Building bridges: a multidisciplinary approach to controlled human hookworm infection

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Chapter 6

Protective efficacy of short-term infection with *Necator americanus* larvae in healthy volunteers in the Netherlands: a single-center, placebo-controlled, randomised controlled trial

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Abstract

Background

Vaccine development against hookworm is hampered by lack of natural immunity, limiting discovery of mechanisms of protective immunity and new vaccine targets. Immunisation with attenuated larvae has proven effective in dogs and partial immunity has been reached using an irradiated larvae model in healthy volunteers. This study investigates the protective efficacy of immunisation with short-term larval infection against hookworm challenge.

Methods

A double-blinded randomized controlled trial was conducted in the Netherlands at Leiden University Medical Center. Healthy volunteers were randomised to three short-term infections with 50 infectious *Necator americanus* L3 larvae or placebo. Infection was abrogated with a three-day course of 400mg albendazole two weeks after each exposure. Subsequently all volunteers were challenged with 2 doses of 50L3 at a two week interval. Primary endpoint was egg load at week 12-16 after challenge. The study is registered at clinicaltrials.gov under NCT03702530.

Findings

Participants were recruited between November 8, 2018 and December 14, 2018. The first immunisation was conducted December 18, 2018. Twenty-three volunteers were randomised, 15 to the intervention group and 8 to placebo. Immunised volunteers showed a trend towards lower eggs per gram (epg) faeces (geometric mean 571 vs 873, p=0.1). Five immunised volunteers developed a severe skin rash which was associated with 40% reduction in worm burden after challenge (geometric mean 441 epg vs 742 epg after challenge, p=0.003) and associated with higher peak IgG titers.

Interpretation

This is to our knowledge the first study to describe a protective effect of short-term exposure to hookworm larvae and show an association with skin response, eosinophilic response and IgG1.These findings open novel avenues for future vaccine discovery.

Funding

This trial was funded by a grant from Dioraphte foundation. The funders had no role in design or conduct of the study, data interpretation or manuscript preparation.

Introduction

Worldwide around 300 million people are infected with hookworms, mostly in tropical or subtropical climates.[1] These nematode helminths of the species *Ancylostoma duodenale, Ancylostoma ceylanicum* or *Necator americanus* are transmitted from human-to-human through the faecal excretion of eggs. Of these, *Necator americanus* is the most prevalent species in humans.[2] The eggs from faeces hatch in warm, humid soil and develop into infectious filariform larvae (L3) which can penetrate the skin of the human host. After invasion of the skin, larvae migrate to the lungs, are coughed up, swallowed and enter the duodenum, where they attach to the duodenal wall and mature into adult worms.[2] Blood loss from the worm intestinal attachment site causes anaemia and malnutrition, especially in high-intensity infections and children and women of childbearing age with inadequate capacity to replenish their iron and protein stores.[2] Mass drug administration programs aim to control the hookworm burden in endemic areas, but due to high levels of re-infection have so far not been successful in eradicating human hookworm infections.[3]

Individuals in endemic areas are repeatedly exposed to hookworm infection, but do not develop protective immunity.[4] Active immune suppression by adult worms has been suggested to prevent the development of protective responses.[5] However, dogs can be immunised through repeated exposure to irradiated hookworm larvae, which cannot mature to adulthood. The irradiated *Ancylostoma* larvae induced a 55-90% reduction in egg output in faeces and a 60% reduction in intestinal worm burden,[6 7] which was replicated in mice.[8] The irradiated larvae are thought to develop until the lung stage where they die, inducing immunity in their lung sojourn.[5] Similarly, short-term infection of hamsters abrogated with antihelminthic treatment before the adult stage resulted in a reduction of intestinal worm burden of 97% upon subsequent exposure.[9] Animal models, however, cannot be directly translated into humans, as hookworm species and immune responses differ between hosts.[4]

Controlled human infection models are unique tools to obtain insight in human immune responses to different pathogens including hookworms, allowing for dissection of (antigen-specific) responses with little interference of coinfections, prior exposure, or simultaneous adult worm immune interference. The controlled human hookworm infection model has been developed with small numbers of larvae to study the possible beneficial effects of hookworm-induced immune regulation in auto-immune diseases.[10-12] Benefitting from this experience, the protective effects of exposure to radiation-attenuated larvae was recently explored in the human host.[13] In this study, exposure to UV-irradiated larvae did not significantly impact egg output after challenge with 30 wild-type L3 as measured by PCR, but a lower number of larvae were recovered after culture of eggs in faeces possibly indicating mildly protective immune responses.[13] However, the high level of variability in egg output in this study, measured on a single stool sample may have hampered the power to detect differences.[12 14] We have previously shown that higher levels of infection (cumulatively 100 larvae) and repeated sampling increases the power of such controlled infection models.[15] In

addition, we hypothesize that a form of chemo-attenuation, using an abrogated infection in which larvae are treated before maturing to the adult stages, in analogy to other parasitic diseases such as malaria, might result in a more homogenous attenuation phenotype. We thus designed a randomized controlled trial investigating the protective efficacy of repeated exposure to short-term larval infection with *Necator americanus*.

Methods

Study design and participants

This study was a randomized, double-blind, placebo-controlled clinical trial investigating the protective efficacy of repeated short-term exposure to hookworm infection. This trial was approved by the LUMC institutional review board (NL66725.058.18) and is registered at clinicaltrials.gov under NCT03702530. All participants provided written informed consent. Healthy male and female volunteers aged 18-45 years were recruited through advertisements on social media and in in publicly accessible areas at Leiden University Medical Center (Leiden, Netherlands). Before inclusion in the trial, potential participants were screened for concomitant illnesses, previous exposure to hookworm or other conditions that could interfere with the trial. Full in- and exclusion criteria can be found in supplement 1.

Randomisation and masking

Volunteers were randomized to the intervention or placebo group in a 2:1 ratio. Treatment was allocated according to a master randomisation list generated using excel with “random number generator” function, which was prepared by an independent data manager and used when preparing the treatment by the manufacturing team. All investigators and participants were blinded to treatment allocation. Individual deblinding envelopes were prepared to allow emergency deblinding for individual participants. Study procedures can be found in the study protocol.

