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Wammes, LJ, Hamid, F, Wiria, AE et al. (14 more authors) (2016) Community deworming alleviates geohelminth-induced immune hyporesponsiveness. Proceedings of the National Academy of Sciences, 113 (44). pp. 12526-12531. ISSN 1091-6490

https://doi.org/10.1073/pnas.1604570113

Community deworming alleviates geohelminth-induced immune hyporesponsiveness

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Submitted to Proceedings of the National Academy of Sciences of the United States of America

In cross-sectional studies, chronic helminth infections have been associated with immunological hyporesponsiveness that can affect responses to unrelated antigens. To study the immunological effects of deworming, we conducted a cluster-randomized double blind placebo-controlled trial in Indonesia and assigned 954 households to receive albendazole or placebo once every three months for two years. Helminth-specific and non-specific whole blood cytokine responses were assessed in 1059 subjects of all ages, while phenotyping of regulatory molecules was undertaken in 121 school-aged children. All measurements were performed before and at 9 and 21 months after initiation of treatment. Anthelmintic treatment resulted in significant increases in pro-inflammatory cytokine responses to *Plasmodium falciparum*-infected red blood cells (PfRBC) and mitogen, with the largest effect on TNF responses to PfRBC at 9 months (estimate and 95% confidence interval 0.37 [0.21-0.53], p-value over time <0.0001). Although the frequency of regulatory T-cells did not change after treatment, there was a significant decline in the expression of the inhibitory molecule CTLA-4 on CD4$^+$ T-cells of albendazole-treated individuals (-0.060 [-0.107 – -0.013] and -0.057 [-0.105 – -0.008] at 9 and 21 months, respectively, p_{time}=0.017). This trial shows the capacity of helminths to upregulate inhibitory molecules and to suppress pro-inflammatory immune responses in humans. This could help to explain the inferior immunological responses to vaccines and lower prevalence of inflammatory diseases in low- compared to high-income countries.

helminths | albendazole | cytokine responses | Indonesia | deworming

Introduction

Soil-transmitted helminths (STH) represent the most common infectious disease worldwide (1). In addition to specific worm-associated morbidities, it has been argued that chronic STH infections may magnify health-related burdens in communities remote from health care facilities, exacerbating anemia, poor nutritional status, and possibly poor cognitive development (1). However, this was not fully supported by the latest analysis of the Cochrane database (2).

Immunologically, cellular immune hyporesponsiveness is a hallmark of chronic helminth infections that may allow parasites' long-term survival (3). The consequences of immunosuppression are manifold with potentially major public health relevance. Immune hyporesponsiveness could curtail effective immune responses, thereby increasing susceptibility to pathogens, and helminths are associated with suboptimal vaccine responses (4-6). The helminth-related dampened immune responses might nevertheless help to prevent immunopathology during coinfections and, possibly, aberrant reactivity to environmental or self-antigens (7). With respect to the latter, there is currently much interest in the use of helminth infections to treat allergies and autoimmune diseases, exploiting their ability to induce immune hyporesponsiveness (8).

Suppressed lymphocyte responses were described in the 1970s (9), but the evidence base has not moved much beyond animal models and cross-sectional studies in humans (10). The cellular mechanisms associated with helminth-related immune hyporesponsiveness are not fully understood. Several regulatory cells and molecules are thought to play an important role in the regulatory network (3). Within T-cell responses, expansion of T-regulatory cells (Treg) is reported in both animal models (10) and some human studies (11, 12). Tregs suppress helminth-specific and bystander proliferative and pro-inflammatory responses. Expression of T-cell–associated molecules, including cytotoxic T-lymphocyte–associated antigen (CTLA)-4 and programmed death (PD)-1, may also be involved in helminth-induced hyporesponsiveness and spill-over suppression (13).

