~~**Anthelmintic treatment increases the *Mycobacterium tuberculosis*-specific CD4+ T-cell response, associated with a decrease of FoxP3+ regulatory T cells in migrants with latent TB infection in London.**~~[LS1]

Mycobacterium tuberculosis-specific CD4+ T-cell reponse is reduced, and regulatory T cells are increased in TB patients after anthelminthic treatment

Mycobacterium tuberculosis-specific CD4+ T-cell response is reduced, and Treg cells increased, in anthelminthic-treated TB patients [LS2]

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~~**Running Title**: Helminth infection affects immune responses to TB infection~~

Abstract:

XXX. [LS4]~~To~~ We investigated the potential impact of helminth infection on immune responses to *Mycobacterium tuberculosis* (Mtb) ~~we recruited~~ in patients with latent Mtb infection (LTBI) with or without helminth infection (Strongyloides or *Schistosoma*), and tested ~~T~~ T-cell [LS5]responses before and after anthelminthic treatment. The study was performed in migrants resident in the UK, where re-exposure and re-infection following anthelminthic treatment would not occur. The frequency of $CD4^+$IFN-γ^+ T cells was measured following stimulation with Mtb PPD or ESAT-6/CFP-10 antigen, and concentrations of IFN-γ in culture supernatants measured by ELISA and multiplex bead array. Helminth infection was associated with a lower frequency of $CD4^+$~~IFNγ~~IFN-γ^+ T cells, which increased following treatment. Patients with helminth infection showed a significant increase in $CD4^+FoxP3^+$ T (Treg) cells [LS6]compared to those without helminth infection. ~~Following anthelminthic treatment th~~There was a decrease in the frequency of ~~$CD4^+$FoxP3~~Treg$^+$ cells, and an associated increase in $CD4^+$~~IFNγ~~IFN-γ^+ T cells after the anthelminthic treatment. ~~This study therefore~~Here, we show~~s~~ ~~the~~ a potential role of ~~regulatory $CD4^+FoxP3^+$~~ Treg cells in reducing the frequency and function of anti-mycobacterial $CD4^+$~~IFNγ~~IFN-γ^+ T cells, and that ~~–~~these effects ~~the reversal of~~are reversed after ~~these effects following~~ anthelminthic treatment~~, in a setting where re-infection would not occur~~.[LS7]

Introduction:

Mycobacterium tuberculosis (*Mtb*) infects a third of the world's population, causing 1.5 million deaths a year [1, 2]. In addition, helminth infections are estimated to affect 1.5 billion people worldwide, with the majority of these infections concentrated in developing countries where TB is endemic [3, 4].

Helminth infections are reported to induce Th2 type immune responses in the host [5], and evidence suggests that Th2 cytokines may play a critical role in reducing the Th1 immune response to other infections. Protection against tuberculosis is not well understood, but Th1 T cell responses involving interferon gamma (~~IFNγ~~IFN-γ), tumour necrosis factor alpha (TNFα), and interleukin 2 (IL-2) are induced in the immune response to *Mtb* [6]. ~~IFNγ~~IFN-γ is thought to play an important role in the protective immune response against tuberculosis (TB) as indicated by the susceptibility of humans with ~~IFNγ~~IFN-γ signalling pathway deficiencies to TB disease [7, 8]. A number of studies have previously investigated the immunomodulatory effect of helminth infection on anti-mycobacterial immunity (reviewed in [9]). For example, in mouse models it has been shown that helminth infection can inhibit the immune response to *Mtb* [10]. A study performed in Ethiopia has shown an impact of deworming on the immune response to *Mtb* Purified Protein Derivative (PPD) [11]. As helminths have been shown to modulate immunity towards Th2 type responses, this may make individuals infected with helminths more susceptible to progression of tuberculosis infection [11-16].

Regulatory T cells (Treg), are another way to control and regulate immune responses. These cells are able to control both Th2 and Th1 type immune responses [17]. Helminth infection can increase the frequency of Treg cells in mice [18, 19], and in man an increase of Treg cells has been observed in Strongyloides infection in patients co-infected with HTLV-1 [20] as well as in

hookworm infected patients [21]. Furthermore it has been shown that Treg cells can modulate the immune response in BCG vaccinated individuals [22, 23] and in patients with active tuberculosis [24]. A recent study showed that in children in Gabon, the proportion of Tregs decreased with anthelminthic treatment [25].

There have been only a few studies that investigated the impact of helminth infections on T cell immunity in patients with latent TB infection. Filaria infections were shown to reduce Th1 responses in Indian patients with LTBI, that were increased with blockage of CTLA-4 or PD-1 [26], while anthelminth treatment increased TLR2 and TLR9 expression with an associated increase in production of pro-inflammatory cytokines. In Indian patients with LTBI, coincident hookworm infection reduced Mtb-specific Th1 and Th17 CD4 T cells [14]. Indian pulmonary tuberculosis patients with Wucheria or Stronglyoides also showed reduced CD4 and CD8 T cells responses to mycobacterial antigens, that could be partly blocked with anti-IL-10 neutralising antibodies [27].

