estimates for treatment effects at 9 and 21 months and 95% confidence intervals are reported. The reported p-values are obtained using likelihood ratio tests by comparing the model with and without the treatment effect. Unless stated otherwise all outcomes were adjusted for area (the two study villages in Ende District: Nangapanda or Anaranda) as covariate in the model. For the continuous outcomes linear mixed-effects models[22] were used with three random effects, namely to model clustering within households a random household specific intercept was used and to model correlation within subjects a random subject specific intercept and slope were used. For the binary outcomes a logistic model was used with random household effects and random subject effects. All models were fitted using the lme4 package (supplementary appendix, p6-7)[23]. For each fever and additional malaria-like symptoms, total number of events and person months are computed for each treatment arm. Hazard ratios for effect of treatment were calculated with Cox regression with robust SE to allow for within-household clustering (STATA 11).

Table 1. Baseline characteristics

	N	Placebo	N	Albendazole
Age (mean in years, SD)	2022	25.7 (18.7)	1982	25.8 (18.7)
Sex (female, n, %)	2022	1090 (53.9)	1982	1042 (52.6)
Area (rural, n, %)	2022	260 (12.9)	1982	253 (12.8)
BMI > 19 years old (mean, SD)	575	22.3 (4.0)	582	21.8 (3.6)
Z score of BMI ≤ 19 years old (mean, SD)	427	-1.20 (1.2)	386	-1.37 (1.3)
Parasite infection (n, %)				
Helminth *(any spp)*	655	571 (87.2)	609	533 (87.5)
Hookworm[1]	683	509 (74.5)	629	486 (77.3)
N. americanus[1]	683	503 (73.7)	629	481 (76.5)
A. duodenale[1]	683	44 (6.4)	629	41 (6.5)
A. lumbricoides[1]	683	238 (34.9)	629	209 (33.2)
S. stercoralis[1]	683	7 (1.0)	629	18 (2.9)
T. trichiura[2]	953	258 (27.1)	852	237 (27.8)
Malarial parasitemia *(any spp)*[2]	1225	60 (4.9)	1187	52 (4.4)
P. falciparum	1225	32 (2.6)	1187	28 (2.4)
P. vivax	1225	26 (2.1)	1187	18 (1.5)
P. malariae	1225	2 (0.2)	1187	7 (0.6)
Malarial parasitemia *(any spp)*[1]	772	195 (25.3)	739	200 (27.1)
P. falciparum	772	106 (13.7)	739	112 (15.2)
P. vivax	772	102 (13.2)	739	93 (12.6)
P. malariae	772	10 (1.3)	739	18 (2.4)
Skin prick reactivity (n, %)				
Any allergen	711	190 (26.7)	653	163 (25.0)
House dust mite	711	88 (12.4)	653	75 (11.5)
Cockroach	711	163 (22.9)	653	140 (21.4)
Specific IgE, kU/L (median, IQR)				
House dust mite	452	0.8 (0.3-2.6)	431	0.8 (0.2-2.4)
Cockroach	452	1.5 (0.4-5.7)	431	1.9 (0.5-5.0)

The number of positive (n) of the total population examined (N).
[1]diagnosed by PCR; [2]diagnosed by microscopy.

Results

At baseline, 954 households with 4004 subjects were registered. Randomization of households resulted in 1982 people assigned to albendazole treatment and 2022 people to placebo (473 and 481 houses respectively). At baseline 87.3% of the individuals were infected with one or more helminth species. The baseline characteristics were similar between the treatment arms (table 1). The overall trial profile is shown in figure 1, and in supplementary appendix (p13-15, figure S1A, S1B, S1C) separately for malaria, allergy and helminth outcomes. The analysis was intention-to-treat and involved all participants as assigned randomly at the start of the trial. During the study, in the albendazole arm 61 people moved to a house that was assigned to placebo while in the placebo arm 62 people moved to a house that was assigned to albendazole. The 44 subjects who died during the trial, included 4 people below the age of 20, 3 between 20 and 40 and the rest above 40 years of age, and were equally distributed between the treatment arms. At 9 months post-treatment full compliance was 77.8% for albendazole treatment and 78.0% for placebo. This was 63.1% and 62.5% respectively at 21 months.

This intensive treatment with albendazole resulted in a reduction but not elimination of STH. There was a decrease both after 9 (OR (95% CI) = 0.07 (0.04-0.11) and 21 months (0.05 (0.03-0.08)) of treatment ($p<0.0001$). Albendazole had the largest effect on hookworm followed by *Ascaris* while the effect on *Trichuris* was less pronounced (figure 2A and supplementary appendix p8, table S1). Treatment also led to statistically significant reduction in the intensity of hookworm and *Ascaris* infection as determined by PCR (figure 2B). The fact that the stool sampling was on a voluntary basis could have created a selection bias. Analyzing baseline characteristics of subjects providing stool samples and those who did not at 9 months follow up, showed no differences in helminth prevalence, age and sex. Although at 21 months post treatment, sex and helminth prevalence were not different, age was slightly but significantly higher in subjects who provided stool samples mean age in years (SD) = 29.9 (20.4) vs 24.3 (17.5), $p=0.006$).

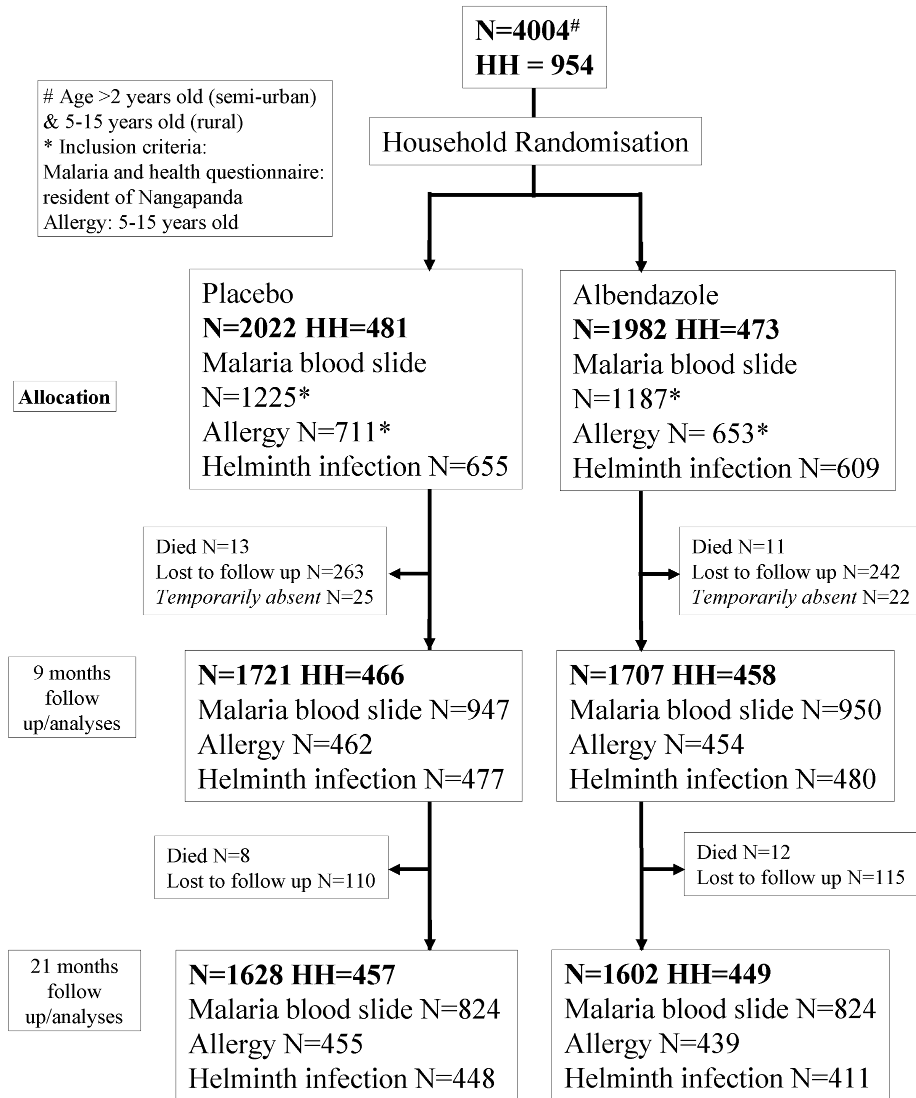
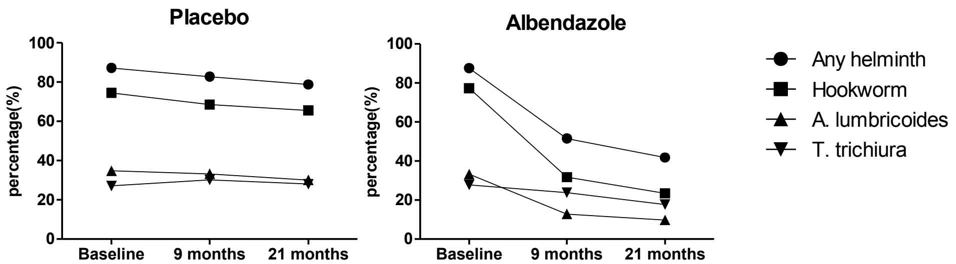


Figure 1. Trial Profile. HH: Household. Lost to follow up implies that the participants have no data from this time point onward. Temporarily absent implies that the participants have no data at this time point but have data available at other time point.

A



B

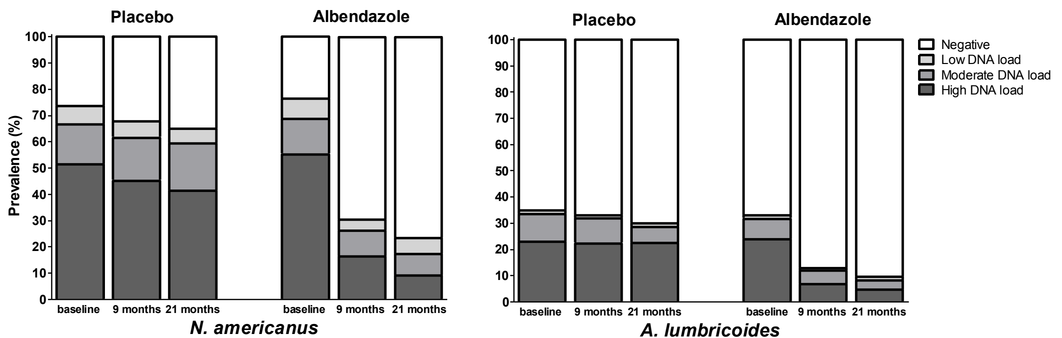


Figure 2. The effect of placebo versus albendazole treatment on prevalence and intensity of helminth infection. (A) The presence of hookworms (by PCR), Ascaris lumbricoides (by PCR) and Trichuris trichiura (by microscopy) or any of these helminth infections in subjects who provided stool samples at baseline, 9 and 21 months post treatment (numbers are given in table S1A). (B) The intensity of hookworm and *Ascaris* infection in positive subjects as determined by PCR is displayed in categories. Negative is when no helminth specific DNA was found. Positive Ct- values were grouped into three categories: Ct<30.0, 30.0≤Ct<35.0 and ≥35.0 representing a high, moderate and low DNA load, respectively.

The overall percentage of subjects with malarial parasitemia, irrespective of treatment arm, decreased over the trial period (table 2A). However, when the data were modelled to assess the effect of albendazole treatment over time, there was a significant (P=0.0064) increase, which might result from the transient four-fold increased risk of malarial parasitemia (OR 4.16 (1.35-12.80)) (table 2B) at 6 months after initiation of treatment (after 2 doses of albendazole). The effect of anthelminthic treatment was assessed in those younger than 15 years of age who would be the prime target of the global deworming programs. The transient

increase in parasitemia was only seen in the older (>15 years) age group (figure 3). Malarial parasites were also assessed by PCR, at 9 and 21 months after initiation of treatment and revealed that albendazole had no effect when all *Plasmodium* species were considered together, but when analyzed separately there was a significant increase in the percentage of subjects positive for *P. falciparum* at 9 months post-treatment (table 2C). There was no difference in the incidence of fever and additional malaria-like symptoms between the two treatment arms (supplementary appendix p10, table S2).

Table 2. Effect of three-monthly albendazole treatment on malaria outcomes

A. Percentage of subjects with malarial parasitemia

	P. falciparum		***P. vivax***		***P. malariae***	
	Placebo n/N (%)	**Albendazole** n/N (%)	**Placebo** n/N (%)	**Albendazole** n/N (%)	**Placebo** n/N (%)	**Albendazole** n/N (%)
Malarial parasitemia by microscopy						
0 month	32/1225 (2.6)	28/1187 (2.4)	26/1225 (2.1)	18/1187 (1.5)	2/1225 (0.2)	7/1187 (0.6)
3 months	41/897 (4.6)	46/910 (5.1)	17/897 (1.9)	22/910 (2.4)	1/897 (0.1)	6/910 (0.7)
6 months	8/815 (1.0)	20/794 (2.5)	4/815 (0.5)	9/794 (1.1)	0	0
9 months	14/947 (1.5)	7/950 (0.7)	4/947 (0.4)	5/950 (0.5)	1/947 (0.1)	1/950 (0.1)
12 months	9/834 (1.1)	9/813 (1.1)	4/834 (0.5)	2/813 (0.2)	0	0
15 months	14/773 (1.8)	13/772 (1.7)	3/773 (0.4)	4/772 (0.5)	1/773 (0.1)	3/772 (0.4)
18 months	3/815 (0.4)	10/803 (1.2)	1/815 (0.1)	1/803 (0.1)	1/815 (0.1)	1/803 (0.1)
21 months	6/824 (0.7)	11/824 (1.3)	6/824 (0.7)	0	3/824 (0.4)	1/824 (0.1)
Malarial parasitemia by PCR						
0 month	106/772 (13.7)	112/739 (15.2)	102/772 (13.2)	93/739 (12.6)	10/772 (1.3)	18/739 (2.4)
9 months	35/656 (5.3)	56/627 (8.9)	56/656 (8.5)	50/627 (8.0)	7/656 (1.1)	9/627 (1.4)
21 months	21/584 (3.6)	31/553 (5.6)	24/584 (4.1)	27/553 (4.9)	10/584 (1.7)	5/553 (0.9)

The number of positives (n) of the total population examined (N).

B. Malarial parasitemia by microscopy

	Placebo n/N (%)	**Albendazole** n/N (%)	**OR (95%CI)***	**OR (95%CI)***
3 months	59/897 (6.6)	72/910 (7.9)	1.54 (0.75-3.16)	
6 months	12/815 (1.5)	29/794 (3.7)	4.16 (1.35-12.80)	
9 months	19/947 (2.0)	13/950 (1.4)	0.57 (0.16-2.04)	
12 months	13/834 (1.6)	10/813 (1.2)	0.62 (0.12-3.15)	
15 months	18/773 (2.3)	20/772 (2.6)	1.17 (0.18-7.65)	
18 months	5/815 (0.6)	12/803 (1.5)	1.84 (0.12-29.03)	
21 months	15/824 (1.8)	12/824 (1.5)	0.26 (0.01-6.59)	
				0.02 0.06 0.25 1.00 4.00 16.00

*The number of positives (n) of the total population examined (N). *Odds ratio and 95% confidence interval are based on mixed effects logistic regression models. OR's and 95% CI are shown for the separate time points on malarial parasitemia. The p-value is generated from the modeled data for the combined effect of albendazole treatment over time, which is significant (P = 0.0064) and might result from the effect of 6 months post treatment time point.*

C. Effect of treatment on malarial parasitemia by PCR

	Placebo n/N (%)	**Albendazole** n/N (%)	OR (95% CI)
Malaria (any spp)			
9 months	95/656 (14.5)	103/627 (16.4)	1.13 (0.77-1.64)
21 months	53/584 (9.1)	59/553 (10.7)	1.09 (0.68-1.76)
P. falciparum			
9 months	35/656 (5.3)	56/627 (8.9)	2.82 (1.29-6.15)
21 months	21/584 (3.6)	31/553 (5.6)	1.63 (0.63-4.22)
P. vivax			
9 months	56/656 (8.5)	50/627 (8.0)	0.84 (0.41-1.71)
21 months	24/584 (4.1)	27/553 (4.9)	1.40 (0.56-3.52)
P. malariae			
9 months	7/656 (1.1)	9/627 (1.4)	0.34 (0.04-2.79)
21 months	10/584 (1.7)	5/553 (0.9)	0.04 (0.00-0.39)

The number of positives (n) of the total population examined (N). Odds ratio and 95% confidence interval based on logistic mixed models. The statistically significant results are given in bold. The p-values are generated from the modeled data for the combined effect of albendazole treatment over time for each of the species separately, which were significant for P. falciparum (P = 0.029) and P. malariae (P = 0.016).

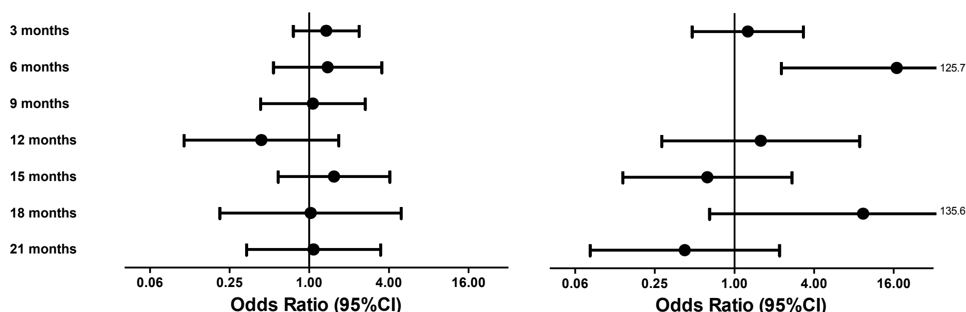


Figure 3. Effect of albendazole treatment on malarial parasitemia based on two age categories. Risk of malarial parasitemia in A) ≤15 and B) >15 years of age. The risk of malarial parasitemia after albendazole treatment compared to placebo is shown as odds ratio with 95% CI. The reference line is set at 1, indicating that symbols at the right of this line represent an increased risk, while symbols at the left of the line would predict decreased risk of malarial parasitemia. Note: at 9 month time point in those >15 years of age, the OR is ∞.

The proportion of subjects with SPT reactivity was 353/1364 (25.9%) at baseline. Albendazole treatment had no statistically significant effect on SPT to any allergen (table 3A), but it was noted that there was an incremental increase in the risk of being SPT positive to any allergen at 9 months and 21 months post initiation of treatment. Moreover, additional analysis on allergens separately, showed a significantly higher SPT to cockroach at 21 months after treatment (1.63 (1.07-2.50)) (table 3B). The levels of IgE to allergens showed that albendazole treatment had no effect (table 3B). No effect of treatment was seen on symptoms of asthma or atopic dermatitis (supplementary appendix p11, table S3).
No significant change in BMI was observed in children or in adults (supplementary appendix p12, table S4). Moreover, there was no adverse effect of treatment reported.

Table 3. Effect of three-monthly albendazole treatment on allergy outcomes

A. Skin prick test to any allergens

	Placebo n/N (%)	Albendazole n/N (%)	OR (95%CI)*	OR (95%CI)*
SPT to any allergen				
9 months	80/462 (17.3)	82/454 (18.1)	1.18 (0.74-1.86)	
21 months	145/455 (31.9)	161/439 (36.7)	1.37 (0.93-2.01)	
				0.50 1.00 2.00

The number of positives (n) of the total population examined (N).
**Odds ratio and 95% confidence interval are based on mixed effects logistic regression models. OR's and 95% CI are shown for the separate time points on SPT to any allergen. The p-value is generated from the modeled data for the effect of albendazole treatment overtime and no significant effects were found (P>0.05).*

B. Skin prick test and specific IgE to aeroallergen

	Placebo	Albendazole	
Skin prick test reactivity*	**n/N (%)**	**n/N (%)**	**OR (95% CI)**
House dust mite			
9 months	36/462 (7.8)	35/454 (7.7)	1.31 (0.52-3.27)
21 months	77/455 (16.9)	76/439 (17.3)	1.37 (0.62-3.02)
Cockroach			
9 months	60/462 (13.0)	65/454 (14.3)	1.27 (0.75-2.15)
21 months	112/455 (24.6)	139/439 (31.7)	1.63 (1.07-2.50)
Specific IgE**	**N (Median, IQR)**	**N (Median, IQR)**	**β (95% CI)**
House dust mite			
9 months	391 (0.46, 0.16-2.35)	381 (0.46, 0.14-1.98)	1.01 (0.91-1.12)
21 months	339 (0.82, 0.27-3.29)	334 (0.65, 0.20-2.69)	0.93 (0.81-1.06)
Cockroach			
9 months	391 (1.47, 0.30-5.01)	381 (1.55, 0.44-4.40)	1.04 (0.93-1.16)
21 months	339 (1.83, 0.47-5.44)	334 (1.64, 0.42-4.82)	0.98 (0.85-1.14)

*The number of positives (n) of the total population examined (N). *Odds ratio and 95% confidence interval based on logistic mixed models; **β (beta) and 95% confidence interval based on generalized linear mixed models from the log-transformed IgE. The values shown are back-transformed. The p-values are generated from the modeled data for the effect of albendazole treatment overtime and no significant effects were found (P>0.05).*

Discussion

This household-based clustered-randomized, double-blind, placebo-controlled trial shows that administering a total of seven single doses of albendazole, at three-monthly intervals, to a population living in an area of Indonesia where STH are highly prevalent, leads to decreased prevalence of helminth infections which although statistically significant, can be taken as an incomplete reduction. The results show a transient increase in malarial parasitemia in the albendazole- compared with the placebo-treated arm in the first six months after initiation of treatment. Albendazole treatment had no statistically significant effect on the designated co-primary outcome, skin prick test reactivity to allergens.

The clinical data collected of fever and additional malaria-like symptoms, were not affected by the deworming. Clinical signs of asthma and atopic dermatitis were also not affected by albendazole treatment.

