Immune modulation by helminths and the impact on the development of type 2 diabetes

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THE EFFECT OF HELMINTHS ON GRANULOCYTE ACTIVATION: A CLUSTER-RANDOMIZED PLACEBO-CONTROLLED TRIAL IN INDONESIA

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ABSTRACT

Background

Eosinophils are a prominent cell type in the host response to helminths, and some evidence suggests that neutrophils might also play a role. However, little is known about the activation status of these granulocytes during helminth infection.

Methods

We analysed the expression of eosinophil and neutrophil activation markers in peripheral blood by flow cytometry, and measured serum levels of eosinophil granule proteins in 300 subjects residing in an area endemic for soil-transmitted helminths (STH). The data generated are on samples before and after 1 year of 3-monthly albendazole treatment.

Results

Anthelmintic treatment significantly reduced the prevalence of STH. While eosinophil numbers were significantly higher in STH-infected subjects compared to those uninfected and significantly decreased following albendazole treatment, there was no effect exerted by the helminths on either eosinophil nor neutrophil activation. Although at baseline, eosinophil granule protein levels were not different between STH-infected and uninfected subjects, treatment significantly reduced the levels of eosinophil-derived neurotoxin (EDN) in those infected at baseline.

Conclusions

These results show that besides decreasing eosinophil numbers, anthelmintic treatment does not significantly change the activation status of eosinophils, nor of neutrophils, and the only effect seen was a reduction on circulating levels of EDN.

Clinical trial registration

http://www.isrctn.com/ISRCTN75636394

INTRODUCTION

Eosinophilia is a well-known hallmark of helminth infections. While these bone marrow-derived, innate cells reside primarily in the tissues where they can survive up to two weeks (1), elevated frequencies are found in peripheral blood during helminth infections and, to a lesser extent, in allergic diseases. Although in vitro, eosinophils have been shown to be able to kill helminths (2-5), their role during helminth infections remains uncertain, as in vivo depletion of eosinophils has shown inconclusive results regarding their protective efficacy (6), and helminths such as *Trichinella* larvae, appear to benefit from the presence of eosinophils (7, 8). Furthermore, eosinophils are increasingly being recognized as cells that contribute to tissue, metabolic and immune homeostasis (9). Adipose tissue eosinophils for example, play a crucial role in maintaining insulin sensitivity through the secretion of the cytokines IL-4 and IL-13 (10).

Whereas increased eosinophil concentrations are characteristic of helminth infections, it is the activation status of these cells that drives the eosinophil mediated effects (11). Eosinophils can exist in different states of activation, and this is reflected by the expression of certain surface markers and by the increased serum levels of eosinophil-specific granule proteins. Upon recruitment to inflammatory sites, eosinophils alter the expression of a number of surface molecules that are involved in tethering, rolling along and adhesion to endothelial cells, followed by trans-endothelial migration into the tissue (12). The presence of these surface molecules (e.g. CD11b, CD35, CD69, CD66b and CD62L) on peripheral blood eosinophils is a useful indicator of cellular activation, during both helminth infections (13-15) and allergic diseases (16-19). Moreover, eosinophils respond to eotaxin, a chemokine responsible for eosinophil recruitment into tissues, by upregulating CD11b and shedding CD62L (20, 21).

Upon eosinophil degranulation, cytotoxic proteins including major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO) are released from secondary granules, and increased serum levels of these granule proteins are also considered to be a measure of eosinophil activation (19).

While neutrophils are known primarily for their potent anti-bacterial properties through the secretion of their granule proteins such as myeloperoxidase (MPO), they have also been shown to kill helminth larvae (22-24), and levels of MPO were significantly elevated in subjects infected with *Strongyloides stercoralis* (25) indicating that neutrophils can also become activated during helminth infections. Similar to eosinophils, neutrophil activation is associated with the upregulation of several surface molecules including CD11b, CD35, CD66b and the shedding of CD62L (16, 18). Although the expression of these markers and their upregulation or shedding in response to *N*-Formyl-Met-Leu-Phe (fMLF) have been used as a very sensitive measure of neutrophil (pre-)activation in patients with allergic asthma (16-18), it has never been studied in the context of helminth infections.

In this study, we assessed the effect of helminth infections on the activation status and responsiveness of both eosinophils and neutrophils. Although previous work has shown

that helminth infections are associated with increased eosinophil activation by assessing either activation markers (13-15) or eosinophil granule proteins (11, 25-28), these studies often had a cross-sectional design, a relatively small sample size, and did not assess eosinophil responsiveness. To this end, we measured the expression of eosinophil and neutrophil activation markers by flow cytometry, both ex vivo and after in vitro stimulation, in subjects infected with soil-transmitted helminths (STH), before and after 1 year of anthelmintic treatment. In addition, serum levels of eosinophil granule proteins were assessed. This study is part of a large cluster-randomized, placebo-controlled trial (29) and therefore the first placebo-controlled trial investigating the effect of helminths on granulocyte activation.

METHODS

Study design

This report describes a nested study within the SugarSPIN trial (29), a household-based cluster-randomized double-blind trial that was conducted in Nangapanda, Ende district of Flores Island (East Nusa Tenggara), Indonesia. After randomisation, all study subjects received either a single tablet of albendazole (400 mg) or matching placebo (tablets from PT Indopharma Pharmaceutical, Bandung, Indonesia) for three consecutive days under direct supervision from the research team members. This treatment regimen was given every three months for a total of four rounds (maximum of 12 tablets in total), between May 2014 and February 2015.

Although the study was aimed at subjects aged 16 and above, all subjects in the study area, except children below 2 years of age and pregnant women, were included in the trial to avoid cross-contamination between household members. Subjects aged 16 and above underwent clinical and laboratory examination, excluding subjects with active treatment for diabetes mellitus and serious concomitant diseases. Written informed consent was obtained from participants prior to the study. The study was approved by the ethics committee of the Faculty of Medicine, Universitas Indonesia (FKUI) (ref: 549/H2·F1/ETIK/2013), and filed by the ethics committee of Leiden University Medical Center (LUMC), the Netherlands. The trial is registered as a clinical trial (Ref: ISRCTN75636394).

Study population

The randomization for the total study was based on 752 households comprising 3698 individuals, resulting in 1825 (377 houses) and 1741 (375 houses) subjects in the placebo and albendazole group, respectively (Supplementary Figure S1). An additional randomization was performed on the 2406 subjects aged 16 and above, in order to study immune mechanisms in more detail (29). For this subgroup, we aimed to select one subject per household and stratified by age group (16-36 years, 36-56 years, and >56 years) to ensure that sufficient numbers of all groups were represented. Randomization was based on households. This resulted in a total of 300 subjects who were included for

immunological studies (152 subjects on placebo and 148 subjects in albendazole group) and randomly selected, paired samples from 195 subjects were subsequently used for flow cytometric analysis.

