Protective efficacy of short-term infection with Necator americanus hookworm larvae in healthy volunteers in the Netherlands: a single-centre, placebo-controlled, randomised, controlled, phase 1trial

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Summary

Background Vaccine development against hookworm is hampered by the absence of the development of protective immunity in populations repeatedly exposed to hookworm, limiting identification of mechanisms of protective immunity and new vaccine targets. Immunisation with attenuated larvae has proven effective in dogs and partial immunity has been achieved using an irradiated larvae model in healthy volunteers. We aimed to investigate the protective efficacy of immunisation with short-term larval infection against hookworm challenge.

Methods We did a single-centre, placebo-controlled, randomised, controlled, phase 1 trial at Leiden University Medical Center (Leiden, Netherlands). Healthy volunteers (aged 18–45 years) were recruited using advertisements on social media and in publicly accessible areas. Volunteers were randomly assigned (2:1) to receive three short-term infections with 50 infectious *Necator americanus* third-stage filariform larvae (50L3) or placebo. Infection was abrogated with a 3-day course of albendazole 400 mg, 2 weeks after each exposure. Subsequently all volunteers were challenged with two doses of 50L3 at a 2-week interval. The primary endpoint was egg load (geometric mean per g faeces) measured weekly between weeks 12 and 16 after first challenge, assessed in the per-protocol population, which included all randomly assigned volunteers with available data on egg counts at week 12–16 after challenge. This study is registered with ClinicalTrials.gov, NCT03702530.

Findings Between Nov 8 and Dec 14, 2018, 26 volunteers were screened, of whom 23 enrolled in the trial. The first immunisation was conducted on Dec 18, 2018. 23 volunteers were randomly assigned (15 to the intervention group and eight to the placebo group). Egg load after challenge was lower in the intervention group than the placebo group (geometric mean 571 eggs per g [range 372–992] *vs* 873 eggs per g [268–1484]); however, this difference was not statistically significant (p=0·10). Five volunteers in the intervention group developed a severe skin rash, which was associated with 40% reduction in egg counts after challenge (geometric mean 742 eggs per g [range 268–1484] *vs* 441 eggs per g [range 380–520] after challenge; p=0·0025) and associated with higher peak IgG1 titres.

Interpretation To our knowledge, this is 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 could inform future hookworm vaccine development.

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Introduction

Around 300 million people are infected with hookworm worldwide, mostly in tropical or subtropical climates.[1] Hookworm are soil-transmitted helminths and the species that infect humans—*Ancylostoma duodenale*, *Ancylostoma ceylanicum*, or *Necator americanus*—are transmitted from person to person through the faecal excretion of eggs. Of these hookworm species, *N americanus* is the most prevalent species in people.[2] The eggs from faeces hatch in warm, humid soil and develop into infectious third-stage filariform larvae (L3) that 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 infections with high worm burden and in children and women of childbearing age with inadequate capacity to replenish their iron and protein stores.[2] Mass drug administration programmes using albendazole aim to control the hookworm burden in endemic areas but, due

Research in context

Evidence before this study

The use of attenuated infections is a well known concept in vaccinology, stemming from smallpox variolation. Vaccines against measles and yellow fever, for example, consist of live attenuated viruses. The antigenic complexity of parasites as a multicellular organism with multiple life stages and paucity of knowledge of underlying mechanisms of protection has slowed the development of anti-parasitic vaccines compared with viruses. However, in malaria vaccine research, immunisation with chemoattenuated parasites has proven to be highly efficacious. For hookworm, little is known about mechanisms of protection and possible vaccine targets. We searched PubMed from database inception until Nov 6, 2023, for research articles published in English, Dutch, German, and French, using the search terms: ("hookworm" OR "Necator americanus" OR "ancylostoma") AND ("controlled infection" OR "human challenge study" OR "attenuated larvae" OR "vaccination"). Our search yielded 151 studies, of which 35 were relevant. Studies in dogs have shown that attenuated hookworm larvae can induce protection against subsequent infection. In humans, one trial has been conducted using irradiated *Necator americanus* larvae, which generated partial immunity against hookworm challenge in healthy volunteers. No studies had been published on the use of a chemoattenuated approach as immunisation against hookworm infection.

Added value of this study

To our knowledge, this is the first study to describe the protective effects of chemoattenuated hookworm larvae against subsequent hookworm challenge. This study demonstrated that the severity of skin responses was associated with lower egg loads; this association has not previously been described to our knowledge. IgG1 and eosinophilic response were also found to be associated with protection against challenge, indicating a mixed T-helper-1-cell (Th1) and Th2 response.

Implications of all the available evidence

Although sterile immunity against hookworm infection has not yet been achieved, the available studies have proven that it is possible to induce at least partial immunity against challenge. Based on the findings in this study, larval stage antigens might potentially yield vaccine targets and warrant further research.

to high rates of re-infection, such programmes 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] It is postulated that active immune suppression by adult worms prevents 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 caninum* 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 and presumably induce protective immunity.[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, since 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 elucidation of (antigen-specific) responses with little interference of co-infections, previous exposure, or simultaneous adult worm immune interference. The controlled human hookworm infection model has previously been developed with small numbers of larvae to study the possible beneficial effects of hookworm-induced immune regulation in autoimmune diseases.[10–12] Benefiting from this experience, the protective effects of exposure to radiation-attenuated larvae were explored in the human host.[13] In this study, exposure to ultraviolet-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 amount of variability in egg output in this study, measured using a single stool sample, might have reduced the power to detect differences.[12,14] We have previously shown that high larvae burden of infection (100 larvae cumulatively) and repeated sampling increases the power of such controlled infection models.[15] Additionally, we hypothesise that a form of chemoattenuation, using an abrogated infection in which larvae are treated before maturing to the adult stages, similar to other parasitic diseases such as malaria, might result in a more homogeneous attenuation phenotype. We aimed to investigate the protective efficacy of repeated exposure to short-term larval infection with *N americanus*.

