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Community deworming alleviates geohelminth-induced immune hyporesponsiveness

Linda J Wammes^{1,§,*}, Firdaus Hamid^{1,2,*}, Aprilianto E Wiria^{1,3,*}, Linda May¹, Maria MM Kaiser^{1,3}, Margaretta A Prasetyani-Gieseler³, Yenny Djuardi³, Heri Wibowo³, Yvonne CM Kruize¹, Jaco J Verweij^{1,4}, Sanne E de Jong¹, Roula Tsoukala⁵, Jeanine J Houwing-Duistermaat^{5,6}, Erliyani Sartono¹, Adrian J F Luty⁷, Taniawati Supali³, Maria Yazdanbakhsh^{1,#}

1. Dept Parasitology, Leiden University Medical Center, Leiden, the Netherlands[§] 2. Dept Microbiology, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia 3. Dept Parasitology, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia 4. Laboratory for Medical Microbiology and Immunology, St Elisabeth Ziekenhuis, Tilburg, the Netherlands 5. Dept Medical Statistics and Bioinformatics, Leiden University Medical Center, Leiden, the Netherlands 6. Dept of Statistics, University of Leeds, Leeds, United Kingdom 7. Department of Medical Microbiology, Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands^{§§} § Currently at Dept Medical Microbiology & Infectious Diseases, Erasmus Medical Center, Rotterdam, the Netherlands^{§§} §§ Currently at Institut de Recherche pour le Développement, MERIT UMR 216, Paris, France *These authors have contributed equally to this work.

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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, $p_{\text{time}}=0.017$). 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)
	<i>N. americanus</i> ¹	335	252 (75.2)	245	188 (76.7)
	<i>A. duodenale</i> ¹	335	25 (7.5)	245	17 (6.9)
	<i>A. lumbricoides</i> ¹	335	105 (31.3)	245	80 (32.7)
	<i>S. stercoralis</i> ¹	335	3 (0.9)	245	14 (5.7)
	<i>T. trichiura</i> ²	415	106 (25.5)	310	62 (20.0)
	Malarial parasitaemia (any spp) ²	567	24 (4.2)	483	24 (5.0)
	<i>P. falciparum</i>	567	16 (2.8)	483	11 (2.3)
	<i>P. vivax</i>	567	8 (1.4)	483	10 (2.1)
	<i>P. malariae</i>	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]
PfrBC	IL-5 (pg/mL)	516	563 [309-840]	435	520 [317-829]
	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]
AscAg	IL-5 (pg/mL)	300	14 [5-26]	239	12 [4-23]
	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.

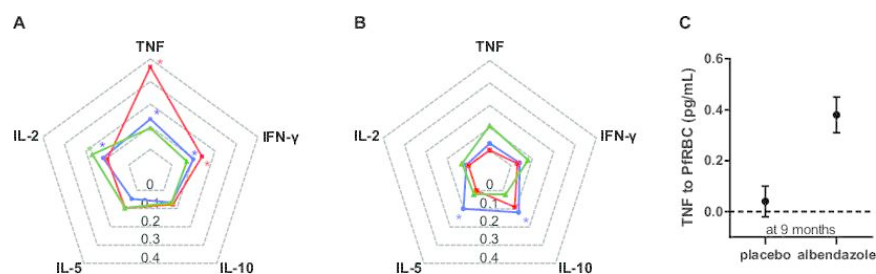


Fig. 1. The effect of anthelmintic treatment on cytokine responses to AscAg, PfrBC and PHA. TNF, IFN-γ, IL-2, IL-5 and IL-10 concentrations were assessed in supernatants of 72h-stimulated whole-blood cultures. The values on the 'y-axis' (the spider web lines) represent the estimated outcome (beta) of 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

Outcome	Effect of treatment at 9 months			Effect of treatment at 21 months			P _{time}
	Placebo N	Albendazole N	β [95%CI]*	Placebo N	Albendazole N	β [95%CI]*	
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.