Longitudinal studies assessing the effect of anthelmintic treatment on cellular immune responsiveness are rare, and either lack placebo controls, target children only, or measure immune responses at one time point post-treatment (14-16). Moreover,

Significance

Chronic helminth infections are accompanied by profound immune regulation. In humans, helminth-induced immune reactivity has not been thoroughly investigated in trial settings. We assessed the effect of anthelmintic treatment on immune responses in a whole community, in a placebo-controlled RCT. We show increased immune responses to helminth-specific as well as unrelated antigens, in parallel with decreased CTLA-4 expression, which is a molecule involved in putting a brake on immune activation. Deworming seems to lead to decreased immunoregulation and increased immune responsiveness. These findings are of importance regarding the suboptimal vaccine responses in helminth-endemic areas, but also in anticipating the future rise in inflammatory diseases when helminth infections are increasingly controlled.

Reserved for Publication Footnotes

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, %)*		572	328 (57.3)	487	279 (57.3)
Area (rural, n, %)*		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 parasitaemia *(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-α (pg/mL)	554	743 [368-1293]	468	769 [339-1318]
	IL-10 (pg/mL)	554	271 [163-441]	468	256 [158-406]
PHA	TNF-α (pg/mL)	516	100 [50-222]	435	103 [50-214]
	IL-10 (pg/mL)	515	76 [41-129]	435	70 [37-116]
	IFN-γ (pg/mL)	516	1625 [584-3983]	435	1270 [538-4340]
	IL-2 (pg/mL)	516	23 [0-101]	432	23 [0-92]
	IL-5 (pg/mL)	516	563 [309-840]	435	520 [317-829]
PfRBC	TNF-α (pg/mL)	299	18 [4-42]	237	14 [3-38]
	IL-10 (pg/mL)	300	10 [5-19]	238	10 [5-20]
	IFN-γ (pg/mL)	300	163 [75-388]	239	176 [70-376]
	IL-2 (pg/mL)	300	50 [5-125]	239	40 [5-112]
	IL-5 (pg/mL)	300	14 [5-26]	239	12 [4-23]
AscAg	TNF-α (pg/mL)	517	5 [0-15]	438	6 [0-14]
	IL-10 (pg/mL)	516	7 [2-15]	438	7 [1-14]
	IFN-γ (pg/mL)	516	19 [6-47]	441	21 [7-47]
	IL-2 (pg/mL)	497	38 [4-114]	426	36 [0-107]
	IL-5 (pg/mL)	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)
SD, standard deviation; BMI, body mass index; IQR, interquartile range.

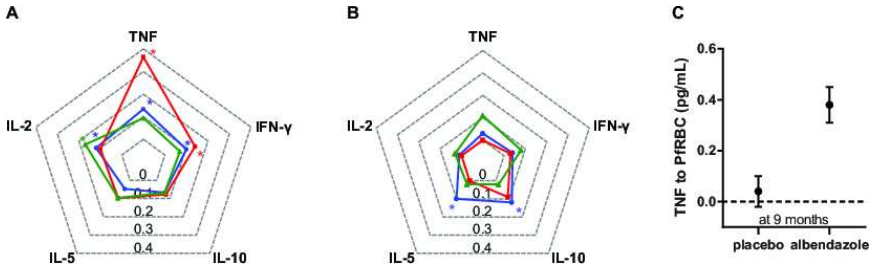

Fig. 1. The effect of anthelminthic treatment on cytokine responses to AscAg, PfRBC and PHATNF, IFN-γ, IL-2, IL-5 and IL-10 concentrations were assessed in supernatants of 72h-stimulated whole-blood cultures. The values on the 'y-axis' (the spider web lines) represent the estimated outcome (beta) of the effect of albendazole treatment on cytokine responses to PHA (blue circles), PfRBC (red squares) and AscAg (green triangles). By comparing the responses in the albendazole versus placebo group, the estimates of the treatment effect in the whole study population after 9 (A) and 21 (B) months of albendazole treatment were obtained using linear mixed models and positive values were plotted in a spider chart. Statistically significant estimates at 9 months were IL-2 responses to AscAg (estimated effect of treatment [95% confidence interval]: 0.17 [0.05–0.28]), TNF (0.37 [0.21-0.53]) and IFN-γ (0.14 [0.03-0.24]) responses to PfRBC and TNF (0.14 [0.05-0.24]), IFN-γ (0.10 [0.01-0.19]) and IL-2 (0.12 [0.01-0.23]) responses to PHA. At 21 months post-treatment, PHA-induced IL-5 (0.10 [0.01-0.19]) and IL-10 (0.12 [0.05-0.19]) were significantly enhanced. As an indication of the magnitude of change in level of cytokines that were significantly different between placebo and albendazole group, geometric mean with standard error for TNF to PfRBC at 9 months (C) is given as an example.

none have examined the changes in regulatory cells or molecules. No large-scale community-based intervention studies to establish whether helminth infections lead to immune hyporesponsiveness in humans have been reported.

