Experimental hookworm infection and gluten microchallenge promote tolerance in celiac disease

John Croese, MD,[a,b]* Paul Giacomin, PhD,[b]* Severine Navarro, PhD,[b] Andrew Clouston, MD,[c] Leisa McCann, RN,[b] Annette Dougall, PhD,[b] Ivana Ferreira, BSc,[b] Atik Susianto, MD,[b] Peter O'Rourke, PhD,[d] Mariko Howlett, MD,[e] James McCarthy, MD,[d,e] Christian Engwerda, PhD,[d] Dianne Jones, BHSc,[f] and Alex Loukas, PhD[b] *Brisbane and Cairns, Australia*

Background: Celiac disease (CeD) is a common gluten-sensitive autoimmune enteropathy. A gluten-free diet is an effective treatment, but compliance is demanding; hence, new treatment strategies for CeD are required.
Objective: Parasitic helminths hold promise for treating inflammatory disorders, so we examined the influence of experimental hookworm infection on the predicted outcomes of escalating gluten challenges in CeD subjects.
Methods: A 52-week study was conducted involving 12 adults with diet-managed CeD. Subjects were inoculated with 20 *Necator americanus* larvae, and escalating gluten challenges consumed as pasta were subsequently administered: (1) 10 to 50 mg for 12 weeks (microchallenge); (2) 25 mg daily + 1 g twice weekly for 12 weeks (GC-1g); and (3) 3 g daily (60-75 straws of spaghetti) for 2 weeks (GC-3g). Symptomatic, serologic, and histological outcomes evaluated gluten toxicity. Regulatory and inflammatory T cell populations in blood and mucosa were examined.
Results: Two gluten-intolerant subjects were withdrawn after microchallenge. Ten completed GC-1g, 8 of whom enrolled in and completed GC-3g. Primary outcomes: median villous height-to-crypt depth ratios (2.60-2.63; $P = .98$) did not decrease as predicted after GC-1g, and the mean IgA-tissue transglutaminase titers declined, contrary to the predicted rise after GC-3g. Secondary outcomes: quality of life scores improved (46.3-40.6; $P = .05$); celiac symptom indices (24.3-24.3; $P = .53$), intra-epithelial lymphocyte percentages (32.5-35.0; $P = .47$), and Marsh scores were unchanged by gluten challenge. Intestinal T cells expressing IFNγ were reduced following hookworm infection (23.9%-11.5%; $P = .04$), with corresponding increases in $CD4^+$ $Foxp3^+$ regulatory T cells (0.19%-1.12%; $P = .001$).
Conclusions: *Necator americanus* and gluten microchallenge promoted tolerance and stabilized or improved all tested indices of gluten toxicity in CeD subjects. (J Allergy Clin Immunol 2015;135:508-16.)

Key words: *Celiac disease, gluten, hookworm, autoimmunity, helminth therapy, desensitization, mucosal immunology, regulatory T cells, intra-epithelial lymphocytes*

Celiac disease (CeD) is an acquired autoimmune enteropathy characterized by an inappropriate immune response to dietary gluten.[1] The pathogenesis is complex: ingested gluten is catalyzed to form glutamine-rich peptides, which in genetically susceptible people are deamidated by tissue transglutaminase (tTG), resulting in high-affinity linkages with HLA-DQ2 or HLA-DQ8 proteins on antigen-presenting cells.[2] Proinflammatory T lymphocytes populate the small intestine, inducing epithelial cell apoptosis and the hallmark villous injury associated with CeD.[3] Autoantibodies against tTG and deamidated gluten (DG) correlate with—and as is the case with tTG, may contribute to—the destruction of the epithelial barrier and mucosal inflammation.[4,5] As the prevalence of autoimmune diseases such as CeD is on the rise in the developed world,[6,7] the need to develop new treatment strategies has increased.

Strict compliance with a gluten-free diet (GFD) is the only effective treatment for CeD, but the diet is inconvenient and expensive, and inadvertent gluten exposure is common. Desensitization immunotherapy that involves controlled microchallenge with a deamidated gluten "superantigen" is being evaluated,[8] and it is similar to strategies being employed for allergic diseases.[9] Whether this approach alone will be an effective therapeutic modality for CeD is unclear. Other approaches exploiting the "hygiene hypothesis"[10] in a variety of inflammatory disorders have evaluated the immunoregulatory activities of parasitic helminths—notably the pig whipworm (*Trichuris suis*) and the human hookworm (*Necator americanus* [NA])—in clinical trials[11-16] but have met with mixed success. Our experiences using NA in Crohn disease and CeD show that hookworms alone do not suppress disease symptoms sufficiently to be considered a worthwhile therapy.[12,13] More specifically, in the lead-in study to the current trial, most subjects developed symptoms and mucosal inflammation immediately following an abrupt reintroduction of gluten, irrespective of whether or not they had been previously infected with NA.[13] Nonetheless, NA

From [a]the Department of Gastroenterology and Hepatology, The Prince Charles Hospital, Brisbane; [b]Center for Biodiscovery and Molecular Development of Therapeutics, Australian Institute of Tropical Health and Medicine, James Cook University, Cairns; [c]Envoi Specialist Pathologists, Brisbane; [d]QIMR Berghofer Medical Research Institute, Brisbane; [e]Royal Brisbane and Women's Hospital, Brisbane; and [f]Logan Hospital, Brisbane.
*These authors contributed equally to this work.
The work was funded by an Australian National Health and Medical Research Council (NHMRC) Program Grant (1037304 to A.L., C.E., and J.M.), NHMRC Overseas Biomedical Fellowship 613718 (to P.G.), NHMRC Principal Research Fellowship (1020114 to A.L.), NHMRC Senior Research Fellowship (1058685 to C.E.), NHMRC Practitioner Fellowship and Government of Queensland Health Research Fellowship (1041802 to J.M.) and a Far North Queensland Hospital Foundation Small Research Grant (P.G. and S.N.).
Disclosure of potential conflict of interest: This work was funded by an Australian National Health and Medical Research Council Program Grant (1037304). The authors declare that they have no other relevant conflicts of interest.
Received for publication April 24, 2014; revised July 14, 2014; accepted for publication July 15, 2014.
Available online September 20, 2014.
Corresponding authors: John Croese, MD, Gastroenterology Unit, The Prince Charles Hospital, Rode Rd, Chermside, Brisbane, QLD 4032, Australia. E-mail: mcroese@bigpond.net.au. Or: Alex Loukas, PhD, Australian Institute of Tropical Health & Medicine, James Cook University, McGregor Rd, Smithfield, Cairns, QLD 4878, Australia. E-mail: alex.loukas@jcu.edu.au.
0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2014.07.022

Abbreviations used
CeD: Celiac disease
CSI: Celiac symptom index
DG: Deamidated gluten
GC: Gluten challenge
GFD: Gluten-free diet
IEL: Intra-epithelial lymphocyte
LPL: Lamina propria lymphocyte
NA: *Necator americanus*
Treg: Regulatory T
QOL: Quality of life
tTG: Tissue transglutaminase
Vh:Cd: Villous height:crypt depth

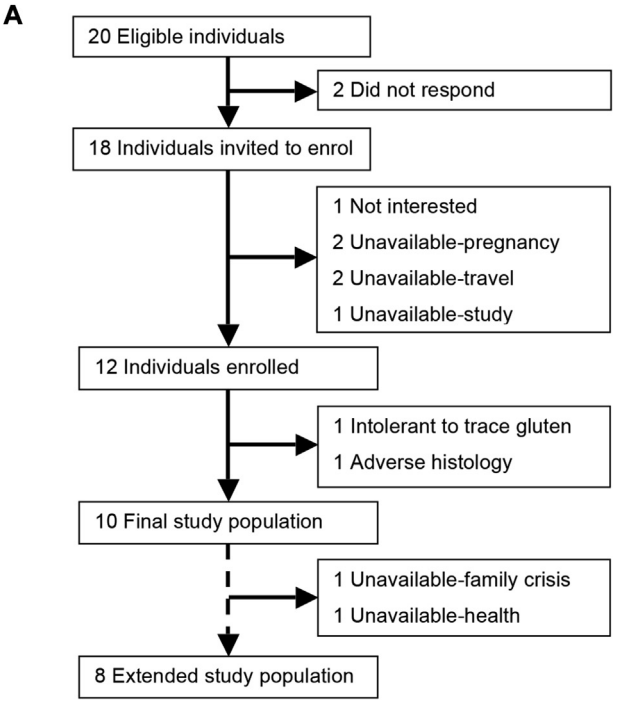
infection did suppress gluten-induced IFNγ, IL-17, and IL-23 expression and upregulated expression of pro-regulatory IL-10, TGFβ, and IL-22 in the gut of patients with CeD.[17,18] In light of these anti-inflammatory hookworm-induced changes, we posed the question of whether NA infection may be more suitable as a tolerizing agent to trace amounts of gluten as opposed to a stand-alone therapy.