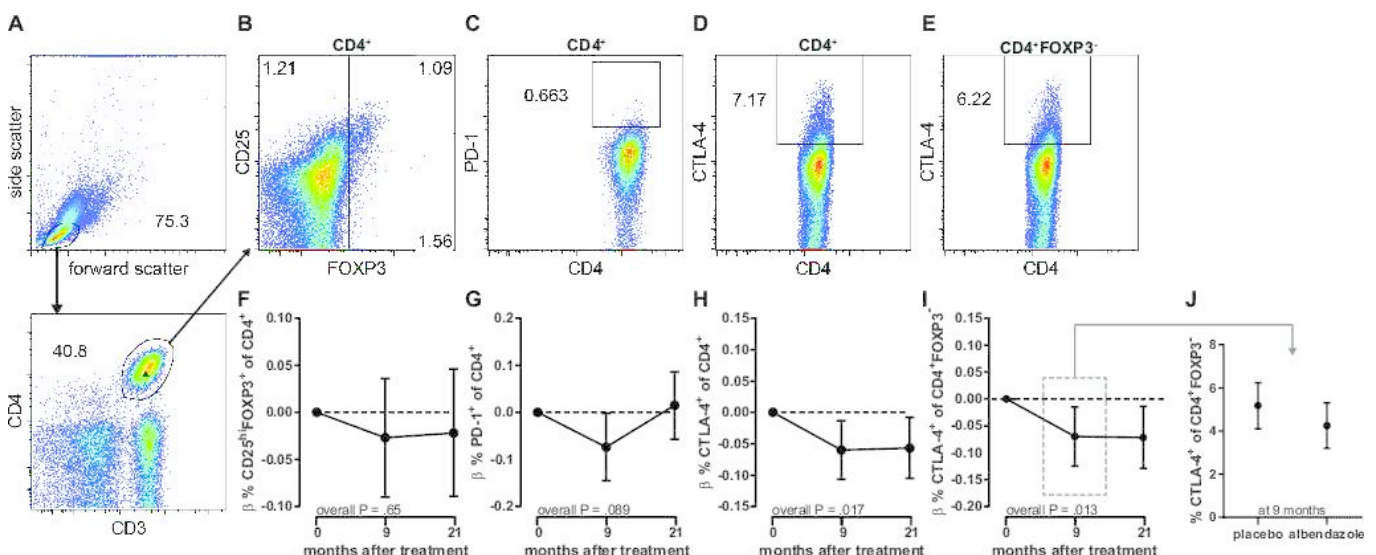


Fig. 2. Effect of deworming on cell subsets and marker expression Flow cytometry was performed on PBMC from a subset of schoolchildren. Gating strategy is shown for (A) lymphocytes and CD4⁺ T-cells, from which (B) CD25^{hi}FOXP3⁺ Treg cells, (C) PD-1- and (D) CTLA-4 expression on CD4⁺ T-cells, were derived. (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 ($p_{\text{time}}=0.018$), 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; $p_{\text{time}} < 0.0001$) and interferon-gamma (IFN- γ ; $p_{\text{time}} = 0.036$) 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 ($p_{\text{time}} = 0.011$ and $p_{\text{time}} = 0.003$ 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 $p_{\text{time}} = 0.076$, IL-2 $p_{\text{time}} = 0.11$, IL-5 $p_{\text{time}} = 0.068$, 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 ($p_{\text{time}} = 0.91$), however TNF production was increased post-treatment ($p_{\text{time}} = 0.018$). 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 ($p_{\text{time}} = 0.0004$ and $p_{\text{time}} = 0.006$, 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 ($p_{\text{time}} = 0.098$, 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]; $p_{\text{time}} = 0.65$, 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]; $p_{\text{time}} = 0.089$, 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; $p_{\text{time}} = 0.017$, 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, $p_{\text{time}} = 0.013$ (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-

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

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

558 With regard to immune regulation, no treatment-related
559 change in Treg frequencies was seen, consistent with the find-
560 ing of similar Treg frequencies in STH-infected and -uninfected
561 children reported from the same study area (12). The proportion
562 of PD-1-expressing CD4⁺ T-cells was not significantly altered by
563 albendazole treatment over two years, although in the first year
564 post-treatment this was significantly lower. This is consistent with
565 studies that show increased PD-1 expression is associated with
566 helminth infections (13,18). The significant decrease in CTLA-
567 4-expressing CD4⁺ T-cells adds support to the important role
568 of this molecule in suppression of immune responses in general,
569 and its suggested role in immune hyporesponsiveness induced by
570 helminths (21). When put in the context of the blockade of CTLA-
571 4 (as well as PD-1) in treatment of melanoma and other cancers
572 (22), these findings lend further support to the suggested simi-
573 larities between immunoregulation in chronic infectious diseases
574 and cancers (23).