We also collected venous blood of 9 healthy volunteers which had not been exposed to helminth infections, hereafter referred to as "Europeans". We used the whole blood samples for flow cytometric analysis to assess granulocyte phenotype and response in naïve subjects.

Parasitological examination

Fresh stool samples were frozen at -20°C in the field centre and subsequently at -80°C at the Department of Parasitology of FKUI. Stool DNA isolation and real-time PCR were performed pairwise (baseline and follow-up). DNA isolation from stool was performed as described elsewhere (30, 31). Multiplex real-time polymerase chain reaction (PCR) was performed to simultaneously detect the presence of hookworm (*Ancylostoma duodenale*, *Necator americanus*), *Ascaris lumbricoides*, *Trichuris trichiura*, and *Strongyloides stercoralis*, using a method described previously (30). Stool samples were considered positive by PCR when cycle threshold (Ct) values were <50. Since the prevalence of *S. stercoralis* at baseline appeared to be very low (1.4% (4/284)), this species was not included in the analysis.

Sample collection

Blood samples were collected and processed as previously described (29, 32). Briefly, from each subject 3 polystyrene tubes containing 200 µL of heparinised venous blood were pre-incubated for 5 minutes in a 37°C waterbath, followed by a 5 minute-stimulation at 37°C with fMLF (10^{-5} M; Sigma, Saint Louis, MO, USA) or eotaxin (10^{-7} M; R&D systems, Abingdon, UK) or left unstimulated. While both Indonesian and European blood samples were stimulated with fMLF, stimulation with eotaxin was only applied to Indonesian samples. Subsequently, 4 mL of FACS lysing solution (BD Biosciences) was added and after an incubation period of 15 minutes at room temperature, cells were washed with RPMI 1640 containing 10% heat-inactivated foetal calf serum (FCS) and resuspended in RPMI 1640 containing 10% FCS and 10% dimethyl sulfoxyde (DMSO). Cryovials containing the cell suspension were placed at -80°C for a minimum of 4 hours, followed by storage in liquid nitrogen until analysis.

Flow cytometry of granulocyte surface markers

While flow cytometric analysis of the samples was randomly divided over multiple measurement days, all samples belonging to one individual were thawed, stained and measured pairwise (baseline and follow-up) on the same day. After thawing, cells were washed in RPMI 1640 containing 10% FCS and resuspended in FACS buffer (PBS supplemented with 0.5% BSA and 2 mM EDTA). Cells were counted using microscopy and 500.000 white blood cells were stained for 30 minutes at 4°C with anti-CD35-FITC (E11,

Biolegend), anti-CD66b-PerCP/Cy5.5 (G10F5, Biolegend), anti-CD193-PE (5e, Biolegend), anti-CD16-PE/CF594 (3G8, BD Biosciences), anti-CD69-PE/Cy5 (FN50, Biolegend), anti-CD274-PE/Cy7 (MIH1, eBioscience), anti-CD3/CD19/CD20/CD56-APC (UCHT1, HIB19, 2H7, 5.1H11, Biolegend), anti-CD11b-APC/eF780 (ICRF44, eBioscience), anti-CD203c-BV421 (NP4D6, Biolegend), anti-CD14-BV510 (M5E2, Biolegend), anti-CD62L-BV605 (DREG-56, BD Biosciences). Antibody dilutions can be found in Supplementary Table S1. For each antibody, a fluorescence-minus-one (FMO) control sample was included using pooled cells from different subjects. Cells were acquired on a LSR Fortessa flow cytometer (BD Biosciences) and before each measurement a performance run was conducted with cytometer setup and tracking (CS&T) beads (BD Biosciences). Data was analysed in FlowJo software (version 9.9.3) and median fluorescence intensity (MFI) data are displayed. Representative gating schemes to select eosinophil and neutrophils are shown in Supplementary Figure S2. As all cells of each sample were acquired, the absolute number of eosinophils and neutrophils which was analysed, would differ per sample.

After gating all leukocyte populations (eosinophils, neutrophils, basophils, monocytes, lymphocytes), percentages of eosinophils and neutrophils were calculated relative to the total amount of white blood cells. The absolute eosinophil count (AEC) reflects the number of eosinophils in 200 µl blood, and was calculated using the proportion of eosinophils and the white blood cell count after thawing.

As described previously (32), the detection of CD62L on eosinophils was impaired as a result of fixation and this marker was therefore not included in the analysis as activation marker for eosinophils.

Serum eosinophil granule proteins

The serum concentrations of eosinophil granule proteins, MBP, ECP, EDN and EPO were measured in a suspension array assay in multiplex as previously described (33). As the levels of EPO were below the limit of detection of 6 ng/ml in every sample, this protein was excluded from analysis. Eosinophil granule proteins were measured in the total subgroup selected for immunological studies (300 and 258 subjects at baseline and follow up, respectively). In 5/558 samples, one of the proteins could not be detected while in 1/558 samples, neither MBP, ECP, nor EDN could be detected.

Statistical analysis

For continuous variables, normally distributed data were presented as mean and standard deviation, while non-normally distributed data (eosinophil count, total IgE, eosinophil granule proteins and eosinophil activation markers) were presented as geometric mean and 95% confidence interval, and log-transformed for analyses. Categorical data such as infection prevalence were expressed as proportions. Comparisons between STH-infected and uninfected subjects at baseline were performed with Student's *t* test. Comparisons between Indonesian STH-infected, Indonesian uninfected and European subjects were

performed with ANOVA followed by Tukey's multiple comparisons test. ANOVA followed by a Dunnett's multiple comparison test was used to test granulocyte counts after stratifying subjects by helminth species. To determine the relationship between serum levels of eosinophil granular proteins and eosinophil counts Spearman's rank correlation was used. Paired *t* tests were performed to assess the responsiveness of granulocytes to eotaxin or fMLF. *P* values < .05 were considered statistically significant.

The effect of anthelmintic treatment on eosinophil counts, eosinophil granule proteins and eosinophil activation markers was assessed using an intention-to-treat approach, applying mixed models to account for the correlation within households. Two random effects were used: to model clustering within households a random household specific intercept was used and to model correlation within subjects random subject-specific intercept was used. Parameter estimates for treatment effect and 95% CIs were reported. The reported *p* values were obtained using a likelihood ratio test comparing the model with and without the treatment effect. For the binary outcome (helminth infection status), a logistic model was used with random household effects and random subject effects. All models were fitted using the lme4 package (R software).

