

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

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Chronic helminth infections induce T-cell hyporesponsiveness, which may affect immune responses to other pathogens or to vaccines. This study investigates the influence of Treg activity on proliferation and cytokine responses to BCG and *Plasmodium falciparum*-parasitized RBC in Indonesian schoolchildren. Geohelminth-infected children's *in vitro* T-cell proliferation to either BCG or pRBC 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 geohelminth-uninfected groups: Ag-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 parasitized RBC was increased after removal of CD4⁺CD25^{hi} T cells. These data demonstrate that geohelminth-associated Treg influence immune responses to bystander Ag of mycobacteria and plasmodia. Geohelminth-induced immune modulation may have important consequences for co-endemic infections and vaccine trials.

Key words: Coinfection · FOXP3 · Geohelminths · Treg · Vaccination

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 toward the possible involvement of natural and inducible Treg 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

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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 *Brugia malayi* parasites [4].

Interestingly, it has also been shown that helminth infections can affect responses to unrelated Ag, 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, 8]). At the immunological level, a recent study has shown higher IL-10 responses to malaria Ag in children infected with *Schistosoma 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 *Plasmodium falciparum*-parasitized RBC (pRBC) in infected and geohelminth-uninfected subjects from a rural area of Flores island, Indonesia. Proliferative responses to BCG and pRBC 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.

Results

Study population

School-age children were recruited from Welamosa primary school. Stools were microscopically examined for soil-transmitted helminth eggs and two groups of ten children, either geohelminth-infected or geohelminth-uninfected were included for immunological studies. Within the geohelminth-infected children, four had *A. lumbricoides*, four had hookworms, one had both *A. lumbricoides* and hookworm and one had both *T. 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 geohelminth-uninfected groups of children.

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

To determine the immunological reactivity of geohelminth-infected versus geohelminth-uninfected children, we analyzed Ag-specific T-cell responses to BCG vaccine, *P. falciparum* pRBC or uRBC. BrdU incorporation by CD4⁺CD25⁺ cells was assessed to measure effector T-cell proliferation. T-cell proliferation to BCG and pRBC was lower in helminth-infected children (Fig. 1A) compared to uninfected children (geomeans 8.7 versus 13.5% and 8.6 versus 15.9%; *p*-values of 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 geohelminth-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 helminth-uninfected individuals. Similar proportions of CD4⁺ T cells from the two groups expressed CD25 (20 versus 25%; *p* = 0.85), and there

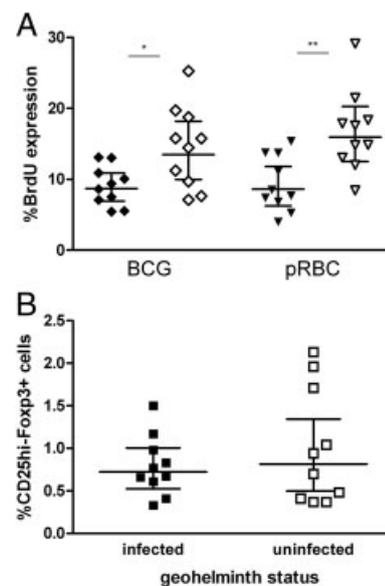


Figure 1. Helminth-infected children show lower T-cell proliferation responses but similar Treg frequency. Donors were grouped by infection status; geohelminth-infected and geohelminth-uninfected (both *n* = 10) groups are shown by filled and open symbols, respectively. (A) Proliferation was analyzed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

were similar populations of CD25^{hi} T expressing cells (5.4 versus 4.7%; $p = 0.57$) as well as of CD25^{hi}FOXP3 co-expressing T cells (0.7 versus 0.8%; $p = 0.68$; Fig. 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 (four infected and seven 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, Fig. 2A) in total CD4⁺ T cells. In three 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%, $p = 0.83$; Fig. 2B). Significantly enhanced proliferation in response to pRBC after Treg