To disentangle the impact of helminths on the immune system from other influences, we conducted a household cluster-randomized double blind placebo-controlled trial of albendazole once every three months in communities with high STH prevalence on Flores island, Indonesia. Here we present results con-

Table 2. – Effect of albendazole treatment on immune responses by helminth infection status at baseline

Out-come	Effect of treatment at 9 months			Effect of treatment at 21 months			
	Placebo N	Albendazole N	β [95%CI]*	Placebo N	Albendazole N	β [95%CI]*	p_{time}
A. Effect of albendazole on cytokine responses in helminth-infected individuals							
PHA							
TNF	261	190	**0.14 [0.01-0.26]**	228	152	0.03 [-0.11-0.17]	0.098
IL-10	260	190	0.08 [-0.00-0.16]	227	152	0.06 [-0.03-0.15]	0.12
PfRBC							
TNF	154	106	**0.42 [0.20-0.64]**	133	84	-0.10 [-0.33-0.14]	**0.0004**
IFN-γ	155	108	0.12 [-0.02-0.26]	134	86	-0.01 [-0.19-0.16]	0.18
AscAg							
IL-2	249	182	**0.25 [0.10-0.41]**	215	146	0.04 [-0.12-0.20]	**0.006**
B. Effect of albendazole on cytokine responses in helminth-uninfected individuals							
PHA							
TNF	31	19	0.02 [-0.40-0.43]	28	19	0.20 [-0.21-0.62]	0.63
IL-10	31	19	0.03 [-0.25-0.31]	28	19	**0.31 [0.01-0.60]**	0.12
PfRBC							
TNF	26	17	0.33 [-0.13-0.78]	22	15	0.15 [-0.38-0.67]	0.35
IFN-γ	26	17	0.34 [-0.03-0.71]	22	15	-0.00 [-0.42-0.41]	0.18
AscAg							
IL-2	31	20	0.08 [-0.36-0.53]	28	19	-0.08 [-0.54-0.39]	0.83

The analysis of the effect of anthelmintic treatment was stratified based on helminth infection status at baseline. By comparing the responses in the albendazole versus placebo group, the estimated outcome (beta) of the treatment effect after 9 and 21 months of albendazole treatment were obtained.The number of the total population examined (N). *β (beta) and 95% confidence interval are based on linear mixed models. An overall p-value (p_{time}) is indicated for the effect of treatment over time. Statistically significant results (p<0.05) are given in bold.