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

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

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

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

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

640 Methods

641 Study design

642 The study was nested within the ImmunoSPIN trial, a double-blind
643 placebo-controlled trial conducted in two villages on Flores island, Indonesia
644 (20). All households were randomized to receive either a single dose of
645 400 mg albendazole or placebo once every three months for two years.
646 Treatment was allocated to households to minimise the risk of reinfection,
647 and was provided to all household members older than two years, except for
648 pregnant women, according to Indonesian guidelines. Intake was observed
649 by field workers. Participants gave written informed or parental consent.
650 The study was approved by the Ethics Committee of the Medical Faculty,
651 University of Indonesia, Jakarta and was filed by the Ethics Committee of the
652 Leiden University Medical Center, the Netherlands. The trial was registered
653 as ISRCTN83830814.

654 Study population

655 Randomization was based on 954 households in total, comprising 2022
656 (481 houses) and 1982 (473 houses) subjects in placebo and albendazole
657 groups, respectively. For immunological studies, 250 households in the main
658 village were randomly selected and individuals older than 4 years of age
659 were invited for venous blood sampling and assessment of anthropometric
660 parameters. Thereby 882 individuals were included, of which 858 provided
661 sufficient blood for whole-blood cultures. In the other village, 250 children
662 were randomly selected from the total population and children from the
663 same households were also included, giving 295 children in total with whole-
664 blood cultures. After cleaning the data (see below), at baseline 839 and 220
665 subjects were included from the two areas, comprising 572 placebo- and 487
666 albendazole-treated individuals.

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

675 Whole-blood culture and cytokine measurements

676 Whole-blood was stimulated *in vitro* as described before (32), for 24h
677 (lipopolysaccharide (LPS) stimulation) and 72h (*Ascaris lumbricoides* antigen
678 (AscAg), *Plasmodium falciparum*-parasitized red blood cells (pRBC), unin-
679 fected (u)RBC and phytohemagglutinin (PHA) stimulations). pRBC and uRBC
680 were prepared according to a standardized procedure (32). AscAg was a
681 homogenate of adult worms *A. lumbricoides* obtained from infected hu-
682 mans. Supernatants were stored at -20°C until quantification using Luminex
683 kits (Biosource, Camarillo, USA) on a Luminex 200® Workstation (Qiagen,
684 Venlo, the Netherlands). Tumor necrosis factor (TNF) and interleukin (IL)-
685 10 were quantified in all supernatants whilst interferon (IFN)- γ , IL-2 and IL-
686 5 were quantified only in 72h supernatants. Samples with TNF levels \geq 250

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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

1. Hotez PJ, et al. (2008) Helminth infections: the great neglected tropical diseases. *The Journal of clinical investigation* 118(4):1311-1321.
2. Taylor-Robinson DC, Maayan N, Soares-Weiser K, Donegan S, & Garner P (2015) Deworming drugs for soil-transmitted intestinal worms in children: effects on nutritional indicators, haemoglobin, and school performance. *Cochrane Database Syst Rev* 7:CD000371.
3. McSorley HJ & Maizels RM (2012) Helminth infections and host immune regulation. *Clinical microbiology reviews* 25(4):585-608.
4. Cooper PJ, et al. (2000) Albendazole treatment of children with ascariasis enhances the vibriocidal antibody response to the live attenuated oral cholera vaccine CVD 103-HgR. *The Journal of infectious diseases* 182(4):1199-1206.
5. Elias D, et al. (2001) Effect of deworming on human T cell responses to mycobacterial antigens in helminth-exposed individuals before and after bacille Calmette-Guerin (BCG) vaccination. *Clinical and experimental immunology* 123(2):219-225.
6. Esen M, et al. (2012) Reduced antibody responses against *Plasmodium falciparum* vaccine candidate antigens in the presence of *Trichuris trichiura*. *Vaccine*.
7. Rook GA (2009) Review series on helminths, immune modulation and the hygiene hypothesis: the broader implications of the hygiene hypothesis. *Immunology* 126(1):3-11.
8. Wammes LJ, Mpairwe H, Elliott AM, & Yazdanbakhsh M (2014) Helminth therapy or elimination: epidemiological, immunological, and clinical considerations. *The Lancet. Infectious diseases* 14(11):1150-1162.
9. Ottesen EA, Hiatt RA, Cheever AW, Sotomayor ZR, & Neva FA (1978) The acquisition and loss of antigen-specific cellular immune responsiveness in acute and chronic schistosomiasis in man. *Clinical and experimental immunology* 33(1):37-47.
10. Danilowicz-Luebert E, O'Regan NL, Steinfeldt S, & Hartmann S (2011) Modulation of specific and allergy-related immune responses by helminths. *Journal of biomedicine & biotechnology* 2011:821578.
11. Ricci ND, et al. (2011) Induction of CD4(+)CD25(+)FOXP3(+) regulatory T cells during human hookworm infection modulates antigen-mediated lymphocyte proliferation. *PLoS neglected tropical diseases* 5(11):e1383.
12. Wammes LJ, et al. (2010) Regulatory T cells in human geohelminth infection suppress immune responses to BCG and *Plasmodium falciparum*. *European journal of immunology* 40(2):437-442.
13. Babu S, et al. (2009) Human type 1 and 17 responses in latent tuberculosis are modulated by coincident filarial infection through cytotoxic T lymphocyte antigen-4 and programmed death-1. *The Journal of infectious diseases* 200(2):288-298.
14. Cooper PJ, et al. (2008) Repeated treatments with albendazole enhance Th2 responses to *Ascaris lumbricoides*, but not to aeroallergens, in children from rural communities in the Tropics. *The Journal of infectious diseases* 198(8):1237-1242.
15. Flohr C, et al. (2010) Reduced helminth burden increases allergen skin sensitization but not clinical allergy: a randomized, double-blind, placebo-controlled trial in Vietnam. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 40(1):131-142.
16. Wright VJ, et al. (2009) Early exposure of infants to GI nematodes induces Th2 dominant immune responses which are unaffected by periodic anthelmintic treatment. *PLoS neglected tropical diseases* 3(5):e433.