The present study combines 2 strategies to mitigate autoimmunity: protracted antigen microchallenge and duodenal immunomodulation with NA infection. A modest gluten challenge was employed to mimic what occurs during inadvertent exposure to gluten. Because gluten is toxic for most people with CeD, we utilized historical control data to compare trial outcomes. Duodenal villous height:crypt depth ratio (Vh:Cd) was the primary outcome in a longitudinal format using a gluten challenge similar to that employed by Catassi et al, who demonstrated significant deterioration of the Vh:Cd following 12 weeks of 50 mg gluten daily.[19] After our preliminary analyses suggested improved gluten tolerance, approval to undertake an additional 2-week intense GC similar to that employed by Leffler et al was granted,[20] with IgA-tTG levels used as the primary outcome. Secondary outcomes included a quality of life questionnaire to evaluate well-being,[21] a celiac-specific symptom record to score adverse responses to gluten ingestion,[22] and IgA- and IgG-DG titers.[5] We also evaluated immunological changes in the intra-epithelial lymphocyte (IEL) and lamina propria lymphocyte (LPL) tissue compartments before and after hookworm infection combined with gluten microchallenge, and after gluten challenge.

METHODS

Patients and regulatory commitments

Twenty participants with documented HLA-DQ2 CeD who were enrolled in a clinical trial that was completed in 2010 were approached.[13] All subjects had been infected with NA between 2008 and 2010 and developed typical celiac enteropathy following a GC in that trial.[13] Two candidates could not be contacted, 1 was not interested, and 5 were unavailable because of pregnancy (2), travel (2), or study commitments (1) (Fig 1, *A*). The 12 enrolled subjects (mean age 53 years, range 39-67; females 9) were adherent to a GFD for an average of 12.5 years except for during the 5-day GC from our earlier trial (Fig 1, *B*).[13] The total length of the trial was 52 weeks. Eight subjects committed to 3 endoscopic procedures (Group 1), including a baseline examination 6 weeks after anthelmintic therapy (200 mg mebendazole twice daily for 3 days), and 4 committed to 2 endoscopies and no anthelmintic treatment (Group 2). The study and the application to extend the challenge, both designated HREC/07/QPAH/115, were approved on June 23, 2012 and



B

ID	Age	Sex	CeD Diagnosis	HLA status	Years on GFD	Group
ID1	44	F	Feb 2000	DQ2	12	1
ID2	39	F	1996	DQ2	16	1
ID3	47	F	2001	DQ2	11	1
ID4	62	F	May 1997	DQ2	15	2
ID5	62	F	March 2005	DQ2	7	2
ID6	39	M	2005	DQ2	7	1
ID7	62	M	July 2001	DQ2	11	1
ID8	59	F	1999	DQ2	13	2
ID9	61	F	2001	DQ2	11	1
ID10	52	M	2001	DQ2	11	1
ID11	47	F	June 2007	DQ2	5	2
ID12	67	F	May 1981	DQ2	31	1

FIG 1. Trial CONSORT diagram and participants. CONSORT chart showing flow of patients through the clinical trial **(A)**. Table of patient identification (ID) numbers **(B)**, displaying age, sex, clinical diagnosis and diet-management details, and whether patient was part of Group 1 (full trial, with anthelmintic drug treatment) or Group 2 (abbreviated trial, no anthelmintic drugs).

September 4, 2013, respectively, by the Human Research Ethics Committee (EC00167) at the Centres for Health Research, Princess Alexandra Hospital, Ipswich Road, Woolloongabba, Queensland 4102, Australia. The trial was registered with ClinicalTrials.gov, Protocol Record AU/3/BOBD012 on August 6, 2012.

NA infection

Hookworm ova were collected from 2 volunteers infected in 2004 with NA infective third-stage larvae (L3) from a line donated by Professor David Pritchard (University of Nottingham) and maintained in-house through re-inoculation. Regular sampling of feces and blood from these donors had consistently tested negative for pathogens. Inocula composed of 10 L3 were

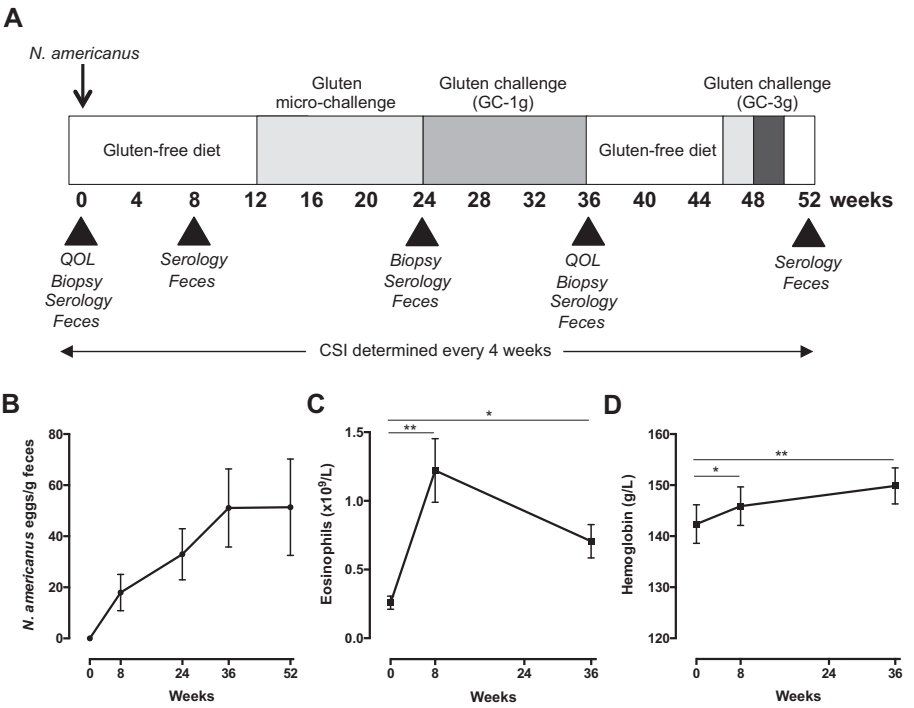


FIG 2. Trial design and successful establishment of NA infection. Trial timeline **(A)**. Quantitative PCR determination of mean NA eggs per gram of feces ± SEM throughout the trial **(B)**. Blood eosinophil counts **(C)** and hemoglobin concentration **(D)** in Group 1 subjects. *$P < .05$ and **$P < .001$.

prepared as previously described.[23] Each volunteer received 10 NA L3 at the trial commencement and a second inoculation with 10 L3 4 to 8 weeks later for a total of 20 L3 per subject (Fig 2, *A*). NA infective status was monitored using quantitative PCR on fecal samples as described previously.[24,25]