Methods

Study design and participants

This study was a randomised, double-blind, placebo-controlled, clinical trial done at Leiden University Medical Center (Leiden, Netherlands). The study consisted of two stages: an immunisation stage, in which the intervention group was exposed to 50 infectious *N americanus* L3 (hereafter referred to as 50L3) on

three occasions at 3-week intervals, and a challenge stage (starting at week 13 of the trial), in which all participants were challenged with 50L3 on two occasions.

Healthy male and female volunteers aged 18–45 years were recruited through advertisements on social media platforms (ie, Facebook and Instagram) and in publicly accessible areas at Leiden University Medical Center. Before inclusion in the trial, potential participants were screened for concomitant illnesses and previous exposure to hookworm or other conditions that could interfere with the trial. Full inclusion and exclusion criteria are in the appendix (p 1).

The trial was approved by the institutional review board at Leiden University Medical Center (NL66725.058.18). All participants provided written informed consent.

Study procedures can be found in the study protocol (appendix pp 8–44).

Randomisation and masking

Volunteers were randomly assigned (2:1) to the intervention or placebo group. Treatment was allocated according to a master randomisation list generated using Microsoft Excel with a 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 masked to treatment allocation. Individual envelopes were prepared to allow emergency unmasking for individual participants, which was permitted in cases of urgent medical need.

Procedures

Infective *N americanus* L3 larvae were cultured following the Good Manufacturing Practice principles and adhere to previously published guidance.[16] Larvae were cultured from faeces provided by a chronically infected donor, according to a modified copro-culture method following previously described procedures.[17] Four chronically infected donors were part of an ongoing study approved by the institutional review board (P20.100). For infection of the chronically infected donors, larvae were originally provided by James Cook University (Cairns, QLD, Australia).[12]

During the immunisation stage, volunteers were exposed to a dose of 50L3 administered over four body sites (both upper arms 10L3, both calves 15L3) for the intervention group or water (0·5 mL) for the placebo group. Immunisation was followed by treatment with albendazole 400 mg for both intervention and placebo groups, ingested with fatty food, during 3 days at a 2-week timepoint after each infection. For the challenge stage, all participants were exposed to 50L3 at week 13 and 15 of the study (7 weeks and 9 weeks after the last immunisation). During the challenge stage participants were followed up for 16 weeks and then treated with albendazole, except for one volunteer who gave written informed consent to remain infected as a chronically infected donor in the ongoing study. The trial schedule was based on previous studies showing stabilising egg excretion after 12 weeks of infection that can be used as a 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.[18]

At each immunisation and treatment timepoint, volunteers visited the trial centre at Leiden University Medical Center. At these timepoints adverse events were recorded, blood samples were taken by clinical trial staff, and stool samples were collected by the participants. Blood and stool samples were collected for safety analyses, all conducted at the study centre. In between these timepoints volunteers reported adverse events through e-mail or telephone contact. During the challenge stage, volunteers visited the study centre weekly for recording of adverse events, and collection of 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). Photographs of skin rash were taken 3 weeks after each immunisation and weekly for 6 weeks after the challenge infections. Severity of rash was defined separately through assessment of photos of skin rash independently by two masked 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 discrepancies, photographs were re-reviewed by the physicians and consensus was reached through debate.

See Online for appendix

During the immunisation stage, stool samples were analysed by Kato-Katz and *N americanus* real-time PCR at weeks 8, 9, and 12 (2, 3, and 6 weeks after the final immunisation) according to previously described protocols.[16] During the challenge stage, Kato-Katz and PCR were performed on stool samples collected weekly from week 18 onwards (5 weeks after challenge). For every sample, two Kato-Katz slides were prepared with 25 mg of homogenised stool, read by two separate technicians; egg counts were added and multiplied by 20 to calculate eggs per g of 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. Lower Ct values indicated a higher quantity of parasite-specific DNA in the sample.

Hatching assays were performed on stool samples collected at weeks 25 and 29 (12 weeks and 16 weeks after challenge). For the hatching assay, eggs from 5 g of collected stool were cultured according to the method previously described.[14,17] 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°C. Samples for antibody analysis were collected before each immunisation, 2 weeks after the last immunisation, before each challenge and at 4, 8, 12, 16, and 24 weeks after the first challenge (appendix p 3). Hookworm-specific IgG1, IgG4, and IgE were measured by ELISA using *N americanus* L3 extract as hookworm antigen (appendix p 2). Data was expressed as arbitrary units (AU)/mL and expressed as fold-change from baseline at study start. Seroconversion was defined as at least a 3-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 (Bio-Rad, Hercules, CA, USA) according to the manufacturer instructions. The following cytokines were measured: fibroblast growth factor, eotaxin, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, 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, IFN-γ-induced protein 10, monocyte chemoattractant protein-1, macrophage inflammatory protein-1α, macrophage inflammatory protein-1β, platelet-derived growth factor, regulated on activation, normal T cell expressed and secreted, tumour necrosis factor-α, and vascular endothelial growth factor using the Bio-plex 200 Luminex (Bio-Rad).