RESULTS

Study population

At baseline, 300 subjects were included for immunological studies (152 subjects and 148 subjects in the placebo and the albendazole group respectively) (Supplementary Figure S1). The loss to follow up was 14% which was mainly due to movement out of the village. Baseline characteristics of the study participants are shown in Table 1, while details of a subset of the study population (n=195), used to study granulocyte activation markers by flow cytometry can be found in Supplementary Table S1.

At baseline 59.2% (168/284) of the individuals were infected with one or more helminth species, with hookworm infection being the most prevalent. In all subjects treated with albendazole in the SugarSPIN trial, the prevalence of helminth infection was 55.4% before, and 11.3% after treatment (30). Similar to this result, we observed that albendazole treatment reduced the percentage of subjects with any helminth (55.9% (80/143) to 9.2% (11/120) in the albendazole arm vs 62.4% (88/141) to 51.2% (62/121) in the placebo arm, *P* < .0001). The highest reduction was seen for hookworm, followed by *A. lumbricoides* and *T. trichiura* infection (Supplementary Figure S3).

Granulocyte counts in peripheral blood

At baseline, the frequency of eosinophils in whole blood was significantly higher in infected subjects compared to non-infected subjects (Geomean (95% CI), STH+ 7.0 (6.3-7.8)% vs STH- 5.5 (4.7-6.5)%, *P* = .01) (Figure 1A). Irrespective of coinfection with other helminths, subjects infected with hookworm showed the highest eosinophil counts (7.9 (6.9-8.9)%) (Figure 1A). As observed in the whole SugarSPIN trial (34), anthelmintic

Table 1. Baseline characteristics of the study population.

	n	Placebo	n	Albendazole
Age (mean in years, SD)	151	46.7 (13.4)	148	46.2 (16.2)
Sex (female, n, %)	151	100 (66.2)	148	86 (58.1)
BMI (kg/m^2) (mean, SD)	149	23.1 (4.2)	147	22.5 (4.2)
Total IgE (IU/mL) (GM, 95% CI)	150	663 (506-870)	148	672 (514-878)
Eosinophil count* (GM, 95% CI)	148	5.5 (4.9-6.2)	146	6.1 (5.5-6.8)
MBP (ng/mL) (GM, 95% CI)	150	978 (859-1154)	147	969 (830-1132)
ECP (ng/mL) (GM, 95% CI)	150	908 (756-1124)	147	935 (786-1111)
EDN (ng/mL) (GM, 95% CI)	149	346 (295-408)	147	380 (326-442)
Helminth infection by PCR (n,%)	141	88 (62.4)	143	80 (55.9)
A. lumbricoides	141	36 (25.5)	143	22 (15.4)
Hookworm	141	62 (44.0)	143	53 (37.1)
T. trichuris	141	50 (35.5)	143	36 (25.2)

*Determined by using a Giemsa-stained peripheral thin blood smear. Abbreviations: BMI body mass index; ECP eosinophil cationic protein; EDN eosinophil-derived neurotoxin; GM geometric mean; IgE immunoglobulin; MBP major basic protein; PCR polymerase chain reaction; SD standard deviation.

treatment effectively reduced eosinophil counts, especially in those infected with helminths at baseline (Estimated treatment effect (95% CI), -0.187 (-0.258 – -0.117), P = .01) (Figure 1C). Neutrophil counts did not differ between uninfected and infected subjects at baseline (P = 0.59, Figure 1B). Interestingly, albendazole treatment significantly increased the frequency of neutrophils in peripheral blood in those who were infected with helminths at baseline (0.041 (0.017 – 0.066), P < .01) (Figure 1D). While Europeans exhibited significantly lower eosinophil frequencies (Geomean (95% CI), 1.9 (0.9-3.7)%) compared to Indonesians, irrespective of current helminth infections, similar frequencies of neutrophils were observed (53 (45-62)%).

Eosinophil activation status and responsiveness

At baseline, the intensity of the eosinophil activation markers CD11b, CD35, CD66b and CD69 was similar in STH-infected and uninfected subjects (Table 2). Albendazole treatment did not influence the expression of these markers (Table 3). Neither at community level, nor when analysing subjects with helminth infection as baseline. The comparison with Europeans revealed a lower intensity of CD35 in Indonesian subjects, both STH-infected and uninfected, while other markers did not differ (Table 2).

Whole blood stimulated with eotaxin or fMLF was used to assess the responsiveness of eosinophils. The intensity of CD11b, CD35, CD66b and CD69 on eotaxin- or fMLF-stimulated eosinophils did not differ between uninfected subjects and those infected with helminths at baseline (Supplementary Figure S4 A-B). Moreover, albendazole treatment did not affect the responsiveness (data not shown). Interestingly, not all individuals responded to eotaxin or fMLF by upregulating the activation markers. In half of the subjects, there

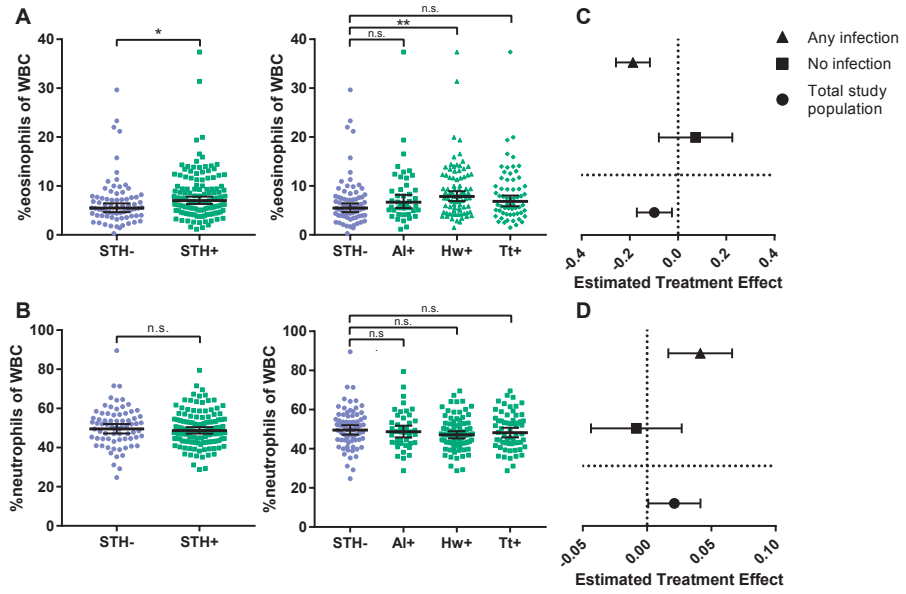

Figure 1. Granulocyte counts. Percentage of eosinophils (*A*) and neutrophils (*B*) relative to the total number of white blood cells (WBC). Counts at baseline are shown displaying geomeans and corresponding 95% confidence intervals (STH- n=73, STH+ n=118, Al+ n=42, Hw+ n=81, Tt+ n=61). The effect of anthelmintic treatment eosinophil (*C*) and neutrophil (*D*) counts is displayed with the corresponding 95% confidence intervals (n=195). Differences between STH- and STH+ subjects were tested with Student's *t* test. Differences between STH-, Al+, Hw+ and Tt+ subjects were tested with ANOVA followed by a Dunnett's multiple comparison test. Treatment effect was assessed using mixed models (see Methods). *P <.05; **P <.01; n.s. not significant; STH Soil-transmitted helminths; Al *A. lumbricoides*; Hw Hookworm; Tt *T. trichuria*.