The prevalence of infection was high (>60%), which reflects the situation in many areas that are being targeted by the global deworming programs. Using a three-monthly treatment regimen, which represents an extreme scenario for helminth control strategy, percentage of subjects positive for STH was reduced by 39% compared to placebo. It should be noted that in our study the sensitive PCR method has been used. The reduction in the proportion of subjects infected with hookworm and *Ascaris* was more pronounced than for *Trichuris* infections, confirming the findings using a single dose of albendazole[24]. Subjects who provided stool samples at 21 months were slightly but significantly older than those who did not. Given that hookworm infection is more prevalent in older subjects, this may have contributed to the poor deworming achieved by albendazole. The reduction achieved in worm loads, did not have any beneficial effect on BMI. Observational studies have reported that helminth infections affect growth; however randomized trials have not been consistent[25,26]. In this regard, our study would support the outcome of a recent Cochrane review of no beneficial effect of deworming programs on nutritional indicators[27] even though it can be argued that in our study the suboptimal reduction in the STH would not allow any beneficial effect of anthelmintic in terms of BMI to be seen in the community. Importantly, the fact that the effect of such an intensive deworming strategy in a community is incomplete, needs to be considered in the agenda for the control and elimination of helminth diseases of humans[28].

Most studies on the effect of helminth infections on malarial parasitemia and clinical malaria episodes have used cross-sectional designs and have been inconclusive[6]. Longitudinal studies of anthelminthic treatment have also reported conflicting results[29,30]. A small study conducted in Madagascar has reported an increase in malarial parasitemia in levimasole treated subjects, older than 5 years of age[29], while in Nigeria, albendazole treatment of pre school children was

associated with lower *P. falciparum* infection and anemia, however, the lost to follow up in this study was very high[30]. The question whether albendazole treatment during pregnancy could affect health outcomes in the offspring, was addressed in a recent report from Uganda[31]. It was found that the incidence of malaria up to one year of age was not different in the offspring of mothers born to those treated with albendazole or placebo. Our study reports the results of a community wide randomized-controlled trial that used three-monthly malarial parasitemia data obtained by microscopy. A significantly higher percentage of subjects positive for malarial parasites in the albendazole compared to the placebo arm was seen but this seemed to be a transient effect and restricted to individuals older than 15 years of age, an age group that is not the main target of the current deworming programs. The question arises as to why this effect was only seen in those >15 years of age. This could be due to the fact that *Ascaris* infection is lower in older age and therefore more easily cleared. It has been suggested that *Ascaris* is the species of helminth that has the most effect on malarial parasitemia and diseases[6]. Therefore by removing *Ascaris* in older age, we might be seeing a more profound effect on malarial parasitemia.

Using PCR, which enables detection of sub-microscopic infections at species level, it was also concluded that albendazole did not affect overall malarial parasitemia. When malaria species were analyzed separately, the percentage of subjects infected with *P. falciparum* but not with *P. vivax* increased significantly in the first 9 months post-treatment in the albendazole-treated arm, which is contrary to our hypothesis that anthelminthic treatment would reduce prevalence of malarial parasitemia[32]. It was expected that by decreasing STH, the immune hyporesponsiveness would be reversed and this would be associated with stronger immune effector responses to malaria parasites. One of the possible explanations for the enhanced malarial parasitemia would be that with a reduction in STH, there is increased nutrient availability for other co infections and their growth.

It has been suggested that there are different malaria outcomes with different species of helminths; *Ascaris* being associated with protection regarding parasitemia and severity of malaria while hookworm with higher incidence of malaria[6].(Nacher 259) Our study was not powered to conduct a stratified analysis, and with the overall gradual decrease in malaria in the study area during our study, the numbers of subjects positive for malaria parasites are too few for an ad-hoc analysis.

The findings concerning allergy outcomes, although not significant, are in line with our hypothesis that anthelminthic treatment would increase SPT reactivity. The risk of SPT reactivity increased incrementally with longer treatment and raises the question whether even longer deworming periods are needed for more pronounced effects on allergic outcomes. In support of this, a recent study reported that 15-17 years of ivermectin treatment for onchocerciasis control in

Ecuador led to a significant increase in SPT reactivity to allergens[12]. In the same country, one year of anthelmintic treatment in schoolchildren did not lead to any change in SPT[10]. The question whether different species of helminths might affect allergic outcomes to a different degree, remains unanswered. It is interesting that one year anthelmintic treatment in Vietnam where hookworm infection was the prominent species, as in our study, resulted in a significant increase in SPT positivity in schoolchildren. This is in contrast to what was seen in Ecuador where *Ascaris lumbricoides* was the most prevalent species. One common feature of the anthelmintic trials seems to be that clinical symptoms of allergy do not change with deworming. However, an important trial in pregnant women in Uganda has shown an increased risk of infantile eczema in infants of mothers treated with anthelminthics compared to those that received placebo[33]. This could indicate that exposure to worms in early life, might affect allergic outcomes more profoundly than when helminths are removed later in life[34].

One of the limitations of this trial is the overall decrease in malarial parasitemia during the two-year study period, most probably caused by actively referring subjects with malaria-like symptoms to puskesmas. Therefore further studies in areas highly endemic for malaria are needed. Treatment in the trial did result in a significant reduction in percentage of subjects infected with STH, but this reduction was incomplete. It is therefore possible that the community was insufficiently dewormed. However, our primary aim was to measure the possible effect of deworming programmes on malaria or allergy. We conclude that despite transient increase in malarial parasitemia as a result of albendazole treatment, there were no clinically relevant changes to outcome measures 21 months after treatment was initiated.

In conclusion, an extremely intensive anthelminthic treatment in a community where STH are highly endemic, does not lead to elimination but reduces both prevalence and intensity of helminths. Such MDA regiment appears safe and does not lead to any significant change with respect to malaria infections or allergies. However, it is worrying that such vigorous community treatment does not have a more pronounced effect on STH burden. Better integrated control strategies would be needed to deworm and subsequently assess whether the risk for malaria infections or allergies change.

References

1. Bethony, J., *et al.* Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. *Lancet* 367, 1521-1532 (2006).
2. Utzinger, J. A research and development agenda for the control and elimination of human helminthiases. *PLoS neglected tropical diseases* 6, e1646 (2012).
3. Lustigman, S., *et al.* A research agenda for helminth diseases of humans: the problem of helminthiases. *PLoS neglected tropical diseases* 6, e1582 (2012).
4. Allen, J.E. & Maizels, R.M. Diversity and dialogue in immunity to helminths. *Nature reviews. Immunology* 11, 375-388 (2011).
5. Maizels, R.M. & Yazdanbakhsh, M. Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nature reviews. Immunology* 3, 733-744 (2003).
6. Nacher, M. Interactions between worms and malaria: good worms or bad worms? *Malaria journal* 10, 259 (2011).
7. Bach, J.F. The effect of infections on susceptibility to autoimmune and allergic diseases. *The New England journal of medicine* 347, 911-920 (2002).
8. Feary, J., Britton, J. & Leonardi-Bee, J. Atopy and current intestinal parasite infection: a systematic review and meta-analysis. *Allergy* 66, 569-578 (2011).
9. Flohr, C., Quinnell, R.J. & Britton, J. Do helminth parasites protect against atopy and allergic disease? *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 39, 20-32 (2009).
10. Cooper, P.J., *et al.* Effect of albendazole treatments on the prevalence of atopy in children living in communities endemic for geohelminth parasites: a cluster-randomised trial. *Lancet* 367, 1598-1603 (2006).
11. Flohr, C., *et al.* Reduced helminth burden increases allergen skin sensitization but not clinical allergy: a randomized, double-blind, placebo-controlled trial in Vietnam. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 40, 131-142 (2010).
12. Endara, P., *et al.* Long-term periodic anthelmintic treatments are associated with increased allergen skin reactivity. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 40, 1669-1677 (2010).
13. Lau, S. & Matricardi, P.M. Worms, asthma, and the hygiene hypothesis. *Lancet* 367, 1556-1558 (2006).
14. Hamid, F., *et al.* A longitudinal study of allergy and intestinal helminth infections in semi urban and rural areas of Flores, Indonesia (ImmunoSPIN Study). *BMC infectious diseases* 11, 83 (2011).
15. Wiria, A.E., *et al.* Does treatment of intestinal helminth infections influence malaria? Background and methodology of a longitudinal study of clinical, parasitological and immunological parameters in Nangapanda, Flores, Indonesia (ImmunoSPIN Study). *BMC infectious diseases* 10, 77 (2010).
16. Elyazar, I.R., Hay, S.I. & Baird, J.K. Malaria distribution, prevalence, drug resistance and control in Indonesia. *Advances in parasitology* 74, 41-175 (2011).
17. Harijanto, P.N. Malaria treatment by using artemisinin in Indonesia. *Acta medica Indonesiana* 42, 51-56 (2010).
18. Szefler, S.J. Advances in pediatric asthma in 2007. *The Journal of allergy and clinical immunology* 121, 614-619 (2008).
19. WHO. Guidelines for the treatment of malaria, 2nd ed. (2010).
20. Adegnika, A.A., *et al.* Microscopic and sub-microscopic Plasmodium falciparum infection, but not inflammation caused by infection, is associated with low birth weight. *The American journal of tropical medicine and hygiene* 75, 798-803 (2006).

21. WHO. WHO Child Growth Standards: Length/height-for-age, weight-for-age, weight-for-length, weight-for-height and body mass index-for-age: Methods and development. (2006).
22. Laird, N.M. & Ware, J.H. Random-effects models for longitudinal data. *Biometrics* 38, 963-974 (1982).
23. R-Forge. lme4 - Mixed-effects models project. (2011).
24. Keiser, J. & Utzinger, J. Efficacy of current drugs against soil-transmitted helminth infections: systematic review and meta-analysis. *JAMA : the journal of the American Medical Association* 299, 1937-1948 (2008).
25. Alderman, H., Konde-Lule, J., Sebuliba, I., Bundy, D. & Hall, A. Effect on weight gain of routinely giving albendazole to preschool children during child health days in Uganda: cluster randomised controlled trial. *BMJ* 333, 122 (2006).
26. Dickson, R., Awasthi, S., Williamson, P., Demellweek, C. & Garner, P. Effects of treatment for intestinal helminth infection on growth and cognitive performance in children: systematic review of randomised trials. *BMJ* 320, 1697-1701 (2000).
27. Taylor-Robinson, D.C., Maayan, N., Soares-Weiser, K., Donegan, S. & Garner, P. Deworming drugs for soil-transmitted intestinal worms in children: effects on nutritional indicators, haemoglobin and school performance. *Cochrane Database Syst Rev* 11, CD000371 (2012).
28. Prichard, R.K., *et al.* A research agenda for helminth diseases of humans: intervention for control and elimination. *PLoS neglected tropical diseases* 6, e1549 (2012).
29. Brutus, L., Watier, L., Hanitrasoamampionona, V., Razanatsoarilala, H. & Cot, M. Confirmation of the protective effect of Ascaris lumbricoides on Plasmodium falciparum infection: results of a randomized trial in Madagascar. *The American journal of tropical medicine and hygiene* 77, 1091-1095 (2007).
30. Kirwan, P., *et al.* Impact of repeated four-monthly anthelmintic treatment on Plasmodium infection in preschool children: a double-blind placebo-controlled randomized trial. *BMC infectious diseases* 10, 277 (2010).
31. Webb, E.L., *et al.* Effect of single-dose anthelmintic treatment during pregnancy on an infant's response to immunisation and on susceptibility to infectious diseases in infancy: a randomised, double-blind, placebo-controlled trial. *Lancet* 377, 52-62 (2011).
32. Specht, S. & Hoerauf, A. Does helminth elimination promote or prevent malaria? *Lancet* 369, 446-447 (2007).
33. Mpairwe, H., *et al.* Anthelminthic treatment during pregnancy is associated with increased risk of infantile eczema: randomised-controlled trial results. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology* 22, 305-312 (2011).
34. Djuardi, Y., Wammes, L.J., Supali, T., Sartono, E. & Yazdanbakhsh, M. Immunological footprint: the development of a child's immune system in environments rich in microorganisms and parasites. *Parasitology* 138, 1508-1518 (2011).

Supplementary Methods

Additional information on the study area and procedures

Ende district, an area highly endemic for STH, is situated near the equator (8°45'S, 121°40'E) and it is characterized by a uniform high temperature, in the range of 23-33.5 °C, with humidity of 86-95%. Average yearly rain fall is 1.822 mm with about 82 rainy days, especially from November to April, with the peak in December until March. The semi-urban village of Nangapanda, endemic for malaria, had a population of 3583 and is located in the coastal area with most villagers being farmers and fishermen with some government officers or private sector employees. The rural village Anaranda had 1631 inhabitants and is located 80 km further inland of Nangapanda. There was poor infrastructure and inhabitants generated income mainly from farming.

Regarding the availability of the anthelminthics in the community, there was no deworming campaign in this area during the study period. Pyrantel pamoat (Combantrin®) and dehydropiperazine (Bintang 7 puyer 17®) were the only available anthelminthics in the market. The local primary health centre (Puskesmas) did not provide the current trial study participants by any anthelminthic treatment but referred them to the trial team.

Malaria control, such as by artemisinin-combination therapy (ACT) treatment and insecticide-treated nets (ITN) or long-lasting insecticide-treated nets (LLIN) although planned, were not implemented yet during our study period. This was due to several difficulties faced in some parts of Indonesia, such as instable drug supply, lack of training on definitive diagnosis of malaria by the laboratory staff, as well as insufficient bednet supply and poor compliance[1]. Malaria drugs such as chloroquine and quinine were available in the shops, however, little information is available on proper self medication. Therefore, before and during the study period, regular training of field workers was undertaken on how to prevent malaria (use of repellent and bednet, irrigation of breeding places) and how to treat malaria (not to self medicate but to visit puskesmas for diagnosis and treatment). Indoor residual spraying was done by the local health authority for dengue control against an outbreak at the beginning of 2008.

The treatment of suspected malaria cases at the puskesmas was chloroquine and primaquine for *P. vivax,* while for *P. falciparum* sulfadoxine/pyrimethamine was commonly used. Subjects in our study with fever and/or any one of the malaria-like symptoms (see below for detailed description) were referred to the puskesmas for assessment and treatment according to local health center policy.

The anthelmintic treatment and placebo were coded and the code was concealed from trial investigators and participants. The tablets were distributed by trained health workers and the intake was directly observed. Labels with the study subject ID were printed from a computer database and attached to the appropriate strip of

treatment by a separate team located in Jakarta without the involvement of the study investigators. In order to assess whether anthelminthic treatment had any adverse effect on the growth of children or on the incidence of allergy, interim analyses were done at one year post-treatment by a monitoring committee. After completion of the study the whole population was treated with albendazole (a single dose of 400mg for three consecutive days).

The malaria slides were read by microscopy at the Department of Parasitology in Jakarta. The quality control for microscopic reading took place in the pilot phase of the project. In cooperation with NAMRU-2 (US Naval Medical Research Unit-2) two microscopists from our team were trained, inter-observer differences were assessed and following satisfactory training they were certified. At the pilot phase, and throughout the study, PCR was used to monitor the microscopy data with a high degree of agreement between microscopy and PCR. In a random sub-sample at 9 months and 21 months post-treatment we measured malarial parasitemia by PCR.

Primers and the *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*-specific probes were used with some modifications in the fluorophore- and quencher-chemistry. Amplification reactions of each DNA sample are performed in white PCR plates, in a volume of 25 µl with PCR buffer (HotstarTaq master mix), 5 mmol/l MgCl2, 12.5 pmol of each Plasmodium-specific primer and 15 pmol of each PhHV-1-specific primer, 1.5 pmol of each *P. falciparum*, *P. vivax*, *P. malariae*-specific XS- probes, and PhHV-1-specific Cy5 double-labeled detection probe, and 2.5 pmol of each *P. ovale*-specific XS-probes (Biolegio), and 5 µl of the DNA sample were used. Amplification consists of 15 min at 95ºC followed by 50 cycles of 15 s at 95ºC, 30 s at 60ºC, and 30 s at 72ºC. Amplification, detection, and analysis are performed with the CFX96 real-time detection system (Bio-Rad laboratories). The PCR output from this system consists of a cycle-threshold value (Ct), representing the amplification cycle in which the level of fluorescent signal exceeds the background fluorescence, and reflecting the parasite-specific DNA load in the sample tested. Negative and positive control samples are included in each amplification run.

Stool samples were collected and preserved in 4% formaldehyde for microscopy examination or frozen (-20°C) unpreserved for PCR detection. The formol-ether acetate concentration method was performed on the formalin preserved stool samples followed by microscopic examination for *Trichuris trichiura* infections. As described in detail before[2], DNA was isolated from approximately 100 mg unpreserved feces and examined for the presence of *Ancylostoma duodenale, Necator americanus, Ascaris lumbricoides* and *Strongyloides stercoralis* DNA by the multiplex qPCR. The qPCR output from this system consisted of a Ct value; negative and positive control samples were included in each run of the amplification. Positive Ct- values were grouped into three categories: Ct<30.0,

30.0≤Ct<35.0 and ≥35.0 representing a high, moderate and low DNA load, respectively.

Data collection on clinical symptoms

A year before the study enrolment, community workers were recruited and trained in taking finger-prick blood for the three-monthly malarial parasitemia survey in Nangapanda, observing drug intake, recording adverse treatment effects, as well as measuring fever and administering monthly malaria-like symptoms questionnaire. These questionnaires were based on WHO definitions[3] and were assessed in all individuals that were present at the time of the survey. Subjects with fever (≥37.5°C) or additional malaria-like symptoms (headache, fatigue and nausea) at the time of visits were referred to the puskesmas for treatment according to local standard protocols. The monthly data on fever (≥37.5, using digital thermometer) and additional malaria-like symptoms were collected at baseline September 2008 and in the months Oct 08, Nov 08, Dec 08, Jan 09, Feb 90, March 09, Apr 09, May 09, June 09, Aug 09, Sept 09, Oct 09, Nov 09, Dec 09, Jan 10, Feb 10, March 10 and Apr 10. At baseline, 1396 individuals were assessed in placebo and 1381 in the albendazole arm and at the last time point, 1165 and 1181 subjects were followed up in the two groups, respectively. Questionnaire data were available for all time points from 45.8% and 47.2% of placebo and albendazole group whereas data for 80% of the time points were available from 83.8% and 87.6% of the two groups, respectively. The number of events was recorded in total of 15259 and 15307 person months at risk for placebo and albendazole groups, respectively.

The modified video-assisted (for asthma symptoms) and illustration-assisted (for atopic dermatitis) ISAAC questionnaire, translated to Bahasa Indonesia and back translated for use in our studies within the EU funded project GLOFAL (www.glofal.org), were administered at baseline and at 21 month time points. Data were available from 629 in placebo and 635 in albendazole arm at baseline, while these numbers were 460 and 445, respectively, at the 21 month time point. These questionnaires were administered to the parents/guardians of subjects who were skin prick tested with allergens: the trial profile is given in supplementary figure 1B. The prevalence of asthma symptoms were obtained from the following questions: (i) has your child ever had asthma? (ii) has your child ever been diagnosed for asthma by a doctor? and (iii) has your child in the past 12 months had wheezing or whistling in the chest?; while the prevalence of atopic dermatitis was obtained from the questions: (i) has your child ever had doctor/paramedic diagnosed allergic eczema and (ii) has your child ever had one or more skin problems accompanied by an itchy rash?

If the answer to one or more of these questions was positive, the subjects were considered to have either asthma or atopic dermatitis symptoms.

Detailed description of the statistical models used

Descriptives were computed for each variable (mean and standard deviation or median and interquartile range for continuous outcomes, numbers and percentages for categorical variables). For children ≤ 19 years, BMI age-standardized z-scores were calculated according to WHO references[4].

Two sources of correlation among observations should be accounted for when modeling these data, namely observations at various time points for a subject are correlated due to subject specific effects and observations within households are correlated due to environmental effects shared within households. To model these correlations we used random effects. For subject effects a random intercept and a random slope were used, i.e. each subject has its own intercept and slope, where the latter models the change of the outcome variable over time. Observations within a household also have a shared random intercept. Thus the intercept for an observation of a specific subject from a specific household is the overall mean plus the subject specific effect plus the household effect. By doing so correlation among observations of the same household was modeled since these observations share the same household effect. To assess treatment effects generalized linear mixed models[5] were used where the term "mixed" corresponds to the used random effects. Unless stated otherwise all models included area as covariate in the model to take into account the differences between the two villages. Generalized linear mixed models provide a flexible and powerful tool to derive valid inference while capturing the data correlations induced by clustering within households and repeated evaluations in time of the same subject.

For continuous outcome variables which were measured at 0, 9 and 21 months, treatment effects were modeled at time point 9 and 21 months, because treatment started at 0 months and the design is a randomized trial no treatment effect should be present at time 0. We allowed for different treatment effects at 9 and 21 months. Beta's and 95% confidence intervals are provided for 9 and 21 months. The betas represent the mean difference between the placebo and treatment group. An overall test for treatment effect over time was performed by using a likelihood ratio test which compares the model with and without the treatment effect (2 df test).

For binary outcome variables measured at 9 and 21 months, the logit link was used (mixed effect logistic regression). In these models only the two random intercepts were included and the random subject specific slope was omitted. Odds ratios and 95% confidence intervals are reported. Analogously to continuous outcome variables two degrees of freedom likelihood ratio tests were performed to assess treatment effects over time. Note that the model based odds ratios are different from crude odds ratios directly computed from the sample due to missing observations and due to the presence of random effects and the covariate area in

the model. Malarial parasitemia by microscopy was measured at a three monthly basis. To model these data, similar models were used. Specifically at each of the seven time points (excluding time zero) a treatment effect was included. The likelihood ratio test for treatment effect over time has therefore 7 degrees of freedom. All generalized linear mixed models were fitted using the lme4 package (Douglas Bates, Martin Maechler and Ben (2011). [lme4: Linear mixed-effects models using S4 classes. R package version 0.999375-42. http://CRAN.R-project.org/package=lme4) in R[6]].

For each malaria-like symptom (fever, headache, fatigue, and nausea), total number of events and person months were computed for each treatment group. We calculated incidence rates for all events. Symptom episodes within three months of an initial presentation with the same symptom were regarded as part of the same episode. Hazard ratios for effect of treatment were calculated with Cox regression with robust SE to allow for within-subject and within household clustering (STATA 12).