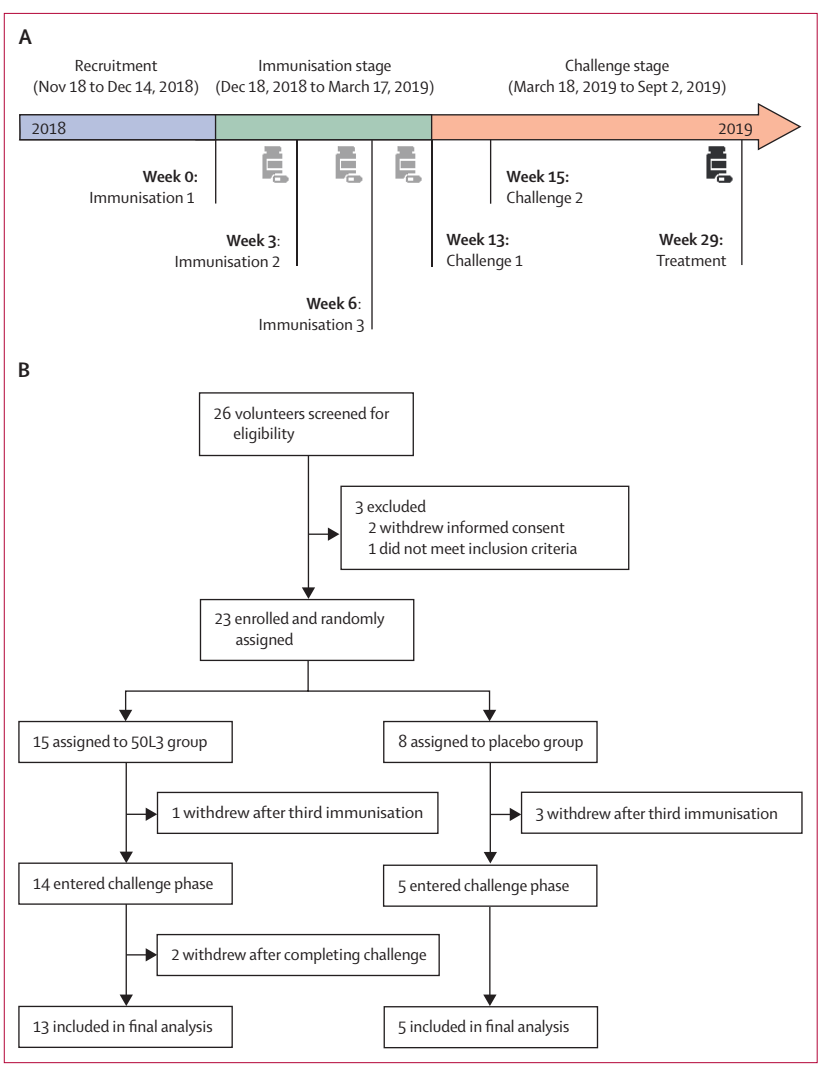


Figure 1: **Trial profile**
Pill bottles represent treatment with albendazole. 50L3=50 *Necator americanus* third-stage filariform larvae.

Outcomes

The primary endpoint was the difference in egg load (defined as the geometric mean [eggs per g faeces]), between the intervention and placebo groups, as measured weekly by Kato-Katz between weeks 12 and 16 after first challenge. Secondary endpoints were the comparison of frequency and severity of adverse events between the immunisation and challenge stage and between placebo and immunisation groups, and changes in cellular and humoral immunological responses after immunisation and challenge in both the placebo and intervention groups.

Statistical analysis

The 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 Kruskal-Wallis test for non-parametric data and χ^2 or Fisher's exact test for categorical data. The primary endpoint was assessed in the per-protocol population, which included all randomly assigned volunteers with available data on egg counts between weeks 12 and 16 after challenge. Safety data was assessed in the intention-to-treat population, which included all randomly assigned participants. A p value of 0·05 was considered to indicate a statistically significant difference. Statistical analyses were performed using IBM SPSS software (version 23.0).

Sample size was based on statistical modelling from our previous controlled infection studies,[14,15] which showed that groups of six volunteers each with five stool samples taken from 12 weeks after challenge and analysed using Kato-Katz would result in 80% power at a one-sided α of 0·05 to detect an expected reduction in egg load of 50%. To anticipate loss to follow-up, we increased sample size to eight volunteers in the placebo group. For immunological dissection of potentially protective responses, we chose to increase the target size of the intervention group to 16 participants.

Data integration was performed through sparse partial least squares (sPLS) regression using the mixOmics package (version 6.12.2) in R software (version 4.0.1). This method finds the combination of measured parameters that has a maximum covariance with the outcome (egg load). sPLS regression is suited for high-dimensional datasets and datasets with multicollinearity among the parameters. Sparsity is induced through a Lasso-like regularisation, whereby most predictive

features are selected. All datasets were included up to week 16 after challenge; antibodies and serum cytokines were normalised 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 eight features in the final model. The leave-one-out average error was used to select the model with the best prediction after regularisation. Plots were made with ggplot2 (version 3.3.5) and pheatmap (version 1.0.12) packages. Heatmap clustering was performed using standard parameters: complete linkage based on Euclidean distance (a hierarchical clustering approach that visualises which populations behave similarly using a dendrogram).

The trial is registered with ClinicalTrials.gov, NCT03702530.