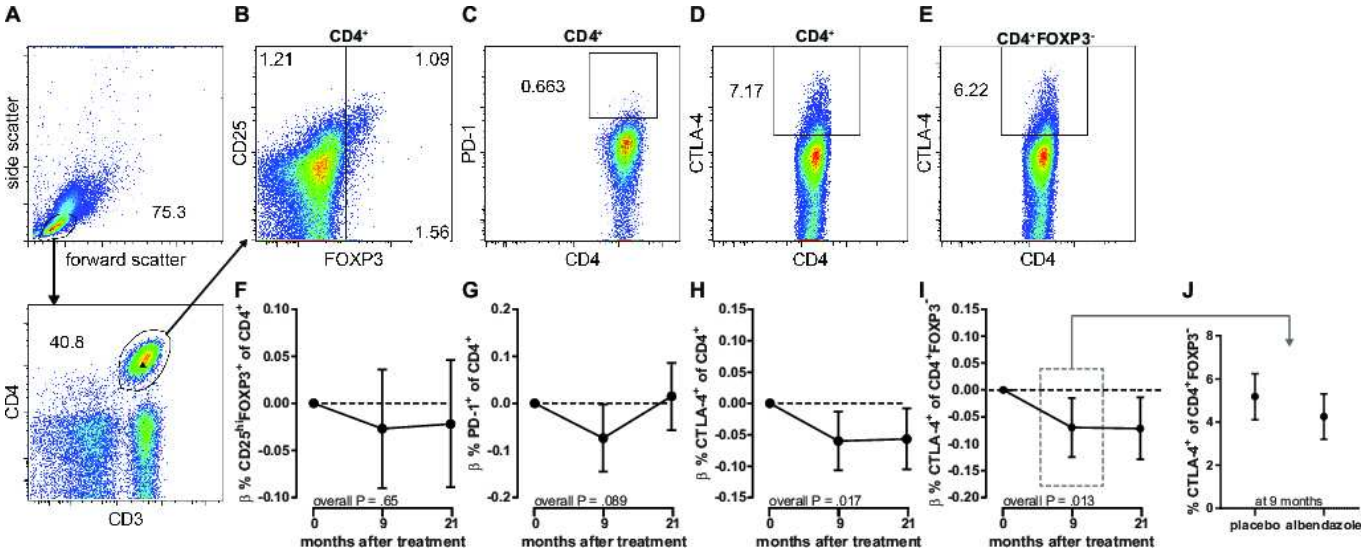

Fig. 2. Effect of deworming on cell subsets and marker expression Flow cytometry was performed on PBMC from a subset of schoolchildren. Gating strategy is shown for (A) lymphocytes and $CD4^+$ T-cells,from which (B) $CD25^{hi}FOXP3^+$ Treg cells, (C) PD-1- and (D) CTLA-4 expression on $CD4^+$ T-cells, were derived. (E) CTLA-4 expression on $CD25^{hi}FOXP3^-$ cells, was gated from B. The estimated effect of albendazole treatment is shown for the time points 9 and 21 months after start of treatment for percentages of $CD25^{hi}FOXP3^+$ (F), PD-1^+ (G), CTLA-4^+ (H) of$CD4^+$ T cells, and CTLA-4^+ of$CD4^+FOXP3^-$cells (I). Estimates, β(beta) were obtained by linear mixed models; 95% confidence intervals and overall p-values over time (p_{time}) are indicated. As an indication of magnitude of change, the actual percentage of CTLA-4^+ of$CD4^+FOXP3^-$cells in placebo and albendazole groups is shown at 9 months (J).

cerning the effects of anthelmintic treatment on cellular immune responses.

Results

Albendazole treatment reduces but does not eliminate helminth infections

Characteristics of the study participants (n=1059) are shown in table 1. At baseline one or more helminth species were found in 88.7% of individuals, hookworm being the most prevalent (77.1% of total). The trial consort diagram with follow-up data can be found in the supplementary information (fig. S1). Albendazole treatment reduced the prevalence of geohelminths after 9 (51.9% vs. 84.1% for placebo) and 21 months (39.2% vs. 80% for placebo) (table S1). In the whole IMMUNOSPIN trial the prevalence of geohelminth infection was 87.3% and albendazole treatment reduced prevalence of geohelminths after 9 (51.4% vs. 82.8% for placebo) and 21 months (41.9% vs. 78.8% for placebo). As for the whole IMMUNOSPIN trial, the greatest effect was on hookworm followed by *Ascaris*, while the effect on *Trichuris* infections was less pronounced. Albendazole also reduced intensities of hookworm and *Ascaris* infections, as assessed by PCR (fig. S2).

Helminth-specific and nonspecific whole blood cytokine responses are increased after albendazole treatment

Figure 1 shows the effect of treatment on cytokine responses at 9 months (A) and 21 months (B).

Regarding helminth-specific cytokines, *Ascaris* antigen (AscAg)-induced interleukin-2 (IL-2) production was significantly enhanced by treatment over the study period (p_{time}=0.018), with a significant increase in the treated group at 9 months (estimate [95% CI]: 0.17 [0.05–0.28], fig. 1A).