References (for supplementary material)

1. Elyazar, I.R., Hay, S.I. & Baird, J.K. Malaria distribution, prevalence, drug resistance and control in Indonesia. *Advances in parasitology* **74**, 41-175 (2011).
2. Wiria, A.E., *et al.* Does treatment of intestinal helminth infections influence malaria? Background and methodology of a longitudinal study of clinical, parasitological and immunological parameters in Nangapanda, Flores, Indonesia (ImmunoSPIN Study). *BMC infectious diseases* **10**, 77 (2010).
3. WHO. Guidelines for the treatment of malaria, 2nd ed. (2010).
4. WHO. WHO Child Growth Standards: Length/height-for-age, weight-for-age, weight-for-length, weight-for-height and body mass index-for-age: Methods and development. (2006).
5. Laird, N.M. & Ware, J.H. Random-effects models for longitudinal data. *Biometrics* **38**, 963-974 (1982).
6. R-Forge. lme4 - Mixed-effects models project. (2011).

Table S1. Effect of three-monthly albendazole treatment on helminth infection

	Placebo n/N (%)	Albendazole n/N (%)	OR (95% CI)
Helminth infection (any spp)			
9 months	395/477 (82.8)	247/480 (51.4)	0.07 (0.04-0.11)
21 months	353/448 (78.8)	172/411 (41.9)	0.05 (0.03-0.08)
Hookworm[1]			
9 months	359/524 (68.5)	161/508 (31.7)	0.02 (0.01-0.04)
21 months	305/466 (65.5)	99/423 (23.4)	0.01 (0.01-0.03)
A. lumbricoides[1]			
9 months	174/524 (33.2)	65/508 (12.8)	0.24 (0.16-0.36)
21 months	140/466 (30.0)	41/423 (9.7)	0.18 (0.11-0.29)
T. trichiura[2]			
9 months	219/726 (30.2)	160/673 (23.8)	0.58 (0.42-0.80)
21 months	177/633 (28.0)	101/571 (17.7)	0.40 (0.28-0.58)

The number of positives (n) of the total population examined (N).
[1]diagnosed by PCR. [2]diagnosed by microscopy. Odds ratio and 95% confidence interval based on logistic mixed models. The p-values are generated from the modeled data for the combined effect of albendazole treatment over time, which were significant (P < 0.001) for any helminth and for each of the species separately.

Table S2. The effect of albendazole on fever and additional malaria like symptoms

	Placebo		Albendazole		Unadjusted IRR	Adjusted IRR
	Events (PM)	Incidence per PM	Events (PM)	Incidence per PM		
Fever	414 (18494)	0.02	429 (18636)	0.02	1.03	1.03
Headache	333 (19067)	0.02	340 (19563)	0.02	1.00	1.00
Fatigue	49 (22362)	0.002	69 (22535)	0.003	1.39	1.41
Nausea	76 (21749)	0.003	55 (22211)	0.002	0.71	0.71
Any symptom	661 (15259)	0.04	690 (15307)	0.05	1.04	1.04

IRR: incidence rate ratio, PM: Person months. Adjusted with age and sex. The p-values are generated from Cox regression of albendazole treatment over time with robust SEs to allow for within-subject and within household clustering and no significant effects were found (P>0.05).

Table S3. Reported clinical symptoms of allergy

	Placebo n/N (%)	Albendazole n/N (%)	OR (95% CI)
Asthma			
21 months	8/461 (1.7)	11/445 (2.5)	1.11 (0.07-17.26)
Atopic dermatitis			
21 months	13/461 (2.8)	9/445 (2.0)	0.57 (0.16-2.02)

The number of positives (n) of the total population examined (N).
The p-values are generated from the modeled data for the effect of albendazole treatment after 21 months and no significant effects were found (P>0.05). At baseline 8/692 (1.2%) and 18/692 (2.6%) in the placebo group reported symptoms of asthma and atopic dermatitis, respectively, while in the albendazole group this was 10/635 (1.6%) and 11/635 (1.7%).

Table S4. Effect of three-monthly albendazole treatment on BMI

	Placebo N (Median, IQR)	Albendazole N (Median, IQR)	β (95% CI)
BMI			
9 months	498 (22.42, 19.91 – 25.54)	499 (22.07, 19.96 – 24.56)	-0.10 (-0.29–0.09)
21 months	430 (22.42, 19.68 – 25.56)	425 (21.56, 19.44 – 24.12)	-0.15 (-0.39–0.10)
z-BMI			
9 months	346 (-0.81, -1.44 – -0.13)	334 (-0.96, -1.56 – -0.30)	-0.04 (-0.17–0.09)
21 months	272 (-1.29, -2.21 – -0.56)	269 (-1.57, -2.32 – -0.74)	-0.07 (-0.23–0.10)

The total population examined (N). IQR = Interquartile range.
β (beta) and 95% confidence interval based on generalized linear mixed models. The p-values are generated from the modeled data for the combined effect of albendazole treatment over time and no significant effects were found (P>0.05). Baseline data are shown in Table 1 of the manuscript.

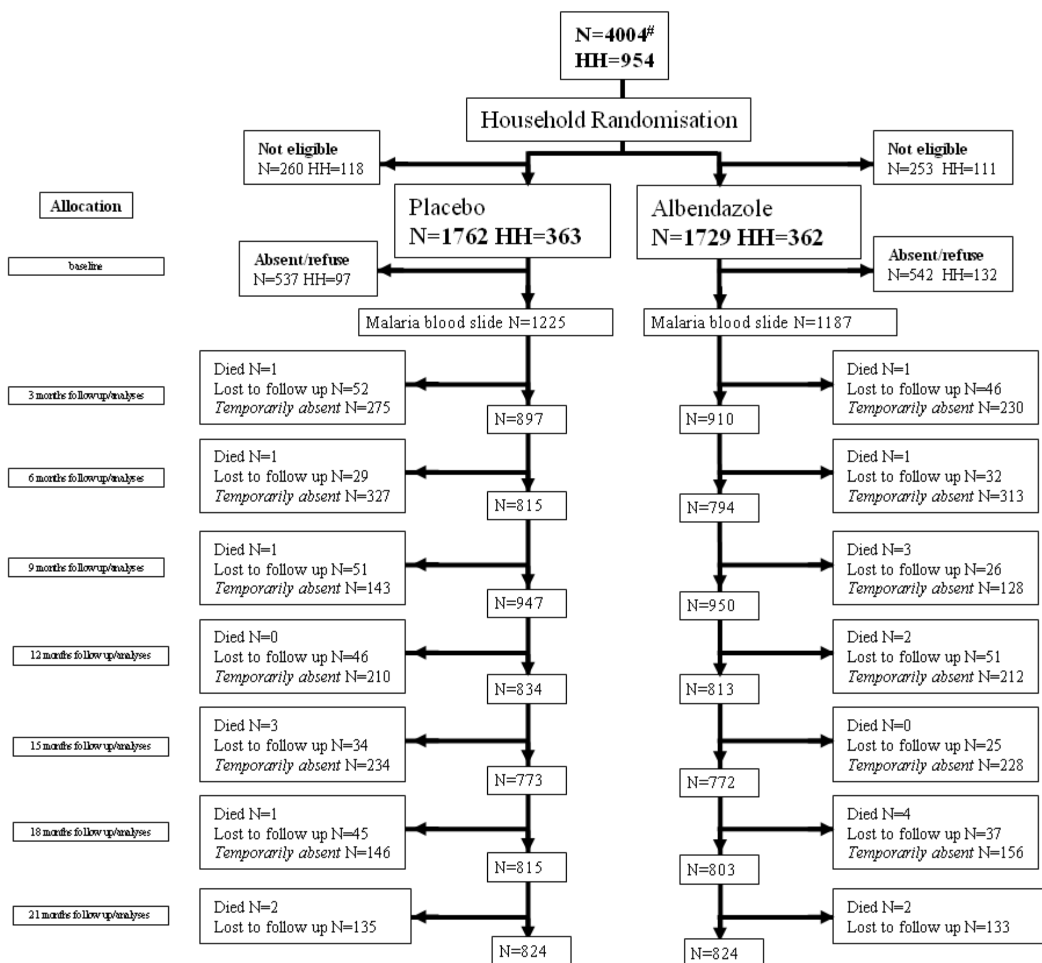


Figure S1A. Profile of trial with malarial parasitemia as outcome in the village of Nangapanda where malaria is endemic.

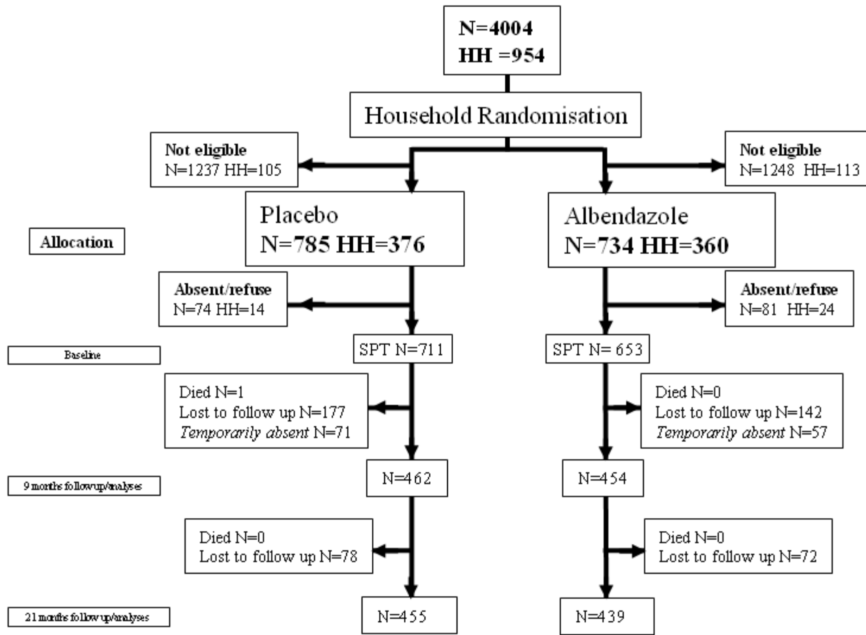


Figure S1B. Profile of trial with skin prick test (SPT) reactivity as outcome in children 5-15 years of age in both Nangapanda and Anaranda.

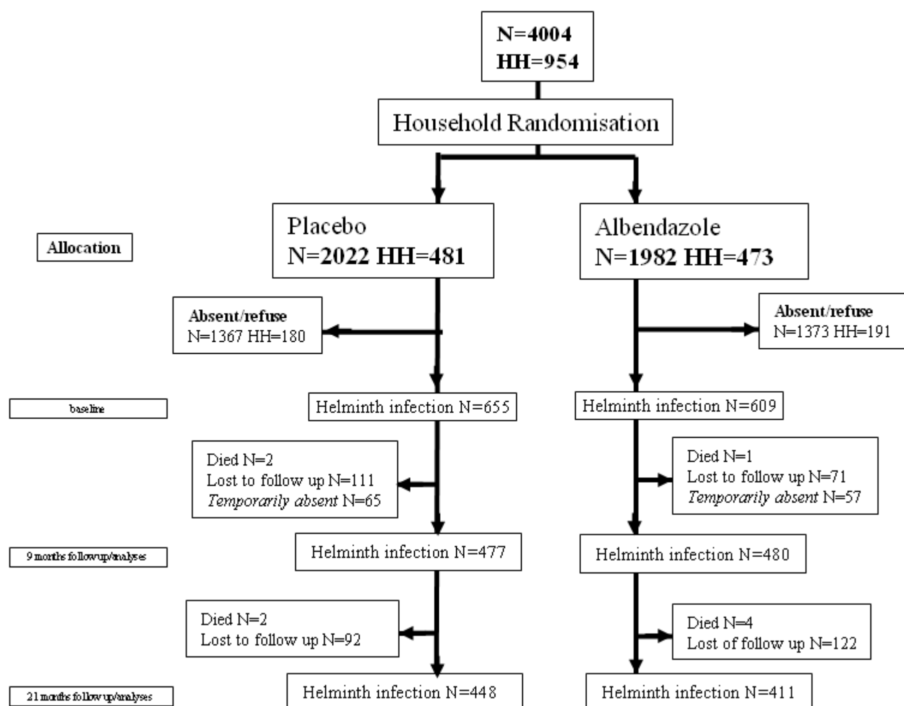


Figure S1C. Profile of trial with helminth infection as outcome in villages of Nangapanda and Anaranda .

CHAPTER 8

Global worming or deworming? Epidemiological, immunological and clinical perspectives

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Summary

Helminth infections still affect billions of people worldwide. Deworming is advocated to prevent the morbidities induced by helminths. At the same time, in resource-rich settings, the possibility of infecting patients with different life cycle stages of worms is being explored, specifically for those suffering from a range of inflammatory diseases. It has been recognized that helminths have immunomodulatory properties, which can impair not only parasite-specific immune responses but also responses to third party antigens. By using these properties, parasitic helminths allow their long-term survival within their human host but at the same time curtail overt immunological responses that lead to allergic, inflammatory bowel or autoimmune diseases. However, this would also suggest that deworming could lead to the development of inflammatory conditions, due to the removal of regulatory mechanisms associated with helminths. This can result in unfavorable situations in countries that are not prepared for the emergence of such pathological conditions. This review summarizes the consequences of deworming versus helminthic therapy.

Background

Parasitic worms have accompanied us throughout human history[1]. The fact that mortality due to helminth infections is rare, and that worm infections are often asymptomatic, with only a minority of infected individuals having intense infections[2], suggests that this is as a result of evolutionary co-adaptation between parasitic worms and man. A key component of this partnership is the immunological interaction between helminths and their mammalian hosts. Helminths modulate immunological processes: multiple mechanisms of helminth-induced immunological tolerance and regulatory pathways have been revealed over the past 50 years, that may explain chronicity of these infections[3]. Helminths also appear to fundamentally affect the host's genetic composition; studies into the recent evolutionary history of human interleukin genes reveal that pathogens have driven selection of certain genetic adaptations and that helminths have had a stronger influence than other classes of pathogens in this process[4]. Pressure from helminth infections seems to have promoted the selection of alleles likely to protect against infection, while predisposing humans to immune-mediated diseases such as allergies.

In the twentieth century, great effort was put into the worldwide control of infectious diseases by improved hygiene, vaccination programs and drug treatment. However, the decline in parasitic and other infectious diseases was associated with a marked increase in prevalence of chronic inflammatory disorders such as asthma, auto-immune disease (type 1 diabetes, multiple sclerosis) and inflammatory bowel disease[5]. Although the prevalence of asthma and allergic disorders now seems to have stabilized in developed countries[6,7], the prevalence is on the increase in developing countries[7,8]. These epidemiological observations accord with the Hygiene Hypothesis[9], which suggests that removal of the regulatory effects of pathogens such as helminths (from populations genetically adapted to live with them) tends to lead to an imbalance in the immune system followed by a number of pathological conditions.

Consequently, the question arises whether helminths should be considered as harmful pathogens or as beneficial commensals. While in low-resource settings deworming is advocated to prevent worm-associated morbidity, several research groups in the Western world are currently investigating the therapeutic potential of worms and their secreted products in inflammatory diseases. Whereas deworming may result in an increase of inflammatory disorders, introduction of helminths experimentally can potentially be harmful. Experimental therapeutic infections using worms and their products may lead to unforeseen immunological and pathological consequences. This review aims to summarize current knowledge on the immunological effects of worms, the beneficial aspects of chronic helminth infections, the possible consequences of global deworming, and the current

evidence as to whether the controlled use of worms or their products for treating patients is beneficial.

T cell responses in infectious and inflammatory diseases

The immune system is equipped with different cell types involved in recognition of pathogens and their elimination. So far, a number of T-cell subsets have been identified that appear to be key to the control of distinct classes of incoming pathogens. T-helper (Th1) cells are mainly involved in defence against intracellular pathogens, Th2 are there to combat helminths and ectoparasites and Th17 cells appear to be important for defence against extracellular bacteria and fungi[10]. However, it is also clear that these cells can inflict damage to tissues and organs if uncontrolled. Thus whereas Th1 and Th17 cells that release pro-inflammatory cytokines are involved in recruitment and activation of macrophages and neutrophils that can attack bacteria, viruses, protozoa and fungi, their overt activation is associated with autoimmune and inflammatory diseases. Th2 cells trigger responses that disable, degrade and dislodge parasites, as recently reviewed[11]. Interestingly, Th2 cytokines seem to be involved in tissue repair as well[12,13], which may encapsulate the parasite and prevent excessive inflammation[14]. However, an overactivated Th2 immune response can lead to allergies and allergic diseases. Therefore it is not surprising that an important component of the immune system is the regulatory network, spearheaded by the regulatory T cells, that are capable of controlling activated effector T cells through expression of inhibitory molecules[15]. It should be noted that additional components of the regulatory network are the so-called modified Th1 and Th2 cells which express the signature cytokines, IFN-γ and IL-4/IL-5/IL-13, respectively, along with IL-10[16]. It is thought that over time, effector Th1 and Th2 cells start to express IL-10 to control the damage that might otherwise be inflicted by these cells.

Characteristics of immune responses in helminth infections

Helminth-induced immune regulatory network

In the late half of the 20th century, Ottesen and others started to elucidate the immunological basis of helminth-host interaction[17-19]. Interestingly, they observed that in helminth-infected individuals, the proliferative response of lymphocytes to specific antigens was lower than in uninfected subjects[17-20]. These and other studies[21] have led to the concept that cellular immune "hyporesponsiveness" induced by helminths is one of the key mechanisms used by these parasites to evade the host immune system. Numerous studies have since contributed to our understanding of a sophisticated immune regulatory network operative during helminth infections[16]. First, the presence of non-lymphocytic adherent cells was

demonstrated in the blood of patients with *Brugia malayi* microfilariae[22]. The adherent cells could suppress anti-filarial immune responses when cultured with patient-derived lymphocytes[22]. These cells are probably the precursors for regulatory antigen-presenting cells that are now studied, such as alternatively activated macrophages and monocytes or regulatory dendritic cells[23-26]. Furthermore, the existence of suppressor $CD8^+$ T cells was shown in individuals with patent *B. malayi* or *B. timori* microfilaremia in Indonesia; antibody-mediated depletion of these cells enhanced proliferative responses to parasite antigens[27]. Thereafter, the focus was shifted to a population of $CD4^+$ T cells, termed regulatory T cells (Tregs)[28]. Tregs are an essential component of the immune system, helping the immune response to reach an optimal balance between sufficiently strong anti-pathogen responses and carefully gauged control of overt and pathogenic inflammation[15]. Helminths are thought to exploit this immune suppressory mechanism to ensure their long-term survival. Natural thymus-derived Tregs are currently characterized as $CD4^+CD25^{hi}FOXP3^+$ cells, but several peripherally inducible subsets of regulatory T cells with variable phenotypes have also been described[29,30]. Several cross-sectional studies in human helminthiases have indeed provided evidence for the expansion of Tregs during infection[31-36]. Not only numbers, but also functional properties of Tregs have been studied. In individuals with patent *Wuchereria bancrofti* microfilaremia, suppressed filarial-specific proliferative T and B cell responses as well as IL-13 production *in vitro* were restored after removal of $CD4^+CD25^{hi}$ Tregs[37].

The mechanisms for Treg expansion by these parasites are not fully understood, but lately a number of helminth-derived molecules have been identified that can directly drive Treg induction. Molecules derived from *Schistosoma mansoni* can condition dendritic cells to induce IL-10 producing Tregs[38]. The excretory/secretory products of *Heligmosomoides polygyrus* as well as omega-1, a glycoprotein of *S. mansoni* eggs, can directly induce Foxp3 expression in murine $CD4^+$ T cells[39,40]. These studies indicate that there might be great potential in utilising helminth-derived molecules in the manipulation of the human immune system such that regulatory T cells are induced or enhanced.

Besides T cells, several other cells appear to be involved in immune regulation, as illustrated by emergence of regulatory B cells, a subset producing IL-10 during human helminthiases[41,42], and the importance of regulatory subsets of antigen-presenting cells as mentioned above.

Induction of type 2 responses by helminths

One of the hallmarks of helminth infections is the expansion of the Th2 responses; elevated cytokines such as IL-4, IL-5 and IL-13, along with high levels of IgE characterize helminth-infected subjects[43]. As there is evidence for a degree of counter-regulation between Th1 and Th2 responses, the strong type 2 inducing

capacity of helminths might also play a role in keeping Th1- (and Th17-) mediated diseases such as inflammatory bowel diseases (IBD) and arthritis at bay. Therefore, understanding the mechanisms whereby helminths lead to Th2 responses might, in analogy with understanding how Tregs are induced, lead to potential therapeutic interventions. Recent studies have identified helminth antigens with the ability to drive Th2 responses in murine models. Th2-inducing properties of molecules derived from *Fasciola hepatica*[44], *S. mansoni*[44] and *H. polygyrus*[45] was demonstrated by injection into mice. ES-62, a glycoprotein secreted by the filarial nematode *Acanthocheilonema viteae*, and omega-1, a molecule found in excretory secretory (ES) products of *S. mansoni* eggs, have been shown to endow DC with the ability to skew immune responses toward type 2 in a mouse model[46,47]. The Th2-inducing capacities of omega-1 may seem in contrast to the previously mentioned Foxp3-inducing activity, however in the latter NOD mice were used[40]. Some of these Th2-inducing capacities have been confirmed in experiments using human cells[46].
More recently, research into tissues at the sites of worm infection has revealed that epithelial cells, the first line of defence in the gut and lung, produce a set of cytokines, IL-25, IL-33 and thymic stromal lymphopoietin (TSLP), so-called 'alarmins'[48-50], which in turn induce innate lymphocytes to produce type 2 cytokines[51,52]. These cells, termed natural helper cells (NHC), are found in peripheral lymphoid clusters and produce IL-5 and IL-13 enhancing B and T cell function[53]; as yet very little is known about the equivalent of these cells in humans.

The association between helminth infections and inflammatory diseases

To date, human studies of the possible influence of worm infections on inflammatory diseases have included cross-sectional studies, anthelmintic trials and trials of helminth therapy. We shall now look at each in turn, discussing the potential pitfalls and advantages of each approach. We will start with summarizing the associations between helminth infections and different inflammatory diseases.

Allergy & asthma

Based on the ability of helminth infections to induce a regulatory network and to modify the Th2 response, we have postulated that helminths have a special capacity to protect against allergic conditions. There is evidence from several murine models that helminth infections might be protective against allergic airway inflammation[54-57]. However, the findings from cross-sectional studies in humans regarding the association between worm infections and allergy-related conditions have so far been inconsistent (reviewed by Leonardi-Bee et al[58] and Flohr et al[59]).