Role of the funding source

The funder had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Between Nov 8 and Dec 14, 2018, 26 volunteers were screened for eligibility, of whom 23 enrolled in the trial on Dec 18, 2018. The trial timeline and flow chart is depicted in figure 1. Six volunteers withdrew informed consent for reasons unrelated to the trial, four in the immunisation stage and two in the challenge stage; three had been randomly assigned to the placebo group and three to the intervention group. All safety data were 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 included in the analysis of egg counts, the other did not. For immunological analysis, only volunteers who completed the trial were included. Baseline characteristics are shown in table 1.

No serious adverse events were reported. During the immunisation stage, the most common adverse events were itching and skin rash (table 1). Severity of itching increased substantially with each subsequent exposure to infective larvae, progressing to severe itching that interfered with sleep in four of 15 volunteers, all in the immunisation group (table 2). Six volunteers were prescribed cortisone topical treatment due to severity of itching after the second and third immunisation. Abdominal adverse events were rarely reported during the immunisation stage.

During the challenge stage, the most frequently reported adverse events were itching and rash after challenge and gastrointestinal symptoms (table 1). Itching increased in severity in both groups; however, the proportion of volunteers in the intervention group who reported grade 3 itching after challenge was higher than the placebo group (tables 1, 2; p=0·018) and a higher proportion of volunteers in the intervention group had skin-related adverse events than in the intervention group (table 1; p=0·0070). Rash after challenge lasted for a longer duration in the intervention group than the placebo group (mean 30·8 days [SD 14·2] *vs* 8·37 days [12·9]; p=0·027 after the first challenge; 46·9 days [SD 23·2] *vs* 17·6 days [8·1]; p=0·0090 after the second challenge; figure 2A). 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

	Intervention group (n=15)	Placebo group (n=8)	All (n=23)	p value
Immunisation stage, n	15	8	23	..
Challenge stage, n	14	5	19	..
Median age, years (IQR)	23 (20–26)	21 (18–28)	22 (20–26)	..
Sex				
Male	7 (47%)	3 (12·5%)	10 (43%)	..
Female	8 (53%)	5 (62·5%)	13 (57%)	..
Adverse events per volunteer, mean (SD)				
Immunisation stage	8·6 (1·7)	1·8 (2·4)	6·7 (3·6)	<0·0001
Challenge stage	12·4 (4·8)	8·6 (5·0)	11·4 (5·0)	0·15
Related skin adverse events (itching and rash) per group, mean (SD)				
Immunisation stage	7·1 (0·8)	0·8 (1·0)	5·3 (3·0)	<0·0001
Challenge stage	4·8 (0·9)	3·6 (0·5)	4·5 (1·0)	0·0070
Volunteers with grade 3 itching after challenge, n (%)	8 (57%)	1 (20%)	9 (47%)	0·018
Related abdominal adverse events per volunteer in challenge stage, mean (SD)	6·4 (4·0)	4·8 (4·3)	6·0 (4·0)	0·46
Volunteers with related grade 3 abdominal adverse events in challenge stage, n (%)	7 (50%)	2 (40%)	9 (47%)	0·55

Ethnicity data were not collected.

Table 1: **Baseline characteristics and adverse events data**

	Intervention group (n=15)				Placebo group (n=8)			
	None	Mild	Moderate	Severe	None	Mild	Moderate	Severe
Immunisation stage								
Immunisation 1	0	10 (67%)	5 (33%)	0	7 (88%)	1 (13%)	0	0
Immunisation 2	0	9 (60%)	6 (40%)	0	7 (88%)	1 (13%)	0	0
Immunisation 3	0	3 (20%)	8 (53%)	4 (27%)	8 (100%)	0	0	0
Challenge stage								
Challenge 1	0	4 (29%)*	6 (43%)*	4 (29%)*	0	4 (80%)†	1 (20%)†	0
Challenge 2	0	1 (7%)*	8 (57%)*	5 (36%)*	0	2 (40%)†	2 (40%)†	1 (20%)†

*One participant withdrew after immunisation 3; therefore the number of participants in the challenge stage for the intervention group became 14. †Three participants withdrew after immunisation 3; therefore the number of participants in the challenge stage for the placebo group became 5.

Table 2: **Participants with mild, moderate, and severe itching after each exposure in intervention and placebo groups**