Gluten challenge

Gluten was provided as wheat pasta (Balducci [Rockville, Md] Spaghettini No. 3; 12% protein content and an estimated 6% dry weight of gluten, assuming an equal prolamins to globulins + albumins ratio) and consumed either dry or after cooking in boiling water. The gluten microchallenge commenced 4 weeks after the second NA inoculation at 1 mg daily and increased to 10 mg daily for 6 weeks, then 50 mg daily for 6 weeks (Fig 2, *A*). The gluten challenge consisted of 25 mg of gluten daily and 1 g twice weekly (20-25 spaghetti straws; GC-1g) for 12 weeks. Ten weeks after the conclusion of GC-1g, during which time participants had notionally returned to a GFD, an extension of the GC was notified (AU/3/BOBD012) to include 10 mg daily for 1 week, 50 mg for 1 week, and 3 g daily for 2 weeks (GC-3g). Celiac symptom index (CSI) and quality of life (QOL) analyses were performed as previously described.[21,22]

Serum analyses

Complete blood counts were measured in an Australian accredited clinical pathology laboratory. Serum IgA-tTG and IgA/IgG-DG titers were measured using a multiplex flow immunoassay (BioPlex 2200 Celiac IgA and IgG; BioRad, Hercules, Calif) 4 weeks prior to GC-1g, immediately after GC-1g, and 2 weeks after GC-3g.[26]

Endoscopy and assessment of duodenal histopathology

Endoscopies were performed by a gastroenterologist (M.H. or J.C.) while subjects were under deep sedation administered by an anesthetist. Of 14 biopsies collected from the mid-duodenum at each endoscopy, 2 were processed for analysis of IEL/100 enterocytes, Vh:Cd score, and Marsh score as previously described (see Fig E1 in this article's Online Repository at www.jacionline.org).[13,27] For intra-individual comparative purposes, a cut-off IEL score of <40% and a Vh:Cd of >2 were arbitrarily assigned based on values used clinically.[28] For Group 2 subjects who did not undergo a baseline biopsy, the most recent duodenal sample collected between 2008 and 2010 was used as a pre-trial reference.

Lymphocyte isolation and flow cytometry

IELs and LPLs were isolated from duodenal biopsy tissue as previously described.[29] Blood leukocytes were isolated using density centrifugation (Lymphoprep; Stemcell Technologies, Vancouver, British Columbia, Canada). Single cell suspensions were cultured in RPMI + 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine in round-bottom 96-well plates in the presence of 25 ng/mL phorbol 12-myristate 13-acetate, 0.5 ng/mL ionomycin, and 10 μg/mL Brefeldin A for 4 hours at 37°C. Cells were stained with fluorochrome-labelled antibodies against CD3 (clone OKT3), CD45 (HI-30), CD4 (OKT4), IFNγ (4S.B3), IL-17A (ebio64DEC17), CD8 (SK1), CTLA-4 (14D3), and Foxp3 (PCH101). Samples were analyzed on a BD LSR-Fortessa flow cytometer (Becton Dickinson, Franklin Lakes, NJ), and data analysis was performed using FlowJo software (Treestar, Ashland, Ore).

Statistical analyses

The pre- versus post-GC-1g Vh:Cd and the pre- versus post-GC-3g IgA tTG titers were the designated primary outcomes. Where applicable, data across 2 time points were compared by Wilcoxon matched-paired *t* tests or unpaired Mann-Whitney *t* tests. Data for multiple time points were analyzed with a general linear model, with subjects and time as factors, and variance was estimated on a within-subjects basis.

RESULTS

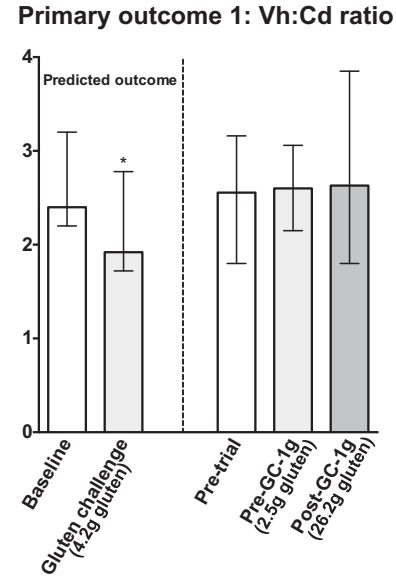
Successful establishment of *Necator americanus* infection

Each participant acquired a patent NA infection, confirmed by the detection of hookworm ova in feces that persisted for the course of the 52-week trial (Fig 2, *B*; and see Fig E2 in this

A

	Vh:Cd (ratio)			IgA-tTG (U/mL)		
ID#	**Pre-trial**	**Pre-GC-1g**	**Post-GC-1g**	**Pre-trial**	**Post-GC-1g**	**Post-GC-3g**
ID1	2.61	4.13	2.60	15.2	10.9	7.8
ID2	3.16	3.03	2.66	1.0	ND	ND
ID3	2.5	2.33	2.34	3.8	4.4	3.0
ID4	1.8#	2.06	1.7	1.4	1.1	ND
ID5	2.5#	3.06	3.01	5.1	2.1	1.7
ID6	2.97	2.28	3.85	0.5	0.5	0.5
ID7	3.42	2.7	2.21	1.3	0.5	0.5
ID8	2.2#	2.15	1.8	1.9	1.5	0.9
ID9	2.62	2.5	4.17	2.3	2.1	ND
ID10	1.4	3.01	3.0	1.6	0.7	0.5
ID11	0.76#	1.07	1.58^	1.0	0.9	ND
ID12	2.93	2.28	ND	3.7	3.3	2.0

B



C

D

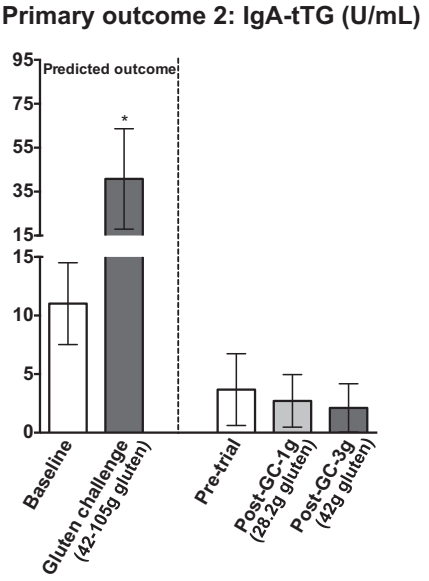


FIG 3. **A,** Primary trial outcomes did not display changes consistent with increased CeD severity following escalating gluten challenges. Vh:Cd values for each individual's duodenal biopsy specimen. **B,** Predicted median Vh:Cd ratio ± 95% CI following a 12-week challenge with 50 mg of gluten/day (historical data from Catassi et al[19]) and the results for the 10 subjects who completed the current GC-1g trial. **C,** Table depicting raw IgA-tTG titers from trial participants. **D,** Predicted mean IgA-tTG titers ± 95% CI following a 2-week challenge with 3 g or 7.5 g of gluten/day (historical data from Leffler et al[20]) and the results from the current GC-1g and GC-3g trials. *Significant ($P < .05$) reductions in Vh:Cd and increases in IgA-tTG were observed in historical control data sets and were used as predicted outcomes for the current trial. ^ This was an exit biopsy done for clinical reasons 12 weeks after recommencing a GFD. **#**Pre-trial reference from 2008 to 2010 while subject was on a GFD. *ND,* Not determined.