For atopy, usually assessed as a positive skin prick test (SPT) response to a panel of allergens, an inverse association with worms has commonly been observed[60-65], with occasional exceptions[66]. For allergy-related clinical syndromes, results have been more mixed. Both negative[67, 68] and positive[69] associations have been reported for eczema. Negative associations have been observed for wheezing in some studies[70,71]; in others, an absence of association between worms and wheeze[71] or asthma[72], or a positive association between worms and asthma[66] have been reported. Factors that may influence the relationships between worms and allergy observed in these studies are likely to be complex but may include the timing, or chronicity of helminth infection – with recent infections having a different effect from chronic, established infections[64] – as well as the intensity of the infections, the specific parasite species and the host genetics[73, 74].

Inflammatory Bowel Diseases

Studies in murine models have shown that infection with worms protects against several forms of experimentally induced colitis[75-78] but studies in humans are hindered by the fact that inflammatory bowel disease (IBD) and parasitic infections do usually not coincide. A small study in India showed that patients with Crohn's disease had lower immune responses to different hookworm antigens compared to healthy controls, suggesting that patients had less often harbored hookworm infections, however the infection was not assessed directly[79].

Multiple sclerosis

A case-control study in 1966 already pointed to the contribution of environmental factors in MS; the presence of piped water, a flush toilet and sharing a room with 1 person or less were more often recorded as environmental factors associated with MS patients compared to healthy controls[80]. Reasoning from the Hygiene Hypothesis, the country prevalences of MS and *T. trichiura* infections were compared and shown to be almost mutually exclusive[81]. Correale and colleagues took this further by assessing a prospective cohort of MS patients. When comparing helminth-infected and -uninfected patients, the appearance of new MRI lesions was much less frequent in the infected individuals over 5 years of follow-up. This difference seemed to be associated with higher production of IL-10 and TGF-β by peripheral blood mononuclear cells (PBMC) and with suppressive activity of Tregs[82]. Furthermore, when a few of the helminth-infected patients were treated with anthelmintics due to intestinal symptoms, the number of clinical relapses and MRI lesions increased, parallel to a decrease in regulatory immune responses[83]. Using the murine model for MS, experimental autoimmune encephalomyelitis (EAE), evidence was further obtained for a protective effect of helminths or their products[84-88] on the clinical course and CNS inflammation.

Diabetes mellitus

Another autoimmune condition that has been linked to immune modulatory properties of helminths is diabetes mellitus. In the non-obese diabetes (NOD) mouse model, it was shown that several types of helminthic infections could prevent type 1 diabetes (T1D)[89-92]. In humans, an inverse association has been observed between prevalence of T1D and neglected tropical diseases, which helminth infections form a substantial part of. They showed an inverse relation between the prevalence of T1D and the access to sanitation and clean water, although this was not statistically tested[93]. Within the Chennai Urban Rural Epidemiology Study (CURES) in India, the prevalence of lymphatic filariasis was lower in T1D subjects[94]. They furthermore measured total anti-filarial IgG and IgG4 as proxies for past and current infections, respectively. Whereas total IgG levels were not different between the groups, IgG4 was higher in the non-diabetic group, indicating that current infections might lead to this inverse relationship.

For type 2 diabetes (T2D), it is more difficult to establish experimental models. This multifactorial disease is currently regarded as an inflammatory disease[95], but is also associated with genetic as well as nutritional and other life style factors. Another report from the CURES study has shown lower prevalence of LF in T2D patients[96]. Moreover, the study showed lower serum levels of pro-inflammatory cytokines in LF-positive versus LF-negative subjects with T2D, suggesting a role for anti-inflammatory properties of LF infection in protection against T2D.

Rheumatoid arthritis

Although some helminth infections can induce reactive arthritis which resembles rheumatoid arthritis[97] several studies in different rodent models have shown that helminth infections or extracts can suppress or prevent arthritis[98-100]. The filarial-derived glycoprotein ES-62 was shown to be effective as a therapy for murine collagen-induced arthritis and furthermore synovial cells from rheumatoid arthritis (RA) patients treated with ES-62 produced less pro-inflammatory cytokines after LPS stimulation[101].

Other autoimmune or inflammatory diseases

Aoyama et al. showed that *Strongyloides stercoralis* infections were less prevalent in patients with autoimmune liver diseases than in other individuals visiting the hospital[102], however there has been no further follow-up study to this. In a mouse model of Graves' disease, an autoimmune disease of the thyroid, *S. mansoni* appeared to have a protective effect[103], but this has not been examined in humans. Furthermore, it has been suggested that helminths might have therapeutic potential in atherosclerosis and other cardiovascular diseases[104], based on a study that showed *S. mansoni* to have anti-atherogenic effects in a mouse model. The

exact mechanism has not been resolved but it might be caused by the consumption of lipids by S. mansoni, rather than by immune-mediated mechanisms[105].

Studies of anthelmintic treatment

Cross-sectional and observational studies are inherently flawed due to difficulty in ascertaining cause and effect, issues of confounding and reverse causation. Therefore, evidence from such studies need to be complemented by other study designs. Since remote areas still suffer from a high burden of helminthic infections[106], mass deworming programs are currently advocated[107]. This creates opportunities to investigate prospectively whether deworming paves the way for the increased prevalence of allergic and other inflammatory diseases.

Effect of deworming on atopic diseases

Anthelmintic trials are designed to study the influence of worms on allergic conditions indirectly, by studying the effects of treating the worms. Such trials have been conducted among school-going children from worm-endemic areas using different anthelmintic drugs for various periods of follow-up. An initial non-randomized study in Venezuela compared treated children with children who declined treatment, and suggested that anthelmintic treatment was associated with increased prevalence of atopy[108]. The first randomized trial was carried out among 317 Gabonese school children using open-label three-monthly praziquantel and mebendazole, versus no treatment, over 30 months; it found that anthelmintic treatment was associated with an increased rate of developing positive skin responses to house dust mites[109]. A cluster-randomized trial among 1632 children from Ecuador using two-monthly albendazole versus placebo over 12 months found no effect on SPT responses[110]. An individually randomized trial, among 1566 rural children from Vietnam using initially three-monthly mebendazole versus placebo but later three-monthly albendazole versus placebo over 12 months, found an increased risk of positive SPT responses to allergens[111]. The latest trial in Indonesia, assessed the effect of three-monthly albendazole treatment compared to placebo on SPT reactivity to different allergens. A significant increase in cockroach reactivity was observed after 21 months, but overall SPT responses were not altered[112]. None of these four trials showed any effects of anthelmintic treatment on clinical allergy outcomes. Given the fact that clinical allergy is relatively rare in these areas, it should be considered that the power of these studies might have been not sufficient to detect significant effects. Moreover, it has to be noted that differences in species of prevalent helminths, co-prevalence of other immunomodulating infections (such as oro-faecal infections[113,114] or malaria[115]), exposure to environmental pollutants and duration and timing of treatment, can have major impact on trial outcomes.

Pertinent to the duration of treatment, a study in Ecuador compared SPT reactivity and allergy-related symptoms among school-age children from communities that had received 15-17 years of periodic ivermectin treatment to school-age children from adjacent communities that had not received treatment[116]. This study observed that the prevalence of skin reactivity to allergens among children from the treated communities was double that for the children from untreated communities. The children from the treated communities also had a higher prevalence of recent eczema symptoms, but not any other allergy-related symptoms[116]. While the design of this study is again limited by the fact that the communities were not randomized relative to the intervention, the results do suggest that long-term intervention against helminths may be required to alter responsiveness to allergens and to influence allergy-related clinical outcomes.
Regarding the timing of treatment, a trial among 2507 pregnant women in Uganda using single doses of albendazole and praziquantel versus matching placebos (in a 2x2 factorial design) found that albendazole during pregnancy was associated with an increased risk of eczema in infancy[117] and in the first five years of life, whether the mother had hookworm infection or not[118]. Praziquantel during pregnancy was associated with an increased risk of eczema in infancy among children whose mothers were infected with *S. mansoni* infection[117]. In this same trial, children themselves were randomized to three-monthly albendazole versus placebo from fifteen months to five years of age; this treatment was not associated with an increased risk of eczema in early childhood[118]. These results suggest that in-utero events may be more important in priming or programming the child's immune system (thereby influencing the risk of eczema, and perhaps other allergy-related conditions) than events in early childhood. Surprisingly, anthelmintic treatment with albendazole and praziquantel during pregnancy had no beneficial effects on the immune responses to BCG, tetanus and measles vaccines in early childhood and none of the anticipated benefits for birth weight, resistance to infectious diseases, or improved child development[119,120], that would have compensated for the observed adverse effect on eczema.
All the above studies examined effects of anthelmintics in general populations. Another approach has been to examine effects of anthelmintic treatment in people already suffering from allergy-related disease. For example, a recent small trial conducted among individuals aged five to fifty years with a history of asthma in the last twelve months from a schistosomiasis-endemic area in Brazil has been published[121]. Study participants received single dose albendazole and praziquantel or placebos. No differences in asthma severity between the two treatment arms were observed over three months. When all participants were treated with both drugs after about three months of follow-up, the study observed the worsening of clinical asthma symptoms at fifteen months[121]. However, this worsening cannot be confidently attributed to anthelmintic treatment since there was no comparison

arm in the second part of the study. Larger studies investigating the effect of treating worms in people with established allergy-related diseases would be warranted.

Effect of deworming on other inflammatory diseases
The effect of deworming on other chronic inflammatory diseases has not extensively been studied, partly because naturally (and inherent to our hypothesis) the presence of helminth infections and these conditions may not overlap. An interesting study by Bager and colleagues did assess the effect of mebendazole treatment retrospectively in a population cohort in Denmark[122]. 14% of the more than 900.000 children had been prescribed mebendazole, for probable infection with *Enterobius vermicularis*, the pinworm that is still endemic in the US and in Europe[123]. The incidence rate ratios for the chronic inflammatory diseases asthma, T1D, juvenile arthritis and IBD were not significantly higher in treated children. However, mebendazole was prescribed based on presumption and not diagnosis of pinworm infection. Moreover, enterobiasis in these children might not have been sufficiently chronic to induce immune regulation[122] and thus the treatment might have masked the possible benefits.

The main drawback of anthelmintic trials in studying the influence of worms on inflammatory diseases is that they are based on a number of assumptions whose validity is currently unknown. For instance, the trials assume that the effect of worms is immediately removed following treatment and that development of allergy symptoms follows soon after anthelmintic treatment. What if the protective effects of worms persist long after anthelmintic treatment? What if the development of clinical allergy and other conditions is not immediate or could only be observed in the next generation? This concern might be answered by longer follow-up and different study designs, such as the Ugandan trial[117], which showed that the effect of treating worms in the mother could be observed in the next generation as increased risk of childhood eczema. Follow-up for this Ugandan study is currently ongoing to determine whether the risk of asthma is also increased as the children grow older. The second drawback in using anthelmintic drugs to study the possible influence of worms on allergy is that any observed effect might be due to the anthelmintic drug itself, or to broader spectrum effects, and not due to the elimination of worms. This seems to be the most likely explanation for the observed effect of albendazole treatment in pregnancy on the increased risk of eczema in the Ugandan trial described above. Albendazole acts by binding to tubulin thereby interfering with the formation of microtubules in the cytoskeleton[124] and hence can affect protozoa[125, 126], fungi[127] and mammalian cells. Whether maternal albendazole increased the risk of childhood eczema through a direct effect or through acting on other organisms, was not established in the

Ugandan study. Therefore, results from anthelmintic trials should be interpreted with caution and it might be helpful to examine effects of a variety of drugs in different trials.

Immunological consequences of deworming

A number of studies have been able to assess immune responses after anthelmintic treatment. Helminth-specific responses have been examined in Vietnamese school children in the context of the larger study on allergy[111]. Cytokine responses were only evaluated in hookworm-positive children, in which albendazole treatment led to lower IL-10 responses to hookworm antigens. A similar effect was seen in *Ascaris* responses in children treated with albendazole in Ecuador, although this study was not placebo-controlled[128]. A decrease in IL-10 might be expected after clearance of immunoregulatory helminth infections, however in earlier studies, treatment of schistosomiasis and STH infections with praziquantel and mebendazole, respectively, had been associated with enhanced IL-10 responses[129,130]. Also a placebo-controlled trial of praziquantel treatment of pregnant women resulted in higher *S. mansoni*-specific IL-10 production[131]. This latter study also found a treatment-induced increase of Th1 and Th2 responses to schistosomal antigens, similar to the enhanced Th1 and Th2 cytokines in response to *Ascaris* after albendazole treatment of school children in Ecuador[128].

Overall, immune responses tend to increase after anthelmintic treatment, which is in line with the results of our randomized controlled trial (RCT) in Indonesia, showing enhanced pro-inflammatory cytokine responses to *Ascaris* and malaria antigens as well as mitogen after albendazole treatment (Wammes et al. manuscript in preparation). However, it is not known how long this immune stimulatory effect may persist and whether this predisposes to hyperinflammatory conditions.

Very few studies have looked at allergen-specific immune responses after anthelmintic treatment. The trial in Ecuador found no differences in cytokine production in response to cockroach and house mite *Dermatophagoides pteronyssinus* antigens after repeated treatment with albendazole[128]. More trials might be needed to confirm this observation.

Helminth therapy in humans

A more direct approach, which avoids the pitfalls described above, is to study the direct effects of helminths through clinical trials using whole helminths or helminth-derived molecules.

The first scientists to undertake clinical evaluation of infecting patients with helminths were Joel Weinstock and David Elliott in the 1990s, who have recently

summarized the progress in translational studies on helminthic therapy in a review[132]. Up to now, *Trichuris suis* and *Necator americanus* worms have been used in a clinical trial setting, as discussed below.

Trichuris eggs

Most clinical trials so far were performed with *Trichuris suis* ova (TSO) in patients with IBD. *T. suis* is a pig whipworm, which is able to colonize the human gut for a short period of time, but without overt pathology. After two open-label trials assessing the safety of *T. suis* infection in IBD patients and showing promising results (about 70% remission in Crohn's disease[133,134]), Summers and colleagues set out to study the effect of TSO in a first placebo-controlled double blind randomized trial including 54 ulcerative colitis (UC) patients[135]. The UC Disease Activity Index (UCDAI) improved more in the TSO group compared to the placebo group, however numbers of remissions were not significantly different. The group of P'ng Loke, working on characterization of the local immune responses surrounding the location of *Trichuris* worms, studied a patient who infected himself with *T. trichiura*[136]. In this patient, during colitis, T cells only producing IL-17 were abundant, while after *Trichuris* infection more multifunctional T cells were induced, mainly producing IL-22. At the same time goblet cell hyperplasia and enhanced mucus production was seen, which suggests that IL-22 is involved in the tissue repair response, possibly together with the canonical Th2 cytokines[136]. In addition to the previous observations of helminth-associated *de novo* induction of regulatory cells, it appears that *Trichuris* worms are able to modify the cytokine signature of local inflammatory cells. In rhesus macaques with idiopathic chronic diarrhoea, which resembles UC in intestinal inflammation, the Loke group further examined the effects of *T. trichiura* treatment[137]. Interestingly, four of the five monkeys showed clinical improvement. Colonic T cells produced more IL-4 but had less Tregs after treatment, however, no data could be generated on IL-17 and IL-22 responses, which makes it difficult to correlate these clinical effects to the previously reported immunological alterations. However, IL-22 might be a promising candidate as correlate of protection and this may be further explored in helminthic therapy trials, with *T. trichiura* or other helminths.

Recently, the results of a safety trial of TSO in MS patients were reported, as a starting point for a planned phase 2 trial (trial identifier NCT00645749). The trial followed 5 patients with relapsing-remitting MS after inoculation with TSO[138]. Although the majority experienced mild gastrointestinal symptoms, the number of new lesions revealed by MRI was lower during TSO treatment than the number before treatment, or after treatment had been discontinued. This was not accompanied by a change in circulating Tregs or monocytes expressing typical molecules of alternatively activated macrophages, raising the question whether it

is difficult to detect these cells in peripheral blood of patients rather than in the intestine.
A relatively large RCT in 100 allergic rhinitis patients showed the induction of gastrointestinal symptoms and immunological response to TSO, but no effect in reducing symptom scores, medication use or skin prick test reactivity[139]. However, this trial has been criticized due to the fact that infection with TSO was too short before the hay fever season started and therefore might not have allowed sufficient time for the treatment to work[140].
As of December 2012, there are a total of 11 clinical trials registered assessing safety and/or efficacy of TSO in IBD, MS, allergies and even autism (Table 1).

Necator americanus larvae
Relatively new is the therapeutic potential of worms known to establish chronic infections in humans. An advantage of this could be that administration is only needed once, whereas TSO should be administered at two- or three-weekly intervals. The safety and efficacy of *Necator americanus* larvae has been assessed in three trials. Inoculation with 50 larvae or more was shown to cause considerable gastrointestinal symptoms in healthy volunteers[141-143]. A dose-ranging trial to establish the dose which would achieve an infection intensity of 50 eggs per gram of faeces in healthy individuals showed that inoculation with 10 larvae was sufficient. This dose also induced a modest immunological response, as measured by eosinophil counts, IgE and hookworm-specific IgG levels[143].
Before starting a RCT of *N. americanus* infection in asthma patients, two other issues were addressed, which could potentially affect the safety of these studies. It was shown in allergic rhinitis patients that the lung passage of hookworm larvae did not cause deterioration in airway reactivity[144] and that hookworm-induced type 2 responses did not potentiate an allergen-specific IgE response[145]. In an asthma trial that followed, although infection using 10 larvae was well tolerated, it did not show any beneficial effect against asthma symptoms[146]. One RCT has been reported on *N. americanus* infection in celiac disease patients under restricted diet[147]. Wheat challenge was performed to assess the effect that helminthic immune modulation would have, however no differences in mucosal T cell counts, gluten-specific IFN-γ production by PBMC or clinical responses to challenge were observed comparing groups of 10 patients. Currently five clinical trials are registered to use *N. americanus* larvae for celiac disease, allergic rhinitis, asthma and MS (Table 1).

Table 1. Overview of registered clinical trials on helminthic therapy

Trial identifier	Sponsor	Phase	Status	Condition	Intervention
ACTRN12608000241336	Asphelia Pharaceuticals	1	not yet recruiting	Crohn's disease	TSO
EUCTR2007-006099-12-DK	Statens Serum Institut, Denmark	2	not recruiting	Allergic rhinitis	TSO
NCT00645749	University of Wisconsin, Madison, US	2	recruiting	MS	TSO
NCT01006941	Rigshospitalet, Copenhagen, Denmark	2	completed	MS	TSO
NCT01040221	Montefiore, New York, US	1	not yet recruiting	Autism	TSO
NCT01070498	Beth Israel,Boston, US	1	completed	Food allergy	TSO
NCT01279577 / EUCTR2006-000720-13-DE	Dr. Falk Pharma, Frankfurt, Germany	2	recruiting	Crohn's disease	TSO
NCT01413243 / EUCTR2009-015319-41-DE	Charite, Berlin, Germany	2	recruiting	MS	TSO
NCT01433471	NYU, New York, US	?	recruiting	Ulcerative colitis	TSO
NCT01434693	Coronado Biosciences, US	1	ongoing	Crohn's disease	TSO
NCT01576471	Coronado Biosciences, US	2	recruiting	Crohn's disease	TSO
NCT01734941	Hadassah Medical Organization, Jerusalem, Israel	2	not yet recruiting	Autism	TSO
NCT00232518	University of Nottingham, UK	1	completed	Allergic rhinoconjunctivitis	N.a. larvae
NCT00469989	University of Nottingham, UK	1	completed	Asthma	N.a. larvae
NCT00671138	Brisbane, Australia	2	unknown	Celiac Disease	N.a. larvae
NCT01470521 / EUCTR2008-005008-24-GB	University of Nottingham, UK	2	recruiting	MS	N.a. larvae
NCT01661933	Brisbane, Australia	1&2	recruiting*	Celiac Disease	N.a. larvae

TSO *Trichuris suis* ova; N.a. *Necator americanus*; *enrolling by invitation only

Helminth-derived molecules
Regarding the fact that helminth species currently used or planned to be used in trials are able to colonize the human intestine or could have clinical and pathological consequences, there has been a shift on focusing on helminth-derived molecules to substitute whole parasite treatment approach (reviewed by William and Margaret Harnett[148]).
As already alluded to earlier, several helminthic products with immune modulating properties have been defined. For several of these, cellular immunological responses induced have been investigated and some have been tested in disease models, however none has been administered to humans. Currently, ES-62 is the most well-characterized candidate molecule for therapeutic trials. This phosphorylcholine-coupled glycoprotein was first identified in 1994[149] and, as discussed, has been used to treat arthritis in a murine model[101]. Furthermore, through inhibition of mast cell histamine release, ES-62 may protect against allergic diseases[150].
The *Heligmosomoides* excreted-secreted (ES) products[76], characterized by the group of Rick Maizels, have been shown to suppress allergic airway inflammation[55]. AvCystatin, another molecule secreted by *A.viteae*, inhibits the development of allergic airway inflammation and acute colitis[151]. Moreover, it has been shown that *in vitro* Th2 responses of PBMC from grass pollen allergic patients are markedly reduced by adding AvCystatin to cultures[152]. Furthermore, there is data indicating that soluble products from *S. mansoni*, *T. suis* and *Trichinella spiralis* can suppress clinical signs of EAE by modulation of DC[88].
In murine colitis, extracts from *S. mansoni* adult worms and ES products of the canine hookworm *Ankylostoma caninum* have displayed beneficial effects[153]. Although treatment with *S. mansoni* extracts did not improve the clinical score, it diminished local inflammation and myeloid cell infiltration in colonic tissue. In parallel, lower mucosal Th1 and Th17 responses and enhanced expression of IL-10 and TGF-β in T cells was found[153]. These results illustrate that altered immune responses do not always lead to clinical improvement, and a longer time period may be needed for a detectable clinically beneficial effect. Lastly, Lewis^X-containing glycan from *S. mansoni* eggs, lacto-N-fucopentaose III (LNFPIII), was tested in mouse models of inflammatory conditions. LNFPIII suppressed EAE by enhancement of IL-10 and Th2 cytokines[154] and was also shown to be beneficial in psoriasis[155]. Taken together these results in animal models encourage further studies, and possibly clinical trials, to evaluate their beneficial effects.