Study procedures

Infective *Necator americanus* L3 larvae were cultured following the principles of Good Manufacturing Practice principles and adhere to the guidance stipulated in the published white paper.[16] Larvae were cultured from faeces provided by a chronically infected donor, according to a modified copro-culture method following procedures previously described.[18] Four chronic donors were part of an ongoing study approved by the institutional review board under P20.100. For infection of the chronic donors, larvae were originally provided by prof A. Loukas (James Cook University, Australia).[12]

The study consisted of an immunisation phase, in which the intervention group was exposed to 50L3 (infective larvae stage L3) on three occasions at three-week intervals and a challenge phase (starting at week 13 of the trial) in which all participants were challenged with 50L3 on two occasions (Figure 1). During the immunisation phase, volunteers were exposed to a dose of 50L3 divided over four sites (both upper arms 10L3, both calves 15L3) for the

intervention group or water for the placebo group. This was followed by treatment with 400mg albendazole for both intervention and placebo group, ingested with fatty food, during three days at a two weeks timepoint after each infection. For the challenge phase, all participants were exposed to 50L3 at week 13 and 15 of the study (7 and 9 weeks after the last immunisation). During the challenge phase participants were followed for 16 weeks and then treated with albendazole except for one volunteer who gave written informed consent to remain infected as a chronic donor in the ongoing study. Trial schedule was based on previous studies showing stabilizing egg excretion after 12 weeks of infection that can be used as primary endpoint, resulting in treatment at week 16.[14] The immunisation schedule was based on previous animal studies using triple immunisations. [6 7] Treatment schedule was determined following national guidelines for the treatment of hookworm infection.[17]

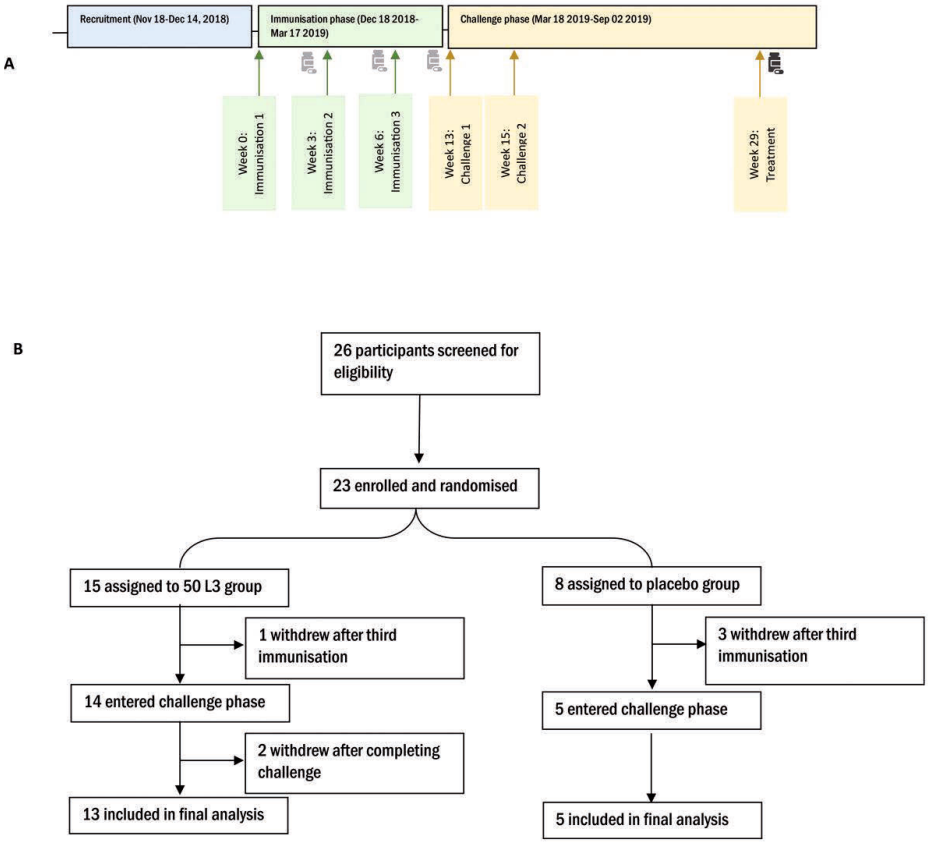


Figure 1. Trial flowchart

At each immunisation and treatment timepoint, volunteers visited the trial centre at Leiden University Medical Center. At these timepoints adverse events were collected, blood samples taken and stool samples collected by the participants. And blood and stool samples taken for safety analyses, all conducted at the study center. In between these timepoints

volunteers reported adverse events through e-mail or phone contact. During the challenge phase volunteers visited the study centre weekly for collection of adverse events, blood and stool samples. Adverse events were classified as unrelated, unlikely related (considered unrelated in dichotomous analyses), possibly related, probably related or definitely related (considered related in dichotomous analyses) and as mild (no impairment to daily life), moderate (some impairment) or severe (unable to carry on daily activities). Photos of skin rash were taken three weeks after each immunisation and weekly for six weeks after the challenge infections. Severity of rash was defined separately through assessment of photos of skin rash independently by two blinded physicians as mild (localised mild erythema) moderate (erythema at site of larval entry without further spread to surrounding skin) or severe (ardent red rash with serpentine lesions extending beyond site of entry or pustules). In case of dispute, photos were re-reviewed by the physicians and consensus was reached through debate.

During the immunisation phase, stool samples were analysed by Kato-Katz and *Necator americanus* real time PCR at week 8, 9 and 12 (2, 3 and 6 weeks after the final immunisation) according to previously described protocols.[16] During the challenge phase Kato-Katz and PCR were performed on weekly stool samples collected from week 18 onwards (5 weeks after challenge). For every sample, two Kato-Katz slides were prepared with 25 milligrams of homogenised stool, read by two separate technicians, egg counts were added and multiplied by 20 to calculate eggs per gram faeces. The quantitative real-time PCR results are expressed as cycle threshold (Ct)-values, which are inversely related with the parasite-specific DNA in the sample.

Hatching assays were performed on stool samples collected at week 25 and 29 (12 and 16 weeks after challenge). For the hatching assay, eggs from 5 gram of collected stool were cultured according to the method previously described.[14 18] Larvae were filtered after culture and washed, after which 250 µL of larval suspension was counted for the number of viable, motile larvae, in triplicate after stimulation with water at 50^{o}C. Samples for antibody analysis were collected before each immunisation, two weeks after the last immunisation, before each challenge and at 4, 8, 12, 16 and 24 weeks after the first challenge (supplementary table 1). Hookworm-specific IgG1, IgG4 and IgE were measured by ELISA using *Necator americanus* L3 extract as hookworm antigen (Supplement 2). Data was expressed as AU/mL and expressed as fold change from baseline level at study start. Seroconversion was defined as at least a three-fold change from baseline.

Serum samples were tested for the presence of cytokines using a commercially available Bio-Rad Bio-Plex Pro Human Cytokine 27-plex assay according to the manufacturer instructions. The following cytokines were measured: FGF, Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17A, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF, RANTES, TNF-α and VEGF using the Bio-plex 200 Luminex (Bio-Rad).

Outcomes and statistical analysis

Primary endpoint was defined as the difference in egg load between the intervention and placebo group, with egg load defined as the geometric mean (GM) epg (eggs per gram faeces) as measured by Kato-Katz between weeks 12-16 after first challenge in the per protocol population (all available data). Geometric mean of egg counts per individual was calculated and compared using a student's t-test. Differences in eosinophil counts, antibody response and circulating cytokines were compared with t-test or Kruskall-Wallis for non-parametric data and Chi-square or Fisher's exact test for categorical data. Safety data was assessed on the intention to treat population. A p-value of 0.05 was considered statistically significant. Statistical analyses were performed using IBM SPSS v23. [19]

Sample size was based on statistical modelling performed on our previous controlled infection studies[14 15] which showed that groups of 6 volunteers each with 5 stool samples taken from 12 weeks after challenge and analysed using Kato-Katz would result in 80% power at α=0.05 to detect an expected reduction in egg load of 50%. To anticipate loss to follow-up we increased sample size to 8 volunteers in the placebo group. For immunological dissection of potentially protective responses, we opted to increase the intervention group to 16 participants.