article's Online Repository at www.jacionline.org). During endoscopy, mature NA worms, often mating, were visualized within the duodenum (see Video E1 in this article's Online Repository at www.jacionline.org). The intensity of infection in 1 subject (ID11) was substantially higher than that in all other subjects at week 24 (724 eggs/g feces); her outlier result was excluded from this analysis (infection intensity) only. In participant ID9, a 3-cm blistering reaction at the inoculation site developed that lasted several days, but it resolved without treatment. Participant ID4 developed intermittent colic between inoculations consistent with the hookworm-induced eosinophilic enteritis we have reported previously.[13] Significant blood eosinophilia, a hallmark of NA infection, was present at week 8 ($P < .001$) but had partially subsided by week 36 (Fig 2, *C*). Hemoglobin levels had significantly (and unexpectedly) increased by week 36 ($P < .001$; Fig 2, *D*), and no participant

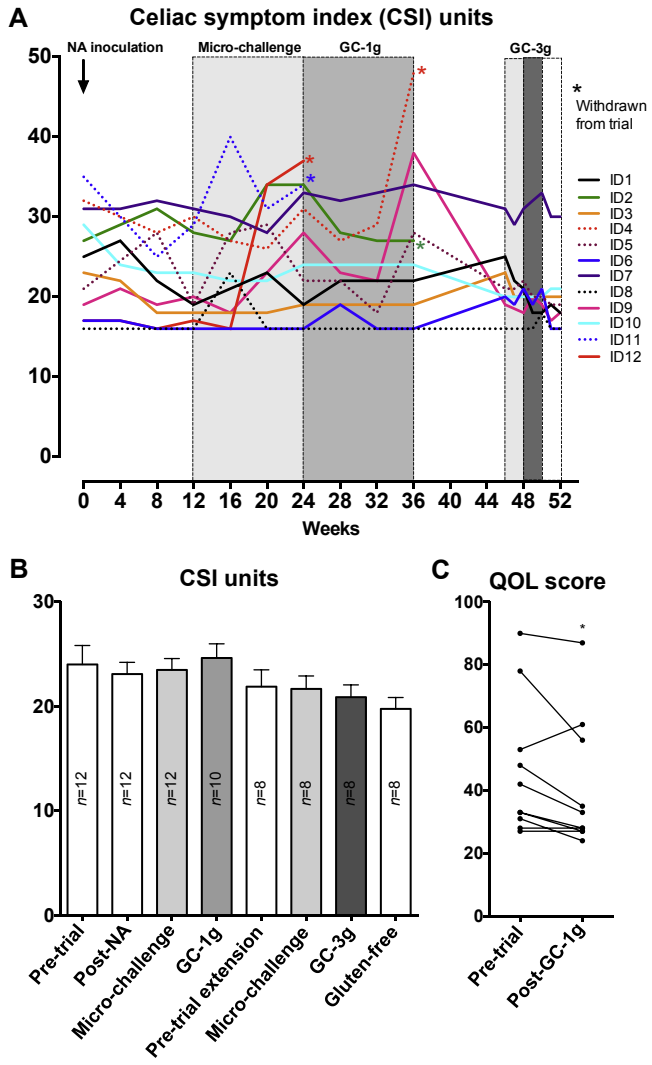


FIG 4. Questionnaire-based assessments of CeD severity remained unaltered or imporved following gluten challenges. **A,** Monthly or weekly CSI values for the 12 subjects and corresponding identification (ID) numbers. **B,** Mean CSI values ± SEM for the 8 to 12 trial subjects, grouped by the nature of the weekly intervention. **C,** QOL score for the 10 subjects who completed GC-1g, in which a lower value indicates greater CeD-related life quality. *$P = .05$.

developed anemia, an expected consequence of heavy hookworm infection in developing countries.[30]

Exclusions and withdrawals

Two subjects (ID11 and ID12) were withdrawn during the microchallenge. ID11 remained indefinably unwell for the duration of the trial and had adverse histology at enrollment and throughout (Marsh 3A),[27] which in retrospect was likely pre-existing and reflective of inadvertent and ongoing gluten ingestion, and was not improved 12 weeks after returning to a notional GFD. ID12 could not tolerate gluten doses exceeding 25 mg daily but retained normal histology. Ten of 10 remaining subjects commenced and completed GC-1g. Two subjects (ID4 and ID9) temporarily discontinued gluten while potential adverse events were being evaluated; ID4 exhibited lower backache and abdominal distension concurrent with a family crisis, and ID9 displayed acute infective enteritis soon after overseas travel. Both recommenced GC-1g within 1 to 2 weeks and completed the program. Eight of 10 subjects participated in GC-3g, while 2 declined; ID2 withdrew on advice from the family doctor because of vitamin D and B_{12} deficiencies, and ID4 withdrew because of depression arising as the result of the family crisis reported during GC-1g.

Primary outcomes were better than predicted

Duodenal Vh:Cd was the primary outcome for evaluation of gluten tolerance following GC-1g. Consensus Vh:Cd scoring (A.C. and J.C.) undertaken in 12 instances of orientation difficulty resulted in 5 changes, the ratio being increased in each case and a lesser Marsh score adjudged in 2 instances. We arbitrarily assigned a Vh:Cd value of <2 as abnormal in the context of established CeD. Three of 12 subjects had a low (<2) Vh:Cd score pre-trial, 1 of 12 displayed a low score post-microchallenge, and 2 of 10 subjects recorded low values post-GC-1g (Fig 3, *A*). Contrary to that previously reported by Catassi et al showing a 20% decline in the median Vh:Cd (the predicted outcome) after a 12-week GC of 50 mg daily (4.2 g in total),[19] the median Vh:Cd levels in our subjects were similar pre-trial and after a 12-week challenge with 2.5 g of gluten (microchallenge, 2.56-2.60; $P = .98$) and the subsequent 12-week challenge with a total of 28.2 g of gluten (GC-1g, 2.60-2.63; $P = .98$) (Fig 3, *B*).

Our subjects closely matched those recently reported by Leffler et al,[20] who had participated in a kinetics study measuring outcomes in response to a 2-week GC. In brief, the investigators reported that IgA-tTG reliably and significantly increased in their participants when measured 2 weeks after completing a 3 g daily or 7.5 g daily GC (total gluten load of 42-105 g), and this outcome correlated with worsening mucosal pathology. Since our analysis of Vh:Cd following GC-1g suggested improved gluten tolerance, we extended the study to include a 2-week re-introduction to gluten (10-50 mg/day), followed by a 2-week challenge with 3 g of gluten per day (GC-3g, total gluten load of 42 g) and analysis of IgA-tTG as the primary outcome 2 weeks later (Fig 3, *C*). Remarkably, and in stark contrast to what was observed in subjects from Leffler et al,[20] (the predicted outcome), kinetic analysis of mean IgA-tTG titers demonstrated a paradoxical linear decline from baseline levels following GC-1g and GC-3g (slope −1.012, 95% CI, −1.166 to −0.363; $P = .005$; Fig 3, *D*). Counter to the declining IgA-tTG, the IgG-DG and IgA-DG levels in 1 subject (ID9) rose 8- and 4-fold, respectively (data not shown; see Fig E3 in this article's Online Repository at www.jacionline.org), but all levels were within the normal range for the other 7 subjects. Together, analysis of these primary outcomes in the context of historical data suggests that NA infection may support tolerance to escalating gluten challenges.