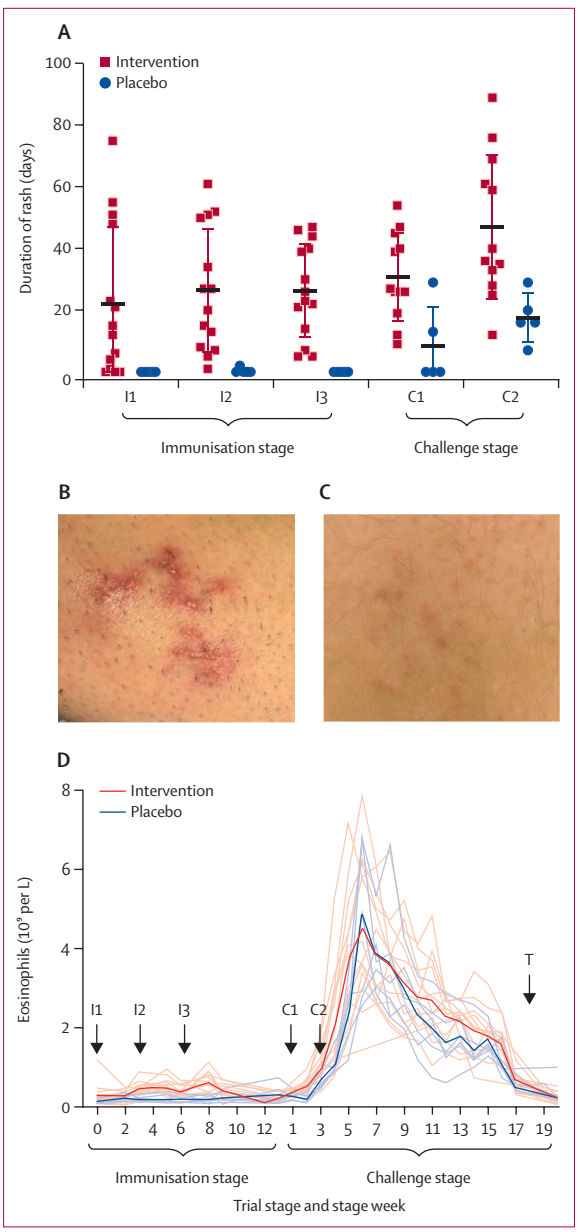


Figure 2: **Skin adverse events and eosinophils**
(A) Duration of rash in days after each hookworm exposure per individual; horizontal black lines show mean and error bars show SD. Representative photographs of severe (B) and mild (C) skin rash at 2 weeks after second challenge. (D) Eosinophil counts in peripheral blood over time in the intervention group and placebo group; the dark red and blue lines represent group mean, all other lines represent individual volunteers. I1=first immunisation. I2=second immunisation. I3=third immunisation. C1=first challenge. C2=second challenge. I=immunisation. C=challenge. T=treatment with albendazole.

(figure 2B, C). Seven volunteers with severe itching were prescribed antihistamines, in addition to the use of cortisone cream. Eight volunteers in the intervention group reported severe abdominal adverse events between 3 weeks and 8 weeks after challenge, either abdominal cramping or nausea and vomiting, all lasting less than 12 h. Severe abdominal adverse events were not correlated with severe skin adverse events and none were reported in the placebo group. No respiratory symptoms indicative of pulmonary infiltration were reported.

During the immunisation stage, volunteers in the intervention group had a small increase in eosinophils to a maximum of $0{\cdot}9\times10^9$ cells per L, which was not observed in the placebo group ($p<0{\cdot}0001$), and returned to baseline 3 weeks after immunisation. Eosinophil counts peaked around week 6 after challenge in all volunteers (figure 2D). No differences in peak eosinophil count after challenge were observed between the intervention and placebo group (mean count $4{\cdot}7\times10^9$ cells per L [SD 1·7] in the intervention group *vs* $5{\cdot}2\times10^9$ cells per L [1·5] in the placebo group; p=0·60). However, counts at 12 weeks and 16 weeks after challenge were higher in the intervention group than the placebo group (mean count $2{\cdot}3\times10^9$ cells per L [0·55] in the intervention group *vs* $1{\cdot}6\times10^9$ cells per L [0·86] in the placebo group at week 12, p=0·069; $1{\cdot}7\times10^9$ cells per L [0·56] *vs* $1{\cdot}1\times10^9$ cells per L [0·44] at week 16, p=0·11), although the differences were not statistically significant. Volunteers with severe skin rash after challenge had higher eosinophil counts than those without, particularly at week 13 after the challenge (mean count $2{\cdot}3\times10^9$ cells per L [0·41] in volunteers with severe skin rash *vs* $1{\cdot}5\times10^9$ cells per L [0·49] in volunteers with mild-to-moderate rash, p=0·0048). Severe abdominal adverse events were not associated with the height or duration of peak eosinophilia (mean count $4{\cdot}8\times10^9$ cells per L in volunteers with severe adverse events *vs* $4{\cdot}9\times10^9$ cells per L for volunteers with non-severe adverse events, p=0·86), nor were severe skin adverse events (mean count $4{\cdot}5\times10^9$ cells per L for volunteers with severe skin rash *vs* $5{\cdot}1\times10^9$ cells per L for volunteers with mild-to-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 weeks 8, 9, and 12 of the immunisation stage, indicating complete abrogation of the infection by repeated albendazole treatment. All volunteers had 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 (data not shown).

Egg load after challenge was lower in the intervention group than the placebo group (geometric mean 571 eggs per g [range 372–992] *vs* 873 eggs per g [268–1484]); however, this difference was not statistically significant, 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 than volunteers with mild-to-moderate rash, with a 40% reduction in egg burden (geometric mean 742 eggs per g [range 268–1484] *vs* 441 eggs per g [380–520]; p=0·0025; figure 3B). No differences in egg load were identified between volunteers with and without grade 3 abdominal adverse events (geometric mean 549 eggs per

g [268–1122] in volunteers with severe adverse events *vs* 728 eggs per g [407–1484] in volunteers with non-severe adverse events; p=0·23). Egg detection by PCR showed similar trends as the microscopy data (mean Ct value for placebo group 27·9 [SD 1·5] *vs* 29·1 [1·2] for the intervention group; p=0·083); however, no differences in Ct values were identified between volunteers with mild-to-moderate rash and severe rash (mean Ct value 28·6 [1·5] for volunteers with mild-to-moderate rash *vs* 29·2 [0·9] for volunteers with severe rash; p=0·36; figure 3C, D). The hatching assay identified no differences between the intervention and placebo groups or between volunteers with or without severe rash (appendix p 4).