Challenges in helminth immunotherapy
There are some drawbacks in the use of helminthic therapy as currently proposed. As mentioned before, infection with *N. americanus* but possibly also *T. suis* could have pathogenic effects in humans. Patients undergoing helminth infection should

be monitored closely for infection intensity and possible extra-intestinal manifestations of the infection[156]. The long-term consequences of this approach have not been assessed; the trials with the longest follow-up time were for 24 weeks. The advantage for introducing hookworm infections would be that only single inoculation is needed, whereas TSO should be provided every two to three weeks. However, this also implies that hookworm infections are less controllable, as they lead to chronic infestations. The timing of infection is another issue, since under natural conditions, the protective effects of helminthic infections might have been acquired in early life. Moreover, possible relevant immunomodulatory effects could need substantial length of time to fully develop.

Another issue is the helminth-induced immune modulation itself. Immune regulatory responses are desirable to counteract inflammatory disorders, but could be detrimental for other immune-associated conditions. Not only defence against incoming pathogens may be impaired, but also anti-tumor immune responses may be compromised. Immunosuppression is a strategy for tumor cells to evade host immune responses and efforts are being made to inhibit Tregs in cancer by immunotherapy[157]. Therefore, non-specific blanket immune suppression, although mild, should be considered critically.

Emerging area – Immunometabolism

Immunometabolism is an emerging concept, which explores the interaction between nutrients, metabolism and the immune system. Since metabolic disorders such as T2D and obesity are associated with immune alterations, they can be regarded as inflammatory diseases[95]. It has become clear that some of the pathways involved in pathogen sensing are also involved in the induction of inflammation that leads to metabolic disorders. Pathogen-associated molecules are known to induce pro-inflammatory cytokine production, such as TNF, through toll-like receptor (TLR) signaling[158]. Large amounts of TNF are found in adipose tissue of obese mice[159]. The intracellular signaling pathways of TNF involve serine kinases, which have been shown to phosphorylate serine residues in insulin receptor substrates [160,161]. As serine phosphorylation is the inhibitory counterpart of tyrosine phosphorylation usually induced by insulin binding to the receptor, the signaling events of TNF lead to inhibition of insulin signaling, resulting in insulin resistance[161].

Combatting microorganisms has high energy demand and moreover, worms and other parasites use host nutrients for their long-term survival. As a consequence, parasitic worms might need to tightly co-regulate immune responses and nutrient metabolism, to avoid depriving their host from resources necessary for survival. Therefore, these metabolic properties of parasitic infections could be of interest. Recently the effects of LNFPIII, which is not only present on *S. mansoni* eggs but

also found in human breast milk[162], was assessed on immune metabolism. LNFPIII was already shown to induce an immunoregulatory phenotype in macrophages[163] but in addition, Bhargava and colleagues reported that this glycoconjugate improves insulin signaling and thereby sensitivity in white adipose tissue in high fat diet-fed mice, in part through IL-10[164]. Furthermore, some detailed studies of the interaction of Th2 cytokines and metabolic homeostasis have shown that IL-4 shifts cellular energy resources from fatty acids to glucose oxidation, by enhancing the activity of insulin[165]. These studies highlight the possible metabolic advantages of harboring helminth infections.

Final remarks

In summary, there is an apparent contrast between efforts to deworm populations in remote areas suffering from helminth-associated morbidities and initiatives to test effects of helminthic therapy on patients with hyperinflammatory diseases (Figure 1).

Murine models have supported the hypothesis that helminths or their products may be beneficial for inflammatory conditions. Human studies in poor-resource settings have been less consistent, which may be explained by the presence of other modulating infections and by the fact that the clinical effects of deworming may take longer to establish. Further deworming trials should take these issues into account and plan for longer follow-up periods. Human studies of the therapeutic use of helminths in resource-rich settings may shed more light on the role of helminths in human physiology and immunology. Although some positive results have been obtained in IBD and MS, not much benefit was seen in treatment of asthma and allergy. Further trials should be less modest in numbers of patients included. Furthermore, assessment of locally – and importantly – systemically induced immune (regulatory) responses deserves further attention, since the underlying mechanisms and consequences of voluntary helminth infection could influence other health outcomes in patients.

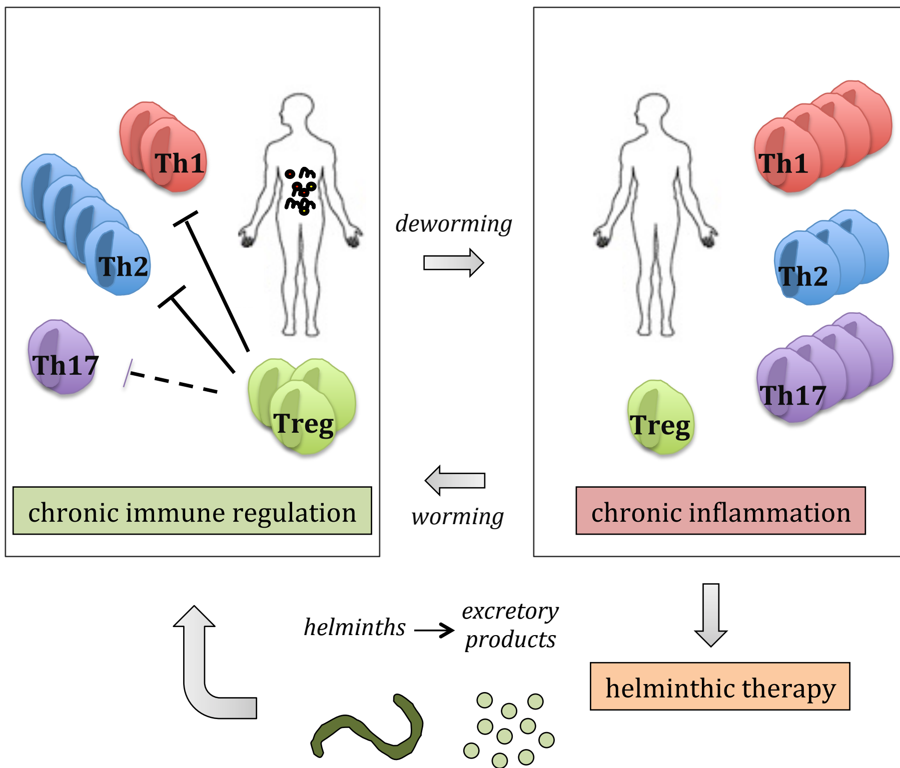


Figure 1. The immunological effects of deworming and worming. Helminth-infected individuals (left panel) harbor enhanced Th2 responses. The number and immunosuppressive capacity of Tregs may also be increased during infection. After deworming, removal of immune suppression could lead to over-inflammation, characteristic of several inflammatory diseases (right panel). Th1, Th2 or Th17 responses are more abundant, whereas Tregs are less in number and function. Treatment with experimental helminth infection or with helminth-derived immunomodulatory molecules could restore the immune regulation as observed during natural chronic helminth infections. The resulting suppression of inflammatory T-cell responses may curtail symptoms of several immune-mediated diseases.

References

1. Hoeppli, R. The knowledge of parasites and parasitic infections from ancient times to the 17th century. *Experimental parasitology* **5**, 398-419 (1956).
2. Bundy, D.A. & Medley, G.F. Immuno-epidemiology of human geohelminthiasis: ecological and immunological determinants of worm burden. *Parasitology* **104 Suppl**, S105-119 (1992).
3. Allen, J.E. & Maizels, R.M. Diversity and dialogue in immunity to helminths. *Nature reviews. Immunology* **11**, 375-388 (2011).
4. Fumagalli, M., *et al.* Parasites represent a major selective force for interleukin genes and shape the genetic predisposition to autoimmune conditions. *The Journal of experimental medicine* **206**, 1395-1408 (2009).
5. Bach, J.F. The effect of infections on susceptibility to autoimmune and allergic diseases. *The New England journal of medicine* **347**, 911-920 (2002).
6. Anderson, H.R., Gupta, R., Strachan, D.P. & Limb, E.S. 50 years of asthma: UK trends from 1955 to 2004. *Thorax* **62**, 85-90 (2007).
7. Pearce, N., *et al.* Worldwide trends in the prevalence of asthma symptoms: phase III of the International Study of Asthma and Allergies in Childhood (ISAAC). *Thorax* **62**, 758-766 (2007).
8. Odhiambo, J.A., Williams, H.C., Clayton, T.O., Robertson, C.F. & Asher, M.I. Global variations in prevalence of eczema symptoms in children from ISAAC Phase Three. *The Journal of allergy and clinical immunology* **124**, 1251-1258 e1223 (2009).
9. Strachan, D.P. Hay fever, hygiene, and household size. *BMJ* **299**, 1259-1260 (1989).
10. Zhu, J. & Paul, W.E. CD4 T cells: fates, functions, and faults. *Blood* **112**, 1557-1569 (2008).
11. Maizels, R.M., Hewitson, J.P. & Smith, K.A. Susceptibility and immunity to helminth parasites. *Current opinion in immunology* **24**, 459-466 (2012).
12. Chen, F., *et al.* An essential role for TH2-type responses in limiting acute tissue damage during experimental helminth infection. *Nature medicine* **18**, 260-266 (2012).
13. Mentink-Kane, M.M., *et al.* Accelerated and progressive and lethal liver fibrosis in mice that lack interleukin (IL)-10, IL-12p40, and IL-13Ralpha2. *Gastroenterology* **141**, 2200-2209 (2011).
14. Allen, J.E. & Wynn, T.A. Evolution of Th2 immunity: a rapid repair response to tissue destructive pathogens. *PLoS pathogens* **7**, e1002003 (2011).
15. Belkaid, Y. Regulatory T cells and infection: a dangerous necessity. *Nature reviews. Immunology* **7**, 875-888 (2007).
16. Maizels, R.M. & Yazdanbakhsh, M. Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nature reviews. Immunology* **3**, 733-744 (2003).
17. Colley, D.G., Todd, C.W., Lewis, F.A. & Goodgame, R.W. Immune responses during human schistosomiasis mansoni. VI. In vitro nonspecific suppression of phytohemagglutinin responsiveness induced by exposure to certain schistosomal preparations. *J Immunol* **122**, 1447-1453 (1979).
18. Lewert, R.M., Yogore, M.G., Jr. & Blas, B.L. Lymphocyte responsiveness to phytohemagglutinin and to worm and egg antigens in human schistosomiasis japonica. *The American journal of tropical medicine and hygiene* **28**, 92-98 (1979).
19. Ottesen, E.A., Weller, P.F. & Heck, L. Specific cellular immune unresponsiveness in human filariasis. *Immunology* **33**, 413-421 (1977).
20. Ottesen, E.A., Hiatt, R.A., Cheever, A.W., Sotomayor, Z.R. & Neva, F.A. The acquisition and loss of antigen-specific cellular immune responsiveness in acute

and chronic schistosomiasis in man. *Clinical and experimental immunology* **33**, 37-47 (1978).
21. Maizels, R.M., Bundy, D.A., Selkirk, M.E., Smith, D.F. & Anderson, R.M. Immunological modulation and evasion by helminth parasites in human populations. *Nature* **365**, 797-805 (1993).
22. Piessens, W.F.*, et al.* Antigen-specific suppressor cells and suppressor factors in human filariasis with Brugia malayi. *The New England journal of medicine* **302**, 833-837 (1980).
23. Babu, S., Kumaraswami, V. & Nutman, T.B. Alternatively activated and immunoregulatory monocytes in human filarial infections. *The Journal of infectious diseases* **199**, 1827-1837 (2009).
24. Semnani, R.T., Mahapatra, L., Moore, V., Sanprasert, V. & Nutman, T.B. Functional and phenotypic characteristics of alternative activation induced in human monocytes by interleukin-4 or the parasitic nematode Brugia malayi. *Infection and immunity* **79**, 3957-3965 (2011).
25. Van den Bossche, J.*, et al.* Alternatively activated macrophages engage in homotypic and heterotypic interactions through IL-4 and polyamine-induced E-cadherin/catenin complexes. *Blood* **114**, 4664-4674 (2009).
26. Li, Z.*, et al.* The phenotype and function of naturally existing regulatory dendritic cells in nematode-infected mice. *International journal for parasitology* **41**, 1129-1137 (2011).
27. Piessens, W.F.*, et al.* Antigen-specific suppressor T lymphocytes in human lymphatic filariasis. *The New England journal of medicine* **307**, 144-148 (1982).
28. MacDonald, T.T. Suppressor T cells, rebranded as regulatory T cells, emerge from the wilderness bearing surface markers. *Gut* **51**, 311-312 (2002).
29. Levings, M.K. & Roncarolo, M.G. T-regulatory 1 cells: a novel subset of CD4 T cells with immunoregulatory properties. *The Journal of allergy and clinical immunology* **106**, S109-112 (2000).
30. Mills, K.H. & McGuirk, P. Antigen-specific regulatory T cells--their induction and role in infection. *Seminars in immunology* **16**, 107-117 (2004).
31. Babu, S., Blauvelt, C.P., Kumaraswami, V. & Nutman, T.B. Regulatory networks induced by live parasites impair both Th1 and Th2 pathways in patent lymphatic filariasis: implications for parasite persistence. *J Immunol* **176**, 3248-3256 (2006).
32. Metenou, S.*, et al.* At homeostasis filarial infections have expanded adaptive T regulatory but not classical Th2 cells. *J Immunol* **184**, 5375-5382 (2010).
33. Nausch, N., Midzi, N., Mduluza, T., Maizels, R.M. & Mutapi, F. Regulatory and activated T cells in human Schistosoma haematobium infections. *PloS one* **6**, e16860 (2011).
34. Ricci, N.D.*, et al.* Induction of CD4(+)CD25(+)FOXP3(+) regulatory T cells during human hookworm infection modulates antigen-mediated lymphocyte proliferation. *PLoS neglected tropical diseases* **5**, e1383 (2011).
35. Teixeira-Carvalho, A.*, et al.* Cytokines, chemokine receptors, CD4+CD25HIGH+ T-cells and clinical forms of human schistosomiasis. *Acta tropica* **108**, 139-149 (2008).
36. Watanabe, K.*, et al.* T regulatory cell levels decrease in people infected with Schistosoma mansoni on effective treatment. *The American journal of tropical medicine and hygiene* **77**, 676-682 (2007).
37. Wammes, L.J.*, et al.* Regulatory T cells in human lymphatic filariasis: stronger functional activity in microfilaremics. *PLoS neglected tropical diseases* **6**, e1655 (2012).

38. van der Kleij, D., *et al.* A novel host-parasite lipid cross-talk. Schistosomal lyso-phosphatidylserine activates toll-like receptor 2 and affects immune polarization. *The Journal of biological chemistry* **277**, 48122-48129 (2002).
39. Grainger, J.R., *et al.* Helminth secretions induce de novo T cell Foxp3 expression and regulatory function through the TGF-beta pathway. *The Journal of experimental medicine* **207**, 2331-2341 (2010).
40. Zaccone, P., *et al.* The S. mansoni glycoprotein omega-1 induces Foxp3 expression in NOD mouse CD4(+) T cells. *European journal of immunology* **41**, 2709-2718 (2011).
41. Correale, J., Farez, M. & Razzitte, G. Helminth infections associated with multiple sclerosis induce regulatory B cells. *Annals of neurology* **64**, 187-199 (2008).
42. van der Vlugt, L.E., *et al.* Schistosomes induce regulatory features in human and mouse CD1d(hi) B cells: inhibition of allergic inflammation by IL-10 and regulatory T cells. *PloS one* **7**, e30883 (2012).
43. Anthony, R.M., Rutitzky, L.I., Urban, J.F., Jr., Stadecker, M.J. & Gause, W.C. Protective immune mechanisms in helminth infection. *Nature reviews. Immunology* **7**, 975-987 (2007).
44. Donnelly, S., *et al.* Helminth 2-Cys peroxiredoxin drives Th2 responses through a mechanism involving alternatively activated macrophages. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **22**, 4022-4032 (2008).
45. Rzepecka, J., *et al.* Calreticulin from the intestinal nematode Heligmosomoides polygyrus is a Th2-skewing protein and interacts with murine scavenger receptor-A. *Molecular immunology* **46**, 1109-1119 (2009).
46. Everts, B., *et al.* Omega-1, a glycoprotein secreted by Schistosoma mansoni eggs, drives Th2 responses. *The Journal of experimental medicine* **206**, 1673-1680 (2009).
47. Whelan, M., *et al.* A filarial nematode-secreted product signals dendritic cells to acquire a phenotype that drives development of Th2 cells. *J Immunol* **164**, 6453-6460 (2000).
48. Fallon, P.G., *et al.* Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. *The Journal of experimental medicine* **203**, 1105-1116 (2006).
49. Humphreys, N.E., Xu, D., Hepworth, M.R., Liew, F.Y. & Grencis, R.K. IL-33, a potent inducer of adaptive immunity to intestinal nematodes. *J Immunol* **180**, 2443-2449 (2008).
50. Hurst, S.D., *et al.* New IL-17 family members promote Th1 or Th2 responses in the lung: in vivo function of the novel cytokine IL-25. *J Immunol* **169**, 443-453 (2002).
51. Koyasu, S., Moro, K., Tanabe, M. & Takeuchi, T. Natural helper cells: a new player in the innate immune response against helminth infection. *Advances in immunology* **108**, 21-44 (2010).
52. Saenz, S.A., *et al.* IL25 elicits a multipotent progenitor cell population that promotes T(H)2 cytokine responses. *Nature* **464**, 1362-1366 (2010).
53. Moro, K., *et al.* Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature* **463**, 540-544 (2010).
54. Mangan, N.E., *et al.* Helminth infection protects mice from anaphylaxis via IL-10-producing B cells. *J Immunol* **173**, 6346-6356 (2004).
55. McSorley, H.J., *et al.* Suppression of type 2 immunity and allergic airway inflammation by secreted products of the helminth Heligmosomoides polygyrus. *European journal of immunology* **42**, 2667-2682 (2012).

56. Smits, H.H., *et al.* Protective effect of Schistosoma mansoni infection on allergic airway inflammation depends on the intensity and chronicity of infection. *The Journal of allergy and clinical immunology* **120**, 932-940 (2007).
57. Wilson, M.S., *et al.* Suppression of allergic airway inflammation by helminth-induced regulatory T cells. *The Journal of experimental medicine* **202**, 1199-1212 (2005).
58. Leonardi-Bee, J., Pritchard, D. & Britton, J. Asthma and current intestinal parasite infection: systematic review and meta-analysis. *American journal of respiratory and critical care medicine* **174**, 514-523 (2006).
59. Flohr, C., Quinnell, R.J. & Britton, J. Do helminth parasites protect against atopy and allergic disease? *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **39**, 20-32 (2009).
60. Araujo, M.I. & de Carvalho, E.M. Human schistosomiasis decreases immune responses to allergens and clinical manifestations of asthma. *Chemical immunology and allergy* **90**, 29-44 (2006).
61. Cooper, P.J., *et al.* Reduced risk of atopy among school-age children infected with geohelminth parasites in a rural area of the tropics. *The Journal of allergy and clinical immunology* **111**, 995-1000 (2003).
62. Flohr, C., *et al.* Poor sanitation and helminth infection protect against skin sensitization in Vietnamese children: A cross-sectional study. *The Journal of allergy and clinical immunology* **118**, 1305-1311 (2006).
63. Mpairwe, H., *et al.* Skin prick test reactivity to common allergens among women in Entebbe, Uganda. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **102**, 367-373 (2008).
64. Rodrigues, L.C., *et al.* Early infection with Trichuris trichiura and allergen skin test reactivity in later childhood. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **38**, 1769-1777 (2008).
65. van den Biggelaar, A.H., *et al.* Decreased atopy in children infected with Schistosoma haematobium: a role for parasite-induced interleukin-10. *Lancet* **356**, 1723-1727 (2000).
66. Palmer, L.J., *et al.* Ascaris lumbricoides infection is associated with increased risk of childhood asthma and atopy in rural China. *American journal of respiratory and critical care medicine* **165**, 1489-1493 (2002).
67. Schafer, T., Meyer, T., Ring, J., Wichmann, H.E. & Heinrich, J. Worm infestation and the negative association with eczema (atopic/nonatopic) and allergic sensitization. *Allergy* **60**, 1014-1020 (2005).
68. Wordemann, M., *et al.* Association of atopy, asthma, allergic rhinoconjunctivitis, atopic dermatitis and intestinal helminth infections in Cuban children. *Tropical medicine & international health : TM & IH* **13**, 180-186 (2008).
69. Haileamlak, A., *et al.* Early life risk factors for atopic dermatitis in Ethiopian children. *The Journal of allergy and clinical immunology* **115**, 370-376 (2005).
70. Dagoye, D., *et al.* Wheezing, allergy, and parasite infection in children in urban and rural Ethiopia. *American journal of respiratory and critical care medicine* **167**, 1369-1373 (2003).
71. Scrivener, S., *et al.* Independent effects of intestinal parasite infection and domestic allergen exposure on risk of wheeze in Ethiopia: a nested case-control study. *Lancet* **358**, 1493-1499 (2001).
72. Cooper, P.J., Chico, M.E., Bland, M., Griffin, G.E. & Nutman, T.B. Allergic symptoms, atopy, and geohelminth infections in a rural area of Ecuador. *American journal of respiratory and critical care medicine* **168**, 313-317 (2003).
73. Peisong, G., *et al.* An asthma-associated genetic variant of STAT6 predicts low burden of ascaris worm infestation. *Genes and immunity* **5**, 58-62 (2004).

