Mechanisms underlying reduced expulsion of a murine nematode infection during protein deficiency

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SUMMARY

Balb/c mice infected with the gastrointestinal nematode *Heligmosomoides bakeri* were fed protein sufficient (PS, 24%) or deficient (PD, 3%) diets to investigate whether diet, infection or dose of larval challenge (0, 100 or 200 larvae) influenced gut pathophysiology and inflammation. Among the PS mice, worms were more posteriorad in the intestine of mice infected with 200 compared with 100 larvae, suggesting active expulsion in the more heavily infected mice. This was consistent with the positive correlation between worm numbers and fluid leakage in PS mice; similar patterns were not detected in the PD mice. Infection also induced villus atrophy, which was more pronounced in PS than in PD mice. Our cytokine screening array indicated that infection in PD mice elevated a wide range of pro-inflammatory cytokines and chemokines. Whereas serum leptin concentrations were higher in PD mice, monocyte chemotactic protein-5 (MCP-5) in serum increased with increasing larval dose and concentrations were lower in PD than PS mice. We suggest that elevated MCP-5 together with villus atrophy may contribute to the apparent dose-dependent expulsion of *H. bakeri* from PS mice but that delayed expulsion in PD mice appeared related to a predominant Th1 cytokine profile that may be driven by leptin.

Key words: *Heligmosomoides bakeri*, nematode, protein deficiency, leptin, villus atrophy, MCP-5, histopathology.

INTRODUCTION

Host protective immunity against gastrointestinal (GI) nematodes, such as the common murine mouse model *Heligmosomoides bakeri* (previously named *Heligmosomoides polygyrus*; see Cable *et al.* 2006) is mediated by the Th2 cytokines, IL-4 and IL-13, as well as IL-3, 5, 6, 9, 10 (Urban *et al.* 1998; Gause *et al.* 2003) that induce Th2 effectors including serum IgE, eosinophils and mucosal mast cells (Finkelman *et al.* 1997). Recent studies have also demonstrated that the timing of peak fluid secretion, ion flux and mucus production correspond with loss of worms from the intestine (Shea-Donohue *et al.* 2001), that IL-13 regulated expression of a Goblet cell derived factor RELM-β disrupts the chemosensory apparatus of GI–dwelling nematodes (Artis *et al.* 2004) and that IL-13 induced intestinal smooth muscle contractility (Vallance *et al.* 1997; Au Yeung *et al.* 2005) and accelerated intestinal epithelial cell turnover (Cliffe *et al.* 2005) may also play an important role in nematode expulsion. Thus, evidence is growing to suggest that Th2-dependent inflammatory responses and altered gut physiology also contribute to worm expulsion.

It is also known that protein deficiency impairs the Th2 responses (Ing *et al.* 2000). We have previously reported reduced IL-4 along with several Th2 effectors including eosinophils, mast cells, and serum IgE, but elevation of the Th1 cytokine, IFN-γ, in protein-deficient mice infected with *H. bakeri* (Ing *et al.* 2000). At that time, we had presumed that the prolonged survival of the parasite in these mice was due to impaired Th2 immunological responses. However, given the recent work indicating that inflammatory and/or pathophysiological effectors secondary to the reduced Th2 response may also contribute to worm expulsion (Shea-Donohue *et al.* 2001; Artis *et al.* 2004; Au Yeung *et al.* 2005; Cliffe *et al.* 2005), the possibility that the impairment of these secondary responses might contribute to prolonged parasite survival in protein-deficient mice must be considered. Moreover, because pathophysiological responses to infection are normally stronger at higher infection doses, we hypothesized that worm expulsion should occur more rapidly when immunocompetent protein-sufficient mice are infected with higher doses of GI nematodes. Thus the objectives of the current study were to determine (1) if *H. bakeri* were expelled more rapidly at higher infection doses, (2) if protein deficiency altered the dose-dependent expulsion, and (3) if delayed expulsion of *H. bakeri* in protein-deficient mice was linked

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with dysregulated inflammatory responses and/or impaired gut pathophysiological responses.

MATERIALS AND METHODS

Mice and diets

Female, 25-day-old BALB/c mice purchased from Charles River Breeding Laboratories (Quebec, Canada) were housed individually in Nalgene cages (Fisher Scientific, Montreal, Canada) with stainless steel covers in a temperature controlled mouse room (22–25 °C) with a 10:14 h light:dark cycle.

Egg-white based semi-purified powdered diets were formulated (Tu *et al.* 2007) such that energy from protein made up 24% of total calories of the protein-sufficient (PS) diet, providing a diet sufficient for maintenance of normal physiology, metabolism and growth of mice (NRC, 1995). In contrast, a 3% protein diet was used as the protein-deficient (PD) diet; previous experiments in our laboratory have shown that *H. bakeri*-infected mice fed this diet have reduced body weight gain, increased parasite burdens (Tu *et al.* 2007) and suppressed Th2 immune responses (Ing *et al.* 2000). Both diets were identical for all nutrients except for protein and carbohydrate, the latter of which was adjusted to ensure that both diets were isoenergetic. Diet composition was based on National Research Council mouse requirements (NRC, 1995) to ensure that a 30% reduction in food intake due to infection or protein deficiency would not generate other nutrient deficiencies. Food and tap water were provided *ad libitum*. All procedures were approved by the McGill Animal Care Committee according to the Canadian Council on Animal Care (1993).