In response to plasmodial antigens (*Plasmodium falciparum*-infected red blood cells; PfRBC), there was an increase over time in pro-inflammatory cytokines tumor necrosis factor (TNF; p_{time}<0.0001) and interferon-gamma (IFN-γ; p_{time}=0.036) after albendazole treatment. As shown in fig. 1A, both TNF and IFN-γ were significantly higher in the albendazole compared to the placebo group at the 9-month time point (0.37 [0.21-0.53] for TNF and 0.14 [0.03-0.24] for IFN-γ). To get an indication of the absolute changes in cytokine levels, TNF production to PfRBC in the two groups at the 9-month time point is shown in fig. 1C. The differences in other statistically significant cytokine changes are shown in fig. S3. None of the significant changes in antigen specific responses were correlated with worm burden before treatment (table S2).

Regarding the general adaptive response (cytokine responses to phytohemagglutinin, PHA), albendazole treatment significantly increased TNF and IL-10 secretion (p_{time}=0.011 and p_{time}=0.003 respectively) over the trial period; for TNF, albendazole treatment resulted in elevated responses at 9 months, whereas for IL-10 the response was significantly higher after 21 months (for TNF at 9 months 0.14 [0.05–0.24], fig. 1A; for IL-10 at 21 months 0.12 [0.05–0.19], fig. 1B). The IFN-γ and IL-2 responses to PHA were transiently increased at 9 months post-treatment and PHA-induced IL-5 was higher at the 21-month time point, but this did not reach statistical significance over the whole trial time period (IFN-γ p_{time}=0.076, IL-2 p_{time}=0.11, IL-5 p_{time}=0.068, fig. 1).

Albendazole did not affect responses to lipopolysaccharide (table S3). Cytokines in unstimulated blood revealed no treatment-related differences (table S3). IFN-γ responses to uninfected RBC (uRBC) were not significantly different between treatment arms (p_{time}=0.91), however TNF production was increased post-treatment (p_{time}=0.018). This was only significant at 9 months (9-month estimate 0.13 [0.01-0.25], at 21 months -0.13 [-0.26-0.003], although to a much lesser extent than the response to PfRBC.

The enhancement of cytokine responses is not a direct albendazole effect

To rule out albendazole as a direct cause of enhanced immune responses, we stratified the analysis on STH infection status at baseline (table 2). Enhanced PfRBC-induced TNF and AscAg-induced IL-2 by albendazole treatment was seen in helminth-infected (p_{time}=0.0004 and p_{time}=0.006, respectively, table 2A) but not in uninfected subjects (table 2B), at 9 months post-treatment. The effect of anthelmintic treatment on PHA-stimulated TNF in the stratified analysis was seen at 9 months post-treatment in the helminth-infected individuals but over the trial period this was not statistically significant (p_{time}=0.098, table 2A). Corresponding background (unstimulated and uRBC-induced) cytokine responses were not increased in either helminth-infected or -uninfected subjects (table S4).

Changes in cell counts after albendazole treatment do not explain changes in cytokine responses

To determine whether increased cellular responses could be explained by higher cell numbers, we analysed complete blood counts and sought associations with cytokine responses. Total leukocytes –most markedly monocytes– were increased in the albendazole group compared to placebo at 9 months post-treatment but not subsequently (table S5). Leukocyte counts were positively associated with IL-2 to AscAg, however the rest were mainly negative associations, of which the one with TNF responses to PfRBC was significant. No association was found between monocyte numbers and cytokine responses to any of the stimuli (table S6). This indicates that increased leukocyte numbers did not account for the general enhancement of cytokine responses. Moreover, when analysis of the treatment effect on cytokine responses was adjusted for leukocyte or monocyte counts, similar effect sizes were observed. No treatment effect was noted on other hematological parameters (table S5).