74. Moller, M., *et al.* Genetic haplotypes of Th-2 immune signalling link allergy to enhanced protection to parasitic worms. *Human molecular genetics* **16**, 1828-1836 (2007).
75. Elliott, D.E., *et al.* Exposure to schistosome eggs protects mice from TNBS-induced colitis. *American journal of physiology. Gastrointestinal and liver physiology* **284**, G385-391 (2003).
76. Khan, W.I., *et al.* Intestinal nematode infection ameliorates experimental colitis in mice. *Infection and immunity* **70**, 5931-5937 (2002).
77. Reardon, C., Sanchez, A., Hogaboam, C.M. & McKay, D.M. Tapeworm infection reduces epithelial ion transport abnormalities in murine dextran sulfate sodium-induced colitis. *Infection and immunity* **69**, 4417-4423 (2001).
78. Melon, A., Wang, A., Phan, V. & McKay, D.M. Infection with Hymenolepis diminuta is more effective than daily corticosteroids in blocking chemically induced colitis in mice. *Journal of biomedicine & biotechnology* **2010**, 384523 (2010).
79. Kabeerdoss, J., Pugazhendhi, S., Subramanian, V., Binder, H.J. & Ramakrishna, B.S. Exposure to hookworms in patients with Crohn's disease: a case-control study. *Alimentary pharmacology & therapeutics* **34**, 923-930 (2011).
80. Leibowitz, U., *et al.* Epidemiological study of multiple sclerosis in Israel. II. Multiple sclerosis and level of sanitation. *Journal of neurology, neurosurgery, and psychiatry* **29**, 60-68 (1966).
81. Fleming, J.O. & Cook, T.D. Multiple sclerosis and the hygiene hypothesis. *Neurology* **67**, 2085-2086 (2006).
82. Correale, J. & Farez, M. Association between parasite infection and immune responses in multiple sclerosis. *Annals of neurology* **61**, 97-108 (2007).
83. Correale, J. & Farez, M.F. The impact of parasite infections on the course of multiple sclerosis. *Journal of neuroimmunology* **233**, 6-11 (2011).
84. Donskow-Lysoniewska, K., Krawczak, K. & Doligalska, M. Heligmosomoides polygyrus: EAE remission is correlated with different systemic cytokine profiles provoked by L4 and adult nematodes. *Experimental parasitology* **132**, 243-248 (2012).
85. Sewell, D., *et al.* Immunomodulation of experimental autoimmune encephalomyelitis by helminth ova immunization. *International immunology* **15**, 59-69 (2003).
86. Walsh, K.P., Brady, M.T., Finlay, C.M., Boon, L. & Mills, K.H. Infection with a helminth parasite attenuates autoimmunity through TGF-beta-mediated suppression of Th17 and Th1 responses. *J Immunol* **183**, 1577-1586 (2009).
87. La Flamme, A.C., Ruddenklau, K. & Backstrom, B.T. Schistosomiasis decreases central nervous system inflammation and alters the progression of experimental autoimmune encephalomyelitis. *Infection and immunity* **71**, 4996-5004 (2003).
88. Kuijk, L.M., *et al.* Soluble helminth products suppress clinical signs in murine experimental autoimmune encephalomyelitis and differentially modulate human dendritic cell activation. *Molecular immunology* **51**, 210-218 (2012).
89. Cooke, A., *et al.* Infection with Schistosoma mansoni prevents insulin dependent diabetes mellitus in non-obese diabetic mice. *Parasite immunology* **21**, 169-176 (1999).
90. Hubner, M.P., Stocker, J.T. & Mitre, E. Inhibition of type 1 diabetes in filaria-infected non-obese diabetic mice is associated with a T helper type 2 shift and induction of FoxP3+ regulatory T cells. *Immunology* **127**, 512-522 (2009).
91. Saunders, K.A., Raine, T., Cooke, A. & Lawrence, C.E. Inhibition of autoimmune type 1 diabetes by gastrointestinal helminth infection. *Infection and immunity* **75**, 397-407 (2007).

92. Robinson, M.W., Dalton, J.P., O'Brien, B.A. & Donnelly, S. Fasciola hepatica: The therapeutic potential of a worm secretome. *International journal for parasitology* (2012).
93. Zaccone, P., Fehervari, Z., Phillips, J.M., Dunne, D.W. & Cooke, A. Parasitic worms and inflammatory diseases. *Parasite immunology* **28**, 515-523 (2006).
94. Aravindhan, V.*, et al.* Decreased prevalence of lymphatic filariasis among subjects with type-1 diabetes. *The American journal of tropical medicine and hygiene* **83**, 1336-1339 (2010).
95. Donath, M.Y. & Shoelson, S.E. Type 2 diabetes as an inflammatory disease. *Nature reviews. Immunology* **11**, 98-107 (2011).
96. Aravindhan, V.*, et al.* Decreased prevalence of lymphatic filariasis among diabetic subjects associated with a diminished pro-inflammatory cytokine response (CURES 83). *PLoS neglected tropical diseases* **4**, e707 (2010).
97. van Kuijk, A.W., Kerstens, P.J., Perenboom, R.M., Dijkmans, B.A. & Voskuyl, A.E. Early-onset polyarthritis as presenting feature of intestinal infection with Strongyloides stercoralis. *Rheumatology (Oxford)* **42**, 1419-1420 (2003).
98. Osada, Y., Shimizu, S., Kumagai, T., Yamada, S. & Kanazawa, T. Schistosoma mansoni infection reduces severity of collagen-induced arthritis via down-regulation of pro-inflammatory mediators. *International journal for parasitology* **39**, 457-464 (2009).
99. Rocha, F.A.*, et al.* Protective effect of an extract from Ascaris suum in experimental arthritis models. *Infection and immunity* **76**, 2736-2745 (2008).
100. Salinas-Carmona, M.C.*, et al.* Spontaneous arthritis in MRL/lpr mice is aggravated by Staphylococcus aureus and ameliorated by Nippostrongylus brasiliensis infections. *Autoimmunity* **42**, 25-32 (2009).
101. McInnes, I.B.*, et al.* A novel therapeutic approach targeting articular inflammation using the filarial nematode-derived phosphorylcholine-containing glycoprotein ES-62. *J Immunol* **171**, 2127-2133 (2003).
102. Aoyama, H.*, et al.* An inverse relationship between autoimmune liver diseases and Strongyloides stercoralis infection. *The American journal of tropical medicine and hygiene* **76**, 972-976 (2007).
103. Nagayama, Y., Watanabe, K., Niwa, M., McLachlan, S.M. & Rapoport, B. Schistosoma mansoni and alpha-galactosylceramide: prophylactic effect of Th1 Immune suppression in a mouse model of Graves' hyperthyroidism. *J Immunol* **173**, 2167-2173 (2004).
104. Magen, E., Borkow, G., Bentwich, Z., Mishal, J. & Scharf, S. Can worms defend our hearts? Chronic helminthic infections may attenuate the development of cardiovascular diseases. *Medical hypotheses* **64**, 904-909 (2005).
105. Doenhoff, M.J., Stanley, R.G., Griffiths, K. & Jackson, C.L. An anti-atherogenic effect of Schistosoma mansoni infections in mice associated with a parasite-induced lowering of blood total cholesterol. *Parasitology* **125**, 415-421 (2002).
106. Hotez, P.J.*, et al.* Helminth infections: the great neglected tropical diseases. *The Journal of clinical investigation* **118**, 1311-1321 (2008).
107. Utzinger, J. A research and development agenda for the control and elimination of human helminthiases. *PLoS neglected tropical diseases* **6**, e1646 (2012).
108. Lynch, N.R.*, et al.* Effect of anthelmintic treatment on the allergic reactivity of children in a tropical slum. *The Journal of allergy and clinical immunology* **92**, 404-411 (1993).
109. van den Biggelaar, A.H.*, et al.* Long-term treatment of intestinal helminths increases mite skin-test reactivity in Gabonese schoolchildren. *The Journal of infectious diseases* **189**, 892-900 (2004).

110. Cooper, P.J., *et al.* Effect of albendazole treatments on the prevalence of atopy in children living in communities endemic for geohelminth parasites: a cluster-randomised trial. *Lancet* **367**, 1598-1603 (2006).
111. Flohr, C., *et al.* Reduced helminth burden increases allergen skin sensitization but not clinical allergy: a randomized, double-blind, placebo-controlled trial in Vietnam. *Clin Exp Allergy* (2009).
112. Wiria, A.E., *et al.* The effect of three-monthly albendazole treatment on malarial parasitemia and allergy: a household-based cluster-randomized, double-blind, placebo-controlled trial. *PloS one* **8**, e57899 (2013).
113. Matricardi, P.M., *et al.* Exposure to foodborne and orofecal microbes versus airborne viruses in relation to atopy and allergic asthma: epidemiological study. *BMJ* **320**, 412-417 (2000).
114. Pelosi, U., *et al.* The inverse association of salmonellosis in infancy with allergic rhinoconjunctivitis and asthma at school-age: a longitudinal study. *Allergy* **60**, 626-630 (2005).
115. Lell, B., Borrmann, S., Yazdanbakhsh, M. & Kremsner, P.G. Atopy and malaria. *Wiener klinische Wochenschrift* **113**, 927-929 (2001).
116. Endara, P., *et al.* Long-term periodic anthelmintic treatments are associated with increased allergen skin reactivity. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **40**, 1669-1677 (2010).
117. Mpairwe, H., *et al.* Anthelminthic treatment during pregnancy is associated with increased risk of infantile eczema: randomised-controlled trial results. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology* **22**, 305-312 (2011).
118. Ndibazza, J., *et al.* Impact of anthelminthic treatment in pregnancy and childhood on immunisations, infections and eczema in childhood: a randomised controlled trial. *PloS one* **7**, e50325 (2012).
119. Ndibazza, J., *et al.* Effects of deworming during pregnancy on maternal and perinatal outcomes in Entebbe, Uganda: a randomized controlled trial. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **50**, 531-540 (2010).
120. Webb, E.L., *et al.* Effect of single-dose anthelmintic treatment during pregnancy on an infant's response to immunisation and on susceptibility to infectious diseases in infancy: a randomised, double-blind, placebo-controlled trial. *Lancet* **377**, 52-62 (2011).
121. Almeida, M.C., *et al.* The effect of antihelminthic treatment on subjects with asthma from an endemic area of schistosomiasis: a randomized, double-blinded, and placebo-controlled trial. *Journal of parasitology research* **2012**, 296856 (2012).
122. Bager, P., Vinkel Hansen, A., Wohlfahrt, J. & Melbye, M. Helminth infection does not reduce risk for chronic inflammatory disease in a population-based cohort study. *Gastroenterology* **142**, 55-62 (2012).
123. Cook, G.C. Enterobius vermicularis infection. *Gut* **35**, 1159-1162 (1994).
124. Lacey, E. Mode of action of benzimidazoles. *Parasitol Today* **6**, 112-115 (1990).
125. Skinner-Adams, T.S., Davis, T.M., Manning, L.S. & Johnston, W.A. The efficacy of benzimidazole drugs against Plasmodium falciparum in vitro. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **91**, 580-584 (1997).
126. MacDonald, L.M., Armson, A., Thompson, A.R. & Reynoldson, J.A. Characterisation of benzimidazole binding with recombinant tubulin from Giardia duodenalis, Encephalitozoon intestinalis, and Cryptosporidium parvum. *Molecular and biochemical parasitology* **138**, 89-96 (2004).

127. Cruz, M.C. & Edlind, T. beta-Tubulin genes and the basis for benzimidazole sensitivity of the opportunistic fungus Cryptococcus neoformans. *Microbiology* **143 (Pt 6)**, 2003-2008 (1997).
128. Cooper, P.J.*, et al.* Repeated treatments with albendazole enhance Th2 responses to Ascaris Lumbricoides, but not to aeroallergens, in children from rural communities in the Tropics. *The Journal of infectious diseases* **198**, 1237-1242 (2008).
129. van den Biggelaar, A.H., Borrmann, S., Kremsner, P. & Yazdanbakhsh, M. Immune responses induced by repeated treatment do not result in protective immunity to Schistosoma haematobium: interleukin (IL)-5 and IL-10 responses. *The Journal of infectious diseases* **186**, 1474-1482 (2002).
130. Wright, V.J.*, et al.* Early exposure of infants to GI nematodes induces Th2 dominant immune responses which are unaffected by periodic anthelminthic treatment. *PLoS neglected tropical diseases* **3**, e433 (2009).
131. Tweyongyere, R.*, et al.* Effect of praziquantel treatment during pregnancy on cytokine responses to schistosome antigens: results of a randomized, placebo-controlled trial. *The Journal of infectious diseases* **198**, 1870-1879 (2008).
132. Weinstock, J.V. & Elliott, D.E. Translatability of helminth therapy in inflammatory bowel diseases. *International journal for parasitology* (2012).
133. Summers, R.W.*, et al.* Trichuris suis seems to be safe and possibly effective in the treatment of inflammatory bowel disease. *The American journal of gastroenterology* **98**, 2034-2041 (2003).
134. Summers, R.W., Elliott, D.E., Urban, J.F., Jr., Thompson, R. & Weinstock, J.V. Trichuris suis therapy in Crohn's disease. *Gut* **54**, 87-90 (2005).
135. Summers, R.W., Elliott, D.E., Urban, J.F., Jr., Thompson, R.A. & Weinstock, J.V. Trichuris suis therapy for active ulcerative colitis: a randomized controlled trial. *Gastroenterology* **128**, 825-832 (2005).
136. Broadhurst, M.J.*, et al.* IL-22+ CD4+ T cells are associated with therapeutic trichuris trichiura infection in an ulcerative colitis patient. *Science translational medicine* **2**, 60ra88 (2010).
137. Broadhurst, M.J.*, et al.* Therapeutic helminth infection of macaques with idiopathic chronic diarrhea alters the inflammatory signature and mucosal microbiota of the colon. *PLoS pathogens* **8**, e1003000 (2012).
138. Fleming, J.O.*, et al.* Probiotic helminth administration in relapsing-remitting multiple sclerosis: a phase 1 study. *Mult Scler* **17**, 743-754 (2011).
139. Bager, P.*, et al.* Trichuris suis ova therapy for allergic rhinitis: a randomized, double-blind, placebo-controlled clinical trial. *The Journal of allergy and clinical immunology* **125**, 123-130 e121-123 (2010).
140. Summers, R.W., Elliott, D.E. & Weinstock, J.V. Trichuris suis might be effective in treating allergic rhinitis. *The Journal of allergy and clinical immunology* **125**, 766-767 (2010).
141. Croese, J.*, et al.* A proof of concept study establishing Necator americanus in Crohn's patients and reservoir donors. *Gut* **55**, 136-137 (2006).
142. Croese, J., Wood, M.J., Melrose, W. & Speare, R. Allergy controls the population density of Necator americanus in the small intestine. *Gastroenterology* **131**, 402-409 (2006).
143. Mortimer, K.*, et al.* Dose-ranging study for trials of therapeutic infection with Necator americanus in humans. *The American journal of tropical medicine and hygiene* **75**, 914-920 (2006).
144. Feary, J.*, et al.* Safety of hookworm infection in individuals with measurable airway responsiveness: a randomized placebo-controlled feasibility study. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **39**, 1060-1068 (2009).

145. Blount, D., *et al.* Immunologic profiles of persons recruited for a randomized, placebo-controlled clinical trial of hookworm infection. *The American journal of tropical medicine and hygiene* **81**, 911-916 (2009).
146. Feary, J.R., *et al.* Experimental hookworm infection: a randomized placebo-controlled trial in asthma. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **40**, 299-306 (2010).
147. Daveson, A.J., *et al.* Effect of hookworm infection on wheat challenge in celiac disease--a randomised double-blinded placebo controlled trial. *PloS one* **6**, e17366 (2011).
148. Harnett, W. & Harnett, M.M. Helminth-derived immunomodulators: can understanding the worm produce the pill? *Nature reviews. Immunology* **10**, 278-284 (2010).
149. Harnett, W., Frame, M.J., Nor, Z.M., MacDonald, M. & Houston, K.M. Some preliminary data on the nature/structure of the PC-glycan of the major excretory-secretory product of Acanthocheilonema viteae (ES-62). *Parasite* **1**, 179-181 (1994).
150. Melendez, A.J., *et al.* Inhibition of Fc epsilon RI-mediated mast cell responses by ES-62, a product of parasitic filarial nematodes. *Nature medicine* **13**, 1375-1381 (2007).
151. Schnoeller, C., *et al.* A helminth immunomodulator reduces allergic and inflammatory responses by induction of IL-10-producing macrophages. *J Immunol* **180**, 4265-4272 (2008).
152. Danilowicz-Luebert, E., *et al.* A nematode immunomodulator suppresses grass pollen-specific allergic responses by controlling excessive Th2 inflammation. *International journal for parasitology* (2012).
153. Ruyssers, N.E., *et al.* Therapeutic potential of helminth soluble proteins in TNBS-induced colitis in mice. *Inflammatory bowel diseases* **15**, 491-500 (2009).
154. Zhu, B., *et al.* Immune modulation by Lacto-N-fucopentaose III in experimental autoimmune encephalomyelitis. *Clin Immunol* **142**, 351-361 (2012).
155. Atochina, O. & Harn, D. Prevention of psoriasis-like lesions development in fsn/fsn mice by helminth glycans. *Experimental dermatology* **15**, 461-468 (2006).
156. Van Kruiningen, H.J. & West, A.B. Potential danger in the medical use of Trichuris suis for the treatment of inflammatory bowel disease. *Inflammatory bowel diseases* **11**, 515 (2005).
157. Zou, W. Regulatory T cells, tumour immunity and immunotherapy. *Nature reviews. Immunology* **6**, 295-307 (2006).
158. Janssens, S. & Beyaert, R. Role of Toll-like receptors in pathogen recognition. *Clinical microbiology reviews* **16**, 637-646 (2003).
159. Hotamisligil, G.S., Shargill, N.S. & Spiegelman, B.M. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* **259**, 87-91 (1993).
160. Law, J.P., *et al.* The importance of Foxp3 antibody and fixation/permeabilization buffer combinations in identifying CD4+CD25+Foxp3+ regulatory T cells. *Cytometry. Part A : the journal of the International Society for Analytical Cytology* **75**, 1040-1050 (2009).
161. Hotamisligil, G.S. Inflammation and metabolic disorders. *Nature* **444**, 860-867 (2006).
162. Stahl, B., *et al.* Oligosaccharides from human milk as revealed by matrix-assisted laser desorption/ionization mass spectrometry. *Analytical biochemistry* **223**, 218-226 (1994).

163. Atochina, O., Da'dara, A.A., Walker, M. & Harn, D.A. The immunomodulatory glycan LNFPIII initiates alternative activation of murine macrophages in vivo. *Immunology* **125**, 111-121 (2008).
164. Bhargava, P.*, et al.* Immunomodulatory glycan LNFPIII alleviates hepatosteatosis and insulin resistance through direct and indirect control of metabolic pathways. *Nature medicine* **18**, 1665-1672 (2012).
165. Ricardo-Gonzalez, R.R.*, et al.* IL-4/STAT6 immune axis regulates peripheral nutrient metabolism and insulin sensitivity. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 22617-22622 (2010).

Chapter 9

Summarizing Discussion

Future directions

While the poorest areas in the world suffer from a high burden of helminth infections, the same parasites are currently proposed to be beneficial in hampering inflammatory diseases in the richest areas of the world. The immune regulatory network induced by helminths may explain both sides of the coin; the chronic presence of worms in the human host and the dampening of pathological inflammatory conditions.
This thesis – in the context of the ImmunoSPIN project (www.immunospin.org) – has contributed to further understanding of immune modulation exerted by parasites. Regarding the aims of this thesis, the main findings were that (i) polarized T-helper cell and Treg subsets can be detected in peripheral blood of parasitized patients, (ii) Tregs functionally contribute to suppression of parasite-specific and bystander responses, (iii) deworming restores helminth-induced general and parasite-specific immune responsiveness and (iv) these immunological observations do not immediately lead to evident clinical consequences, leaving the question whether they may elicit relevant effects in the longer term, unanswered.

Characterization of T-helper & Tregs during parasitic infection

The thesis starts with the characterization of Tregs as well as T-helper subsets during parasitic infections and the role that Tregs have in controlling immune responses. In **chapter 2** we show that the proportion of $CD4^{+}CD25^{hi}FOXP3^{+}$ Tregs is higher in individuals with detectable *Loa loa* microfilaria (MF) compared to uninfected endemic controls. Interestingly, the Treg population is also increased in the group with no patent infection, yet positive for filaria-specific IgG4 and with a recent history of eye worm passage, which indicate that these subjects are infected but amicrofilaremic. Both infected groups displayed lower Th1 and Th17 responses to filarial antigens compared to the control group. Interestingly, in the infected but amicrofilaremic group, higher Th2 and IL-10 responses were measured compared to MF-positives and endemic controls. This suggests that immune regulatory mechanisms are induced during different stages of *Loa loa* infection, but are possibly more profound in individuals carrying MF. When we assessed Treg phenotype in asymptomatic malaria infections (**chapter 3**), the $CD4^{+}CD25^{hi}FOXP3^{+}$ subset was not different in parasitemic individuals, however the TNFRII-expressing Tregs were significantly elevated in the infected, suggesting a role for this specific subtype of Tregs in malaria infection. Surprisingly, malaria-induced Th1 responses were not altered in the infected group, whereas Th2 responses were lower. These findings were confirmed after treatment of malaria-positive children, revealing a decrease in TNFRII expression by Tregs and an increase in malaria-specific IL-13 production, in parallel to an expansion in the $GATA3^{+}$ subset. These data are in line with the view that Tregs are involved in suppression of different T-helper responses. With respect to the malaria study in **chapter 3**, it is interesting that malarial parasites, similar to helminths, are also able to induce the expansion of Tregs, supported by earlier studies[1,2]. However, previous studies have not assessed Th2 cells in any great detail. The results of our study along with the observation that the Fulani, a West-African tribe that is more resistant to malaria infection, express higher levels of *IL4* and *GATA3* genes[3], suggest that Th2 responses might need to be considered as important players, worth investigating in malaria immunity.