IgG1 titres at challenge were significantly higher than at baseline in volunteers with severe skin rash (fold increase 4·5 *vs* 1·2; p=0·03). Furthermore, IgG1 titres after challenge increased to markedly higher levels in the intervention group than the placebo group (fold change 12 weeks after the challenge 4·0 AU/mL in the intervention group *vs* 0·8 AU/mL in the placebo group, p=0·0020; fold change 16 weeks after challenge 3·6 AU/mL *vs* 0·9 AU/mL, p=0·026). Similarly, volunteers with severe rash had higher peak IgG1 after challenge than volunteers with mild-to-moderate rash (fold change 12 weeks after the challenge 6·7 AU/mL in volunteers with severe rash *vs* 1·7 AU/mL in volunteers with mild-to-moderate rash, p=0·026; fold change 16 weeks after challenge 6·2 *vs* 1·5 AU/mL, p=0·019; figure 4A, B).

Six volunteers in the intervention group had IgG1 seroconversion after challenge, of whom four had severe skin rash. Seroconversion was more frequent in volunteers with severe rash (n=5) than volunteers with non-severe rash (n=13; 67% *vs* 11%; p=0·013) and was associated with duration of rash after second challenge (mean 57·5 days [SD 20·8] in volunteers who seroconverted *vs* 27·7 days [18·8] in volunteers who did not; p=0·0040), but not with severe abdominal adverse events (p=1·00) or peak eosinophil count (p=0·24; appendix p 4). In the group who had seroconversion, the mean egg load tended to be lower than the group who did not have seroconversion (geometric mean 507 eggs per g [186] *vs* 838 eggs per g [369]; p=0·085; appendix p 4).

Changes in hookworm-specific IgG4 were insignificant—ie, only one volunteer had seroconversion. No differences were identified between IgG4 titres in volunteers in the placebo group and intervention group or between volunteers with mild-to-moderate skin rash and severe skin rash (appendix p 5). IgE titres did not increase in any of the volunteers over the course of the study.

Circulating cytokines measured in serum showed considerable interindividual variation (appendix pp 6–7). No differences in circulating cytokines were identified between treatment groups. IL-4, a T-helper-2 cell (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.

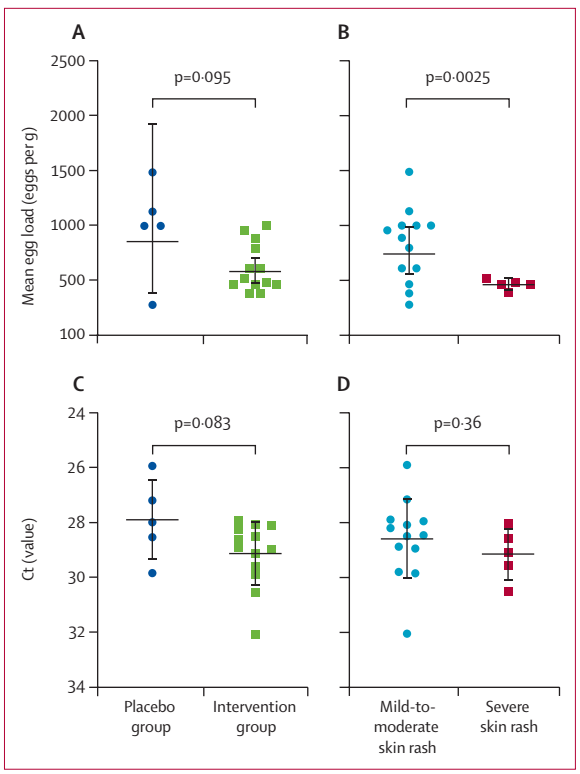
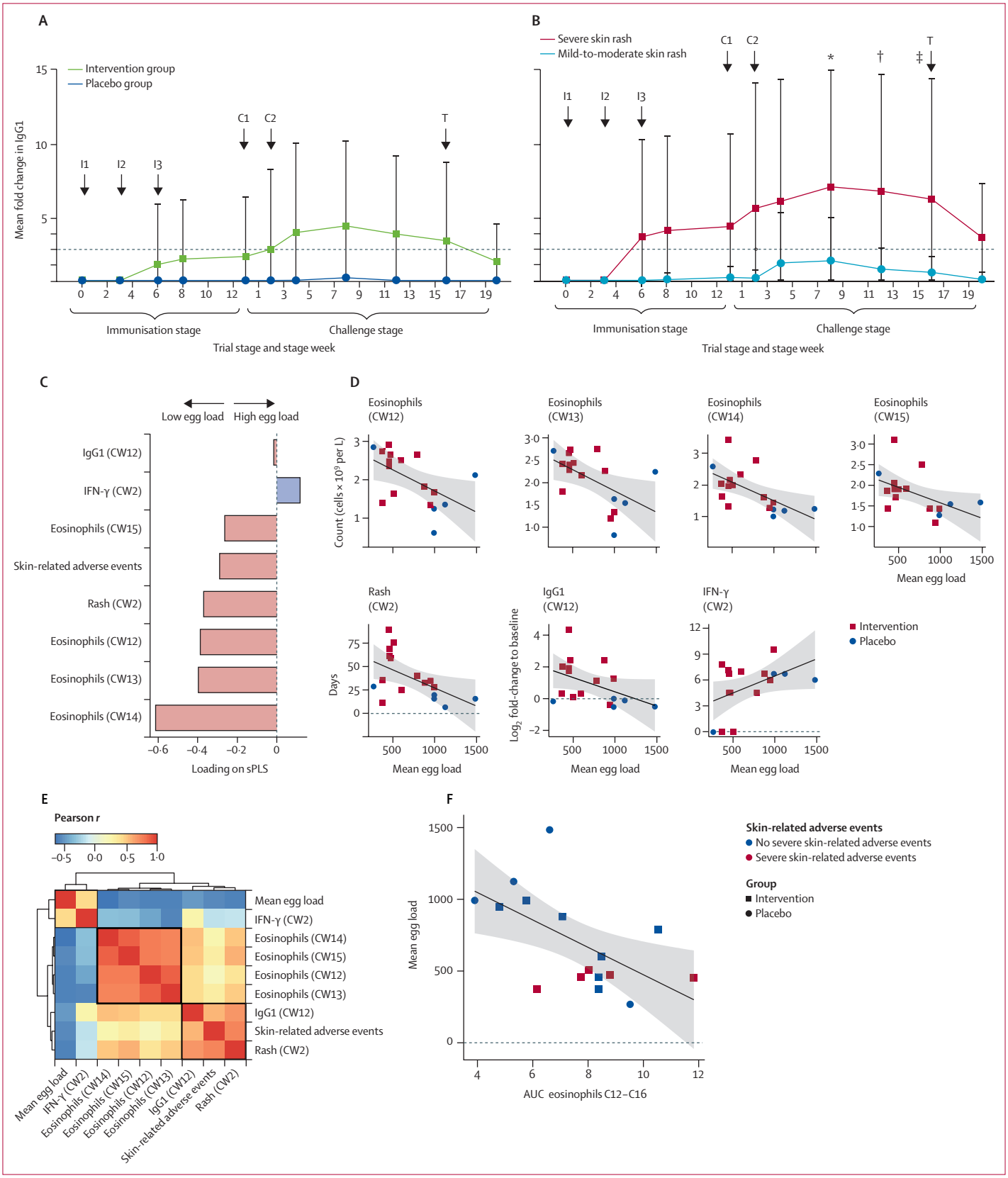