In this study we measured the impact of anthelmintic treatment on the immune response to *Mtb* in LTBI in a migrant cohort in London. As the UK is not ~~an~~a helminth endemic area, reinfection is not likely in this setting. This provides a unique opportunity to study the effect of anthelminth treatment on the immune response in helminth infected patients. We also measured the frequency of ~~$CD4^{+}FoxP3^{+}$~~ Treg cells in order to assess the potential impact ~~of Treg cells~~ in helminth infected patients. [LS8]FoxP3 is currently the best known single marker of the major population of Treg cells [17, 28, 29] and previous studies have shown that assessing $CD4^{+}FoxP3^{+}$ T cells is a convenient way to measure the Treg-cell population in human PBMCs [30, 31]. The results obtained in this study demonstrate the influence of helminths in reducing the immune responses to

Mtb, and the importance that $CD4^{+}FoxP3^{+}$ T cells play in this modulation.

Results

Impact of helminth infection on T cell responses to *Mtb*.

We measured the frequency of $CD4^+$ ~~IFNγ~~IFN-γ$^+$ T cells in PBMCs from uninfected individuals, helminth infected patients, LTBI individuals and patients co-infected with helminths and LTBI (Figure 1). The results show a significant reduction of the frequency of $CD4^+$ ~~IFNγ~~IFN-γ$^+$ T cells in helminth infected versus non helminth infected individuals after stimulation of cells with PPD (Figure 1A). The reduction is even more significant (p=0.001) in the group with LTBI. There was no difference in frequency of $CD4^+$ ~~IFNγ~~IFN-γ$^+$ T cells between the helminth only vs. the helminth+LTBI group. We did not observe any significant difference in responses between Strongyloides and Shistosomiasis infected patients (data not shown). PBMC were also stimulated with the *M.tuberculosis* fusion protein ESAT-6/CFP10, peptides from which form the basis of the QuantiFeron Gold InTube (QFT-GIT) test. The results show that, as observed with PPD stimulation, helminth infected patients have a significant reduction in the frequency of $CD4^+$ ~~IFNγ~~IFN-γ$^+$ T cells (Figure 1B). As controls, patients with negative QFT-GIT were also tested but did not respond to ESAT-6/CFP10 (data not shown). Furthermore, we did not observe any significant expression of ~~IFNγ~~IFN-γ in the $CD8^+$ population after stimulation with PPD or ESAT-6/CFP10.

Anthelmintic treatment increases the frequency of $CD4^+$ ~~IFNγ~~IFN-γ$^+$ T cells after stimulation.

Patients were requested to come back at least 4 months after receiving anthelmintic treatment (V2). We stimulated PBMC from these treated patients using the same protocol as used for cells from the first clinic visit prior to treatment (V1). The results show that anthelmintic treatment

induces a significant increase in the frequency of $CD4^+$ ~~IFNγ~~IFN-γ^+ T cells after stimulation with PPD (Figure 2A) or ESAT-6/CFP10 (Figure 2B), in both the helminth (p=0.001) and the helminth+LTBI (p=0.001 for PPD and p=0.002 for ESAT-6/CFP10) groups, compared to the same groups before treatment. The control group and the LTBI group were also analysed at least 4 months after the initial visit, but without receiving any anthelmintic treatment. In these groups we did not observe any significant modifications of the frequency of $CD4^+$ ~~IFNγ~~IFN-γ^+ T cells after stimulation by PPD or ESAT-6/CFP10 compared to the initial test results.

Increased frequency of $CD4^+FoxP3^+$ T cells in helminth infected patients.

We next analysed the frequency of $CD4^+FoxP3^+$ T cells in cells after overnight incubation, without any stimulation as described in earlier studies [30, 31]. The results show that helminth infected patients have a significantly higher proportions of $CD4^+FoxP3^+$ T cells compared to the non-helminth-infected control group (p=0.0001) (Figure 3). Furthermore, helminth-LTBI co-infected patients had significantly higher proportions of $CD4^+FoxP3^+$ T cells compared to the LTBI group (p=0.0001). However, there were no significant differences in the frequency of $CD4^+FoxP3^+$ T cells between the control and LTBI groups (p=0.124), or between the helminth infected and helminth-LTBI groups (p=0.87).