Albendazole does not affect Treg frequencies however does expand CTLA-4-expressing $CD4^+$ T cells

To identify potential mechanisms of immune hyporesponsiveness and their reversal by anthelmintics we examined Treg (defined as $CD4^+CD25^{hi}FOXP3^+$ T-cells) as well as $CD4^+$ cells expressing the suppressive molecules PD-1 and CTLA-4 in $CD4^+$ T-cells (fig. 2). The frequency of Tregs did not change in the albendazole group compared to placebo (estimates [95% CI] at 9 months -0.027 [-0.090 – 0.036], at 21 months -0.022 [-0.089 – 0.046]; p_{time}=0.65, fig. 2B & 2F). Similarly, treatment did not alter the expression of PD-1 expressing $CD4^+$ T-cells over the whole trial period, although at 9 months there was a significant decrease (-0.074 [-0.145 – -0.002] and 0.015 [-0.057 – 0.086]; p_{time}=0.089, fig. 2C & 2G). However, the proportion of CTLA-4-expressing $CD4^+$ T-cells decreased after treatment and was significantly lower in the albendazole group at both time points post-treatment (-0.060 [-0.107 – -0.013] and -0.057 [-0.105 – -0.008] respectively; p_{time}=0.017, fig. 2D & 2H). Similar to total $CD4^+$ T cells, the frequency of CTLA-4-expressing $CD4^+FOXP3^-$ effector T cells decreased significantly after treatment with albendazole (-0.07 [-0.125 – -0.015] and -0.072 [-0.129 – -0.014] respectively, p_{time}=0.013 (fig. 2E & 2I). The absolute change in CTLA-4 expression on effector T cells is shown in fig. 2J.

Discussion

This is the first report of cytokine responses as well as regulatory cells and molecules analysed in a community before and after repeated long-term placebo-controlled anthelmintic treatment. We show that treatment of STH infections ablates their immunosuppressive effects, enhancing immune responses to helminth and unrelated antigens as well as to mitogen. Most pronounced were elevated pro-inflammatory cytokine responses after stimulation with plasmodial antigens and mitogen. In addition, we observed a reduction in CTLA-4-expressing $CD4^+$ T-cells in albendazole-

treated children, indicating that immuno-inhibitory mechanisms could be affected by deworming.

The strongest effect of anthelmintic treatment was on anti-plasmodial responses. These had not been specifically investigated in anthelmintic treatment RCTs. However, in cross-sectional studies examining the effect of helminths on malaria-specific cytokine responses, results are inconsistent (17-19). The increase in response to malaria antigens, could be due to a concurrent increase in malarial parasitemia in the albendazole-treated group 6 months after initiation of treatment (20), coincident with peak transmission season. By performing the analysis without malaria-positive subjects, we ruled out that this could explain the enhanced plasmodial-specific cytokine responses.

With regard to immune regulation, no treatment-related change in Treg frequencies was seen, consistent with the finding of similar Treg frequencies in STH-infected and -uninfected children reported from the same study area (12). The proportion of PD-1-expressing $CD4^+$ T-cells was not significantly altered by albendazole treatment over two years, although in the first year post-treatment this was significantly lower. This is consistent with studies that show increased PD-1 expression is associated with helminth infections (13,18). The significant decrease in CTLA-4–expressing $CD4^+$ T-cells adds support to the important role of this molecule in suppression of immune responses in general, and its suggested role in immune hyporesponsiveness induced by helminths (21). When put in the context of the blockade of CTLA-4 (as well as PD-1) in treatment of melanoma and other cancers (22), these findings lend further support to the suggested similarities between immunoregulation in chronic infectious diseases and cancers (23).

Three-monthly albendazole treatment over a two-year period did not eliminate helminths. In earlier reports, the efficacy of one-time single or double doses of albendazole and/or mebendazole treatment has been low for *Ascaris* and *Trichuris* (24). Here we show that this is the case even after 7 doses of albendazole at three-monthly intervals. By using a household-clustered randomization design, repeated treatments and observed intake, we expected a more effective reduction in prevalence of STH. For better deworming results, more intensive treatment or inclusion of environmental control would be needed. However, it is clear that even a 50% reduction in helminth infections in the community can start to reverse immune hyporesponsiveness and that more effective deworming might give even more pronounced immunological effects.