Figure 3: **Parasitological analyses after challenge**
(A) Hookworm eggs detected in faeces by Kato-Katz-method microscopy in intervention and placebo groups. (B) Volunteers with mild-to-moderate skin rash and severe skin rash and their associated egg load detected by Kato-Katz-method microscopy. (C) Real-time quantitative PCR Ct values in intervention and placebo groups. (D) Real-time quantitative PCR Ct values for stool samples from volunteers with mild-to-moderate skin rash and severe skin rash. (C, D) Black horizontal lines show pooled geometric mean and error bars show 95% CIs. Ct=cycle threshold.

To integrate analyses of measured parameters (eosinophils, antibodies, cytokines, and adverse events) and identify the parameters that most strongly associated with protection in this study, we did an sPLS regression.[19] Seven features were associated with low egg counts after challenge, all from the challenge stage (figure 4C).

Eosinophil counts during egg production (at challenge stage weeks 12–15) were associated with protection. We also confirmed that only adverse events of the skin, but not other adverse events, were associated with protection, as were increases in IgG1 subclass at its peak 12 weeks after challenge (figure 4C, D). Correlation analysis of the selected features identified three main clusters of correlated features: egg load with IFN-γ at 2 weeks after first challenge (although not significantly correlated), the eosinophil levels during egg production, and the skin-related adverse events with IgG1 at week 12 after challenge (figure 4E). Area under the curve was calculated for eosinophil numbers in the challenge stage weeks 12–16, which was correlated with lower egg loads


A
Intervention group
Placebo group
Mean fold change in IgG1
I1
I2
I3
C1
C2
T
Immunisation stage
Challenge stage
Trial stage and stage week
B
Severe skin rash
Mild-to-moderate skin rash
*
†
‡
C
Low egg load
High egg load
IgG1 (CW12)
IFN-γ (CW2)
Eosinophils (CW15)
Skin-related adverse events
Rash (CW2)
Eosinophils (CW12)
Eosinophils (CW13)
Eosinophils (CW14)
Loading on sPLS
D
Eosinophils (CW12)
Eosinophils (CW13)
Eosinophils (CW14)
Eosinophils (CW15)
Count (cells × 10⁹ per L)
Rash (CW2)
Days
IgG1 (CW12)
Log2 fold-change to baseline
IFN-γ (CW2)
Mean egg load
Intervention
Placebo
E
Pearson r
Mean egg load
IFN-γ (CW2)
Eosinophils (CW14)
Eosinophils (CW15)
Eosinophils (CW12)
Eosinophils (CW13)
IgG1 (CW12)
Skin-related adverse events
Rash (CW2)
F
Mean egg load
AUC eosinophils C12–C16
Skin-related adverse events
No severe skin-related adverse events
Severe skin-related adverse events
Group
Intervention
Placebo

(r_s=–0·59, p=0·012, Pearson test; figure 4F). Skin-related adverse events and eosinophil numbers together separated volunteers with high egg load from those with lower egg loads.