Table 2. Expression of granulocyte activation markers at baseline.

	STH- (n=73)	STH+ (n=118)	EU (n=9)
Eosinophils			
CD11b	1444 (1337-1559)	1376 (1297-1459)	1700 (1551-1864)
CD35	3269 (3144-3435)	3254 (3150-3360)	3915 (3688-4155)[a,b]
CD66b	2100 (2031-2172)	2055 (2004-2108)	2046 (1825-2295)
CD69	783 (735-834)	772 (737-809)	677 (632-725)
Neutrophils			
CD11b	1151 (1107-1196)	1172 (1141-1203)	1387 (1238-1554)[a,b]
CD35	1571 (1457-1695)	1618 (1519-1722)	1413 (1101-1816)
CD66b	581 (553-609)	592 (568-617)	590 (532-654)
CD62L	748 (676-827)	788 (726-856)	1358 (1262-1462)[a,b]

Geomean of MFI and corresponding 95% confidence intervals are shown. [a] P <.05 analysed using ANOVA Tukey statistical test for comparison with Indonesian STH- subjects. [b] P <.05 analysed using ANOVA Tukey statistical test for comparison with Indonesian STH+ subjects. STH soil-transmitted helminths.

Table 3. The effect of anthelmintic treatment on granulocyte activation markers.

	All (n=195)	STH- (n=73)	STH+ (n=118)
Eosinophils	Estimated treatment effect (95% CI), p value		
CD11b	0.024 (-0.010-0.058), p=0.18	0.012 (-0.045-0.069), p=0.69	0.039 (-0.005-0.082), p=0.09
CD35	0.009 (-0.006-0.024), p=0.23	0.008 (-0.018-0.034), p=0.54	0.015 (-0.004-0.034), p=0.13
CD66b	0.003 (-0.008-0.014), p=0.55	0.004 (-0.016-0.024), p=0.73	0.007 (-0.007-0.020), p=0.32
CD69	-0.016 (-0.038-0.007), p=0.19	-0.029 (-0.067-0.009), p=0.14	0.003 (-0.026-0.031), p=0.86
Neutrophils			
CD11b	0.003 (-0.018-0.023), p=0.81	0.021 (-0.014-0.055), p=0.25	-0.007 (-0.032-0.018), p=0.60
CD35	-0.010 (-0.041-0.021), p=0.53	0.001 (-0.058-0.061), p=0.96	-0.010 (-0.045-0.025), p=0.59
CD66b	0.015 (-0.002-0.032), p=0.09	0.023 (-0.006-0.052), p=0.12	0.010 (-0.012-0.032), p=0.39
CD62L	0.029 (-0.029-0.087), p=0.33	0.053 (-0.041-0.146), p=0.27	-0.009 (-0.082-0.065), p=0.82

Treatment effect was assessed using mixed models (see Methods). Estimated treatment effects are displayed with the corresponding 95% confidence intervals. *P* values were obtained using a likelihood ratio test comparing the model with and without the treatment effect. STH soil-transmitted helminths.

was an increase in the MFI of the activation markers whereas in the other half the MFI of markers decreased after stimulation (Supplementary Figure S4 C-D). Of note, in those who had a low intensity of markers before stimulation, the expression of the markers went up whereas the opposite was seen in subjects who had a high marker expression (Supplementary Figure S4 C-D). However, this observation could not be associated with the infection status, since in both infected and uninfected subjects the same was seen. In Europeans, most subjects responded to fMLF by upregulating the activation markers, also reflected in a stronger responsiveness (Supplementary Figures S4 B).

Neutrophil activation status and responsiveness

We found no difference in the expression levels of neutrophil activation markers (CD11b, CD35, CD66b, CD62L) between STH-infected and uninfected subjects at baseline (Table 2), and treatment did not alter the intensity of these markers (Table 3). When compared to Europeans, levels of both CD11b and CD62L were lower in Indonesians, irrespective of their current infection status (Table 2). Although neutrophils strongly responded to fMLF by upregulating CD11b, CD35 and CD66b, while shedding CD62L, the responsiveness was similar in all subjects, irrespective of their helminth infection status (Supplementary

Figure S5). Albendazole treatment did not affect neutrophil responsiveness (data not shown). When neutrophils in Europeans were considered, the responsiveness was more marked compared to Indonesians (Supplementary Figure S5).

Eosinophil granule proteins

Serum levels of MBP, ECP and EDN were similar in STH-infected and uninfected subjects at baseline (Geomean, (95% CI), MBP, STH+ 979 (864-1156) ng/ml vs STH- 910 (766-1063) ng/ml, *P* = .52; ECP, STH+ 892 (748-1077) ng/ml vs STH- 920 (764-1129) ng/ml, *P* = .82; EDN, STH+ 362 (313-423) ng/ml vs STH- 353 (297-419) ng/ml, *P* = .83) (Figure 2A). No relation was found between protein concentrations and different helminth species or the total number of helminth species a subject was infected with (data not shown). Levels of EDN correlated with absolute eosinophil counts (AEC) at baseline (r=0.54, *P* < .01), however, the correlation coefficient for MBP, although statistically significant, was weak (MBP: r = 0.27, *P* < .01) and when considering ECP no correlation could be found with eosinophil counts (ECP: r = 0.03, *P* = .69) (Supplementary Figure S6). Albendazole treatment decreased the level of MBP (Estimated treatment effect (95% CI), -0.070 (-0.124 – -0.015), *P* = .01) and EDN (-0.060 (-0.121 – 0.0002), *P* = .05),