Questionnaire-based assessments remained unaltered during GC-1g

A disease-specific symptom scoring index, validated in adults with CeD that identifies adverse responses to ingested gluten,[22] was recorded at monthly intervals during lead-in, NA-uptake phase, and microchallenge. The CSI incorporates symptoms such as abdominal pain and altered bowel habit, which we have previously reported in volunteers when first infected with NA.[12] Week zero CSI scores in all cases were low (range,

A

	M0	MI	M2	M3A	M3B	M3C

B

	Marsh score			IEL/100 enterocytes (%)		
ID#	Pre-trial	Pre-GC-1g	Post-GC-1g	Pre-trial	Pre-GC-1g	Post-GC-1g
ID1				35	24	57
ID2				27	58	31
ID3				35	23	35
ID4	#			46#	56	50
ID5	#			31#	26	23
ID6				21	31	35
ID7				19	19	8
ID8	#			20#	8	14
ID9				46	50	56
ID10				45	39	41
ID11	#		^	45#	54	50^
ID12			ND	18	14	ND

C

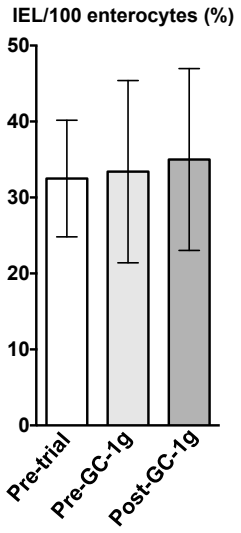


FIG 5. A, Intestinal histopathology remained normal during GC-1g. Heat map displaying individual Marsh scores. **B,** Table displaying IEL counts/100 enterocytes for each individual's biopsy specimen. **C,** Median IEL % values ± 95% CI for the 10 subjects who completed the trial. **#** Pre-trial reference from 2008 to 2010 while subject was on a gluten-free diet. ^ This was an exit biopsy done for clinical reasons 12 weeks after recommencing a GFD. *ND*, Not determined.

16-35), consistent with GFD compliance (Fig 4, *A*). Hookworm infection and gluten microchallenge did not substantially alter CSI scores in 10 of 12 subjects. Eight of 10 subjects who completed GC-1g, and all 8 subjects who completed GC-3g, remained stable, while the 2 subjects who withdrew prior to GC-1g (ID4 and ID9) had developed abrupt and transient increases in CSI (Fig 4, *A*). Mean CSI scores did not significantly change from pre-trial levels following microchallenge or GC-1g (Fig 4, *B*); however, for the 8 subjects who went on to complete GC-3g, mean CSI values unexpectedly demonstrated a linear decline during the 6-week trial extension (slope −0.283 CSI units, 95% CI, −0.476 to −0.091; $P = .005$) (Fig 4, *B*). At week zero and after GC-1g, subjects completed a detailed and validated CeD-specific questionnaire to evaluate general well-being.[21] The celiac quality of life index following GC-1g challenge improved, indicated by a significantly lower mean QOL score ($P = .05$; Fig 4, *C*).

Intestinal histopathology remained normal during GC-1g

No substantial change in histopathological grading score, either individually or collectively, was evident in the 10 subjects after consuming progressively increased quantities of gluten over 24 weeks. The intra-individual variation in Marsh score was minimal and consistent with artefact (Fig 5, *A*).[27] We arbitrarily assigned an IEL value of 40% or greater as abnormally elevated in the context of established CeD. Four of 12 subjects displayed an elevated IEL % score pre-trial, or at their most recent pre-trial endoscopy (Fig 5, *B*). Following GC-1g, 4 of 10 subjects recorded an elevated IEL score, including 3 subjects who had elevated pre-trial values (Fig 5, *B*). The mean IEL % scores were similar before and after GC-1g and were consistent with those recorded pre-trial (Fig 5, *C*).

NA infection and gluten microchallenge alter intestinal proinflammatory and regulatory T cell subsets

A hallmark of CeD is infiltration of T lymphocytes into the duodenal intra-epithelial tissue, which promotes epithelial damage via the production of the proinflammatory cytokines IFNγ, IL-21, and IL-17A.[2] We therefore assessed the presence of pro- and anti-inflammatory T cells within the duodenum. Intestinal T lymphocytes were identified by flow cytometry by co-staining with CD45 (hematopoietic cell marker) and CD3 (a pan T cell marker) (Fig 6, *A*) and subsequently gated for analysis of IFNγ expression (Fig 6, *B*). Mean frequencies of $IFN\gamma^+$ T cells within the IEL population were significantly reduced following NA infection and gluten microchallenge ($P = .04$), and frequencies of these cells remained lowered following GC-1g (Fig 6, *C*). Similar trends toward suppressed IFNγ expression were observed in the LPL, while frequencies of $IFN\gamma^+$ T cells in the blood were unaltered (Fig 6, *C*). Frequencies of T cells expressing IL-17A in the IEL or blood did not change from pre-trial levels, although there was a trend toward reduced $IL\text{-}17A^+$ T cells in the LPL ($P = .06$; Fig 6, *D*). To assess whether the reduction in proinflammatory T cells was accompanied by concurrent increases in anti-inflammatory cell populations, we assessed the frequencies of $CD4^+$ T cells expressing the regulatory T (Treg) cell marker Foxp3. While $CD4^+$ $Foxp3^+$ T cells within the IEL were rare pre-trial, we observed progressive expansion of this cell population pre- and post-GC-1g (Fig 6, *E*), in which mean frequencies of $CD4^+$ $Foxp3^+$ T cells significantly increased