Discussion

To our knowledge, this is the first study 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 are important for 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 volunteers with severe skin rash. This contrasts with natural infections, in which IgG4 is the most prominent immunoglobulin subclass, with higher levels of IgG4 being observed with higher worm burdens.[20] IgG4 is less pronounced after experimental infection, indicating this subclass might 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 cells 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 than the placebo group. In our previous study, we identified an association between eosinophilic response and abdominal adverse events and a non-significant association between lower egg counts in volunteers and 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 *N americanus* hookworm infections with 50L3, where we abrogated the infection at a later stage (week 20), severe skin responses were not observed. This finding indicates that the early destruction of larvae, similar to the radiation-attenuated larvae used in animal models and ultraviolet-irradiated larvae in the controlled human infection model described by Chapman and colleagues,[13] is crucial for the induction of protective immunity, which then attacks the invading larvae in the skin stage on subsequent infections. The involvement of eosinophils and IgG1 in such a response is supported by previous in-vitro studies that demonstrated their ability to kill schistosomula.[21] The skin rash and severe nightly itching are reminiscent of the symptoms observed after human infection with canine hookworms (*Ancylostoma braziliense* and *A caninum*), which can be similarly erythematous, vesicular, and serpentine[22] 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. The lungs were thought to be the primary site for immune induction in models using irradiated hookworm larvae in dogs and murine infection experiments.[7,23] 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.[24] These initially regulatory responses were thought to be the reason why cercarial dermatitis is usually mild.[24] 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.[25] In these models, a large number of neutrophils were observed to swarm *N braziliensis* in murine skin,[26] 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 the survival of some larvae. Obtaining 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-related adverse events were also found in one previous study where the immunising effects of ultraviolet-irradiated larvae were tested in a controlled human infection model described by Chapman and colleagues.[13] Specifically, the attenuation process was targeted to have the larvae cause a mild-to-moderate rash, which therefore might have induced a weaker immunological response than that observed in our study. Based on a previous dose-escalation study,[15] we decided to select higher challenge doses. Our primary endpoint included multiple samples

Figure 4: **IgG1 titres and sPLS analysis**
Fold-change in hookworm-specific IgG1 from baseline in the intervention group and placebo group (A) and in volunteers with mild-to-moderate skin rash and severe skin rash (B); error bars show SD and dashed lines indicate threshold for seroconversion, set at 3-fold increase from baseline. (C) Features associated with decreased or increased egg load in the sPLS regression model; loading on the first principal component is shown per each individual measure included; individuals were considered to have a low egg count (ie, protected) if their egg count was <1 SD of the geometric mean egg count; all other individuals were considered to have a high egg count. (D) Correlation between mean egg load and eosinophil counts, duration of rash, and fold-change from baseline in IgG1 and IFN-γ at different timepoints after challenge; black lines represent linear regression result and shaded areas show 95% CIs. (E) Correlation matrix of selected outcomes by sPLS regression; colours indicate the strength and direction of the Pearson's *r* value. (F) Correlation of egg load with AUC of eosinophil counts during the egg production phase weeks C12–16; the black line represents the linear regression result and shading shows 95% CIs. I1=immunisation 1. I2=immunisation 2. I3= immunisation 3. C1=challenge 1. C2=challenge 2. T=treatment with albendazole. sPLS= sparse partial least squares. CW=challenge stage week. AUC=area under the curve. *p=0·030. †p=0·026. ‡p=0·019.

obtained 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 the recovery of 20 times more larvae per g of faeces in the hatching assay and due to multiple sampling a more robust outcome that accounts for variability in egg excretion.

Although an attenuated larvae approach to vaccination is not feasible on a large scale, the skin and associated initial larval stages might enable novel vaccine candidates to be identified. The only vaccine currently in clinical development, Na-GST-1/Na-APR-1 (Aeras Global Vaccine Foundation, Rockville, MD, USA; Walter Reed Army Institute of Research, Silver Spring, MD, USA), targets adult worms.[27] A previous larval antigen candidate, Na-ASP-2 (Walter Reed Army Institute of Research), showed potential to inhibit larval migration in the skin[28] underscoring the possibilities of vaccines that target early larval stages. However, this vaccine was not successful in early clinical development due to the induction of IgE-mediated allergic responses in a pre-exposed population.[29] Our study shows that early larval-stage antigens do not induce specific IgE responses in non-immune populations but might 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 done before 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. The occurrence of strong eosinophilic responses after challenge suggests that antigen-specific allergic responses might occur, particularly in response to later stage antigens.

For the **code used for the sparse partial least squares regression analysis** see https://github.com/spjochems/ITTCHI_spls

The repeated infection treatment protocol was specifically designed to enhance the development of protective responses that might be diluted in natural infections due to interfering coinfections or previous infections. The controlled infection setting thus allows for a more robust characterisation of immune responses to early infection, thereby elucidating a hitherto uncharacterised 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.

Due to a high loss to follow-up, the placebo group was reduced from eight volunteers originally to five participants, which substantially impacted the study power to detect differences between the intervention and placebo groups. Moreover, the apparent skin rash in some volunteers undid the intervention masking for both trial physicians and volunteers. However, all laboratory evaluations, including Kato-Katz slides, PCR, and ELISA measurements were performed by personnel unaware of treatment allocation, minimising bias. The findings in this study are specific to *N americanus*, the most prevalent hookworm species, but might not be generalisable to *Ancylostoma* infection.

In conclusion, this study is, to our knowledge, 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 a reliable correlate of protection for vaccine efficacy.

Contributors

M-AH, LvL, MY, and MR conceptualised and designed the study. M-AH, JJ, VK, PHV-M, and JK collected clinical data, M-AH, JJ, VK, RvS, YK, JS, and BN performed laboratory procedures. IW and PM supervised the investigational product, EB and LvL supervised the laboratory procedures, and LV, MY, and MR supervised the study. MY and MR acquired funding. M-AH, SJ, and MR performed the statistical analysis. MH and SJ created the figures. JJ and SJ curated the data. M-AH, JJ, VK, SJ, and MR have directly accessed, analysed, and verified the data. M-AH and SJ wrote the original draft. All authors had access to the data and critically reviewed the manuscript before publication.

Declaration of interests

We declare no competing interests.

Data sharing

The data can be made available on request by contacting the corresponding author. All code used for the sparse partial least squares regression analysis can be found online.

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