Data integration was performed through sparse Partial Least Squares (sPLS) regression using the 'mixOmics' package (v6.12.2) in R software (4.0.1).[20] PLS regression is suited for high-dimensional datasets and datasets with multicollinearity among the parameters. Sparsity is induced through a Lasso-like regularization, whereby most predictive features are selected. All datasets were included up to week 16 post challenge, antibodies and serum cytokines were normalized to baseline and $\log_2$-transformed. We filtered out features with a variance below 0.1 to reduce the change of spurious, but not necessarily meaningful, results. To determine the number of features to retain within the sPLS regression, we used leave-one-out validation from 1 to 50 features and selected the number of features giving the lowest mean average error, using the 'tune.spls' function, including 8 features in the final model. The leave one out average error was used to select the model with the best prediction after regularization. Plots were made with 'ggplot2' (v3.3.5) and 'pheatmap' (v1.0.12) packages. Heatmap clustering was performed using standard parameters: complete linkage based on Euclidean distance.

Role of the funding source

This trial was funded by a grant from Dioraphte foundation. The funders had no role in the design or conduct of the study, interpretation of data or manuscript preparation.

Results

Between November 8, 2018 and December 14, 2018, 26 volunteers were screened for eligibility, of whom 23 enrolled in the trial on December 18, 2018. The trial flow chart is depicted in figure 1. Six volunteers withdrew informed consent for reasons unrelated to the trial, four in the immunisation phase and two in the challenge phase. Three had been randomised to the placebo group and three to the intervention group. All safety data was included in the intention to treat analysis for adverse events. All available data from Kato-Katz and PCR analyses were taken forward in the per protocol analysis. One volunteer who withdrew after the challenge had enough faecal samples to be taken forward in the analysis of egg counts, the other did not. For immunological analysis only those volunteers who completed the trial were included. Baseline characteristics are included in table 1.

There were no serious adverse events. During the immunisation phase the most common adverse events (AEs) were itching and skin rash (table 1). Severity of itching increased significantly with each subsequent exposure to infective larvae, progressing to severe itching interfering with sleep in 4 of 15 volunteers, all in the immunisation group (figure 2A). Six volunteers were prescribed cortisone topical treatment due to severity of itching after the second and third immunisation. Abdominal AEs were rarely reported during the immunisation phase.

Table 1. Baseline characteristics and adverse events data. *: p<0.05

	Intervention group	**Placebo group**	**All**	
Immunisation phase n=	15	8	23	
Challenge phase n=	14	5	19	
Median age in years (IQR)	23 (20-26)	21 (18-28)	22 (20-26)	
Sex **Male** **Female**	7 (47%) 8 (53%)	3 (12.5%) 5 (62.5%)	10 (43%) 13 (57%)	
Mean Nº AEs per volunteer, related (SD) **Immunisation phase:** **Challenge phase:**	8.6 (1.7) 12.4 (4.8)	1.8 (2.4) 8.6 (5.0)	6.7 (3.6) 11.4 (5.0)	**p=<0.001*** p=0.15
Mean Nº skin AEs, related (itching and rash) (SD) **Immunisation phase:** **Challenge phase:**	7.1 (0.8) 4.8 (0.9)	0.8 (1.0) 3.6 (0.5)	5.3 (3.0) 4.5 (1.0)	**p=<0.001*** **p=0.01***
Nº of volunteers with grade 3 itching after challenge (%)	8 (57%)	1 (20%)	9 (47%)	**p=0.02***

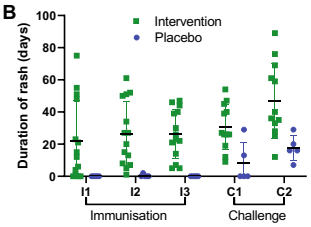
Table 1. Baseline characteristics and adverse events data. *: p<0.05 *(continued)*

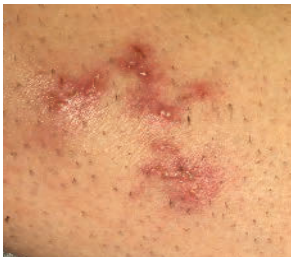
	Intervention group	Placebo group	All	
Mean Nº related abdominal AEs per volunteer in challenge phase (SD)	6.4 (4.0)	4.8 (4.3)	6.0 (4.0)	p=0.46
Nº of volunteers with related grade 3 abdominal AEs in challenge phase (%)	7 (50%)	2 (40%)	9 (47%)	p=0.55

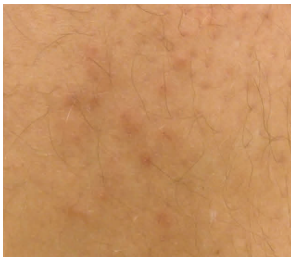
During the challenge phase the most frequently reported AEs were itching and rash after the challenge and gastro-intestinal symptoms (table 1). Itching further increased particularly in the intervention group with a significantly higher number of volunteers reporting grade 3 itching after challenge compared to the placebo group (table 1, figure 2A) and significantly more skin-related AEs in the intervention group (table 1). Rash after challenge lasted significantly longer in the intervention group compared to the placebo group (mean number of days (SD): 30.8 (SD 14.2) vs 8.37 days (12.9), p=0.03 for the first challenge and 46.9 (SD 23.2) vs 17.6 days (8.1) p=0.009 for the second challenge) (figure 2B). Five immunised volunteers developed a grade 3 rash with erythema, blistering, fluid exudate or serpentine eruptions. Such severe rash was not observed in participants in the placebo group (Figure 2C + 2D). Seven volunteers with severe itching were prescribed antihistamines, next to the use of cortisone cream. Eight volunteers in the intervention group reported severe abdominal AEs ranging from three to eight weeks after challenge, either abdominal cramping or nausea and vomiting, all lasting less than 12 hours. Severe abdominal AEs were not correlated with severe skin AEs and were not reported in the placebo group. Respiratory symptoms indicative of pulmonary infiltration were not reported.