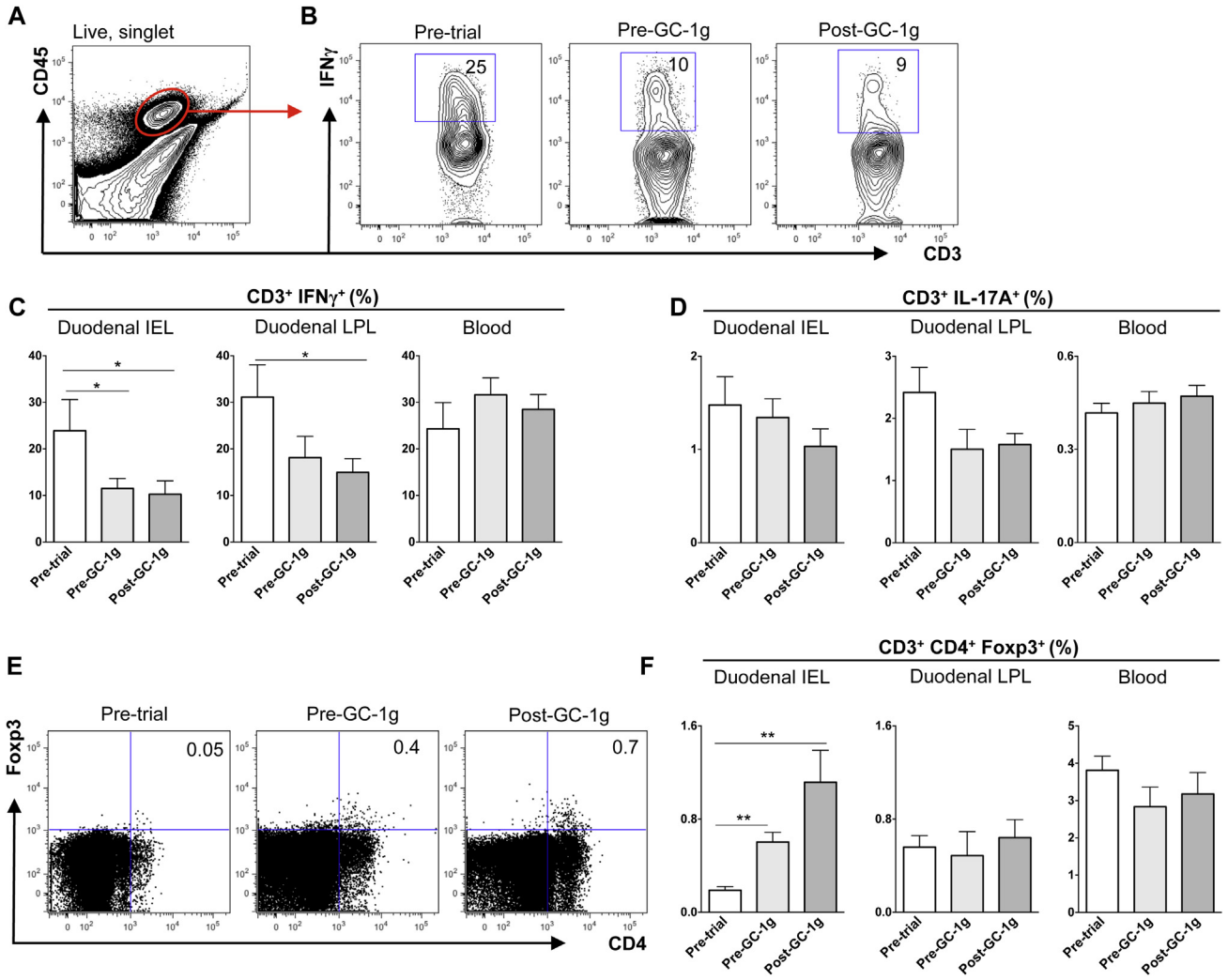


FIG 6. NA infection and gluten microchallenge alter intestinal proinflammatory and regulatory T cell subsets. **A,** Representative flow cytometric plots demonstrating gating strategy for identifying $CD45^+$ $CD3^+$ T cells; data from duodenal IEL tissue is shown. **B,** Representative plots showing IFNγ expression in gated $CD3^+$ $CD45^+$ cell population from part A. **C,** Mean frequencies of $IFN\gamma^+$ T cells ± SEM in IEL, LPL, and blood. **D,** Mean frequencies of IL-17A$^+$ T cells ± SEM. **E,** Representative flow cytometric plots showing frequency of $CD4^+$ and $Foxp3^+$ cells, gated on $CD45^+$ $CD3^+$ cells from the IEL. **F,** Mean frequencies of $CD4^+$ $Foxp3^+$ T cells ± SEM in each tissue. *$P < .05$ and **$P < .005$.

from pre-trial levels selectively within the IEL compartment ($P = .002$; Fig 6, *F*). Gated $CD4^+$ $Foxp3^+$ T cells from all tissues examined co-expressed CTLA-4 (see Fig E4 in this article's Online Repository at www.jacionline.org), a critical factor regulating the immunosuppressive function of Treg cells.

DISCUSSION

We previously reported that although hookworm infection influenced gluten-specific intestinal immune responses in CeD patients,[18] it did not result in a clinical benefit following an aggressive gluten challenge.[13] In the current proof-of-concept trial, we combined 2 strategies: biologic immunomodulation induced by a helminth (herein coined *parabiotic*) and desensitization by sustained antigen microchallenge. The initial gluten challenge (GC-1g), designed to mimic inadvertent gluten ingestion, balanced conflicting priorities by providing sufficient gluten to cause predictable damage while not significantly compromising health. Catassi et al had previously reported a significant 20% decline of the Vh:Cd following 50 mg of gluten daily for 12 weeks.[19] Our challenge far exceeded this in time and quantity and was remarkable for its lack of gluten-induced pathology.

Even more striking, a subsequent, more intense 2-week gluten challenge (GC-3g) structured to match that reported by Leffler et al,[20] improved symptomatic tolerance of gluten and reduced anti-tTG levels. Our subjects were closely matched in age and disease duration to those reported by Leffler et al, thus were capable of developing symptoms of gluten intolerance, which had been confirmed in a prior challenge and accompanied by an anti-tTG increase within the 2-week time frame after completing the challenge.[13] Also consistent with our assertion that the serial GCs did not reactivate CeD, the IgG-DG levels remained normal and unchanged in all but 1 who completed GC-3g, the exception

being the subject who had the only elevated IgA-DG and the highest IgG-DG pre-trial.[5] We elected not to encourage our subjects to undergo a further endoscopy to test for mucosal pathology post-GC-3g, because elevated anti-tTG correlates with the deterioration in mucosal pathology.[20] When taken in the context of historical data in which gluten exposures of similar duration and intensity in the absence of a helminth infection resulted in significant morbidity and intestinal pathology,[19,20] the results indicate that our approach significantly improves tolerance to gluten.

People with CeD should not interpret these findings as an alternative therapy to a GFD. Two subjects were withdrawn immediately after the gluten microchallenge, a decision that clearly selected for a better outcome for the group progressing. In 1 case, the subject had Marsh 3A histology before enrollment, after microchallenge, and 12 weeks after returning to a notional GFD, a histological score not appreciated at the outset, and likely due to either inadvertent gluten ingestion or refractory CeD. The decision to withdraw this subject was clinical, as it was deemed inappropriate to continue gluten in the context of active disease. The other withdrawn subject could not comfortably tolerate trace amounts of gluten, but despite persisting with the microchallenge, she retained normal histology.

The study group for this trial was small and the design open, with clinical staff and participants aware of when and how much gluten was consumed. While we appreciate the advantages of incorporating a gluten or NA placebo, it is worth noting that any placebo-provoked symptoms could only enhance confidence that the CSI data were unaffected, or in the case of the GC-3g challenge, improved. This is particularly relevant as our volunteers had been primed to anticipate becoming ill after eating gluten as a consequence of inadvertent exposures, and as occurred during a lead-in to the current trial when most of them were temporarily ill after an abrupt GC.[13] Further, we appreciate that the gluten doses used in the challenges do not equate to those that occur in a standard Western diet (10-40 g/day), and that by having only 2 duodenal biopsy samples available per assessment, histology scores were possibly subject to under-challenge and orientation artefact.[27] Nonetheless, the absence of any significant changes in histology and serology was highly apparent and remarkable for its clarity. Larger placebo-controlled, double-blinded trials are required to confirm not only the efficacy and durability of the combined immunotherapy, but also to ascertain the relative importance of hookworm infection versus gluten microchallenge. However, since neither NA infection nor naturally occurring ingestion of small amounts of gluten alone predicably restores gluten tolerance,[13,19] it follows that both elements are required. If these results are confirmed through future trials, such a therapy may represent an important social benefit for people with CeD complying with a GFD who are always at risk of inadvertent gluten exposure, particularly when eating outside of the home.

While the mechanism by which combined NA infection and trace gluten exposure influenced gluten tolerance is unclear, the progressive expansion of Treg cells and reductions in proinflammatory IFNγ- and IL-17A-expressing T cells in the duodenal IEL tissue may have been a key factor in restoring intestinal immune homeostasis. NA infection of humans is associated with increased Treg cell responses[31] and mucosal expression of regulatory cytokines.[17] Consequently, when provided in combination with antigen microchallenge, NA infection may have facilitated expansion of a population of gluten-specific Treg cells that localize to the epithelium.

In addition to the expansion of $Foxp3^+$ Treg cells, we observed an unexpected and paradoxical decline in the titers of tTG antibodies as gluten exposure was increased. Serologic testing for IgA-tTG has a long-established clinical role in CeD that has contributed greatly to disease recognition.[6] There is mounting evidence that both tTG and anti-tTG *per se* are involved with CeD pathogenesis, and they are viewed as potential therapeutic targets,[4] hence a decline in anti-tTG may be especially relevant to the lack of gluten-induced pathology in the present trial. This is consistent with the anti-DG levels, which apart from the one case were unchanged following gluten ingestion, suggesting that notionally weak gluten antigens were not being deamidated.

Together, these findings suggest that hookworm infection combined with incrementally increased amounts of gluten promotes immune regulation and tolerance in CeD. What applicability this helminth-induced immunomodulation has for other autoimmune diseases remains untested, but based on our successful strategy it may represent a beneficial and complementary approach to current efforts to mitigate autoimmunity by antigen-specific immunotherapy.[32] The potency of hookworm-secreted factors in regulating immune responses[33-35] indicates that the efforts directed toward developing recombinant protein-based immunotherapies for autoimmune or allergic disorders are also warranted.[36] Despite the apparent safety of clinical infections with NA, the concept of using it as a biologic immunomodulator (or parabiotic) will be difficult for some. Clinical trials testing its acceptability and the merits of this strategy would be required. Our experience has been that our motivated CeD volunteers have found it acceptable and have consistently declined anthelmintic treatment.