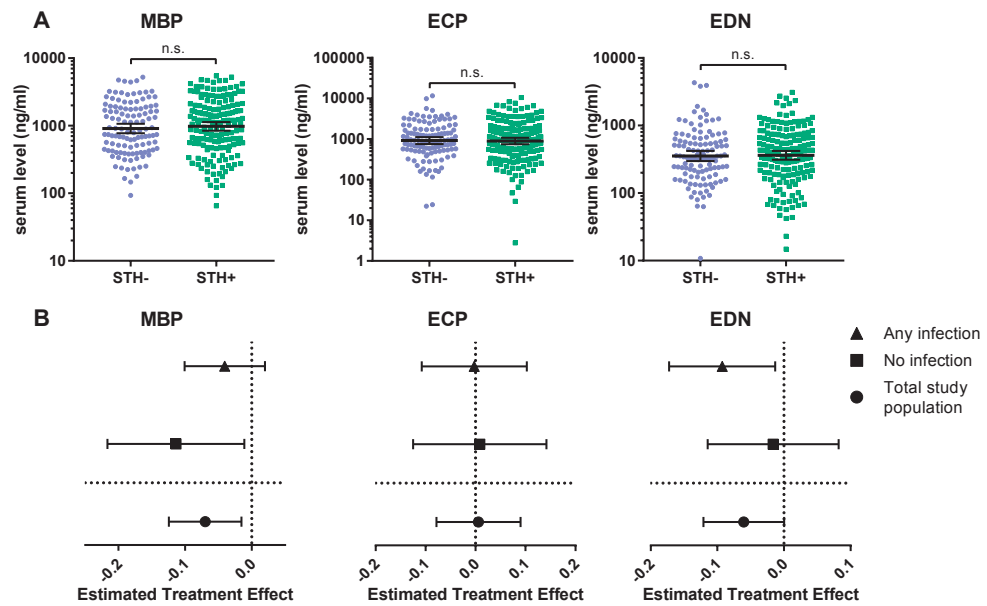

Figure 2. Eosinophil granule proteins. Major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN) concentrations in serum at baseline (*A*) and the effect of albendazole treatment (*B*) are shown. Lines represent geomeans and corresponding 95% confidence intervals (STH- n=116, STH+ n=168 (*A*)). The effect of anthelmintic treatment is displayed with the corresponding 95% confidence intervals (n=299 (*B*)). Differences between STH- and STH+ subjects were tested with Student's *t* test. Treatment effect was assessed using mixed models (see Methods). n.s. not significant; STH Soil-transmitted helminths.

whereas the level of ECP did not change (0.006 (-0.078-0.090), *P* = .93) at community level (Figure 2B). When analysing those infected with helminths at baseline, EDN levels significantly decreased after treatment (-0.093 (-0.172 – -0.013), *P* = .02).

DISCUSSION

Eosinophil numbers are associated with helminth infections. We hypothesized that not only eosinophil numbers, but also their activation status would be affected by the presence of helminths. As a measure of activation, a range of activation markers in peripheral blood eosinophils and neutrophils were assessed, as well as the responsiveness to stimulation. In addition, circulating levels of eosinophil granule proteins were measured as a marker for the activation status of eosinophils, not only in blood but also in tissues. This study was nested within a cluster-randomized, double-blind placebo-controlled trial, conducted in a rural area in Indonesia (29). To our knowledge, this is the first placebo-controlled trial studying the effect of helminth infections on granulocyte activation and derives strength from its design and large sample size.

Although the number of eosinophils was higher in STH-infected compared to uninfected subjects and decreased significantly after albendazole treatment, the intensities of eosinophil, as well as neutrophil, activation markers were not affected by helminths and did not change upon anthelmintic treatment. This is in contrast to what has been reported (11, 13-15, 25-28). Previously, Mawhorter et al. showed an elevated percentage of CD69, CD66 and CD81 positive eosinophils in 18 subjects recruited in the USA, infected with one or more species of seven helminths, and found CD66 to be decreased in the five subjects that were followed after short term anthelmintic treatment (13). In a study conducted in Brazil, the observation that the frequency of $CD23^{+}$ eosinophils was increased while that of $CD62L^{+}$ cells was decreased in subjects infected with *Schistosoma mansoni*, led to the conclusion that eosinophils were chronically activated during infection (15). However, this conflicted with a lower frequency of $CD69^{+}$ eosinophils, indicating little early activation of eosinophils (15). In another study in Brazil, Fujiwara et al. demonstrated a highly activated state of eosinophils in subjects infected with hookworms compared to uninfected individuals (14). None of these studies, in contrast to ours, included uninfected subjects from the endemic regions. Moreover, they included a relatively small number of study subjects (varying from 23 to 35 individuals), and perhaps more importantly, the antibody staining was directly performed on fresh blood. As we were limited by the infrastructure at the field study site with no direct access to a flow cytometer, we developed a method to analyse granulocyte activation markers in cryopreserved, fixed whole blood (32). As previously described, it was observed that marker intensities varied when comparing fresh and fixed granulocytes, most likely due to intracellular staining and increased eosinophil autofluorescence as a consequence of fixation (32). However, we showed that the responsiveness to stimuli could still be clearly measured after fixation. Nevertheless, it is possible that the use of fresh cells allows smaller differences to be detected.

By taking along European subjects, we could compare the results with naïve eosinophils and neutrophils from individuals with lower exposure to microorganisms and helminths. The eosinophils from Indonesians had a lower expression of the activation marker CD35 and a lower responsiveness to fMLF. With respect to neutrophils, there was an interesting observation that both CD62L and CD11b showed a lower expression in Indonesians. While lower CD11b on neutrophils from Indonesians would indicate a lower activation status of these cells, the lower expression of CD62L, which is shed by activated neutrophils, would suggest a higher activation status of neutrophils in Indonesians, contradicting the CD11b data. However, the loss of CD62L has also been associated with aged neutrophils (35) and therefore, it is possible that in Indonesia, the higher exposure of the granulocytes to microbes or inflammation, results in more aged granulocytes with lower responsiveness. Indeed, this is supported by the lower activation of neutrophils from Indonesian subjects by fMLF compared to the response of neutrophils from Europeans. However, future studies are needed to clarify this further.

Whereas the expression of activation markers was assessed in circulating eosinophils, mature eosinophils are predominantly tissue dwelling cells and serum levels of eosinophil granule proteins are thought to be an indirect measure of degranulation in the tissues. MBP, being stored in the core of secondary granules is the most abundant protein and its release is toxic to helminths (1). EDN and ECP, also known as RNase 2 and RNase 3 respectively, both have ribonuclease activity and can be found in the granule matrix (1). Whereas ECP is cytotoxic to helminth larvae, EDN seems to be less efficient in killing helminths (2). In contrast to our hypothesis, we observed no differences in the levels of eosinophil granule proteins between helminth infected and uninfected subjects. It should be noted that the study area was highly endemic for STH infections (36) and perhaps exposure to an environment contaminated with parasite eggs or infective larvae can lead to altered eosinophil homeostasis and maturity/activation, thereby masking the potential difference between currently infected and uninfected subjects.