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.

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17. Diallo TO, et al. (2010) Schistosomiasis coinfection in children influences acquired immune response against *Plasmodium falciparum* malaria antigens. *PLoS one* 5(9):e12764.
18. Hartgers FC, et al. (2009) Responses to malarial antigens are altered in helminth-infected children. *The Journal of infectious diseases* 199(10):1528-1535.
19. Metenou S, et al. (2011) Filarial infection suppresses malaria-specific multifunctional Th1 and Th17 responses in malaria and filarial coinfections. *J Immunol* 186(8):4725-4733.
20. Wiria AE, et al. (2013) The effect of three-monthly albendazole treatment on malarial parasitemia and allergy: a household-based cluster-randomized, double-blind, placebo-controlled trial. *PLoS one* 8(3):e57899.
21. Steel C & Nutman TB (2003) CTLA-4 in filarial infections: implications for a role in diminished T cell reactivity. *J Immunol* 170(4):1930-1938.
22. Riley JL (2013) Combination checkpoint blockade--taking melanoma immunotherapy to the next level. *The New England journal of medicine* 369(2):187-189.
23. Hotchkiss RS & Moldawer LL (2014) Parallels between cancer and infectious disease. *The New England journal of medicine* 371(4):380-383.
24. Namwanje H, Kabatereine NB, & Olsen A (2011) Efficacy of single and double doses of albendazole and mebendazole alone and in combination in the treatment of *Trichuris trichiura* in school-age children in Uganda. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 105(10):586-590.
25. Wilson S, et al. (2014) Posttreatment changes in cytokines induced by *Schistosoma mansoni* egg and worm antigens: dissociation of immunity- and morbidity-associated type 2 responses. *The Journal of infectious diseases* 209(11):1792-1800.
26. Mizuno K, Toyoda Y, Fukami T, Nakajima M, & Yokoi T (2011) Stimulation of pro-inflammatory responses by mebendazole in human monocytic THP-1 cells through an ERK signaling pathway. *Archives of toxicology* 85(3):199-207.
27. Chandra RK (2002) Nutrition and the immune system from birth to old age. *European journal of clinical nutrition* 56 Suppl 3:S73-76.
28. Mulyanjia E, et al. (2014) Immune activation alters cellular and humoral responses to yellow fever 17D vaccine. *The Journal of clinical investigation* 124(7):3147-3158.
29. Obiero JM, et al. (2015) Impact of malaria pre-exposure on anti-parasite cellular and humoral immune responses after controlled human malaria infection. *Infection and immunity*.
30. Elliott DE & Weinstock JV (2012) Helminth-host immunological interactions: prevention and control of immune-mediated diseases. *Annals of the New York Academy of Sciences* 1247:83-96.
31. Endara P, et al. (2010) Long-term periodic anthelmintic treatments are associated with increased allergen skin reactivity. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 40(11):1669-1677.
32. Wiria AE, et al. (2010) Does treatment of intestinal helminth infections influence malaria? Background and methodology of a longitudinal study of clinical, parasitological and immunological parameters in Nangapanda, Flores, Indonesia (ImmunoSPIN Study). *BMC infectious diseases* 10:77.
33. WHO (2006) WHO Child Growth Standards: Length/height-for-age, weight-for-age, weight-for-length, weight-for-height and body mass index-for-age: Method and development. Geneva: World Health Organization:312.

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