A

	Intervention			Placebo			
	Mild	Moderate	Severe	None	Mild	Moderate	Severe
I1	67	33	0	88	12	0	0
I2	60	40	0	88	12	0	0
I3	20	53	27	100	0	0	0
C1	29	42	29	0	80	20	0
C2	7	57	36	0	40	40	20







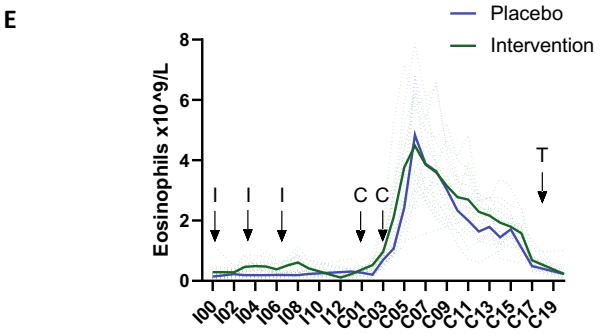


Figure 2. Skin adverse events and eosinophils. Percentage of volunteers with mild, moderate and severe itching after each exposure (A). Duration of rash per group in days after each hookworm exposure (B), individual data in green (intervention) and blue (placebo) with means (black) and SD. I1=first immunisation, I2=second immunisation, I3=third immunisation, C1=first challenge, C2=second challenge. Representative pictures of skin rash at two weeks after second challenge, example of severe rash (C) and mild rash (D).Eosinophil counts in peripheral blood over time (E) in the intervention group (green) and placebo group (blue), solid lines represent group mean, dotted lines represent individual volunteers. X-axis: week of the trialI=immunisation, C=challenge, T=treatment with albendazole

During the immunisation phase intervention group volunteers showed a mild elevation of eosinophils to a maximum of $0.9x10^9$/L which was not found in the placebo group ($p<0.001$) and returned to baseline levels at check-up three weeks after immunisation. Eosinophil counts peaked around week 6 after challenge in all volunteers (figure 2E). There were no differences in peak eosinophil count after challenge between the intervention and placebo group (mean intervention group 4.7, placebo 5.2 $x10^9$/mL, $p=0.60$). However, counts at 12 and 16 weeks after challenge were higher in the intervention group as compared to the placebo group (week 12: mean intervention 2.3, placebo 1.6 $x10^9$/mL, week 16: 1.7 vs 1.1 $x10^9$/mL), although this did not reach statistical significance ($p=0.07$ and $p=0.1$ respectively).

Volunteers with severe skin rash after challenge showed higher eosinophil counts than those without, particularly at week 16 after the challenge (mean severe skin rash 2.3 $x10^9$/mL, mild-moderate rash 1.5 $x10^9$/mL, p=0.005). Severe abdominal adverse events were not associated with the height or duration of peak eosinophilia (mean 4.8 $x10^9$/mL for severe AEs, 4.9 for non-severe, p=0.86), nor were severe skin AEs (mean 4.5 $x10^9$/mL for severe skin rash, 5.1 $x10^9$ for mild-moderate rash, p=0.57).

Kato-Katz and PCR for *N. americanus* on stool were performed on the per protocol population (n=13 for immunisation group, n=5 for placebo group) and were all negative at week 8, 9 and 12 of the immunisation phase, proving complete abrogation of the infection by repeated albendazole treatment. All volunteers showed detectable secretion of eggs in faeces by Kato-Katz, detected for the first time at week 7 (n=2), week 8 (n=15) or week 9 (n=1) after challenge.

Egg load after challenge was lower in the intervention group (geometric mean 571 epg, range 372-992) as compared to the placebo group (873 epg, range 268-1484), however this did not reach statistical significance, possibly due to the small sample size with large variability in the placebo group (p=0.10) (Figure 3A).

Volunteers with severe rash had a markedly lower egg load compared to volunteers with mild to moderate rash with a 40% reduction in egg burden (geomean 441 epg vs 742 epg, p=0.003, figure 3B). A difference in egg load between those with and without grade 3 abdominal AEs was not found (GM for severe AEs 549 epg, for non-severe 728 epg, p=0.23). Egg detection by PCR showed similar trends as the microscopy data (mean Ct value for placebo group 27.9, for intervention group 29.1, p=0.08), however the difference between mild to moderate and severe rash was not detected (mean Ct-value for mild to moderate rash 28.6, for severe rash 29.2, p=0.36, Figure 3C and 3D). The hatching assay showed no differences between groups, both intervention and placebo group and those with or without severe rash (supplementary table 2).

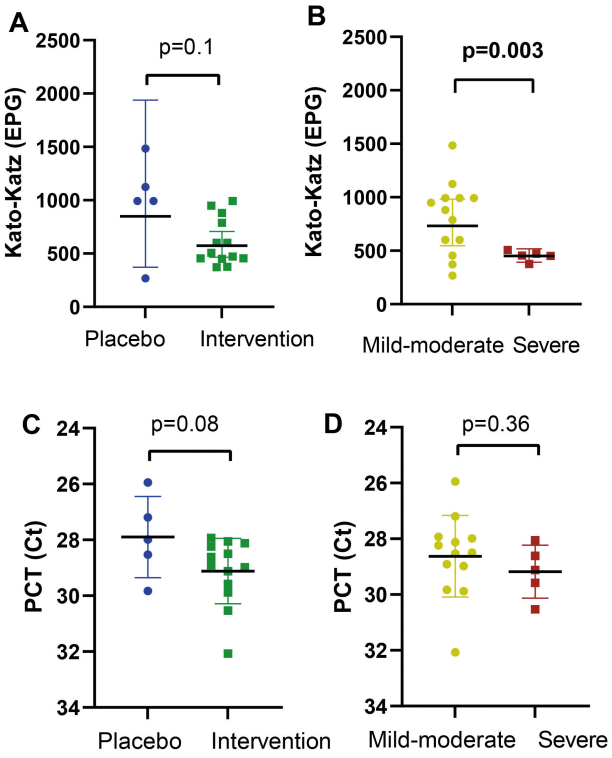


Figure 3. Parasitological analyses after challenge. Hookworm eggs detected in faeces by microscopy, reported as eggs per gram (EPG) (Kato-Katz, A+B) or real-time qPCR (Ct-values, C+D), for volunteers from the intervention group (green) and placebo group (blue) in left panels and volunteers with mild-to-moderate skin rash (yellow) and severe skin rash (red) in right panels. Symbols indicate individual values for egg count or DNA load, lines (black) indicate group GM with 95% CI bars.

IgG1 titers at challenge were significantly increased compared to baseline in those with severe skin rash (fold increase 4.5 vs 1.2, p=0.03). Furthermore, IgG1 titers after challenge peaked to much higher levels in the intervention as compared to placebo group (fold change at weeks 12 after the challenge: 4.0 vs 0.8 AU/mL, p=0.002 for intervention and placebo group respectively; week 16: 3.6 vs 0.9 AU/mL, p=0·03). Similarly, those volunteers with severe rash had higher peak IgG1 after challenge as compared to those with mild-to-moderate rash (week 12: 6.7 vs 1.7 AU/mL, p=0.003; week 16: 6.2 vs 1.5 AU/mL, p=0.02). (figure 4A+B).

Six volunteers showed IgG1 seroconversion after challenge, all were in the intervention group. Four of these had severe skin rash. Seroconversion was significantly more frequent in those with severe rash versus non-severe rash (67% vs. 8%, p=0.02) and was related to duration of rash after second challenge (mean 57.5 days in those who seroconverted vs 27.7

days in those who did not, p=0.003) but not to severe abdominal adverse events (p=1.00) or peak eosinophil count (p=0.24) (supplementary table 3). In the seroconverted group, the mean egg load trended to be lower (GM 507 vs 838 epg, p=0·09) (supplementary table 3).

Changes in hookworm-specific IgG4 were insignificant, e.g. only one volunteer seroconverted. There were no differences between placebo and intervention group in IgG4 titers or between those volunteers with mild-moderate and severe skin rash (Supplementary figure 1). IgE titers did not increase in any of the volunteers over the course of the study.