The frequency of $CD4^+FoxP3^+$ T cells is decreased after anthelmintic treatment in helminth infected patients.[LS9]

The patients were requested to return for a follow up visit more than 4 months after taking anthelmintic treatment. The frequencies of $CD4^+FoxP3^+$ T cells were analysed after overnight incubation, without any stimulation as previously described [30]. The frequencies of $CD4^+FoxP3^+$

T cells after treatment were compared to the frequencies measured in the same patients before treatment (V1). The results in Figure 4 show the frequency of $CD4^+FoxP3^+$ cells, measured in patients before treatment (V1) compared with the frequency in the same patients at least 4 months after treatment (V2). Helminth infected patients showed a significant decrease in the frequency of $CD4^+FoxP3^+$ T cells after anthelmintic treatment ($p=0.0001$ for the helminth group and $p=0.002$ for the LTBI-helminth group, compared respectively to the control group and to the LTBI group). However, there were no such differences in the frequencies of $CD4^+FoxP3^+$ T cells between the control and LTBI groups. There were no significant differences in the frequency of $CD4^+FoxP3^+$ T cells between the helminth and the LTBI+helminth groups after treatment.

Depletion of $CD4^+CD25^+$ T cells reduces $CD4^+FoxP3^+$ T cell frequency and increases the frequency of $CD4^+INF\gamma^+$ T cells induced by PPD stimulation, in helminth infected patients.[LS10]

To test the importance of $CD4^+FoxP3^+$ T cells in regulation of mycobacterial antigen-specific T cell responses, we depleted the $CD25^+$ cells from six untreated helminth infected patients. The representation of 2 typical experiments are illustrated in Figure 5A. The pooled result (Figure 5B) shows that in PBMC from the 6 patients, after depletion of the $CD25^+$ T cells, there was a significant decrease in the frequency of $CD4^+FoxP3^+$ T cells. We next measured, as described above, the response of the PBMC after stimulation with PPD. In all six patients there was an increase in the frequency of $CD4^+$~~IFNγ~~IFN-γ^+ T cells after stimulation with PPD in the $CD25^+$ depleted PBMC compared to the control PBMC. After depletion of $CD25^+$ cells the frequency of $CD4^+$~~IFNγ~~IFN-γ^+ T cells increased from 0.179% to 0.391% for patient#1, from 0.152% to 0.324% for patient#2, from 0.335% to 0.793% for patient#3, from 0.469% to 0.865% for pa-

tient#4, from 0.147% to 0.512% for patient#5 and from 1.43% to 2.37% for patient#6. The results in Figure 5B, show for each patient's PBMC stimulated with PPD, there was an increase in the frequency of $CD4^+$ ~~IFNγ~~IFN-γ$^+$ T cells associated with a decrease of $CD4^+FoxP3^+$ T cells. It is important to note that we also observed a small increase of $CD4^+$ ~~IFNγ~~IFN-γ$^+$ T cells in the CD25 depleted fraction in non-stimulated control PBMC. Patient#6 showed a high frequency of $CD4^+$ ~~IFNγ~~IFN-γ$^+$ T cells, but this patient was also latently infected with *Mtb* (LTBI).

Anthelmintic treatment increases the secretion of ~~IFNγ~~IFN-γ in blood samples from helminth infect patients after stimulation with PPD.[LS11]

We stimulated fresh diluted whole blood from patients with control medium, PPD (10µg/ml) or ESAT-6/CFP10 (10µg/ml) for 1 day and 5 days and then collected supernatants for analysis using Luminex bead array technology. We compared the difference in responses between the first visit (V1) and the post treatment visit (V2). From the supernatant 12 cytokines were measured (IL-2, IL-4, IL-5, IL-10, IL-13, IL-17A, IL-17F, IL-21, IL-22, ~~IFNγ~~IFN-γ, IP10, TNFα). From day 1 supernatant analysis, we did not observe any significant secretion, or difference of expression between groups for all the cytokines measured (data not shown). At Day 5, only ~~IFNγ~~IFN-γ secretion showed significantly higher concentrations in the different groups after both PPD and ESAT6/CFP10 stimulation (Figure 6). The same patients were tested before anthelmintic treatment and 4 months after this first visit. Stimulation with medium only gave <400pg/ml ~~IFNγ~~IFN-γ for each group (data not shown). Stimulation of cells from uninfected control and helminth patient groups with ESAT-6/CFP10 also gave negative responses of <400 pg/ml ~~IFNγ~~IFN-γ.