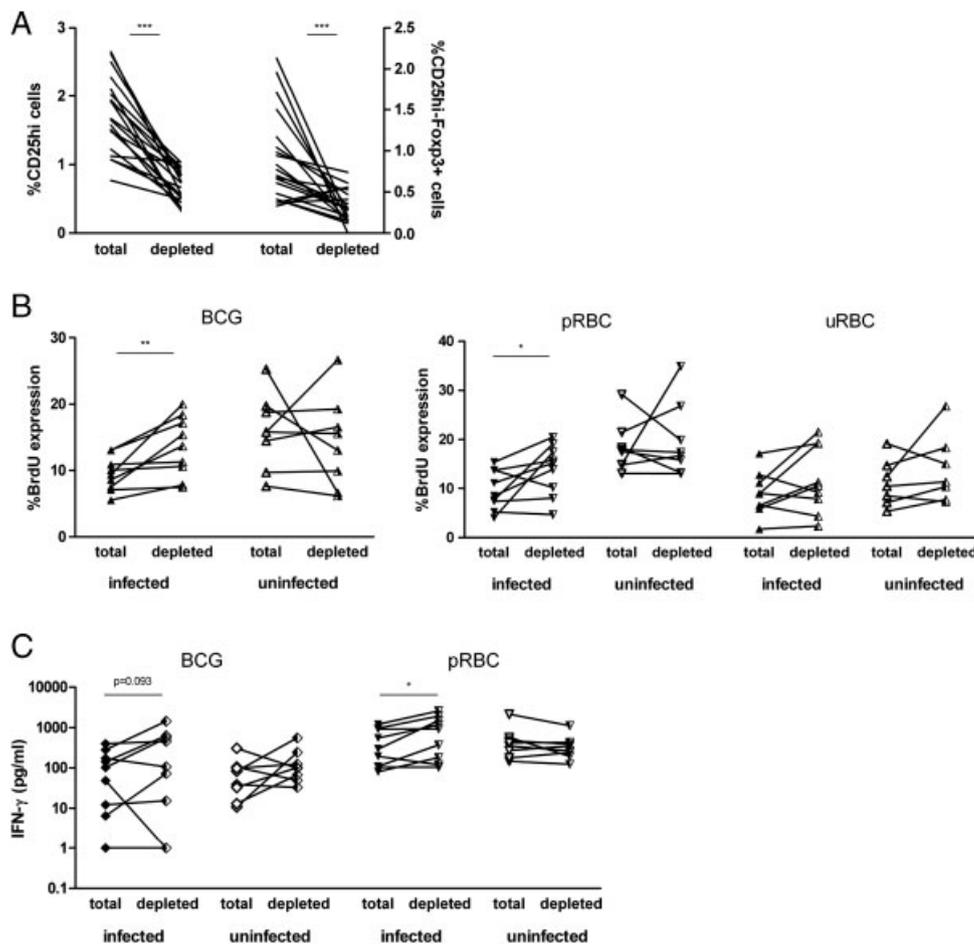


Figure 2. Treg depletion restores BCG and pRBC-induced proliferation and enhances Ag-specific IFN- γ production in geohelminth-infected children. (A) CD4⁺CD25^{hi} T cells were isolated by magnetic bead separation. The “total” and “depleted” data points and the connecting line represent paired data within 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 (nine infected and eight uninfected donors). (B) Effect of Treg depletion is shown for proliferation in response to BCG, pRBC and uRBC. Only in the helminth-infected groups cell proliferation increased significantly after depletion, in response to both BCG stimulation (infected $p = 0.008$ versus uninfected $p = 0.83$) and pRBC stimulation ($p = 0.038$ versus $p = 0.87$). For uRBC no differences were found ($p = 0.17$ versus $p = 0.16$). (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 pRBC 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.

depletion was also seen in samples from helminth-infected (geomeans 8.8 to 12.7%; $p = 0.038$) but not in those from helminth-uninfected children (geomeans 17.9 and 18.7%, $p = 0.87$; Fig. 2B). No such differences were seen in response to uRBC (Fig. 2B). In geohelminth-infected subjects, proliferative responses to BCG and pRBC in depleted PBMC were equivalent to levels found in uninfected children. Interestingly, enhanced IFN- γ production in response to either BCG stimulation or pRBC 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 pRBC 313.8 to 574.3 pg/mL; Fig. 2C), while IL-5 or IL-13 production was unchanged (data not shown).

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 led 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 geohelminth-uninfected school children living in a rural area of Flores Island, Indonesia. The data presented here show lower proliferative responses to BCG and to pRBC 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 pRBC 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 pRBC. 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 Ag such as vaccine Ag 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.

Materials and 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 *A. lumbricoides*, *T. 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

PBMC were obtained by gradient centrifugation of heparinized venous blood over Ficoll. CD4⁺CD25^{hi} T cells were isolated by

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 three 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, 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) Ab.

Bromo-deoxyuridine (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% FBS (Greiner Bio-One GmbH, Frickenhausen, Germany) and 10 μM BrdU. BCG (Bio Farma, Bandung, Indonesia, 0.5 μg/mL), 1 × 10⁶ *P. falciparum* pRBC or 1 × 10⁶ uninfected RBC (uRBC) were used for stimulation. After 96 h cells were fixed in 2% formaldehyde (Sigma-Aldrich, CA, USA) and preserved at -80°C. After thawing, cells were permeabilized and incubated with DNase (Sigma-Aldrich), labeled with anti-BrdU, anti-CD4 and anti-CD25 Ab (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 IFN-γ, IL-5, and IL-13 according to the supplied protocol (Biosource, Invitrogen, Carlsbad, CA, USA). Samples were acquired with Luminex 100™ xMAP technology (Luminex, Austin, TX, USA). Half the detection limit supplied by the manufacturer was used, relevant background values (control medium for BCG, uRBC for pRBC) 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.

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Abbreviations: BrdU: bromo-deoxyuridine · pRBC: parasitized RBC · uRBC: uninfected RBC

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