We thank Fabian de Labastida Rivera and Stacey Llewellyn for technical assistance; Soraya Gaze for discussions relating to trial design; Ann Vandeleur and John Murray on behalf of their staff for clinical assistance at The Prince Charles Hospital; Marie Hetherington at Queensland Medical Laboratories for assistance with serology; and Jeffrey Bethony from George Washington University for helpful discussions.

Clinical implications: Hookworm infection, combined with micro-exposure to gluten, promotes immune regulation and limits gluten-induced pathology in celiac disease. Helminth-based therapies hold promise for celiac disease and potentially other autoimmune or inflammatory disorders.

REFERENCES

1. Qiao SW, Iversen R, Raki M, Sollid LM. The adaptive immune response in celiac disease. Semin Immunopathol 2012;34:523-40.
2. Meresse B, Malamut G, Cerf-Bensussan N. Celiac disease: an immunological jigsaw. Immunity 2012;36:907-19.
3. Abadie V, Discepolo V, Jabri B. Intraepithelial lymphocytes in celiac disease immunopathology. Semin Immunopathol 2012;34:551-66.
4. Di Sabatino A, Vanoli A, Giuffrida P, Luinetti O, Solcia E, Corazza GR. The function of tissue transglutaminase in celiac disease. Autoimmun Rev 2012; 11:746-53.
5. Spatola BN, Kaukinen K, Collin P, Maki M, Kagnoff MF, Daugherty PS. Persistence of elevated deamidated gliadin peptide antibodies on a gluten-free diet indicates nonresponsive coeliac disease. Aliment Pharmacol Ther 2014;39: 407-17.
6. Ludvigsson JF, Rubio-Tapia A, van Dyke CT, Melton LJ 3rd, Zinsmeister AR, Lahr BD, et al. Increasing incidence of celiac disease in a North American population. Am J Gastroenterol 2013;108:818-24.

7. Rubio-Tapia A, Kyle RA, Kaplan EL, Johnson DR, Page W, Erdtmann F, et al. Increased prevalence and mortality in undiagnosed celiac disease. Gastroenterology 2009;137:88-93.
8. Anderson RP, Jabri B. Vaccine against autoimmune disease: antigen-specific immunotherapy. Curr Opin Immunol 2013;25:410-7.
9. O'Mahony L, Akdis M, Crameri R, Akdis CA. Novel immunotherapeutic approaches for allergy and asthma. Autoimmunity 2010;43:493-503.
10. Strachan DP. Hay fever, hygiene, and household size. BMJ 1989;299:1259-60.
11. Bager P, Arnved J, Ronborg S, Wohlfahrt J, Poulsen LK, Westergaard T, et al. *Trichuris suis* ova therapy for allergic rhinitis: a randomized, double-blind, placebo-controlled clinical trial. J Allergy Clin Immunol 2010;125:123-30, e1-3.
12. Croese J, O'Neil J, Masson J, Cooke S, Melrose W, Pritchard D, et al. A proof of concept study establishing *Necator americanus* in Crohn's patients and reservoir donors. Gut 2006;55:136-7.
13. Daveson AJ, Jones DM, Gaze S, McSorley H, Clouston A, Pascoe A, et al. Effect of hookworm infection on wheat challenge in celiac disease–a randomised double-blinded placebo controlled trial. PloS One 2011;6:e17366.
14. Feary JR, Venn AJ, Mortimer K, Brown AP, Hooi D, Falcone FH, et al. Experimental hookworm infection: a randomized placebo-controlled trial in asthma. Clin Exp Allergy 2010;40:299-306.
15. Summers RW, Elliott DE, Urban JF Jr, Thompson RA, Weinstock JV. *Trichuris suis* therapy for active ulcerative colitis: a randomized controlled trial. Gastroenterology 2005;128:825-32.
16. Fleming JO, Isaak A, Lee JE, Luzzio CC, Carrithers MD, Cook TD, et al. Probiotic helminth administration in relapsing-remitting multiple sclerosis: a phase 1 study. Mult Scler 2011;17:743-54.
17. Gaze S, McSorley HJ, Daveson J, Jones D, Bethony JM, Oliveira LM, et al. Characterising the mucosal and systemic immune responses to experimental human hookworm infection. PLoS Pathog 2012;8:e1002520.
18. McSorley HJ, Gaze S, Daveson J, Jones D, Anderson RP, Clouston A, et al. Suppression of inflammatory immune responses in celiac disease by experimental hookworm infection. PloS One 2011;6:e24092.
19. Catassi C, Fabiani E, Iacono G, D'Agate C, Francavilla R, Biagi F, et al. A prospective, double-blind, placebo-controlled trial to establish a safe gluten threshold for patients with celiac disease. Am J Clin Nutr 2007;85:160-6.
20. Leffler D, Schuppan D, Pallav K, Najarian R, Goldsmith JD, Hansen J, et al. Kinetics of the histological, serological and symptomatic responses to gluten challenge in adults with coeliac disease. Gut 2013;62:996-1004.
21. Dorn SD, Hernandez L, Minaya MT, Morris CB, Hu Y, Leserman J, et al. The development and validation of a new coeliac disease quality of life survey (CD-QOL). Aliment Pharmacol Ther 2010;31:666-75.
22. Leffler DA, Dennis M, Edwards George J, Jamma S, Cook EF, Schuppan D, et al. A validated disease-specific symptom index for adults with celiac disease. Clin Gastroenterol Hepatol 2009;7:1328-34, 34 e1-3.
23. Croese J, Wood MJ, Melrose W, Speare R. Allergy controls the population density of *Necator americanus* in the small intestine. Gastroenterology 2006;131:402-9.
24. Lambert SB, Whiley DM, O'Neill NT, Andrews EC, Canavan FM, Bletchly C, et al. Comparing nose-throat swabs and nasopharyngeal aspirates collected from children with symptoms for respiratory virus identification using real-time polymerase chain reaction. Pediatrics 2008;122:e615-20.
25. Verweij JJ, Brienen EA, Ziem J, Yelifari L, Polderman AM, Van Lieshout L. Simultaneous detection and quantification of *Ancylostoma duodenale*, *Necator americanus*, and *Oesophagostomum bifurcum* in fecal samples using multiplex real-time PCR. Am J Trop Med Hyg 2007;77:685-90.
26. Holding S, Wilson F, Spradbery D. Clinical evaluation of the BioPlex 2200 Celiac IgA and IgG Kits - A novel multiplex screen incorporating an integral check for IgA deficiency. J Immunol Methods 2014;405:29-34.
27. Taavela J, Koskinen O, Huhtala H, Lahdeaho ML, Popp A, Laurila K, et al. Validation of morphometric analyses of small-intestinal biopsy readouts in celiac disease. PloS one 2013;8:e76163.
28. Walker MM, Murray JA. An update in the diagnosis of coeliac disease. Histopathology 2011;59:166-79.
29. Lundqvist C, Hammarstrom ML, Athlin L, Hammarstrom S. Isolation of functionally active intraepithelial lymphocytes and enterocytes from human small and large intestine. J Immunol Methods 1992;152:253-63.
30. Bethony J, Brooker S, Albonico M, Geiger SM, Loukas A, Diemert D, et al. Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. Lancet 2006;367:1521-32.
31. Ricci ND, Fiuza JA, Bueno LL, Cancado GG, Gazzinelli-Guimaraes PH, Martins VG, et al. Induction of CD4(+)CD25(+)FOXP3(+) regulatory T cells during human hookworm infection modulates antigen-mediated lymphocyte proliferation. PLoS Negl Trop Dis 2011;5:e1383.
32. Sabatos-Peyton CA, Verhagen J, Wraith DC. Antigen-specific immunotherapy of autoimmune and allergic diseases. Curr Opin Immunol 2010;22:609-15.
33. Ferreira I, Smyth D, Gaze S, Aziz A, Giacomin P, Ruyssers N, et al. Hookworm excretory/secretory products induce interleukin-4 (IL-4)+ IL-10+ CD4+ T cell responses and suppress pathology in a mouse model of colitis. Infect Immun 2013;81:2104-11.
34. Geiger SM, Fujiwara RT, Freitas PA, Massara CL, Dos Santos Carvalho O, Correa-Oliveira R, et al. Excretory-secretory products from hookworm l(3) and adult worms suppress proinflammatory cytokines in infected individuals. J Parasitol Res 2011;2011:512154.
35. Ruyssers NE, De Winter BY, De Man JG, Loukas A, Pearson MS, Weinstock JV, et al. Therapeutic potential of helminth soluble proteins in TNBS-induced colitis in mice. Inflamm Bowel Dis 2009;15:491-500.
36. Navarro S, Ferreira I, Loukas A. The hookworm pharmacopoeia for inflammatory diseases. Int J Parasitol 2013;43:225-31.