The results shown in Figure 6 show that the secretion of ~~IFNγ~~IFN-γ after PPD stimulation is sig-

nificantly lower (p=0.006) in the helminth infected patients compared to the control group. Furthermore the secretion of ~~IFNγ~~IFN-γ, after stimulation with PPD, is significantly lower (p=0.017) in the co-infected Helminth+LTBI group of patients compared to the LTBI group. After anthelmintic treatment, retesting the same patients showed that there was a significant increase in the production of ~~IFNγ~~IFN-γ in the helminth infected group; those infected with both helminths and LTBI showed an increase that was statistically significant (p=0.002). We also measured the secretion of ~~IFNγ~~IFN-γ after stimulation of the diluted blood with the *Mtb* fusion protein ESAT-6/CFP10. The results in Figure 6B show that before anthelmintic treatment, the ~~IFNγ~~IFN-γ concentrations measured were significantly lower in the co-infected helminth-LTBI group than in the LTBI group. After anthelmintic treatment, the same patients were re-tested in the same way, revealing a significant increase in ~~IFNγ~~IFN-γ concentrations (p=0.038).

For the other cytokines measured, IL-4 and IL-5 were not detected in the supernatants collected at either day 1 or day 5. Other cytokines showed a very low level of secretion at day 1, with less than 10pg/ml for IL-13, less than 20pg/ml for IL-17A and IL-21 and less than 40pg/ml for IL-10 and IL-13. No significant variations were observed between the groups with different conditions of stimulation (data not shown). On day 5 there was no significant variation in concentrations of IL-2 TNFα, IL-22, IL17F and IP10 between the groups and between samples tested post and pre-treatment. The results for these cytokines are presented on supplemental Figure 2 and Figure 3.

Discussion

Helminth infections are highly prevalent in populations where *Mtb* infection is endemic, and it has been widely conjectured that the strong mucosal Th2 and T regulatory cell responses elicited by these parasites could down-modulate protective immune responses against *Mtb*. Some studies have specifically demonstrated the importance of regulatory T cells in suppression of the immune response to BCG during helminth infection [32, 33] and the modulation of the TB immune response during helminth infection [10, 14, 26]. A number of previous studies have investigated the immunomodulatory effect of helminth infections on anti-mycobacterial immunity [11, 34]. In this study we took advantage of an opportunity provided by a London cohort of immigrants who had presented with helminth infection with/without LTBI to evaluate the impact of deworming on the *in vitro* T cell memory response to *Mtb* in these patients. Our cohort gave us the ability to study these responses in a setting where the risk of re-exposure and re-infection was negligible, and so provided an ideal environment in which to study the impact of anthelmintic treatment on the anti-mycobacterial immune response.

We compared groups of immigrants who presented with different diagnoses: infected by helminths (mainly Strongyloides with a few Schistosome infections), infected by *Mtb* and diagnosed as LTBI (defined as Quantiferon IGRA test positive), co-infected with *Mtb* and helminths, or those uninfected with either helminths or *Mtb*. We first measured the immune response to *Mtb*, by measuring the frequency of $CD4^{+}IFN\gamma^{+}$ producing T cells in these different groups of patients after stimulation with PPD or ESAT-6/CF10. We observed that the helminth infected groups had lower responses to PPD and ESAT-6/CFP10 than those without diagnosed helminth infection. This suggests that the presence of helminths reduced the capacity of the PBMC to respond to *Mtb* stimulation. This impact of worms on the TB immune response was recently shown

with hookworm infection, where the worm infection modulates the Th1 and Th17 response in patients with latent TB [14].

Following this first observation, we next compared the memory immune response to TB before anthelmintic treatment and after receiving treatment. Because these patients are resident in the UK, we do not expect reinfection with helminths during this period. Further clinical samples were tested after treatment, to confirm clearance of the helminth infections. We showed that anthelmintic treatment increased the memory immune response to *Mtb* (the helminth-LTBI group showed a significant increase in the frequency of $CD4^+$ ~~IFNγ~~IFN-γ$^+$ T cells after treatment). We also stimulated PBMC from *Mtb* infected patients with the *Mtb* specific fusion protein ESAT-6/CFP10, and observed an increase in the frequency of $CD4^+$ ~~IFNγ~~IFN-γ$^+$ T cells after anthelmintic treatment in the helminth-LTBI group only, but not in the non LTBI group, confirming the specificity of the ESA-T6/CFP10 antigen.

We also carried out similar experiments by stimulating directly fresh diluted blood with either PPD or the *Mtb* fusion protein ESAT-6/CFP10, and by measuring the ~~IFNγ~~IFN-γ secretion after 5 days of stimulation (Figure 6). We observed a similar result as had been observed with PBMC for helminth infected patients. The helminth infected group, or co-infected helminth-LTBI patients, secreted lower levels of ~~IFNγ~~IFN-γ after PPD stimulation than the non-helminth infected group or the LTBI group. Furthermore after ESAT-6/CFP10 stimulation we also observed lower ~~IFNγ~~IFN-γ secretion in the co-infected group with LTBI compared to the LTBI alone group. These results confirmed the observations made by flow cytometry. In the visit 2 group (V2), we only observed significant differences compared to pre-treatment in the helminth infected group when PBMC were stimulated with PPD. The helminth-LTBI group did not show a statistically significant difference in response on the first and second visits for either PPD or ESAT-6/CFP10

simulation. This result differs from the observation made by flow cytometry. Two hypotheses can explain why these results differ. First, in the flow cytometry experiments the stimulation with antigen is only overnight compared to the 5 day stimulation for the diluted whole blood assay. Secondly, we compared the PBMC response by flow cytometry rather than by diluted whole blood for the bead array assay. These two differences may explain the difference in response to ESAT6/CFP10 stimulation.