Elevated eosinophil cationic protein levels have previously been described in subjects infected with filaria (*Onchocerca volvulus* (26), *Loa Loa* (37), *Wuchereria bancrofti* (26)), soil-transmitted helminths (*A. lumbricoides* (27), hookworm (27), and *S. stercoralis* (25)) and *S. mansoni* (26, 28). Whereas most studies had a cross-sectional design, two reports described a significant decline in granular protein levels in subjects infected with *S. mansoni* or *S. stercoralis*, respectively, after anthelmintic treatment, indicating a decrease in eosinophil degranulation (25, 28). In our study, albendazole treatment significantly decreased EDN levels in subjects that were infected at baseline, whereas the levels of MBP and ECP did not change. Out of the three proteins, EDN also showed the strongest correlation with the number of eosinophils at baseline and therefore its decrease is likely to reflect the decrease in eosinophil numbers after treatment.

The aim of this study was to investigate the effect of soil-transmitted helminths on granulocyte activation. Based on our results, we can conclude that helminths affect eosinophil numbers in the circulation, but the activation status and responsiveness of

these cells is similar between infected and uninfected subjects, and was not influenced by anthelmintic treatment. The same applies to neutrophils. However, this study should be repeated in an endemic setting with access to a flow cytometer that would allow the analysis of fresh granulocytes to assess activation of these cells, as fixing cells might not allow subtle differences to be detected between helminth infected and uninfected or before and after treatment.

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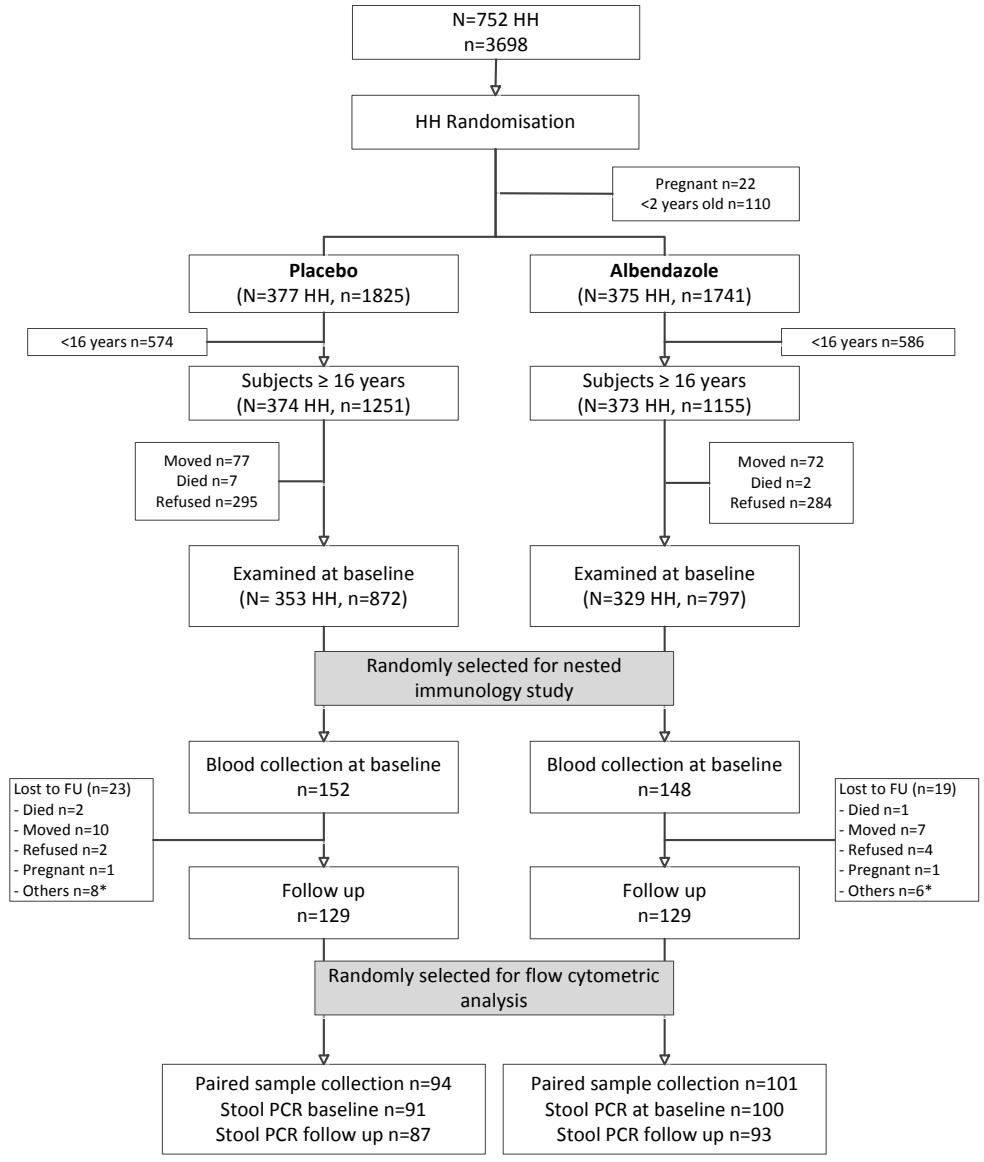
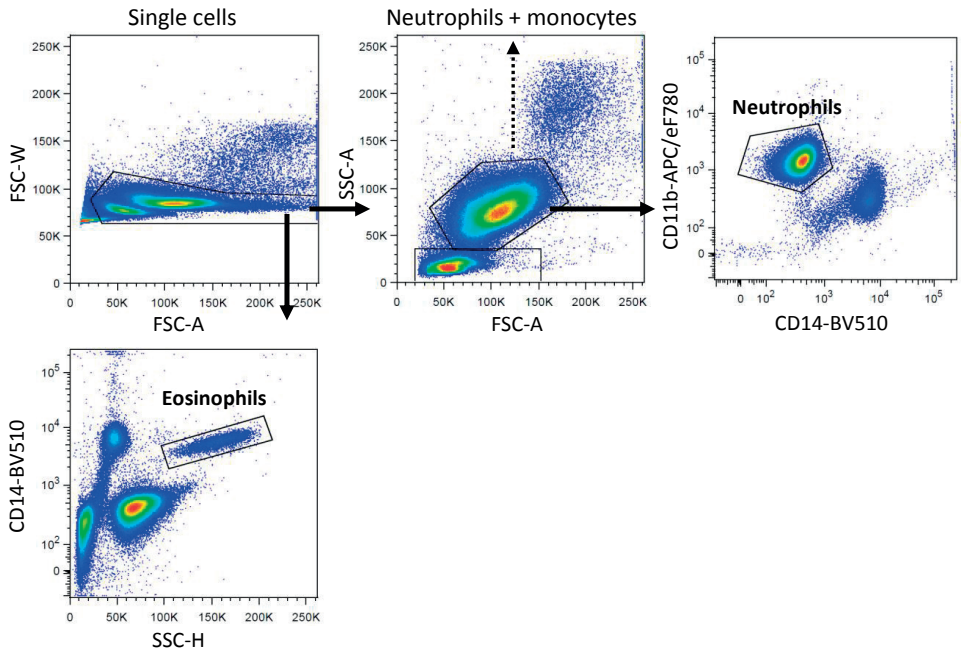
SUPPLEMENTAL DATA