The field of Treg characterization is rapidly expanding. Within a few years, assessment of Tregs in parasitic diseases developed from indirect immunofluorescence of PBMC[4], through measuring mRNA expression of regulatory molecules and cytokines in PBMC[5-8], to distinguishing cell subsets co-expressing Treg markers by flow cytometry[9-12]. Currently it has become possible to measure up to 17 cell markers at a single cell level by flow cytometry. Staining of surface molecules and intracellular cytokines can be combined to define functional T cell subsets[13,14], but also transcription factors and even proliferative responses can be assessed simultaneously[15]. The phenotypic definition of Tregs remains a matter of

debate. Even in the few studies on Tregs in human helminth infections, markers used to distinguish Tregs differ considerably (Table 1). Along the way there has been confusion on the discrimination of regulatory and activated T cells. CD25, component of the IL-2 receptor, was already known as an activation marker for T cells and more confusing was the observation that FOXP3 expression was also seen after activation of naive T cells, without associated suppressive activity[16]. Several other markers have been proposed to be specific for Tregs, among others, increased expression of cytotoxic T cell antigen (CTLA-)4[17], glucocorticoid receptor tumor necrosis factor receptor (GITR)[18,19], GARP[20], Helios[21] and absence of CD127[22,23]. However, the expression of all these markers appears to be largely dynamic and strictly speaking not Treg-specific[24]. The current view on identification of human Tregs is based on FOXP3, CD45RA and CD45RO, in which naive ($CD25^{high}FOXP3^{low}CD45RA^{+}CD45RO^{-}$) Tregs are distinguished from effector ($CD25^{high}FOXP3^{high}CD45RA^{-}CD45RO^{+}$) Tregs, both capable of suppression[25,26]. Activated effector T cells are then characterized by low FOXP3, low or high CD25 and CD69 expression in combination with IL-2 production[26]. Recently, it has been shown that the methylation of the *FOXP3* locus might be an important marker for Tregs with strong suppressive function. It has been proposed that the amount of FOXP3 demethylation can distinguish $FOXP3^{+}$ Tregs from $FOXP3^{+}$ activated conventional T cells, since only Tregs seem to display demethylation and activated T cells not[27].

Table 1. Overview of phenotypic definition of Tregs in human helminth studies by flow cytometry

Helminth species	Disease stage	Study population	Study site	Treg definition	Reference
Wuchereria bancrofti	microfilaremic, CA-positive	adults	India	CD4^{+}IL-10^{+} *	No.5
Wuchereria bancrofti and/or *Mansonella perstans*	microfilaremic	adolescents	Mali	CD4^{+}CD25^{+}FOXP3^{+}CD127^{-} CD4^{+}FOXP3^{-}IL-10+ **	No.14
Schistosoma haematobium	chronic infection	children & adults	Zimbabwe	CD4^{+}CD25^{+}FOXP3^{+} CD4^{+}CD25^{hi} CD4^{+}FOXP3^{+}CD127^{-}	Nausch et al. *PLoS One 2011 (6):e16860*
Schistosoma mansoni	chronic infection	adults	Kenya	CD4^{+}CD25^{hi} CD4^{+}CD25^{hi}CD45RO+	No.12
Schistosoma mansoni	chronic infection chronic pathology	all ages	Brazil	CD4^{+}CD25^{hi} CD4^{+}IL-10^{+} *	Teixeira-Carvalho et al. *Acta Trop 2008;108:139-49*
Schistosoma mansoni and/or *Plasmodium falciparum*	chronic infection	school children	Mali	CD4^{+}CD25^{hi}FOXP3^{+}	Lyke et al. *PLoS One 2012;7:e31647*
intestinal parasites and/or protozoa	chronic infection	school children	Mexico	CD4^{+}CD25^{hi} CD4^{+}FOXP3^{+} CD4^{+}CTLA-4^{+} CD8^{+}CD28^{-}	No.9
geohelminths	chronic infection	school children	Indonesia	CD4^{+}CD25^{hi}FOXP3^{+}	Wammes et al. *Eur J Imm 2010;40:437-42*
Necator americanus	chronic infection		Brazil	CD4^{+}CD25^{+}FOXP3^{+}	No.11
Strongyloides stercoralis and/or HTLV	chronic infection	adults	Peru	CD4^{+}CD25^{hi}FOXP3^{+} CD4^{+}CD25^{hi}	No.10

** after PBMC stimulation with parasite antigens*
*** determined in unstimulated whole blood*

Moreover, the possibilities of cell sorting have allowed scientists to separate cell subsets based on surface markers, including positive selection for CD25 and negative selection for CD127[23] and CD49d[28], after which expression levels of other surface and/or intracellular components and functional capacities can be characterized more directly. The functional studies are based on suppression assays in which Tregs and T effector cells are co-cultured to evaluate inhibitory capacities of Tregs[29]. In translational research this has been extended to *in vitro* expansion of isolated Tregs for the purpose of immunotherapy[30].
However, when studying Tregs and T-cell immunology in parasite-endemic areas, experimental possibilities are limited. We, along with others, have shown that functional capacity of Tregs can nevertheless be studied in the field where facilities are restricted. By depleting $CD4^+CD25^{hi}$ cells from PBMC, freshly isolated from peripheral blood, it is possible to compare the proliferative and cytokine production capacity of PBMC with and without Tregs. This method has been used in chapters 4 and 5. In **chapter 4** we studied three clinical groups in an area endemic for lymphatic filariasis caused by *Wuchereria bancrofti*. We report suppressed Th1, Th2, Th17 and T-helper cell proliferative responses to filarial antigen in microfilaremic individuals compared to endemic controls. In contrast, subjects with chronic pathology displayed similar or enhanced Th1 and Th17 cytokine levels in comparison to the other two groups. After depletion of Tregs, proliferative and Th2 cytokine responses of MF-positives were restored to levels observed in endemic controls, indicating that indeed in microfilaremics, functional regulatory T cells exist that suppress some of the effector T cell responses. Whether the parasites induce Tregs to hide from the immune system or the host-generates Tregs in order to prevent the pathological consequences of infection, remains to be determined.

Immune correlates for infectious and inflammatory diseases

We were furthermore interested in the phenomenon of spill-over suppression. Downmodulation of responses to third party antigens by helminths could on the one hand lead to impaired responses to important infections[31,32] or vaccines[33], but on the other protect against excessive inflammatory responses observed in allergies, asthma and autoimmune diseases[34,35].
Chapter 5 presents the results of our study on bystander responses during geohelminth infections. Although the proportion of Tregs was not altered in infected children, Tregs from geohelminth-positive individuals displayed greater suppressive capacity deduced from the stronger enhancement of proliferation and IFN-γ secretion by PBMC in response to BCG vaccine and *P. falciparum*-infected red blood cells, after Treg depletion. Since this effect was not observed in helminth-

uninfected classmates, we concluded that Tregs with potent suppressory capacity are specifically triggered by the presence of helminths. The most elegant method of assessing the direct effect of helminths is to perform a randomized placebo-controlled study of helminth treatment. Few small-scale studies have previously shown that albendazole treatment increases effector T cell responses to BCG[36] and cholera[37] vaccination, and that it may also boost helminth-specific immune responses[38], although the latter study was not placebo-controlled. Two trials of the anti-schistosomal drug praziquantel have been carried out. A placebo-controlled study in pregnant women showed improved schistosome-specific cytokine production after treatment[39] and similarly, in an open-label trial, praziquantel administration to school children led to increased schistosomal antigen-induced IL-5 responses[40]. So far, no large-scale community anthelmintic treatment trials have been undertaken to assess the impact of deworming on immune responses and clinical outcomes. In **chapter 6** we have analyzed the effect of community-based deworming on cytokine responses to different stimuli. Three-monthly albendazole treatment only partly reduced the burden of STH infections in the community after 7 doses, however it strongly enhanced pro-inflammatory cytokine responses, especially to malaria antigens and mitogen. To ascertain that this is a helminth-specific and not a drug effect, we stratified the analysis based on helminth infection status, which revealed a prominent and in some cases stronger effect of treatment in the helminth-positive group. However, when interpreting these data, it should be noted that the number of helminth-uninfected individuals was relatively small. We also considered other possible confounders in this analysis. Helminths affect the nutritional status of an individual and malnutrition can be a cause of immune hyporesponsiveness[41]. We showed that body mass index did not change after treatment and moreover, helminth infection or albendazole treatment did not alter fasting blood glucose levels (unpublished data). This indicates that the alleviation of immune hyporesponsiveness is most likely due to the clearance of helminth infections, reducing their immune modulatory capacity. However, since our study was not designed to assess nutritional markers, we did not measure other, possibly more informative, parameters to fully exclude the possibility of improved nutritional status as a confounder.

In parallel we assessed the effect of deworming on several clinical outcomes: malaria prevalence and symptoms, SPT reactivity to allergens and symptoms of allergy, as presented in **chapter 7**. Malarial parasitemia was transiently increased in the treated group, although the prevalence of symptoms did not change. In contrast to our finding, earlier studies have indicated that helminth infection could lead to increased malaria parasitemia, but may protect from severe malarial disease[32]. An important limitation of our study was that during our trial, the prevalence of malaria decreased substantially, which might affect the results obtained. For allergic responses, there was an incremental increase in the

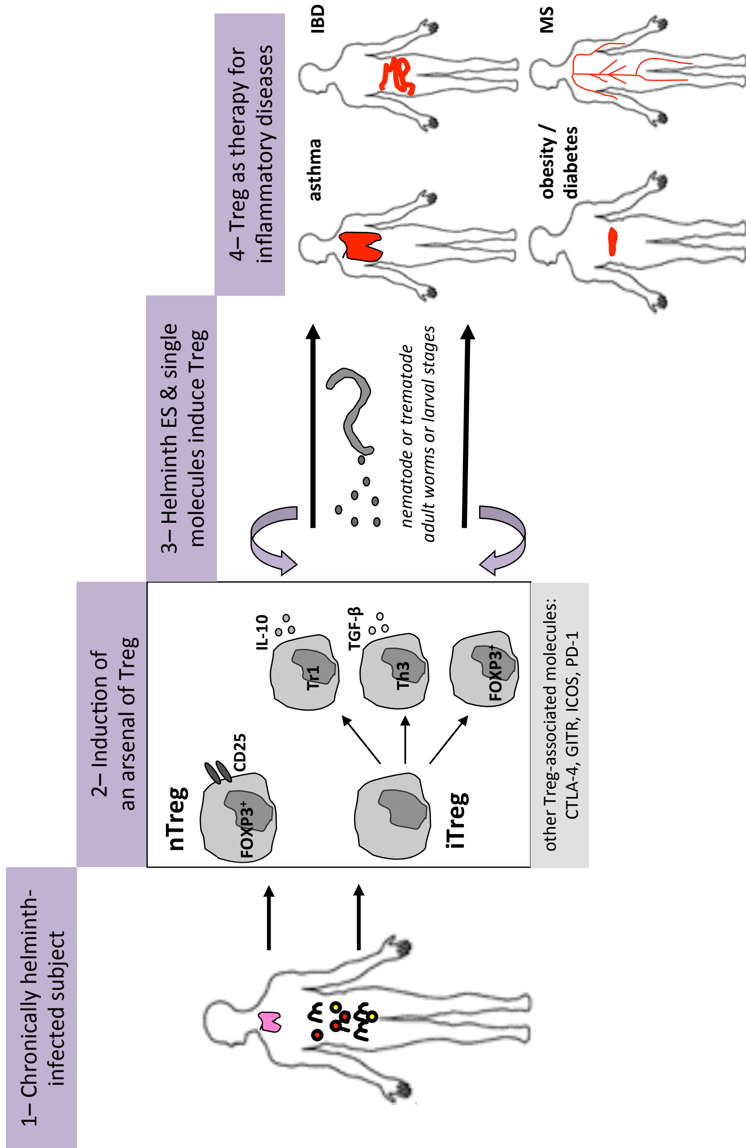
proportion of children with SPT reactivity to any of the allergens in the albendazole arm after 9 and 21 months, which did not reach statistical significance. However, when cockroach allergen was considered separately, a significant increase of SPT positivity was observed after treatment. It must be noted that deworming in this trial was not sufficient, suggesting that even more intensive anthelmintic treatment, possibly a combination of drugs, or drugs and environmental control, is needed for future studies. The fact that deworming was not complete also indicates that more profound changes in immune responses and clinical outcomes might be anticipated when more intensive deworming is achieved.
Comparing the results of **chapter 6** and **chapter 7**, we may conclude that *in vitro*-stimulated immune responses are not reflected in the clinical outcomes, at least not within our trial period. Clinically evident effects of deworming may take longer to develop, as illustrated by a study in Ecuador, which showed a major increase in SPT reactivity and possibly more eczema in communities that were enrolled in more than 15 years of regular treatment with ivermectin[42]. An earlier study by the same group showed that one year of two-monthly albendazole treatment of school children resulted in enhanced Th2 responses to helminth antigens and bacterial superantigen, but no change in allergen responses, SPT reactivity or allergic symptoms[38,43]. Although not in any great detail, immune responses have also been characterized in experimental helminth infections of humans, in the context of helminthic therapy for inflammatory diseases. Similarly, in these trials immunological responses did not always coincide with clinical findings. The report of an RCT of *Trichuris suis* ova (TSO) in allergic rhinitis patients showed increased Th2 and IL-10 responses to the introduced *Trichuris* infection[44], but no change in allergic outcomes were seen[45], supported by lack of any change in allergen-specific cytokine responses. This may imply that helminth-induced immunity may first be directed at helminth-specific responses, followed by modulation of bystander responses, leading to clinical effects at a later stage.

Future directions

First of all, there is no doubt that deworming campaigns should be further advocated[46]. However, it is of utmost importance to follow-up communities where mass drug administration (MDA) has taken place. Deworming may negatively affect coinfections[32], positively affect inflammatory diseases[42,47] and moreover the effect of anthelmintic treatment of pregnant women can become apparent in their offspring, leading to an increased prevalence of eczema[48]. However, some studies have provided evidence for beneficial effects of anthelmintic treatment, for example on malaria incidence[48] and CD4 counts in HIV infection[49]. Since the possibilities of performing placebo-controlled trials are complicated in some countries, further studies are needed that compare treated and untreated communities, to determine long-term effects of mass treatment. In areas where MDA is operational, coinfections and possible development of inflammatory conditions should be monitored while measurement of immunological markers might help understand causation.

At the same time, several clinical trials are running on the use of helminthic therapy to treat inflammatory diseases, as summarized in **chapter 8**. As treatment with full infections might not be the most ideal option, some research groups have been focusing on characterization of helminth-derived molecules that may be able to substitute the whole parasite approach[50]. With the current possibilities of preparing antigen mixtures or isolating single molecules from helminths, several products with immune modulating properties have been defined, however none has been administered to humans. Currently, the filarial product ES-62 is the best-characterized candidate molecule for therapeutic trials; it has shown promising data in murine models[51,52] and in a human *in vitro* model for arthritis[51]. Another product from filarial worms, AvCystatin, has also been shown to be protective for murine allergic airway inflammation[53] and moreover, *in vitro* it suppresses exaggerated Th2 responses of PBMC from grass pollen-allergic patients[54]. Excreted-secreted (ES) products of intestinal worms from *H. polygyrus* (HES) and ES from canine hookworm *Ankylostoma caninum*, have been shown to suppress allergic airway inflammation[55] and colitis[56] in mice, respectively. With regard to schistosomes, soluble worm extracts of *S. mansoni* have had beneficial effects against murine colitis[56], while a Lewis^{X}-containing glycan from *S. mansoni* eggs, lacto-N-fucopentaose III (LNFPIII), was able to suppress EAE[57] and psoriasis[58].

Taken together, these studies and results in animal models encourage the use of helminth-derived molecules, which might reproduce or even surpass the results of a full helminth infection. In particular, molecules associated with Tregs may be promising[59,60], since there is accumulating evidence that Tregs orchestrate the helminth-induced immune regulatory network (Figure 1).



1– Chronically helminth-infected subject
2– Induction of an arsenal of Treg
nTreg
CD25
FOXP3+
iTreg
IL-10
Tr1
TGF-β
Th3
FOXP3+
other Treg-associated molecules: CTLA-4, GITR, ICOS, PD-1
3– Helminth ES & single molecules induce Treg
nematode or trematode adult worms or larval stages
4– Treg as therapy for inflammatory diseases
asthma
IBD
obesity / diabetes
MS

Concluding remarks

Helminths are potent immune modulators and this can have both beneficial and detrimental consequences. Immune hyporesponsiveness is – at least partly – exerted by suppressive capacities of Tregs (**chapters 4 & 5**). Assessment of Treg phenotype and function is possible in areas with limited resources (**chapters 2 – 5**) and Tregs may have favorable properties in terms of dampening the excessive inflammation observed in allergies and autoimmune diseases. The immune regulatory network has gained significant attention as an important part of the web of immune responses, genetics and environmental factors that explain the current disease patterns seen upon epidemiological transition. Future clinical studies of deworming as well as helminthic therapy should encompass studies that will monitor different aspects of this regulatory network. Furthermore, larger and longer anthelmintic treatment RCTs might be needed to assess the immunological and clinical consequences of deworming. Given that there is some evidence on the possible detrimental effects of helminth elimination on the prevalence of allergies, asthma and other inflammatory diseases, it would be important to implement proper monitoring of MDA programs, specifically for altered immunological and clinical outcomes. Together, these strategies may help us to properly anticipate the double burden, of infectious and inflammatory diseases, in resource-poor settings.

Figure 1 (left page). Helminth-associated Tregs: lessons learnt and future directions. A schematic representation of the role of Tregs in infectious and inflammatory diseases. Helminths are associated with expansion of Tregs, which allows their long-term survival within their host (1). Tregs are comprised of both natural Tregs (nTregs), derived from the thymus, which are $FOXP3^{+}$ and express high levels of CD25, as well as different inducible (i)Tregs (2). So far, Tr1 and Th3 cells have been described, which do not always express FOXP3. Moreover, FOXP3 expression has also been seen in cells that do not express other Treg markers and are induced in the periphery. It should be noted that various markers such as CTLA-4, GITR, ICOS or PD-1 have been reported to be expressed on Tregs. Several helminth-derived compounds, such as excreted-secretory (ES) products and even single molecules, have been shown to either directly induce FOXP3 expression in T cells or condition dendritic cells to stimulate the expansion of Tregs (3). Although this is largely based on murine studies, it holds promise for future human studies. Ultimately, it may be possible to apply isolated Tregs clinically in a range of diseases (4), all characterized by high levels of inflammation. Future mechanistic studies may be needed to determine which Treg subsets are particularly suited for the different clinical conditions.

References

1. Minigo, G., *et al.* Parasite-dependent expansion of TNF receptor II-positive regulatory T cells with enhanced suppressive activity in adults with severe malaria. *PLoS pathogens* 5, e1000402 (2009).
2. Walther, M., *et al.* Upregulation of TGF-beta, FOXP3, and CD4+CD25+ regulatory T cells correlates with more rapid parasite growth in human malaria infection. *Immunity* 23, 287-296 (2005).
3. Torcia, M.G., *et al.* Functional deficit of T regulatory cells in Fulani, an ethnic group with low susceptibility to Plasmodium falciparum malaria. *Proceedings of the National Academy of Sciences of the United States of America* 105, 646-651 (2008).
4. Piessens, W.F., *et al.* Antigen-specific suppressor T lymphocytes in human lymphatic filariasis. *The New England journal of medicine* 307, 144-148 (1982).
5. Babu, S., Blauvelt, C.P., Kumaraswami, V. & Nutman, T.B. Regulatory networks induced by live parasites impair both Th1 and Th2 pathways in patent lymphatic filariasis: implications for parasite persistence. *J Immunol* 176, 3248-3256 (2006).
6. Babu, S., Kumaraswami, V. & Nutman, T.B. Transcriptional control of impaired Th1 responses in patent lymphatic filariasis by T-box expressed in T cells and suppressor of cytokine signaling genes. *Infection and immunity* 73, 3394-3401 (2005).
7. Correale, J. & Farez, M. Association between parasite infection and immune responses in multiple sclerosis. *Annals of neurology* 61, 97-108 (2007).
8. Finney, O.C., Nwakanma, D., Conway, D.J., Walther, M. & Riley, E.M. Homeostatic regulation of T effector to Treg ratios in an area of seasonal malaria transmission. *European journal of immunology* 39, 1288-1300 (2009).
9. Garcia-Hernandez, M.H., *et al.* Regulatory T Cells in children with intestinal parasite infection. *Parasite immunology* 31, 597-603 (2009).
10. Montes, M., *et al.* Regulatory T cell expansion in HTLV-1 and strongyloidiasis co-infection is associated with reduced IL-5 responses to Strongyloides stercoralis antigen. *PLoS neglected tropical diseases* 3, e456 (2009).
11. Ricci, N.D., *et al.* Induction of CD4(+)CD25(+)FOXP3(+) regulatory T cells during human hookworm infection modulates antigen-mediated lymphocyte proliferation. *PLoS neglected tropical diseases* 5, e1383 (2011).
12. Watanabe, K., *et al.* T regulatory cell levels decrease in people infected with Schistosoma mansoni on effective treatment. *The American journal of tropical medicine and hygiene* 77, 676-682 (2007).
13. Duhen, T., Duhen, R., Lanzavecchia, A., Sallusto, F. & Campbell, D.J. Functionally distinct subsets of human FOXP3+ Treg cells that phenotypically mirror effector Th cells. *Blood* 119, 4430-4440 (2012).
14. Metenou, S., *et al.* At homeostasis filarial infections have expanded adaptive T regulatory but not classical Th2 cells. *J Immunol* 184, 5375-5382 (2010).
15. Zielinski, C.E., *et al.* Pathogen-induced human TH17 cells produce IFN-gamma or IL-10 and are regulated by IL-1beta. *Nature* 484, 514-518 (2012).
16. Gavin, M.A., *et al.* Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proceedings of the National Academy of Sciences of the United States of America* 103, 6659-6664 (2006).
17. Wing, K., *et al.* CTLA-4 control over Foxp3+ regulatory T cell function. *Science* 322, 271-275 (2008).
18. McHugh, R.S., *et al.* CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* 16, 311-323 (2002).