Helminth infections are largely associated with Th2 immune responses [5], and few studies have shown that helminth infection can be associated with an increase of regulatory T cells (Tregs)[18, 23, 25, 35, 36]. In this study we analysed the frequency of $CD4^+FoxP3^+$ T cells. As shown in a previous study of HTLV-1 infection, $CD4^+FoxP3^+$ T cells can be an excellent marker of regulatory T cell function [30, 37], as the CD25 activation marker may be modified by the infection [30, 38]. In our cohort we observed an increase of $CD4^+FoxP3^+$ T cells in the helminth infected groups, with both the helminth and helminth-LTBI groups showing an increase in the frequency of $CD4^+FoxP3^+$ T cells compared to the control group and the LTBI only groups. It is important to note that in our cohort we did not observe any difference in the frequency of $CD4^+FoxP3^+$ T cells between the control group and the LTBI group as has been shown in other studies [39, 40], although our control group had a frequency of $CD4^+FoxP3^+$ T cells close to the frequency measured in another study using PBMC cells [30].

We next compared the variation of the frequency of $CD4^+FoxP3^+$ T cells before and after anthelmintic treatment as we had for the *Mtb* specific T cell immune response. The results showed that anthelmintic treatment reduced the frequency of $CD4^+FoxP3^+$ T cells in the helminth infected and helminth-LTBI groups. There are very few reports showing an impact of anthelmintic treatment on the modulation of the frequency of $CD4^+FoxP3^+$ T cells, apart from a recent study

in which patients with Schistosomiasis in Gabon showed a marked reduction in the frequency of FOXP3+ T regs post treatment[25]. Interestingly, in our study after treatment the frequencies of $CD4^+FoxP3^+$ T cells measured in the helminth and helminth-LTBI groups were similar to the frequencies measured in the control group and the LTBI group. There was no significant variation in the frequency of $CD4^+FoxP3^+$ T cells in the control group or the LTBI group between the first visit (V1) and the follow-up visit (V2). Because we asked patients to return more than 4 months after the anthelmintic treatment, we do not think the treatment itself has a direct impact on the frequency of $CD4^+FoxP3^+$ T cells measured, or on the other immune responses measured at V2.

The question arises: what is the role of $CD4^+FoxP3^+$ T cells in helminth infection and what role does this population play in the inhibition of the memory immune response to *Mtb*? To answer this question we selected 6 helminth infected patients, to test if the $CD4^+FoxP3^+$ T cells were directly involved in the reduction in the frequency of $CD4^+$~~IFNγ~~IFN-γ^+ T cells after stimulation with PPD. As FoxP3 is a nuclear marker, it cannot be used to purify or deplete $FoxP3^+$ T cells, however CD25 is a surface marker, that can be used to purify or deplete $CD4^+FoxP3^+$ T cells [37]. We observed that when CD25+ cells were depleted, resulting in a reduction of $CD4^+FoxP3^+$ T cells, this was associated with an increase in the frequency of $CD4^+INF\gamma^+$ producing T cells after stimulation with PPD. This result suggests that $CD4^+FoxP3^+$ T cells could be directly involved in reducing the memory immune response to *Mtb*, as described in this study, and confirms the earlier findings in a group of Chinese TB patients where depletion of $CD4^+CD25^+$ $FoxP3^+$ regulatory T cells increased secretion of ~~IFNγ~~IFN-γ and IL-10 [24].

It is difficult to evaluate if the $CD4^+FoxP3^+$ cells are specific for an antigen. *In vitro* observations suggest a nonspecific impact of $CD4^+FoxP3^+$ T cells on antigen-specific responses. For example

in Figure 6, the control unstimulated PBMC cultures also showed higher $CD4^+$ ~~IFNγ~~IFN-γ^+ T cell frequencies in the FoxP3 depleted fractions than in the non-depleted cultures. Furthermore, some studies suggest that intracellular ~~IFNγ~~IFN-γ does not correlate with protection against Mtb infection [41, 42], although the measurement of ~~IFNγ~~IFN-γ still a relevant marker of the immune response [7, 43, 44]. Tregs are thought to exert their function via a number of different mechanisms including IL-10 and/or TGF-β production, IL-2 consumption, or cell-cell contact where inhibitory molecules such as CTLA-4 and PD-1 are key [45, 46]. In a study on the impact of FoxP3 T cells in the modulation of immune responses in LTBI patients [47], the authors observed a specific restoration of immune response after depletion of FoxP3+ cells, but not after blockage of specific immune suppressor molecules such as CTLA-4.