Circulating cytokines measured in serum showed considerable interindividual variation (supplementary figure 2). No statistically significant differences between groups could be detected. Both IL-4, a Th2-cytokine, and IL-1b, a pro-inflammatory cytokine, showed a decreasing trend after the challenge, whereas the pro-inflammatory cytokine IL-8 showed an increasing trend in all groups.

To integrate analyses of measured parameters (eosinophils, antibodies, cytokines, adverse events) and identify those which most strongly associated with protection in this study, we performed a sparse Partial Least Squares (sPLS) regression. [20] This method finds the combination of measured parameters that has a maximum covariance with the outcome (egg load). Seven features were associated with low egg counts after challenge, all from the challenge phase (figure 4C).

Eosinophil counts during egg production (at challenge phase weeks 12-15) were associated with protection. We moreover confirmed that only adverse events of the skin but not other AEs were associated with protection, as were increases in IgG1 subclass at its peak 12 weeks after challenge (figure 4C and 4D). Correlation analysis of the selected features revealed 3 main clusters of correlated features: egg load with IFNg at two weeks after first challenge (although these were not significantly correlated), the eosinophil levels during egg production and the skin adverse events with IgG1 at week 12 after challenge (Figure 4E). An AUC was calculated for eosinophil numbers in the challenge phase weeks 12-16, which correlated significantly with lower egg loads (rho=-0.59, p=0.012, Pearson test) (Figure 4F). Skin adverse events and eosinophil numbers together separated those with high egg load with those from lower egg loads.

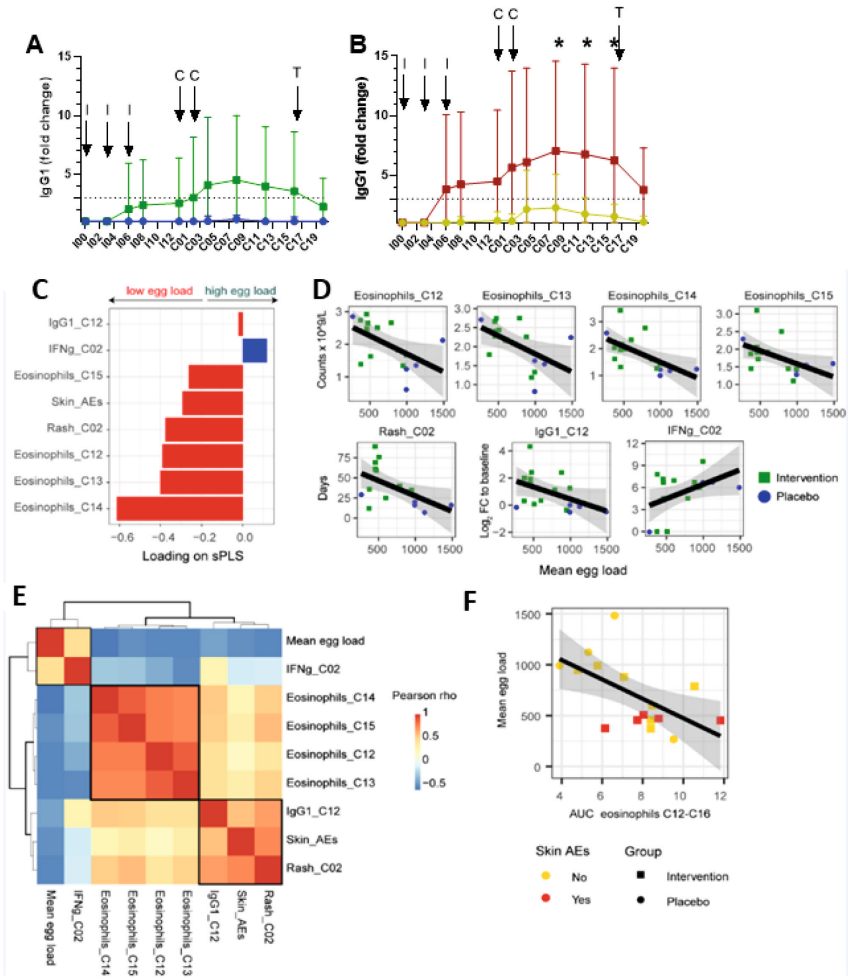


Figure 4. IgG1 titers and sPLS analysis Increases in hookworm-specific IgG1 plotted as fold-change over baseline for placebo (blue circles) versus intervention group (green squares) (A), and volunteers with mild-moderate skin rash (yellow circles) versus severe skin rash (red squares) (B). Symbols indicate mean, error bars SD, dashed line indicate threshold for seroconversion, set at 3-fold rise from baseline. *=p<0.05, I=immunisation, C=challenge, T=treatment with albendazole (C): Features associated with decreased or increased egg load in the sPLS regression model. Loading on the first principal component is shown per feature, with red bars associated with low egg load and blue bars associated with high egg load. (D) Correlation of egg load on the x-axis with selected variables on the y-axis. Green squares indicate participants in the intervention group, blue dots indicate placebo group. Black line and shaded area represent linear regression result and 95% confidence intervals. (E) Correlation matrix of selected features by sPLS regression. Colors indicate the strength and direction of Pearson rho value. (F) Correlation of egg load on the y-axis with the area under curve (AUC) of eosinophil counts during the egg production phase weeks C12-C16. Individuals are depicted with (red) or without (golden) severe skin adverse events. Individuals in placebo and intervention group are depicted by circles and squares, respectively. Black line and shaded area represent linear regression result and 95% confidence intervals. Timepoints are indicated as week of the trial. C: challenge phase

Discussion

To our knowledge, this study is the first to describe the protective effects of immunisation with short-term infections using hookworm larvae. We demonstrated that protection from subsequent challenge is associated with severe skin reactions, eosinophilic response and parasite specific IgG1 production. These results suggest that antibody-mediated effector mechanisms in the skin may play an important role in the protection induced by short-term, abrogated larval infection.

In our study, IgG1 was the predominant immunoglobulin subclass directed to parasite antigen, particularly prominent in those with severe skin rash. This contrasts with natural infections, where IgG4 is the most prominent immunoglobulin subclass, with higher levels of IgG4 being observed with higher worm burdens. [21] IgG4 is less pronounced after experimental infection, indicating this subclass may be associated with chronic trickling infections rather than infrequent, high-dose short-term exposures. The integrative analysis confirmed that IgG1 and eosinophilic responses were correlated with lower egg loads. This points to a mixed Th1 and Th2 response mediating protection, although the exact contribution of each component remains to be further elucidated.