Subsequent to the increased pro-inflammatory responses after 9 months, IL-5 and IL-10 responses increased 21 months post-treatment. Stratified analyses revealed that the increased mitogen-stimulated IL-5 and IL-10 was not specific to helminth-infected subjects, suggesting that factors other than the elimination of helminths may be responsible. This increased IL-10 response after two years of treatment might account for the fact that immune responses are not higher in the albendazole versus placebo at this time point.

Enhanced cytokine responses could also be the result of a boosted immune response due to the release of antigens from dying or dead worms. However, the strongest increases in responses were not to worm antigen but to the unrelated malarial antigen. Moreover, using pre-treatment worm burden as a proxy for antigen release, the modest increase seen to Ascaris antigen was not correlated with burden of *A. lumbricoides* at pre-treatment, nor were responses to non-related antigens correlated with baseline worm burden. These argues that observed boosted immune responses would not be due to release of antigens from dying worms, which has been shown to account for part of the increase in immune responses after treatment in schistosomiasis (25), but rather due to the decrease in immune regulation.

A number of factors other than reduction in helminths could contribute to the findings of this study, such as a direct effect of albendazole, alterations in immune cell counts or changes in nutrients. Albendazole has been shown to affect cytokine responses *in vitro* (26). The higher effect sizes in the stratified analysis of helminth-positives than those in the total group indicate that the enhancement of pro-inflammatory cytokine responses is unlikely to be due to albendazole directly affecting the immune system. Immune hyporesponsiveness could stem from alteration in cell counts and changes in nutrients essential to functioning of the immune system (27). Although cell counts were affected by treatment, cell numbers did not account for cytokine responses. Since improved energy resources can enhance immune responses, we assessed BMI, and fasting glucose level as proxies for nutritional status, but these parameters were not affected by deworming (20).

Our study shows significant effects of deworming on the immune system. The effects could lead to enhanced immune responses to other pathogens and vaccines. With respect to vaccines there is increasing concern regarding poor immunogenicity in rural areas of developing countries (28, 29), therefore any measure to alleviate hyporesponsiveness would have major public health impact. It is also important to consider the long-standing evolutionary coexistence between humans and helminths, the disturbance of which might lead to the emergence of pathological conditions (30), However, for this, long- rather than short-term treatment courses are expected to reveal any clinical impact (31). Considering this, it will be important to include immunological measurements in future deworming programs and anthelmintic therapy trials, to better understand and predict clinical outcomes.

Methods

Study design

The study was nested within the ImmunoSPIN trial, a double-blind placebo-controlled trial conducted in two villages on Flores island, Indonesia (20). All households were randomized to receive either a single dose of 400 mg albendazole or placebo once every three months for two years. Treatment was allocated to households to minimise the risk of reinfection, and was provided to all household members older than two years, except for pregnant women, according to Indonesian guidelines. Intake was observed by field workers. Participants gave written informed or parental consent. The study was approved by the Ethics Committee of the Medical Faculty, University of Indonesia, Jakarta and was filed by the Ethics Committee of the Leiden University Medical Center, the Netherlands. The trial was registered as ISRCTN83830814.

Study population

Randomization was based on 954 households in total, comprising 2022 (481 houses) and 1982 (473 houses) subjects in placebo and albendazole groups, respectively. For immunological studies, 250 households in the main village were randomly selected and individuals older than 4 years of age were invited for venous blood sampling and assessment of anthropometric parameters. Thereby 882 individuals were included, of which 858 provided sufficient blood for whole-blood cultures. In the other village, 250 children were randomly selected from the total population and children from the same households were also included, giving 295 children in total with whole-blood cultures. After cleaning the data (see below), at baseline 839 and 220 subjects were included from the two areas, comprising 572 placebo- and 487 albendazole-treated individuals.

Since STH infection and associated immunological changes were anticipated to be most prevalent in school-age children, detailed analyses of regulatory components were only performed in this age group (4-12 years old). From a randomized selection separate from the above-mentioned subset, 145 children were included (71 randomized for placebo; 74 for albendazole) of which 121 (61 and 60, respectively) had sufficient numbers of cells. After 9 and 21 months 116 (56/60) and 107 (52/55) were followed up, respectively.