Finally, these results suggest an important role for $CD4^+FoxP3^+$ T cells in the control of the *Mtb* immune response induced by helminths. Although the majority of our patients were infected with Strongyloides, a subset was infected with Schistosomes. A recent study also showed an impact of *Schistosoma mansoni* infection in a mouse model, where Treg cells were shown to induce suppression of allergic airway immune response [35]. More interestingly another recent study on human Schistosomiasis patients showed an increase of $CD4^+CD25^+FoxP3^+$ T cells in these patients, and this high frequency of Treg cells was reduced after anthelmintic treatment [25]. In this study we only focused on the ~~IFNγ~~IFN-γ response in LTBI and did not have an opportunity to measure the impact of helminth infection and anthelmintic treatment on TB patients with active clinical disease. Furthermore, due to a lack of Strongyloides antigen, it was only possible to measure the *Mtb* specific immune response; further studies could assess the helminth-specific immune response, and the level of Th2 cytokines that could be induced by these infections. Further studies are now needed to investigate the impact on these Treg responses on progression to

TB. Although a recent study in India failed to show any effect on the rate of progression to active TB associated with filaria or hookworm infections [48] reinfection with both helminths and *M.tuberculosis* would be common in this setting[49].

The next question that now needs to be answered is to understand how helminths induce this population of $CD4^{+}FoxP3^{+}$ T cells. Some studies have already investigated the potential function of CTLA-4 and PD-1 [26, 47] in the immune response against *Mtb*, and some recent studies in the mouse show that helminths can control the $FoxP3^{+}$ T cell population via ICOS stimulation [50]. It also remains to be investigated how $CD4^{+}FoxP3^{+}$ T cells inhibit the T cell mediated immune response, and whether in LTBI patients co-infected with helminths this inhibition is *Mtb* specific or reflects a more general immunosuppression in the co-infected human host.

Materials and Methods

Subjects.

Peripheral venous blood samples, anti-coagulated with sodium heparin, were donated by subjects at the Hospital for Tropical Diseases in London, and at Spitalfields Primary Care Trust clinic in East London. The patients were included in the study after being diagnosed with a high eosinophil count, with a history of living in the tropics, older than 18 years and with or without untreated latent TB infection (LTBI) (see Table I). We excluded HIV-positive, immunocompromised patients, and patients with previous treatment for helminths. All our patient were BCG vaccinated.

Serum samples were analysed by a commercial ELISA for anti-Strongyloides antibodies (Bordier, France) according to the manufacturer's protocol which uses a variable cut-off based on a low positive serum. For anti-schistosome antibodies, we used an in-house assay (IgG antibodies to Schistosoma egg antigens with a standard cut-off of 0.26 OD at 490nm), using the standard clinical diagnostic tests currently in use in the National Parasite Reference Laboratory of the UK, based at the Hospital for Tropical Diseases (HTD). Stool analysis was performed following formol ether/ethyl acetate faecal concentration using a Midi Parasep faecal concentrator. Faeces samples were also cultured to detect Stronglyoides or hookworm using the charcoal culture method. Urine analysis was performed by observation of Schistosoma eggs in the deposit of a filtered terminal urine sample. The presence of ova and cysts in stool samples was used to exclude other helminth infections and these patients were excluded from further study. Filaria serology was performed if the patient was from West Africa or had clinical evidence of filarial infection, with further tests if filarial serology was positive; no filarial infections were diagnosed in the patients studied here. In our cohort 84% of patients presented with a Strongyloides infec-

tion, 10% with Schistosomiasis and 4% with both helminth infections. All the patients were tested for *M. tuberculosis* infection, using the QuantiFeron-Gold In-Tube assay (QFT-GIT). Patients with clinical symptoms of tuberculosis were screened for TB disease and excluded from this study if active TB disease was diagnosed. None of the LTBI patients included in this study were on treatment for their LTBI. Ethical permission for the study was obtained from the Ethics Committee at the London School of Hygiene and Tropical Medicine (ref: 5870) and the NHS (ref: 11/H0713/12). All the patients gave informed consent. Patient were recruited on their first visit to the clinic (V1) when they received anthelmintic treatment if required, and were requested to come back at least 4 months later for a follow-up visit (V2). Those not treated for helminths were also asked to come back 4 months later. The time interval between V1 and V2 is included as Δ V1-V2 in Table 1. The rate of follow up at visit2 was 15% for control, 26% for Helminth, 92% for LTBI, and 41% for Helminth+LTBI.

