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.

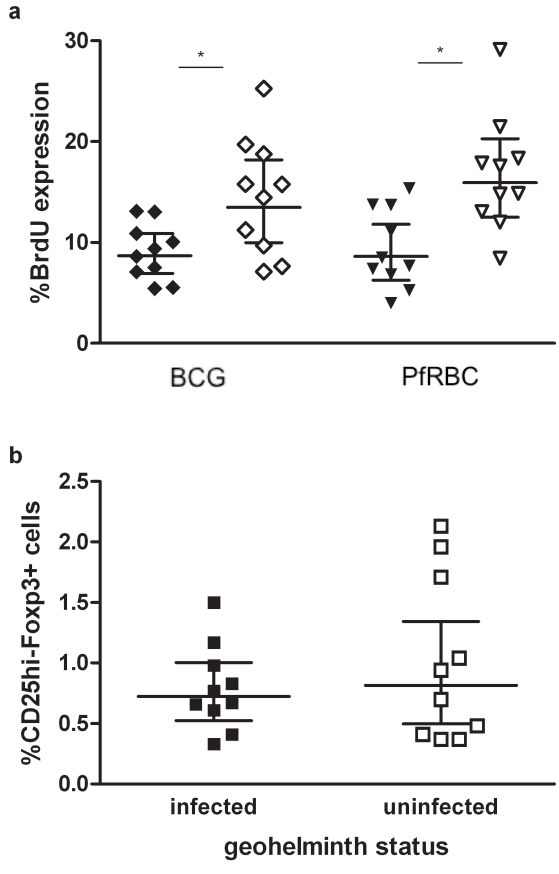

Figure 1. Helminth-infected children show lower T cell proliferation responses but similar Treg frequency. Donors were grouped by infection status; geohelminth-infected and -uninfected (both n=10) groups are shown by filled and open symbols respectively. (a) Proliferation was analyzed by flow cytometric 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).

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).

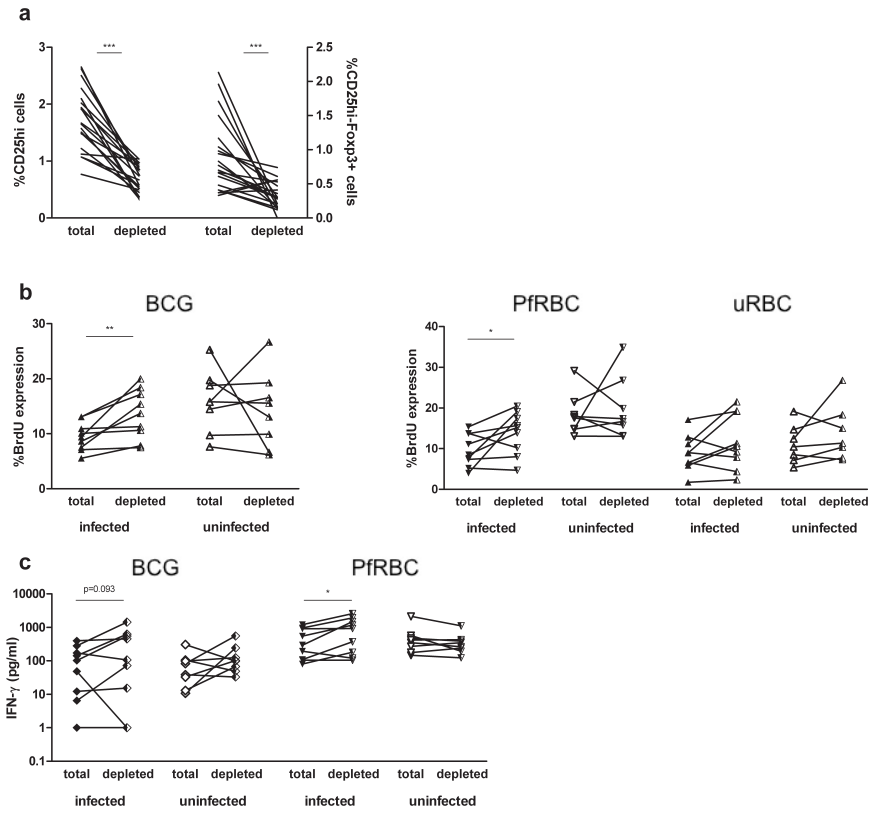

Figure 2. Treg depletion restores BCG and PfRBC-induced proliferation and enhances antigen-specific IFN-γ production in geohelminth infected children. (a) $CD4^+CD25^{hi}$ T cells were isolated by magnetic bead separation. The 'total' and 'depleted' data points and the connecting line represent paired 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). *$p\leq0.05$ **$p\leq0.01$ ***$p\leq0.001$; 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.

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