Whole-blood culture and cytokine measurements

Whole-blood was stimulated *in vitro* as described before (32), for 24h (lipopolysaccharide (LPS) stimulation) and 72h (*Ascaris lumbricoides* antigen (AscAg), *Plasmodium falciparum*-parasitized red blood cells (PfRBC), uninfected (u)RBC and phytohemagglutinin (PHA) stimulations). PfRBC and uRBC were prepared according to a standardized procedure (32). AscAg was a homogenate of adult worms *A. lumbricoides* obtained from infected humans. Supernatants were stored at -20°C until quantification using Luminex kits (Biosource, Camarillo, USA) on a Liquichip 200® Workstation (Qiagen, Venlo, the Netherlands). Tumor necrosis factor (TNF) and interleukin (IL)-10 were quantified in all supernatants whilst interferon (IFN)-γ, IL-2 and IL-5 were quantified only in 72h supernatants. Samples with TNF levels ≥250

pg/mL in unstimulated blood were excluded from the analyses, as they were considered possibly contaminated. This cut-off value was derived from outliers in the data distribution. Cytokine concentrations below the assay's detectable range were replaced by half the detection limit provided by the manufacturer.

Stool examination by microscopy and PCR

Stool samples were collected annually. *Trichuris trichiura* was detected by microscopy after formol-ether concentration, whilst multiplex real-time PCR detected hookworm (*Ancylostoma duodenale*, *Necator americanus*), *A. lumbricoides* and *Strongyloides stercoralis* DNA, as described previously (32).

Complete blood counts

Complete blood counts (CBC) before and one year post-treatment were determined using heparinized blood on a cell counter (Coulter® Ac-T™ diff Analyser, Beckman Coulter Inc., Fullerton, USA), while CBC 2 years post-treatment were determined on a Sysmex KX-21N hematology analyser (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.

Flow cytometry

Peripheral blood mononuclear cells (PBMC) from 121 schoolchildren were isolated by Ficoll gradient centrifugation. PBMC were fixed with FOXP3 Staining Buffer (eBioscience Inc., San Diego, USA) and cryopreserved until further analysis. After thawing, cells were permeabilized and stained with anti-CD3, anti-CD4, anti-FOXP3, anti-CD25, anti-CTLA-4, anti-PD-1 and anti-Ki67 antibodies (table S7). Data were acquired on a FACSCanto (BD Biosciences) and analysed using FlowJo software (Treestar Inc., Ashland, USA).

Statistical analysis

Log transformation was used for cytokines (log10(concentration+1)) and most flow cytometry (log10(value)) data to obtain normally distributed variables. For children's BMI age-standardized z-scores were calculated according to WHO references (33). To assess treatment effects, linear mixed models were used; these are described in more detail in the supplement. Parameter estimates and 95% confidence intervals for treatment effects at 9 and 21 months are reported. The analysis was intention-to-treat, and involved all participants as assigned randomly at the start of the trial.

Acknowledgements

The authors thank all people that were part of the research team, the health staff from the Puskesmas Primary Health Centers of Nangapanda and Welamosa, but most of all the participants from Nangapanda and Anaranda.

Authors' contributions MY developed the study and is the Dutch coordinator of the ImmunoSPIN program. TS developed the study as the Indonesian coordinator of ImmunoSPIN. ES contributed to the study coordination and advised on data collection. LJW, FH and AEW contributed to setting up the field study, recruitment, follow-up and data collection as well as clinical care of the study population. LM contributed to the management of the database and the statistical analysis of all collected data. JJV led the work on PCR detection of parasites and MMMK contributed to data collection and parasitological investigation. MAP contributed to the follow-up data collection. YD, SEdJ and YCMK contributed to immunological data collection. JJH and RT performed the statistical analysis and modelling. AJFL advised on malaria and immunological data collection. HW contributed to safeguarding randomization codes and privacy of the study subjects. LJW and MY drafted the manuscript, with contributions from AEW, FH, JJH, AJFL, ES, and TS who helped to interpret results and prepare the manuscript. All authors reviewed the final manuscript. **Conflicts of interest** All authors declare that they have no conflict of interest.

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