19. Shimizu, J., Yamazaki, S., Takahashi, T., Ishida, Y. & Sakaguchi, S. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nature immunology* 3, 135-142 (2002).
20. Wang, R., *et al.* Expression of GARP selectively identifies activated human FOXP3+ regulatory T cells. *Proceedings of the National Academy of Sciences of the United States of America* 106, 13439-13444 (2009).
21. Thornton, A.M., *et al.* Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J Immunol* 184, 3433-3441 (2010).
22. Liu, W., *et al.* CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *The Journal of experimental medicine* 203, 1701-1711 (2006).
23. Seddiki, N., *et al.* Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *The Journal of experimental medicine* 203, 1693-1700 (2006).
24. Hori, S. Developmental plasticity of Foxp3+ regulatory T cells. *Current opinion in immunology* 22, 575-582 (2010).
25. Miyara, M., *et al.* Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity* 30, 899-911 (2009).
26. Sakaguchi, S., Miyara, M., Costantino, C.M. & Hafler, D.A. FOXP3+ regulatory T cells in the human immune system. *Nature reviews. Immunology* 10, 490-500 (2010).
27. Baron, U., *et al.* DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells. *European journal of immunology* 37, 2378-2389 (2007).
28. Kleinewietfeld, M., *et al.* CD49d provides access to "untouched" human Foxp3+ Treg free of contaminating effector cells. *Blood* 113, 827-836 (2009).
29. McMurchy, A.N. & Levings, M.K. Suppression assays with human T regulatory cells: a technical guide. *European journal of immunology* 42, 27-34 (2012).
30. Hippen, K.L., Riley, J.L., June, C.H. & Blazar, B.R. Clinical perspectives for regulatory T cells in transplantation tolerance. *Seminars in immunology* 23, 462-468 (2011).
31. Abruzzi, A. & Fried, B. Coinfection of Schistosoma (Trematoda) with bacteria, protozoa and helminths. *Advances in parasitology* 77, 1-85 (2011).
32. Nacher, M. Interactions between worms and malaria: good worms or bad worms? *Malaria journal* 10, 259 (2011).
33. Borkow, G. & Bentwich, Z. Chronic parasite infections cause immune changes that could affect successful vaccination. *Trends in parasitology* 24, 243-245 (2008).
34. Rook, G.A. Review series on helminths, immune modulation and the hygiene hypothesis: the broader implications of the hygiene hypothesis. *Immunology* 126, 3-11 (2009).
35. Smits, H.H., Everts, B., Hartgers, F.C. & Yazdanbakhsh, M. Chronic helminth infections protect against allergic diseases by active regulatory processes. *Current allergy and asthma reports* 10, 3-12 (2010).
36. Elias, D., *et al.* Effect of deworming on human T cell responses to mycobacterial antigens in helminth-exposed individuals before and after bacille Calmette-Guerin (BCG) vaccination. *Clinical and experimental immunology* 123, 219-225 (2001).
37. Cooper, P.J., *et al.* Human infection with Ascaris lumbricoides is associated with suppression of the interleukin-2 response to recombinant cholera toxin B subunit following vaccination with the live oral cholera vaccine CVD 103-HgR. *Infection and immunity* 69, 1574-1580 (2001).

38. Cooper, P.J., *et al.* Repeated treatments with albendazole enhance Th2 responses to Ascaris Lumbricoides, but not to aeroallergens, in children from rural communities in the Tropics. *The Journal of infectious diseases* 198, 1237-1242 (2008).
39. Tweyongyere, R., *et al.* Effect of praziquantel treatment during pregnancy on cytokine responses to schistosome antigens: results of a randomized, placebo-controlled trial. *The Journal of infectious diseases* 198, 1870-1879 (2008).
40. van den Biggelaar, A.H., Borrmann, S., Kremsner, P. & Yazdanbakhsh, M. Immune responses induced by repeated treatment do not result in protective immunity to Schistosoma haematobium: interleukin (IL)-5 and IL-10 responses. *The Journal of infectious diseases* 186, 1474-1482 (2002).
41. Chandra, R.K. Nutrition and the immune system from birth to old age. *European journal of clinical nutrition* 56 Suppl 3, S73-76 (2002).
42. Endara, P., *et al.* Long-term periodic anthelmintic treatments are associated with increased allergen skin reactivity. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 40, 1669-1677 (2010).
43. Cooper, P.J., *et al.* Effect of albendazole treatments on the prevalence of atopy in children living in communities endemic for geohelminth parasites: a cluster-randomised trial. *Lancet* 367, 1598-1603 (2006).
44. Bourke, C.D., *et al.* Trichuris suis ova therapy for allergic rhinitis does not affect allergen-specific cytokine responses despite a parasite-specific cytokine response. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 42, 1582-1595 (2012).
45. Bager, P., *et al.* Trichuris suis ova therapy for allergic rhinitis: a randomized, double-blind, placebo-controlled clinical trial. *The Journal of allergy and clinical immunology* 125, 123-130 e121-123 (2010).
46. Utzinger, J. A research and development agenda for the control and elimination of human helminthiases. *PLoS neglected tropical diseases* 6, e1646 (2012).
47. Correale, J. & Farez, M.F. The impact of parasite infections on the course of multiple sclerosis. *Journal of neuroimmunology* 233, 6-11 (2011).
48. Ndibazza, J., *et al.* Impact of anthelminthic treatment in pregnancy and childhood on immunisations, infections and eczema in childhood: a randomised controlled trial. *PloS one* 7, e50325 (2012).
49. Walson, J.L., *et al.* Albendazole treatment of HIV-1 and helminth co-infection: a randomized, double-blind, placebo-controlled trial. *AIDS* 22, 1601-1609 (2008).
50. Harnett, W. & Harnett, M.M. Helminth-derived immunomodulators: can understanding the worm produce the pill? *Nature reviews. Immunology* 10, 278-284 (2010).
51. McInnes, I.B., *et al.* A novel therapeutic approach targeting articular inflammation using the filarial nematode-derived phosphorylcholine-containing glycoprotein ES-62. *J Immunol* 171, 2127-2133 (2003).
52. Melendez, A.J., *et al.* Inhibition of Fc epsilon RI-mediated mast cell responses by ES-62, a product of parasitic filarial nematodes. *Nature medicine* 13, 1375-1381 (2007).
53. Schnoeller, C., *et al.* A helminth immunomodulator reduces allergic and inflammatory responses by induction of IL-10-producing macrophages. *J Immunol* 180, 4265-4272 (2008).
54. Danilowicz-Luebert, E., *et al.* A nematode immunomodulator suppresses grass pollen-specific allergic responses by controlling excessive Th2 inflammation. *International journal for parasitology* (2012).
55. McSorley, H.J., *et al.* Suppression of type 2 immunity and allergic airway inflammation by secreted products of the helminth Heligmosomoides polygyrus. *European journal of immunology* 42, 2667-2682 (2012).

56. Ruyssers, N.E., *et al.* Therapeutic potential of helminth soluble proteins in TNBS-induced colitis in mice. *Inflammatory bowel diseases* 15, 491-500 (2009).
57. Zhu, B., *et al.* Immune modulation by Lacto-N-fucopentaose III in experimental autoimmune encephalomyelitis. *Clin Immunol* 142, 351-361 (2012).
58. Atochina, O. & Harn, D. Prevention of psoriasis-like lesions development in fsn/fsn mice by helminth glycans. *Experimental dermatology* 15, 461-468 (2006).
59. Grainger, J.R., *et al.* Helminth secretions induce de novo T cell Foxp3 expression and regulatory function through the TGF-beta pathway. *The Journal of experimental medicine* 207, 2331-2341 (2010).
60. Zaccone, P., *et al.* The S. mansoni glycoprotein omega-1 induces Foxp3 expression in NOD mouse CD4(+) T cells. *European journal of immunology* 41, 2709-2718 (2011).

Addendum

Nederlandse samenvatting voor niet-ingewijden

List of publications

Curriculum vitae

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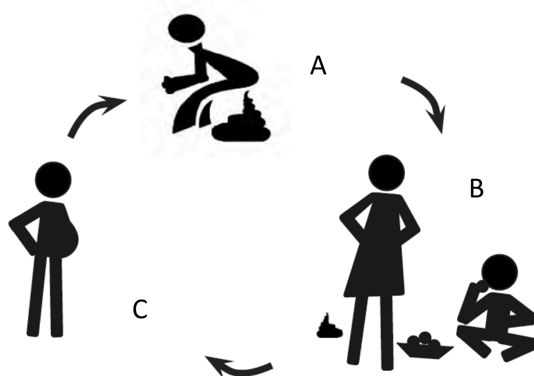
Parasitaire worminfecties

In lage-inkomenslanden komen veel infecties met parasieten voor, bijvoorbeeld worminfecties. Meer dan een miljard mensen zijn besmet met wormen in het maag-darmkanaal, vooral in delen van Afrika, Azië en de Amerika's. Deze wormen kunnen soms tot tien jaar in de darmen aanwezig blijven, waar ze voedingsstoffen en soms bloed en weefsels van hun gastheer consumeren. Behalve tot klachten van het maag-darmkanaal, kan dit – met name bij kinderen – tot een groei- en/of ontwikkelingsachterstand leiden. Infectie met wormen komt het meest voor in gebieden met slechte hygiënische omstandigheden. Wormeieren van de *Ascaris* (spoelworm) en *Trichuris* (zweepworm) kunnen via ontlasting van een geïnfecteerd persoon in de omgeving komen. Door afwezigheid van sanitaire voorzieningen of matige hand-hygiëne kunnen deze eieren in het voedsel van andere personen geraken. Sommige wormlarven, zoals die van mijnworm, kunnen de intacte huid binnendringen. Als kinderen – of volwassenen – geen schoenen dragen, kunnen zij op deze wijze geïnfecteerd raken (zie figuur 1). Andere veelvoorkomende wormen zijn van de soort filaria, waarvan de larven (microfilariae) door muggen worden overgebracht. Hieronder vallen de *Loa loa* worm die in bindweefsel achterblijft en de verschillende soorten die in de lymfvaten leven en elefantiasis (olifantsbenen) veroorzaken. Er zijn aanwijzingen gevonden dat mensen al in het prehistorische tijdperk leden aan worminfecties. We leven dus al heel lang 'samen' met wormen. Hoewel worminfecties in de Westerse wereld vrijwel uitgeroeid zijn, is in landen zoals Indonesië in sommige gebieden bijna 100% van de schoolkinderen geïnfecteerd met wormen in de darmen. Omdat infecties vaak zonder klachten verlopen, wordt niet veel aandacht besteed aan de bestrijding van worminfecties. Ze worden in de medische wereld dan ook aangeduid als één van de 'neglected' ofwel verwaarloosde ziekten.



Figuur 1: Cyclus van spoel-, zweep- en mijnwormen

A. Geïnfecteerd persoon contamineert de bodem met wormeieren. B. Andere personen krijgen wormeieren binnen door besmet voedsel, vieze handen of binnendringen van larven door de huid. C. In de darmen van een geïnfecteerd persoon ontwikkelen eieren en larven zich in volwassen wormen, die eieren produceren.

Bron: www.who.int/intestinal_worms/epidemiology/en/

Infectie met wormen beïnvloedt het afweersysteem

Normaliter zal het afweersysteem infecties proberen tegen te gaan. Op de plaats waar bijvoorbeeld een worm zit worden bepaalde afweercellen aangetrokken en geïnstrueerd om eiwitten en moleculen uit te scheiden, die wormen kunnen doden of helpen verwijderen. Omdat dit soort afweerreacties ontsteking bevorderen en ook schade aan omliggende weefsels kunnen aanrichten, is er een bepaalde mate van regulatie nodig. Dit is beter voor de gastheer, omdat schade wordt voorkomen, maar helaas ook beter voor de worm, omdat deze niet wordt aangevallen door een sterke afweerreactie. De regulatie van het afweersysteem – ook wel *immuunregulatie* genoemd – wordt aangestuurd door verschillende cellen en moleculen, waarvan wij één bepaald type nader hebben onderzocht in dit project: de regulatoire T cel. Deze regulatoire T cellen (of 'Treg' cellen) zijn rond het jaar 2000 voor het eerst duidelijk beschreven in onderzoek naar auto-immuunziekten, ofwel ziekten waarbij het afweersysteem onnodig reageert op lichaamseigen stoffen. De Treg cellen in muizen en mensen met deze auto-immuunziekten bleken niet goed te functioneren. Daaruit blijkt dat je Treg cellen nodig hebt voor een goede regulatie van afweerreacties. Immuunregulatie bleek verder belangrijk te zijn voor onnodige reacties tegen stoffen uit de omgeving, zoals bij allergie en astma.

Onderzoek van Treg cellen in patiënten met parasitaire infecties

In dit proefschrift is een aantal experimenten gedaan om Treg cellen aan te tonen bij mensen met diverse parasitaire infecties. In Gabon zijn jong-volwassenen met *Loa loa* infectie bekeken en in Indonesië zijn kinderen met een milde vorm van malaria onderzocht. Personen met *Loa loa* infectie bleken inderdaad meer Treg cellen te hebben in het bloed dan mensen zonder infectie, wat mogelijk leidt tot andere afweerreacties bij deze mensen (**hoofdstuk 2**). Ook kinderen met malaria infectie lieten meer Treg cellen zien en vooral van een bepaald type, met TNFRII (tumor necrosis factor receptor 2) op het celoppervlak (**hoofdstuk 3**). TNFRII kan ontstekingsbevorderende eiwitten zoals TNF verminderen en dit speelt een belangrijke rol bij malaria. Na het onderzoeken van het *aantal* Treg cellen, zijn wij verder gaan kijken naar de *functie* van Treg cellen. Bij volwassenen met filaria infectie in Indonesië werd gezien dat bepaalde afweercellen minder celdeling ondergingen en minder signaalstoffen maakten dan de afweercellen bij mensen zonder infectie (**hoofdstuk 4**). Wanneer wij in dit experiment in het lab de Treg cellen weghaalden, werden de afweerreacties weer vergelijkbaar met die van mensen zonder infectie. Dit betekent dat Treg cellen actief betrokken zijn bij de onderdrukking van afweer tegen filaria infectie.

Omdat bekend is dat worminfecties ook de afweerreactie kunnen onderdrukken tegen andere infecties of andere stoffen (zoals vaccinaties of stoffen die allergie veroorzaken), hebben wij in **hoofdstuk 5** de afweerreacties tegen malaria en tegen tuberculose-vaccinatie (BCG) onderzocht bij Indonesische schoolkinderen. Het bleek dat kinderen met darmwormen inderdaad een verminderde afweerreactie hadden tegen malaria en het BCG vaccin, maar dat deze reactie verbeterd was nadat Treg cellen in het lab weggehaald waren. Het lijkt er dus op dat Treg cellen een belangrijke rol spelen bij de verminderde afweerreacties tijdens worminfecties.

Het effect van ontwormen op afweerreacties, malaria en allergie

De beste manier om het pure effect van worminfecties wetenschappelijk te bekijken, is door de ene helft van een onderzoeksgroep te behandelen tegen worminfecties en de andere helft met een placebo. Dit hebben wij gedaan in het 'ImmunoSPIN' project op Flores, in Indonesië. De mensen die behandeld werden tegen wormen hadden sterk verbeterde afweerreacties tegen wormen zelf, maar ook tegen malaria (**hoofdstuk 6**). Dit wijst erop dat wormen de afweer inderdaad onderdrukken. Aan de ene kant zou dit negatief kunnen uitpakken voor patiënten met worminfecties; door verminderde afweer kunnen andere infecties makkelijker binnendringen. Echter, het heeft ook een positieve kant, want onnodige reacties tegen lichaamseigen of normale stoffen, zoals bij auto-immuunziekten en astma of allergie, kunnen ook geremd worden door de aanwezigheid van wormen. Inderdaad, in **hoofdstuk 7** hebben wij laten zien dat behandeling van wormen de kans vergrootte op het krijgen van allergische reacties bij schoolkinderen op Flores, maar dit was niet significant. Dit moet misschien nog langer onderzocht worden. Ook kwam er juist iets vaker malaria voor bij bewoners van Flores, hoewel dit maar een tijdelijk effect was. Dit was tegen onze verwachtingen in en moet nader onderzocht worden.

Behandelen *tegen* of *mèt* wormen?

Gezien het effect dat wormen hebben op het afweersysteem, zijn artsen gaan onderzoeken of deze effecten misschien als behandeling gebruikt kunnen worden bij ziekten waarbij het afweersysteem ontregeld is, zoals auto-immuunziekten, astma en allergieën (figuur 3). In **hoofdstuk 8** staat samengevat waar deze studies toe geleid hebben; bij patiënten met chronische darmontsteking lijkt een infectie met een *Trichuris* worm redelijk goed te werken tegen de symptomen. Hoewel sommige studies veelbelovend zijn, is het nog niet duidelijk of dit in de toekomst een behandeling kan zijn tegen dit soort ziekten. Bovendien brengt dit een dilemma naar voren: moeten we mensen in de tropen wel behandelen tegen worminfecties? Natuurlijk, want alle mensen hebben recht op medische behandeling. Raakt hun afweersysteem dan niet over-geactiveerd, wat weer kans geeft op andere ziekten? Onderzoeken in de komende jaren naar therapie mèt wormen in de Westerse wereld en therapie tégen wormen in lage inkomenslanden zullen daar meer duidelijkheid over brengen.

Figuur 3 © Marvin Double www.monkeezemarketing.blogspot.com

Wiria AE*, Hamid F*, **Wammes LJ***, Kaisar MM, May L, Prasetyani MA, Wahyuni, S, Djuardi Y, Ariawan I, Wibowo H, Lell B, Sauerwein R, Brice GT, Sutanto I, van Lieshout EA, de Craen AJ, van Ree R, Verweij JJ, Tsonaka R, Houwing-Duistermaat JJ, Luty AJ, Sartono E, Supali T, Yazdanbakhsh M. *The effect of three-monthly albendazole treatment on malarial parasitemia and allergy: a household-based cluster-randomized double blind, placebo-controlled trial.* PLoS One 2013;8:e57899.
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Wammes LJ, Wiria AE, Toenhake CG, Hamid F, Liu KY, Suryani H, Kaisar MMM, Verweij JJ, Sartono E, Supali T, Smits HH, Luty AJ, Yazdanbakhsh M. *Asymptomatic plasmodial infection is associated with increased TNFRII-expressing Tregs and suppressed type 2 immune responses.* J Inf Dis 2013;207:1590-9.

Wiria AE, **Wammes LJ**, Hamid F, Dekkers OM, Prasetyani M, May L, Kaisar MM, Verweij JJ, Tamsma JT, Partono F, Sartono E, Supali T, Yazdanbakhsh M, Smit JW. *Relationship between carotid intima media thickness and helminth infections on Flores island, Indonesia.* PLoS One 2013;8:e54855.

Mbow M, Larkin BM, Meurs L, **Wammes LJ**, de Jong SE, Labuda L, Camara M, Smits HH, Polman K, Dieye TN, Mboup S, Stadecker MJ, Yazdanbakhsh M. *Th17 cells are associated with pathology in human schistosomiasis.* J Inf Dis 2013;207:186-95.

Wammes LJ, Hamid F, Wiria AE, Wibowo H, Sartono E, Maizels RM, Smits HH, Supali T, Yazdanbakhsh M. *Regulatory T cells in human lymphatic filariasis: stronger functional activity in microfilaremics*. PLoS Negl Trop Dis 2012;6:e1655.

Pasha SM, Wiria AE, **Wammes LJ**, Smit JW, Partono F, Supali T, Yazdanbakhsh M, Tamsma JT. *Blood pressure class and carotid artery intima–media thickness in a population at the secondary epidemiological transition.* J Hypertens 2011;29:2194-200.

Djuardi Y, **Wammes LJ**, Supali T, Sartono E, Yazdanbakhsh M. *Immunological footprint: the development of a child's immune system in environments rich in microorganisms and parasites.* Parasitology 2011;138:1508-18.

Hamid F, Wiria AE, **Wammes LJ**, Kaisar MMM, Lell B, Ariawan I, Uh HW, Wibowo H, Djuardi Y, Wahyuni S, Schot R, Verweij JJ, van Ree R, May L, Sartono E, Yazdanbakhsh M, Supali T. *A longitudinal study of allergy and intestinal helminth infections in semi urban and rural areas of Flores, Indonesia (ImmunoSPIN Study).* BMC Infect Dis 2011;11:83.

Supali T, Verweij JJ, Wiria AE, Djuardi Y, Hamid F, Kaisar MM, **Wammes LJ**, van Lieshout L, Luty AJ, Sartono E, Yazdanbakhsh M. *Polyparasitism and its impact on the immune system.* Int J Parasitol 2010;40:1171-6.

Wiria AE, Prasetyani MA, Hamid F, **Wammes LJ**, Lell B, Ariawan I, Uh HW, Wibowo H, Djuardi Y, Wahyuni S, Sutanto I, May L, Luty AJ, Verweij JJ, Sartono E, Yazdanbakhsh M, Supali T. *Does treatment of helminth infection influence malaria? Background and methodology of a longitudinal study of clinical, parasitological and immunological parameters in Nangapanda, Flores, Indonesia (ImmunoSPIN Study).* BMC Infect Dis 2010;10:77.

Wammes LJ, Hamid F, Wiria AE, de Gier B, Sartono E, Maizels RM, Luty AJ, Fillie Y, Brice G, Supali T, Smits HH, Yazdanbakhsh M. *Regulatory T cells in human geohelminth infection suppress immune responses to BCG and Plasmodium falciparum.* Eur J Immunol 2010;40:437-42.

de Vries JF, **Wammes LJ**, Jedema I, van Dreunen L, Nijmeijer BA, Heemskerk MH, Willemze R, Falkenburg JH, Barge RM. *Involvement of caspase-8 in chemotherapy-induced apoptosis of patient derived leukemia cell lines independent of the death receptor pathway and downstream from mitochondria.* Apoptosis 2007;12:181-93.

Linda Judith Wammes was born on 28th August 1980 in Woerden. After completing grammar school in Utrecht (Utrechts Stedelijk Gymnasium) in 1998, she started to study Biomedical Sciences at the University of Leiden (later Leiden University Medical Center, LUMC). After an internship on determinants of perinatal mortality in Kigoma, west Tanzania and a research project on cellular apoptosis pathways at the LUMC department of Hematology, she obtained her Master's (MSc) in 2005. Meanwhile Linda had started her medical degree at LUMC, which she finished in 2007. Looking for a lab research project preferably in the tropics, Linda set foot into the Parasitology department of LUMC. Soon a match was found between the candidate and an already set-up PhD programme in the group of Professor Maria Yazdanbakhsh. For this project we set up a field laboratory and carried out a large immunoepidemiological research project on Flores island, East Indonesia. During this project several poster presentations and oral talks were given nationally and internationally. Linda Wammes was also involved in teaching students in Leiden and for projects at the Medical Research Unit of the Albert Schweitzer Hospital in Lambaréné, Gabon. Next to her work, Linda was engaged in activities such as the group Uniting Streams, a platform for young researchers in the tropics under the Netherlands Society of Tropical Medicine and International Health, the PhD research committee of the LUMC Center for Infectious Diseases and volunteer work at Unicef and Den Haag Cares. Recently, Linda has started her residency for Clinical Microbiology (arts-microbioloog in opleiding) at the Erasmus MC in Rotterdam under supervision of Professor Henri Verbrugh.

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