Flow cytometry. PBMCs were isolated by density centrifugation on Histopaque (Sigma, UK) and cryopreserved until used. Cells were cultured in complete medium (RPMI-1640, 10% FCS, penicillin/streptomycin, L-glutamine) at 37ºC in 5% CO_2. One millions PBMC were stimulated for 4h with medium alone, PPD batch RT 50 (Statens Serum Institute, Copenhagen, at 10µg/ml) or with the fusion protein ESAT-6/CFP10 (kindly provided by Kees Franken, Dept. of Infectious Diseases, Leiden University Medical Centre at 10µg/ml) in a volume of 300µl. After 4h, 600µl of complete medium containing Golgi plug (1µl/ml) and anti-CD28 (1µg/ml) (BD Bioscience) were added and PBMCs incubated for 18h. The cells were then stained with live dead stain (Invitrogen) to measure the viability. The viability measured was more than 95% in the experi-

mentsincluded here. We then surface-stained with monoclonal antibodies to CD3-APC-cy7, CD4-PerCP-Cy5.5 and CD8-Pacific Blue (BD Bioscience, UK). Cells were then fixed and permeabilized with a commercial kit (Insight Biotechnology, Wembley, UK), following the manufacturer's protocol. Finally, cells were stained intracellularly with an anti-human FoxP3-PE antibody (clone 236A/E7; Insight Biotechnology) in permeabilization buffer (Insight Biotechnology) following the manufacturer's protocol, or with anti-human ~~IFNγ~~IFN-γ-APC (BD Bioscience, UK).

After staining, cells were analysed on a LSRII flow cytometer. One million events were routinely collected. Doublets were excluded and $CD3^+$ viable lymphocytes were gated and from this gate CD4 and CD8 population were discriminated for further analysis using Flow Jo. The complete gating strategy is presented in Supplemental figure 1.

Cytokine and chemokine quantification by Luminex. Fresh heparinised blood samples were diluted 1:4 in RPMI medium. The diluted blood was stimulated with medium only, PPD (at 10µg/ml) or the fusion protein ESAT-6/CFP10 (at 10µg/ml). Supernatants were collected after 5 days of stimulation. Using a Luminex multibead antibody kit (Biorad, UK), we quantified the concentration of 12 cytokines using two kits: a Th1/Th2 8 plex kit detecting IL-2, IL-4, IL-5, IL-10, IL-13, ~~IFNγ~~IFN-γ, IP10, TNFα, and a Th17 4 plex kit detecting IL-17A, IL-17F, IL-21 and IL-22.

Purification of $CD25^+$ cells. $CD4^+CD25^+$ cells were purified with antibody-coupled magnetic microbeads following the manufacturer's instructions (Miltenyi Biotec).

Statistical analysis. Non-parametric statistical tests were used, using SPSS software (v20).

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Conflict of Interest: All authors: No reported conflicts.

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Figure ~~Titles and~~ Legends:[LS12]

Figure 1: Frequencies of $CD4^+$ ~~IFNγ~~IFN-γ^+ T cells induced by PPD (A) or ESAT-6/CFP10 (B), are reduced in helminth infected patients.

(A) PBMCs from Control (n=19), Helminth (n=48), LTBI (n=34) and co-infected Helminth-LTBI (n=17) patients were stimulated with PPD. ~~These boxplot graph represent the frequencies of $CD4^+$ $IFN\gamma^+$ cells in the $CD4^+$ T cell population measured in PBMC after stimulation with PPD, from Control (n=19), Helminth (n=48), LTBI (n=34) and co-infected Helminth-LTBI (n=17) patients.~~ (B) PBMCs from Control (n=6), Helminth (n=10), LTBI (n=31) and co-infected Helminth-LTBI (n=15) patients were stimulated with ESAT-6/CFP10. (A and B) The frequencies of $CD4^+$IFN-γ^+ cells in the $CD4^+$ T cell population in PBMCs was measured by XXX. [LS13]Boxplots show the median, 25th and the 75th percentile (boxes) and minimum and maximum values (whiskers). ~~These~~ [LS14]~~boxplot graph represent the frequency of $CD4^+$ $IFN\gamma^+$ cells in the $CD4^+$ T cell populations measured in PBMC after stimulation with ESAT-6/CFP10, from Control (n=6), Helminth (n=10), LTBI (n=31) and co-infected Helminth-LTBI (n=15)~~ patients. Data shown are representative of XXXX independent experiments. [LS15]P values were determined by a non-parametric Mann-Whitney test.