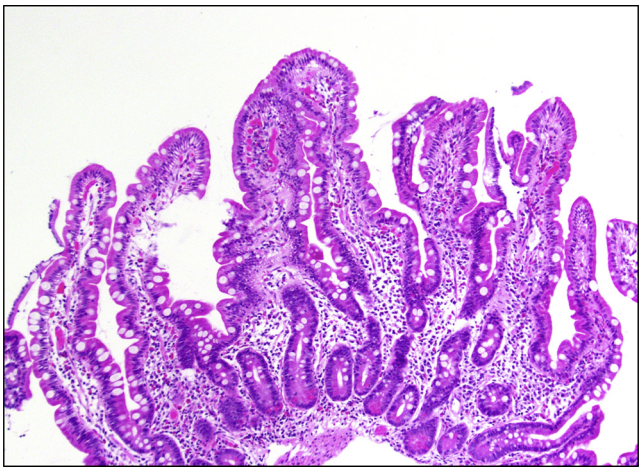


FIG E1. Representative duodenal histology image and description of histological grading methods. Mid-duodenal mucosal biopsy scored Vh:Cd 3.1 and Marsh 0. Good orientation of the mucosal sample is essential to accurately grade celiac disease pathology. The orientation of this section was typical for Vh:Cd and Marsh grading: the long axes of villi were easily identified, and most crypts were sectioned along a longitudinal axis. To the right of this section, crypts sectioned across the longitudinal axis are evident, which creates uncertainty, with a tendency to falsely underscore the Vh:Cd. In clinical practice, it is recommended that 4 samples be provided so as to improve the likelihood of an acceptable section. In our study, 2 biopsies were provided for histology, a decision based on a need to share a limited resource for immunological evaluations. To avoid creating 2 primary outcomes, sampling from the proximal duodenum—as is often suggested in clinical practice to avoid missing localized pathology—was not done.

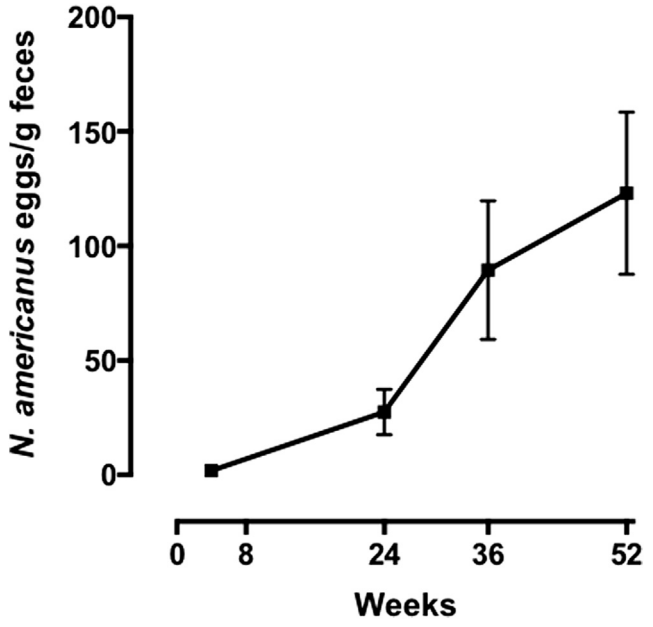


FIG E2. Establishment of NA infection in Group 2 subjects. Quantitative PCR determination of mean NA eggs per gram of feces ± SEM throughout the trial in Group 2 subjects (n = 4).

	IgG-deamidated gluten (DG) titre (U/mL)		
ID#	**Pre-trial**	**Pre-GC-1g**	**Post-GC-1g**
ID1	0.8	1.1	0.6
ID3	3.5	13	2.4
ID5	0.7	0.4	0.4
ID6	0.6	0.4	0.4
ID7	1.1	0.8	0.4
ID8	5.8	8.5	6.9
ID9	9.2	31.1	36.6
ID10	1.9	1.9	2.0

FIG E3. IgG-DG titers remained stable in the 8 subjects who completed both GC-1g and GC-3g trials, aside from 1 subject (ID9).

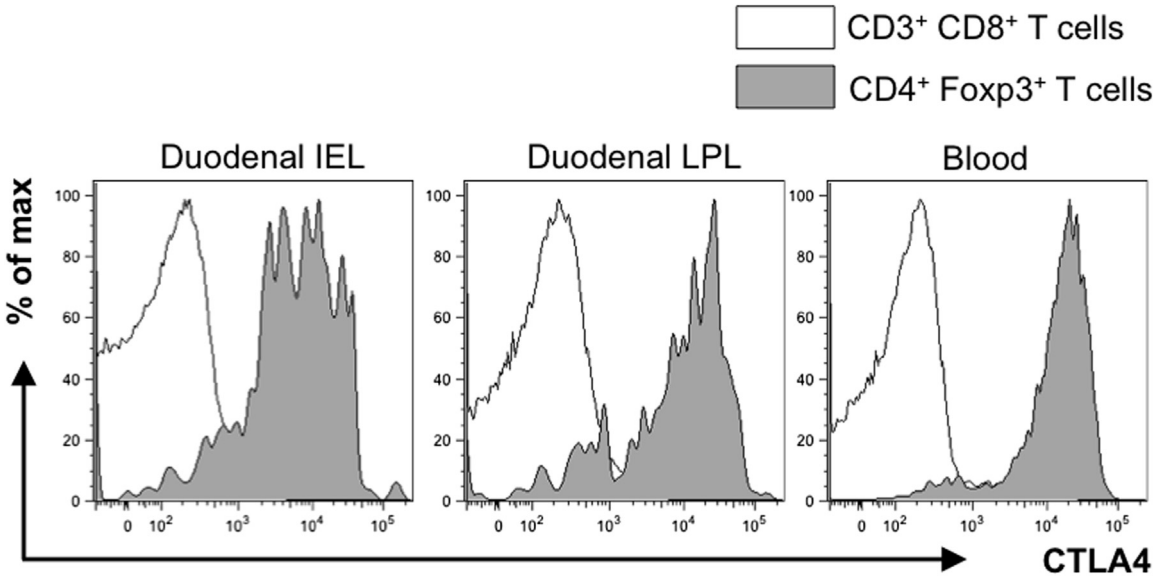


FIG E4. Foxp3$^+$ Treg cells co-express the immunoregulatory molecule CTLA-4. Gated CD3$^+$ CD45$^+$ Foxp3$^+$ cells from duodenal IEL, duodenal LPL, and blood were analyzed for the expression of the Treg marker CTLA-4 *(grey histograms)*. CD8$^+$ CD3$^+$ cells were used as negative controls for CTLA-4 staining *(white histograms)*. Representative data from samples derived post-GC-1g are displayed.

VIDEO E1. Visualization of mature *Necator americanus* adult worms within the duodenum during endoscopy of a CeD trial subject.