Although not significant, we observed more frequent abdominal adverse events in the intervention group. In our previous study, we noticed an association between eosinophilic response and abdominal adverse events and a non-significant trend to lower egg counts in those with more abdominal adverse events. [15] Although the observation in the current study is less clear, these combined findings warrant speculation about a possible eosinophilic enteric response to hookworm antigens. In our previous study[15] using repeated controlled *Necator americanus* hookworm infections with 50L3, where we abrogated the infection at a later stage (week 20), we did not observe the severe skin responses. This indicates that the early destruction of larvae, much alike the radiation-attenuated larvae used in animal models and UV-irradiated larvae in the controlled human infection model described by Chapman et al.,[13] is critical in inducing protective immunity, which then attacks the invading larvae in the skin stage upon subsequent infections. The involvement of eosinophils and IgG1 in such a response is supported by earlier in vitro studies showing their ability to kill schistosomula. [22] The skin eruptions and severe nightly itching are reminiscent of the symptoms seen after human infection with canine hookworms (*Ancylostoma braziliense* and *caninum*), which can be similarly erythematous, vesicular and serpentine[23] and are thought to occur when larvae get trapped in the human skin.

The central role of the human skin in protective immune responses to hookworms has not been described before. Rather, the lungs were thought to be the primary site for immune induction in models using irradiated hookworm larvae in dogs and murine infection experiments.[7 24] In other human helminths such as schistosomes, we have previously found regulatory rather than inflammatory responses in *ex vivo* human skin models, with the

increased expression of IL-10 and PD-L1 by antigen-presenting cells in the skin.[25] These initially regulatory responses were thought to be the reason why cercarial dermatitis is usually mild.[25] It is interesting to observe that the induction of immunity can reverse such natural immune tolerance in the skin. In repeated helminth infection models using the murine helminth *Nippostrongylus braziliensis*, entrapment of larvae in skin has also been demonstrated after repeated infections [26] In these models, a large number of neutrophils were observed to swarm *Nippostrongylus braziliensis* in murine skin,[27] forming neutrophil extracellular traps to capture and aid the killing of larvae, although the larvae themselves could escape the traps by releasing deoxyribonucleases, resulting in survival in some. Taking skin biopsies after controlled infections can elucidate whether the effector cells in human hookworm infection models are the same.

Although not as pronounced as in our study, skin adverse events were also found in one prior study where the immunising effects of uv-irradiated larvae were tested in a controlled human infection model described by Chapman et al.[13] Specifically, the attenuation process was targeted to have the larvae cause a mild to moderate rash, which therefore may have induced a weaker immunological response than that seen in our study. Based on a prior dose escalation study, [15] we decided to select higher challenge doses. Our primary endpoint included multiple samples taken over several weeks when egg excretion is relatively stable instead of a single measurement, which greatly enhances the power of the challenge model. [14 15] The higher challenge dose resulted in twenty-fold higher larvae per grams of faeces recovered in the hatching assay and due to multiple sampling a more robust outcome that takes the variability in egg excretion into account.

Although an attenuated larvae approach to vaccination is not feasible on a large scale, the skin and associated initial larval stages may open up novel avenues for target discovery. The only vaccine currently in clinical development (Na-GST-1/Na-APR-1) targets adult worms.[28] A previous larval antigen candidate, Na-ASP-2, showed potential to inhibit larval migration in the skin[29] underscoring the possibilities of vaccines that target early larval stages. However, this vaccine failed in early clinical development due to the induction of IgE-mediated allergic responses in a pre-exposed population.[30] Our study now shows that early larval-stage antigens do not induce specific IgE responses in non-immune populations but may be efficacious in inducing protective immunity, which is why we would argue that these should be considered as vaccine candidates. Serological studies in endemic areas, to be performed prior to initiating phase 1b studies for any larval antigen vaccine, could be done to exclude the presence of pre-existing IgE and support its continued clinical development. Interestingly, the occurrence of strong eosinophilic responses after challenge suggests that antigen specific allergic responses may occur particularly to later stage antigens.

The repeated infection-treatment protocol was specifically designed to enhance the development of protective responses which may be diluted in natural infections due to interfering co-infections or prior infections. The controlled infection setting thus allows for

a more robust characterization of immune responses to early infection, thereby elucidating a hitherto uncharacterized response that cannot be studied in endemic areas. We have shown that this work is feasible and safe and can move to endemic areas to further assess immune responses in pre-exposed populations.

Limitations
Due to a high loss to follow-up the placebo group was reduced from an original eight to five participants. This has significantly impacted the study power to detect differences between the intervention and placebo group. Moreover, the very apparent skin eruptions in some volunteers broke the blinding for both trial physicians and volunteers. However, all laboratory evaluations, including Kato-Katz slides, PCR and ELISA measurements were performed by blinded personnel, minimizing bias. The findings in this study are specific to *Necator americanus*, the most prevalent hookworm species, but may not be generalizable to *Ancylostoma* infection.

In conclusion, this study is the first to describe protective skin-mediated IgG1 responses against infection with hookworm larvae. This finding supports the investigation of larval antigens as possible vaccine targets and confirms IgG1 as reliable correlate of protection for vaccine efficacy.

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Conflict of interest statement

The authors have no conflict of interest to declare.

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Supplementary material

Supplementary material 1. In- and exclusion criteria

Inclusion criteria

In order to be eligible to participate in this study, a participant must meet all of the following criteria:

1. Participant is aged ≥ 18 and ≤ 45 years and in good health.
2. Participant has adequate understanding of the procedures of the study and agrees to abide strictly thereby.
3. Participant is able to communicate well with the investigator and is available to attend all study visits.
4. Participant agrees to refrain from blood donation to Sanquin or for other purposes throughout the study period.
5. For female participants: participant agrees to use adequate contraception and not to breastfeed for the duration of study.
6. Participant agrees to refrain from travel to a hookworm endemic area during the course of the trial.
7. Participant has signed informed consent.

Exclusion criteria

A potential Participant who meets any of the following criteria will be excluded from participation in this study:

1. Any history, or evidence at screening, of clinically significant symptoms, physical signs or abnormal laboratory values suggestive of systemic conditions, such as cardiovascular, pulmonary, renal, hepatic, neurological, dermatological, endocrine, malignant, haematological, infectious, immune-deficient, psychiatric and other disorders, which could compromise the health of the volunteer during the study or interfere with the interpretation of the study results. These include, but are not limited to, any of the following:

- positive HIV, HBV or HCV screening tests;
- the use of immune modifying drugs within three months prior to study onset (inhaled and topical corticosteroids and oral anti-histamines exempted) or expected use of such during the study period;
- having one of the following laboratory abnormalities: ferritine <10ug/L, transferrine <2.04g/L or Hb <6.5 mmol/L for females or <7.5 mmol/L for males.
- history of malignancy of any organ system (other than localized basal cell carcinoma of the skin), treated or untreated, within the past 5 years;
- any history of treatment for severe psychiatric disease by a psychiatrist in the past year;
- history of drug or alcohol abuse interfering with normal social function in the period of one year prior to study onset;

- inflammmatory bowel syndrome;
- regular constipation, resulting in bowel movements less than three times per week.

2. Known hypersensitivity to or contra-indications for use of albendazole, including co-medication known to interact with albendazole metabolism (e.g. carbamazepine, phenobarbital, phenytoin, cimetidine, theophylline, dexamethasone).
3. Known allergy to amphotericin B or gentamicin.
4. For female participants: positive urine pregnancy test at screening.
5. Positive faecal qPCR for hookworm at screening, any known history of hookworm infection or treatment for hookworm infection.
6. Being an employee or student of the department of Parasitology of the LUMC.
7. Current or past scars, tattoos, or other disruptions of skin integrity at the intended site of larval application.