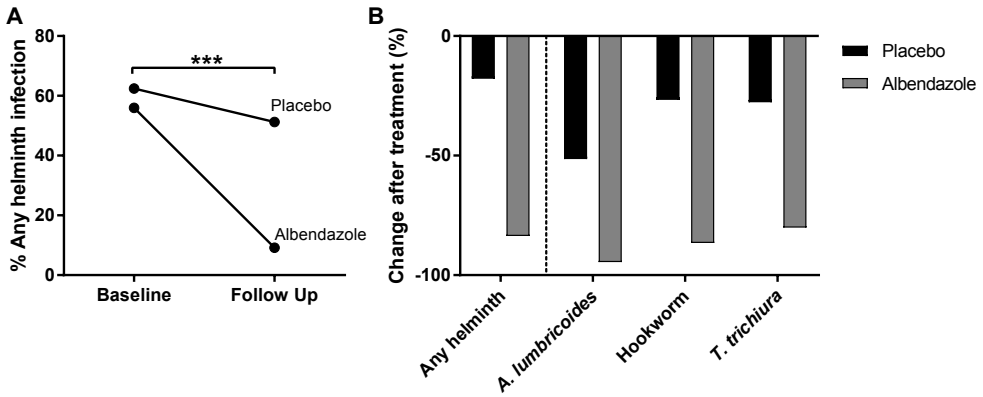


*Other reasons of lost to follow-up were harvesting crops, working on funeral ceremonies, severely ill, hospitalized, nursing mother, etc

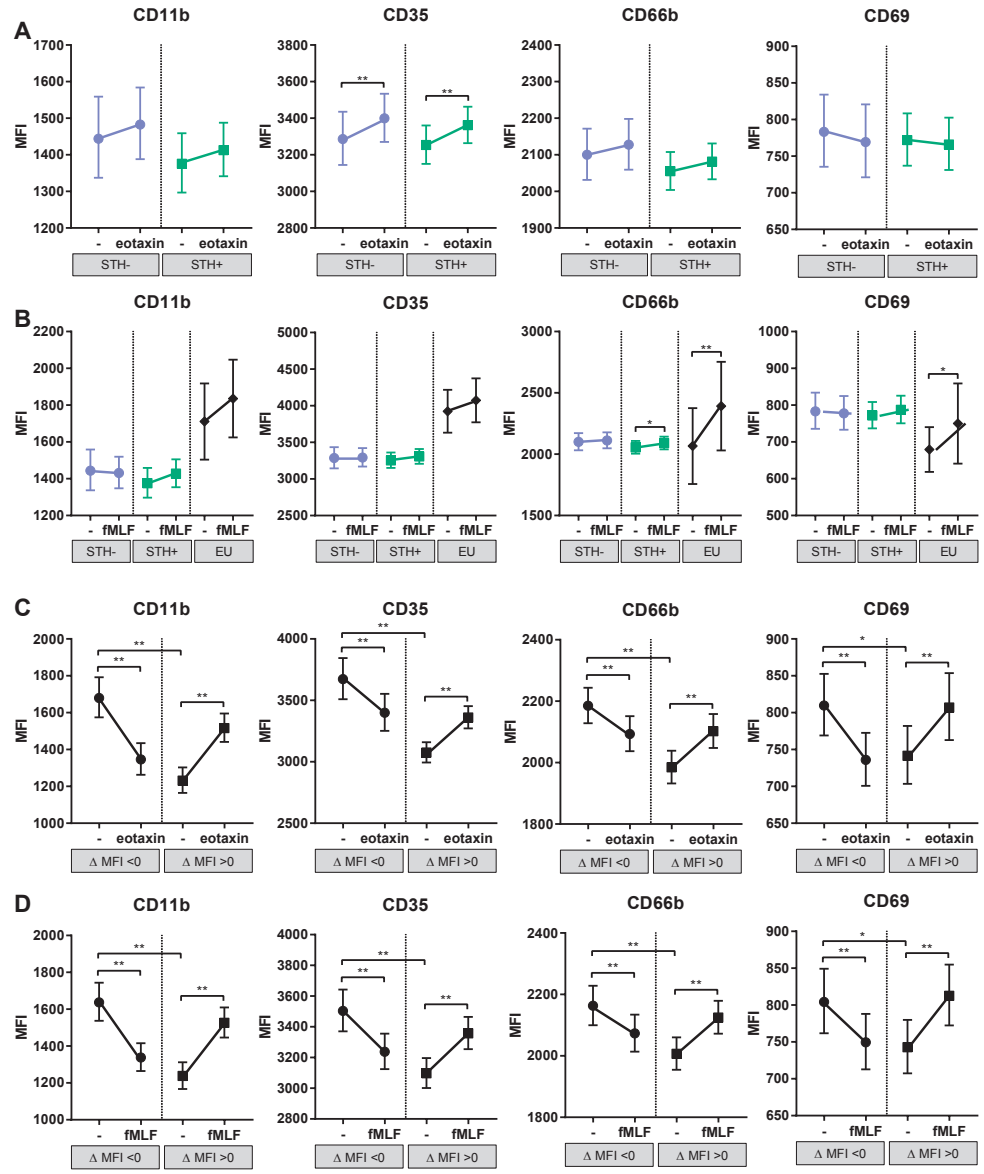
Supplementary Figure S1. Consort diagram.



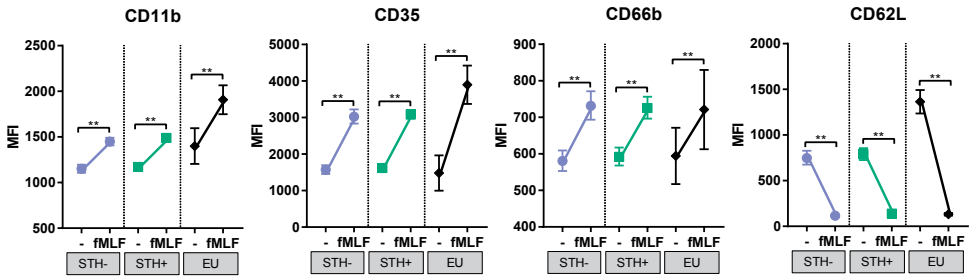
Supplementary Figure S2. FlowJo gating strategy of eosinophils and neutrophils. Eosinophils were selected from single cells and subsequently gated on autofluorescence (CD14-BV510/SSC-H plot). Neutrophils were selected from single cells, then distinguished from monocytes based on their CD11b and CD14 staining.