Figure 2: Anthelmintic treatment increases the frequency of $CD4^+$ ~~IFNγ~~IFN-γ^+ T cells in helminth infected patients

~~These line graph represent the frequency of $CD4^+$ $IFN\gamma^+$ cells in~~ (A nad B) $CD4^+$ cells ~~after stimulation with PPD (A) or ESAT-6/CFP10 (B)~~ from Control (n=5), Helminth (n=13), LTBI (n=30) and co-infected Helminth-LTBI (n=10) patients were stimulated with (A) PPD or (B)

ESAT-6/CFP10. The frequency of CD4$^+$IFN-γ$^+$ cells was measured by xxxx. [LS16]The value plot on the left was obtained from patients on the first visit (V1), the value plot on the right was obtained on the follow up visit (V2), either more than 4 months after visit one, or more than 4 months after anthelmintic treatment. Each line ~~represent~~represents an individual. P values were determined by a non-parametric paired Wilcoxon test. Data shown are representative of XXXX independent experiments. [LS17]

Figure 3: The frequency of CD4$^+$FoxP3$^+$ T cells is increased in helminth infected patients

~~The box plots represent the frequency of CD4$^+$FoxP3$^+$ cells in the CD4$^+$ T cell population, measured in PBMC incubated for 18h without stimulation~~ PBMCs from Control (n=20), Helminth (n=51), LTBI (n=34) and co-infected Helminth-LTBI (n=17) patients were incubated for 18h without stimulation. The frequency of CD4$^+$FoxP3$^+$ cells in the CD4$^+$ T cell population in PBMCswas measured by XXXX. [LS18]Boxplots show the median, 25th and the 75th percentile (boxes) and minimum and maximum values (whiskers).[LS19] Data shown are representative of XXXX independent experiments. [LS20] P values were determined by a non-parametric Mann-Whitney test.

Figure 4 Anthelmintic treatment reduces the frequency of CD4$^+$FoxP3$^+$ T cells in helminth infected patients.

~~The line graph represent the frequency of CD4$^+$FoxP3$^+$ cells in the CD4$^+$ T cell population, measured in~~ PBMCs ~~incubated for 18h without stimulation~~ from Control (n=7), Helminth (n=14), LTBI (n=35) and co-infected Helminth-LTBI (n=14) patients incubated for 18h without

stimulation. The frequency of $CD4^+FoxP3^+$ cells in the $CD4^+$ T cell population in PBMCs was measured by XXXX[LS21]. The value plot on the left was obtained from patients on the first visit (V1), the value plot on the right was obtained on the follow up visit (V2), either more than 4 months after visit one, or more than 4 months after anthelmintic treatment. Each line represent the variation of expression per individual. Data shown are representative of XXXX independent experiments. [LS22]The P values were determined by a non-parametric paired Wilcoxon test.

Figure 5 [LS23]**Depletion of $CD4^+CD25^+$ T cells reduces $CD4^+FoxP3^+$ frequency and increases the frequency of $CD4^+$~~IFNγ~~IFN-γ$^+$ T cells induced by PPD in helminth infected patients.**

(A) The density plot shows the frequency of $FoxP3^+$ cells in the $CD4^+$ population. The values in each density plot represent the frequency of $CD4^+FoxP3^+$ within the $CD4^+$ population for each condition (no depletion, $CD25^+$ bead depleted, $CD25^+$ fraction). The graph represents the frequency of $CD4^+$~~IFNγ~~IFN-γ$^+$ cells in PBMCs with no stimulation (light grey bar) or after PPD stimulation (black bar) for each cell population. (B) The line graphs represents the pooled variation without (control) or with CD25 depletion (depleted) for 6 helminth infected patients. Are plotted the frequency of $CD4^+FoxP3^+$ T cells, the frequency of expression of $CD4^+$~~IFNγ~~IFN-γ$^+$ cells in PBMC unstimulated, and the frequency of $CD4^+$~~IFNγ~~IFN-γ$^+$ cells in PBMC after PPD stimulation. P values were determined by a non-parametric Mann-Whitney test. Data shown are representative of XXXX independent experiments. [LS24]

Figure 6 [LS25]**Measurement of ~~IFNγ~~IFN-γ by Luminex bead array in supernatants from fresh blood after 5 days stimulation with PPD or ESAT-6/CFP10.**

The box plots represent the concentration of ~~IFNγ~~IFN-γ in pg per ml, measured in supernatants

from blood diluted, after 5 days of stimulation with either PPD (A), or ESAT6/CFP10 (B). Are represented the concentration measured from Control (n=21), Helminth (n=53), LTBI (n=38) and co-infected Helminth-LTBI (n=34) patients. The white bars represent the concentrations of ~~IFNγ~~IFN-γ measured after blood stimulation before treatment (V1), the grey bars represent concentrations measured after blood stimulation of the patient's blood after anthelmintic treatment or at follow up (V2). The P values were determined by a non-parametric paired Wilcoxon test. Data shown are representative of XXXX independent experiments. [LS26]