Supplementary material 2. ELISA procedure
Hookworm antigen: Necator americanus L3 extract.

Cultured Hookworm L3 larvae were collected in sterile water and stored in 50ml tubes at -80°C after which they were freeze dried and stored at -80°C again. After thawing, the larvae were suspended in PBS, transferred to a glass homogenizer and kept on ice whilst washing 4 times. The solution in the homogenizer was crushed for 15 minutes and left for one hour on ice. Crushing and resting was repeated three times. Crushed hookworm larvae were transferred to a glass tube, and the homogenizer was washed once with PBS. The crushed hookworm larvae were sonified (Branson Sonifier) 6 times for 30 seconds with an interval of 20 seconds. The samples were kept overnight at -80°C. The frozen samples were thawed and centrifuged for 25 minutes, 13.000 rpm at 4^{o}C. The supernatant as well as the resuspended pellet were centrifuged again, after which supernatants from both tubes were collected and pooled. Antigen concentration was determined using a BCA kit (ThermoFisher 23225) following manufacturers instruction.

IgG1 and IgG4 ELISA: High-binding C96-wells maxisorp plates (Nunc-Immuno™ 430341) were coated with 5ug/ml hookworm antigen in 0.1M Na-carbonate pH 9.6. After overnight incubation at 4^{o}C, plates were washed 4 times with 0.05% Tween 20 (Sigma-Aldrich, 27, 434-8) in PBS wash buffer. Plates were blocked with 5% BSA/PBS for one hour at 37^{o}C. After washing, a mix of positive serum was used in a serial dilution of 1:2 as positive controls, samples were at least 1:2 diluted in PBS 0.05% Tween 20 assay buffer. Plates were incubated overnight at 4^{o}C for IgG1 and IgG4, and 1 hour at 37^{o}C for total IgG. For IgG1 detection, conjugated monoclonal antibody of HRP-labelled anti human IgG1 (Fc) (clone MH161-1, HP6188, Sanquin; cat no: M1328) was added and for IgG4 detection HRP-labelled anti human IgG4 (CH3) (clone MH164-1, HP6196, Sanquin; cat no: M1331) was added, both at a concentration of 1.8uL diluted in 5.5 ml PBS 0.05% tween and both 50 uL/well. For total IgG detection, 50 uL/well 1.8 uL alkaline phosphate conjugated anti-human IgG (Sigma A9544,

4°C) in 5.5 ml PBS 0.05%Tween-4% BSA was added. Plates were incubated at 37°C (one hour for IgG, four hours for IgG1 and IgG4). After washing, TMB substrate was added (TMB Microwel substrate system(KPL, 50-76-00) for IgG1 and IgG4, 6mg p p-nitrophenylphosphate (p-NPP) in 6 ml diethylanolamine (DEA) buffer for IgG)) and development was stopped with 18M H2SO4 in water, after which plates were read at by 450 nm at the ELISA plate reader. Total IgG plates were incubated in the dark for one hour at room temperature and then read at wavelength 405 nm.

IgE ELISA: Polysorp F96-wells plates (Nunc-Immuno™ 475094) were coated with 5ug/ml hookworm antigen in 0.1 M Na-carbonate buffer, pH 9.6. After overnight incubation at 4°C wells plates were washed 4 times with 0.05% Tween 20 (Sigma-Aldrich, 27, 434-8) in PBS wash buffer. Blocking with 5% BSA/PBS was performed for one hour at room temperature. After washing four times, a mix of positive sera were added in a serial dilution of 1:2 in PBS 0.05% Tween 20 assay buffer and serum samples were diluted at least 1:2 in assay buffer. Plates were incubated overnight at 4°C. After five washings 100uL/well of 22uL goat anti human IgE HRP (Invitrogen A18793, 1 mg/ml) diluted in 11 ml PBS 0.05%Tween was added and plates were incubated for three hours at 37°C. Samples were flicked off and washed five times. After washing, TMB substrate was added (TMB Microwel substrate system(KPL, 50-76-00) incubated in the dark for one hour at room temperature and development was stopped with 18M H2SO4 in water, after which plates were read at by 450 nm at the ELISA plate reader. All Ig analyses: OD values are converted to AU/ml using a standard curve with unknown concentration, enabling comparison of a rise or fall in serum concentration within the same subject.

Supplementary table 1. Overview of study visits and sampling

Week	Timepoint	Study procedures	Samples collected for:
Immunisation phase			
Week 0	I00	First immunisation	Eosinophils, Ig, cytokines
Week 1	I01		
Week 2	I02	Albendazole treatment	Eosinophils
Week 3	I03	Second immunisation	Eosinophils, Ig, cytokines
Week 4	I04		
Week 5	I05	Albendazole treatment	Eosinophils
Week 6	I06	Third immunisation	HW PCR, Eosinophils, Ig, cytokines
Week 7	I07		
Week 8	I08	Albendazole treatment	Kato-Katz, HW PCR, Eosinophils, Ig, cytokines
Week 9	I09		Kato-Katz, HW PCR, Eosinophils
Week 10	I10		
Week 11	I11		
Week 12	I12		Kato-Katz

Supplementary table 1. Overview of study visits and sampling *(continued)*

Week	Timepoint	Study procedures	Samples collected for:
Challenge phase			
Week 13	C00	First challenge	Eosinophils, Ig, cytokines
Week 14	C01		Eosinophils
Week 15	C02	Second challenge	Eosinophils, Ig, cytokines
Week 16	C03		Eosinophils
Week 17	C04		HW PCR, Eosinophils, Ig, cytokines
Week 18	C05		HW PCR, Kato-Katz, Eosinophils
Week 19	C06		HW PCR, Kato-Katz, Eosinophils
Week 20	C07		HW PCR, Kato-Katz, Eosinophils
Week 21	C08		HW PCR, Kato-Katz, Eosinophils, Ig, cytokines
Week 22	C09		HW PCR, Kato-Katz, Eosinophils
Week 23	C10		HW PCR, Kato-Katz, Eosinophils
Week 24	C11		HW PCR, Kato-Katz, Eosinophils
Week 25	C12		HW PCR, Kato-Katz, Eosinophils, Ig, cytokines
Week 26	C13		HW PCR, Kato-Katz, Eosinophils
Week 27	C14		HW PCR, Kato-Katz, Eosinophils
Week 28	C15		HW PCR, Kato-Katz, Eosinophils
Week 29	C16	Albendazole treatment	HW PCR, Kato-Katz, Eosinophils, Ig, cytokines
Week 30	C17		HW PCR, Kato-Katz, Eosinophils
Week 32	C19		HW PCR, Kato-Katz, Eosinophils
Week 37	C24		Eosinophils, Ig, cytokines