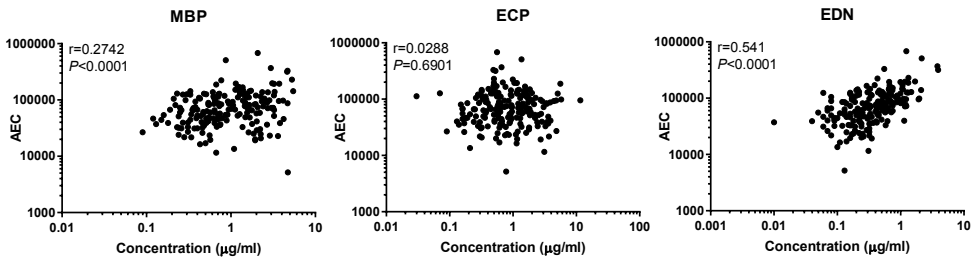
Supplementary Figure S3. The effect of albendazole treatment on the prevalence of soil-transmitted helminths. Percentage of helminth-infected subjects at baseline and following treatment, in placebo (n=141) and albendazole (n=143) treatment arms, as detected by PCR (*A*). Statistical analysis by a logistic model with random household effects and random subject effects; ****P* <.001. Fold change in infection prevalence stratified by helminth species (*B*).



Supplementary Figure S4. Eosinophil responsiveness to eotaxin and fMLF. Median fluorescent intensity (MFI) of CD11b, CD35, CD66b and CD69 of eosinophils left unstimulated (-) and after stimulation with eotaxin (*A, C*) or fMLF (*B, D*). Indonesian subjects were either stratified by helminth infection (*A,B*; STH- (n=73) vs STH+ (n=118)) or by responsiveness (*C,D*; ΔMFI <0 vs ΔMFI >0). Europeans were not included in D, as most responded to fMLF. Geomeans and corresponding 95% confidence intervals are shown. Differences between before and after stimulation were tested with paired *t* tests. Differences between ΔMFI <0 and ΔMFI >0 before stimulation were tested by unpaired *t* tests. **P* <.05; ***P* <.01; n.s. not significant; STH Soil-transmitted helminths.



Supplementary Figure S5. Neutrophil responsiveness. Median fluorescent intensity (MFI) of CD11b, CD35, CD66b and CD62L of neutrophils left unstimulated (-) and after stimulation with fMLF. Indonesian subjects were stratified by helminth infection (STH- (n=73) vs STH+ (n=118)). Geomeans and corresponding 95% confidence intervals are shown. Differences between before and after stimulation were tested with paired *t* tests. ***P* <.01.



Supplementary Figure S6. Association between eosinophil granule proteins and eosinophil counts. Major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN) concentrations in serum at baseline and their association with absolute eosinophil counts (AEC). Spearman's rank correlation was used for statistical analysis (n=195).

Supplementary table S1. Antibody panel used for flow cytometry

Fluorochrome	Specificity	Clone	Vendor	Cat.no.	Dilution	Stock conc. (µg/mL)
FITC	CD35	E11	Biolegend	333404	300x	200
PerCP-Cy5.5	CD66b	G10F5	Biolegend	305107	100x	50
PE	CD193	5e8	Biolegend	310705	100x	100
PE-CF594	CD16	3G8	BD Biosciences	562320	4000x	100
PE-Cy5	CD69	FN50	Biolegend	310907	50x	20
PE-Cy7	CD274	MIH1	eBioscience	25-5983-41	80x	50
APC	CD3, CD19, CD20, CD56	UCHT1, HIB19, 2H7, 5.1H11	Biolegend	363601	150x	5, 6.25, 1.5, 17.5
APC-eF780	CD11b	ICRF44	eBioscience	47-0118-41	100x	50
BV421	CD203c	NP4D6	Biolegend	324611	100x	25
BV510	CD14	M5E2	Biolegend	301841	100x	150
BV605	CD62L	DREG-56	BD Biosciences	562720	100x	50

Supplementary table S2. Baseline characteristics of a subset of the study population, used to study granulocyte activation markers by flow cytometry.

	n	Placebo	n	Albendazole
Age (mean in years, SD)	94	48.6 (12.4)	101	47.4 (15.6)
Sex (female, n, %)	94	64 (68.1)	101	58 (57.4)
BMI (kg/m^2) (mean, SD)	93	23.3 (4.2)	100	22.5 (4.4)
Total IgE (IU/mL) (GM, 95% CI)	94	547 (376-794)	101	572 (410-797)
Eosinophil count (GM, 95% CI)	94	6.3 (5.6-7.1)	101	6.5 (5.6-7.5)
MBP (ng/mL) (GM, 95% CI)	93	816 (691-1010)	101	966 (814-1146)
ECP (ng/mL) (GM, 95% CI)	93	758 (610-981)	101	836 (698-1000)
EDN (ng/mL) (GM, 95% CI)	92	403 (238-352)	101	376 (316-448)
Eosinophil activation markers				
CD11b (GM, 95% CI)	94	1451 (1362-1547)	101	1366 (1276-1462)
CD35	94	3270 (3155-3391)	101	3267 (3147-3391)
CD66b	94	2103 (2047-2160)	101	2046 (1988-2105)
CD69	94	791 (752-833)	101	762 (723-804)
Neutrophil activation markers				
CD11b (GM, 95% CI)	94	1188 (1155-1222)	101	1143 (1107-1181)
CD35	94	1679 (1107-1181)	101	1532 (1436-1635)
CD66b	94	601 (576-628)	101	577 (552-602)
CD62L	94	747 (678-823)	101	794 (729-864)
Helminth infection by PCR (n,%)	91	63 (69.2)	100	55 (55.0)
A. lumbricoides	91	26 (28.6)	100	16 (16.0)
Hookworm	91	44 (48.4)	100	37 (37.0)
T. trichuris	91	34 (37.4)	100	27 (27.0)

Abbreviations: BMI body mass index; ECP eosinophil cationic protein; EDN eosinophil-derived neurotoxin; GM geometric mean; IgE immunoglobulin; MBP major basic protein; PCR polymerase chain reaction; SD standard deviation.