Ig: Immunoglobulins, HW PCR: fecal hookworm PCR

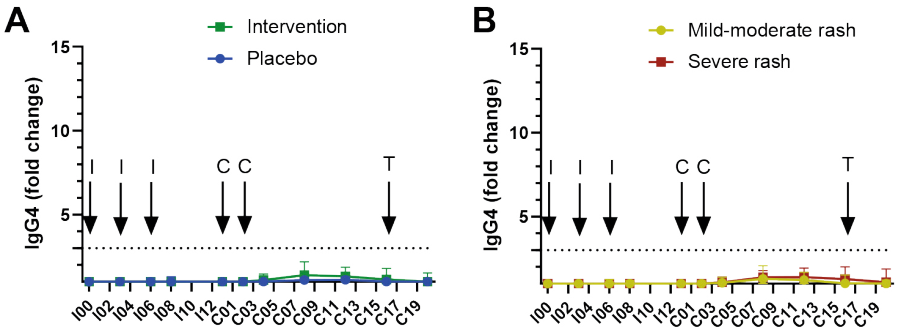
Supplementary table 2. Hatching assay

	Hatching week 12 (mean (SD), larvae per gram faeces)	Hatching week 16 (mean (SD), larvae per gram faeces)
Placebo group (n=5)	164 (146)	248 (122)
Intervention group (n=15)	96 (68)	207 (116)
	p=0.51	p=0.72
Severe skin AEs (n=5)	109 (102)	191 (136)
Non-severe skin AEs (n=12)	119 (100)	230 (110)
	p=0.72	p=0.57

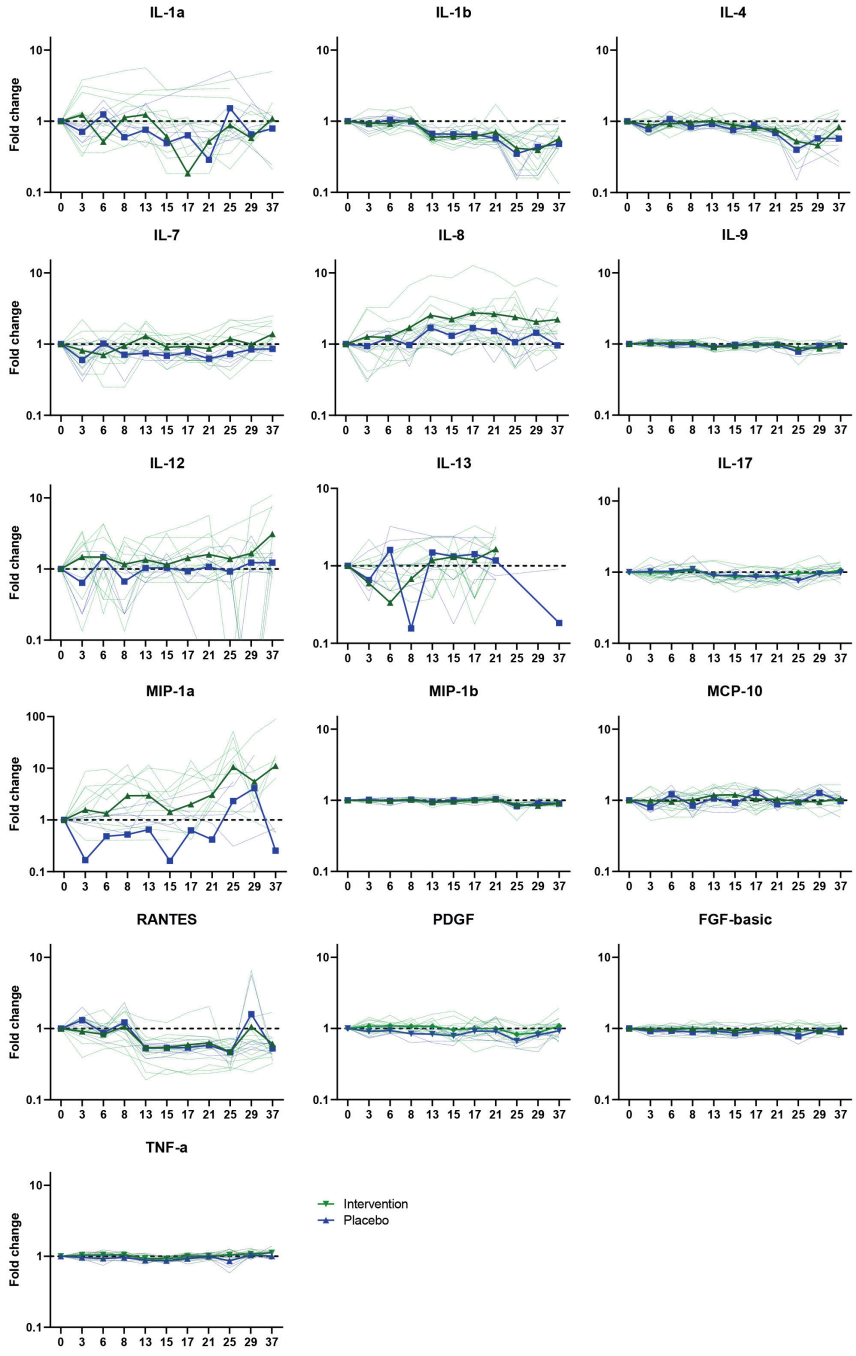
Supplementary table 3. Outcomes for volunteers with and without seroconversion for IgG1.

	IgG1		
	Seroconversion⁺ (n=6)	**No seroconversion (n=12)**	
Placebo Intervention	0 6 (100%)	5 (42%) 7 (58%)	p=0.09
Non-severe skin rash Severe skin rash	2 (33%) 4 (67%)	11 (92%) 1 (8%)	**p=0.02***
Eggload (GM epg, SD)	507 (186)	838 (369)	p=0.09
Grade 3 itching - Yes - No	2 (33%) 4 (67%)	5 (42%) 7 (58%)	p=0.32
Grade 3 abdominal AEs - Yes - No	3 (50%) 3 (50%)	6 (50%) 6 (50%)	p=1.00
Duration of rash after third immunisation (days) (mean) (SD)	30.9 (9.4)	14.0 (18.9)	**p=0.02***
Duration of rash after first challenge (days) (mean) (SD)	32.6 (13.4)	19.6 (17.5)	p=0.13
Duration of rash after second challenge (mean) (SD)	57.5 (20.8)	27.7 (18.8)	**p=0.01***
Peak eosinophil count (x10^9/L, mean (SD))	5.1 (1.9)	4.8 (1.6)	p=0.75

⁺Seroconversion is defined as fold change >3 compared to baseline *p<0.05



Supplementary figure 1. IgG4 titers. Increases in hookworm-specific IgG4 plotted as fold-change over baseline for placebo (blue circles) versus intervention group (green squares) (A), and volunteers with mild-moderate skin rash (yellow circles) versus severe skin rash (red squares) (B). Symbols indicate mean, error bars SD, dashed line indicate threshold for seroconversion, set at 3-fold rise from baseline. I=immunisation, C=challenge, T=treatment with albendazole



Supplementary figure 2. Cytokine measurements by Luminex. Bold lines indicate mean per group, dotted lines indicate individual participants. Green triangles = intervention group, blue circles = placebo group. Data expressed as fold change from baseline.