Experimental design and protocol

Using a 2 × 3 factorial design, a total of 120 mice were randomized into 2 diet groups (PS and PD) at the beginning of the experiment. They were further subdivided into 3 groups at the time of challenge infection (challenged with either 100 L_3 or 200 L_3, or a sham challenge with no larvae). Ten mice per subgroup were killed at both day 7 and day 14 post-challenge infection (pci).

Initially, all mice were fed commercial Mouse Chow 5015 (Agribands Canada, Ontario, Canada) for 3 days and then acclimatized for 4 days to the PS diet provided in Mouse Powder Feeders (Lab Products, Quebec, Canada) designed to reduce spillage. Following acclimatization to the powder feeders, mice were fed either the PD or PS diet for 1 week and then each mouse was given 100 L_3 by oral gavage. L_3 were obtained by faecal culture of stock parasites maintained in outbred CD-1 mice (Charles River) and counted in 10 sham doses for accuracy before infecting the mice. At days 9 and 14

post-primary infection (ppi), each mouse was treated with pyrantel pamoate (172 mg/kg bwt, Pfizer Canada Inc., Montreal, Canada) to kill the adult worms. One week later, mice were re-infected with 0, 100 or 200 L_3. This drug-abbreviated immunizing protocol normally induces a strong protective immune response (Behnke and Robinson, 1985) leading to parasite expulsion within 18 to 21 days after challenge infection (Ing *et al.* 2000). Just prior to necropsy on days 7 and 14 pci, mice were anaesthetized using Ketamine/Xylazine (50/5 mg/kg body weight), then exsanguinated by cardiac puncture; sera were frozen at −20 °C until later analysis for cytokines and chemokines. At both times, 7 mice per subgroup were anaesthetized 1 h before they were killed and 0·1 ml of 2% Evan's Blue in saline was injected into the tail vein for later measurement of fluid leakage into the duodenum (Ohishi and Odagiri, 1984). Intestinal tissues from the 3 remaining mice per subgroup were used for histopathology and measurement of gut myeloperoxidase (MPO) activity. Body weight was recorded weekly and food intake was measured every 4 days.

Parasites and fluid leakage into the intestine

At necropsy, the intestine of mice injected with Evan's Blue was removed and cut into 8 equal length sections. The first 3 cm of duodenum was rinsed with 1 ml of saline, any adult worms were removed, and then the flushed fluid was frozen at −20° for later detection of Evan's Blue (Ohishi and Odagiri, 1984), an index of intestinal mucosal leakage. Briefly, thawed extravasation fluid was made up to 3 ml by adding 0·5% sodium dodecyl sulphate and 10% Na_2CO_3 to dissolve the mucus. The solution was then transferred to a 1 cm cuvette and the absorbance was measured using a Beckman spectrophotometer (Bio-Tek Instruments, Inc. Winooski, Vermont) at 620 nm. One extravasation unit was defined as 0·001 absorbance units. Each sample was measured in duplicate.

Fourth-stage larvae (L_4) embedded in the intestinal mucosa and adult worms were counted and their spatial distribution along the intestine was recorded. At day 14 pci, worm viability was recorded based on response of non-moving worms to the touch of a needle. The median location of L_4 and adult worms, the percentage recovery of L_4 and adults, and percentage viability were expressed as mean ± S.E.M.

Gut histopathology and MPO activity

Histopathology was assessed in the proximal and distal duodenum, defined by Solaiman *et al.* (2001) as 0–1·0 and 3·5–4·5 cm from the pyloric sphincter, respectively. In each region, two 0·5 cm segments were removed from 3 mice per subgroup immediately after the mouse was killed, and fixed in either

4% paraformaldehyde or Carnoy's fixative. The fixed tissues were dehydrated and embedded in paraffin following standard histological procedures. Sections (5 μm) of paraformaldehyde-fixed tissues were stained with haematoxylin and eosin; eosinophils, Goblet cells, and Paneth cells were counted and villus height and crypt depth were recorded. Toluidine blue staining was done on sections from Carnoy-fixed tissues and mucosal mast cells were counted. Each indicator was expressed as the mean $\pm$ S.E.M. per 10 villus crypt units (vcu).

In addition, two 2 cm segments (1·5 and 4·5 cm along the intestine from the pyloric sphinter) were immediately removed, blotted dry and weighed. Gut MPO activity was detected using an MPO assay kit (Cytostore, Calgary, Alberta, Canada, Cat. ASA-001) as a biochemical marker of intestinal inflammation (Bradley *et al*. 1982). Briefly, the segment was put in hexadecyltrimethyl ammonium bromide buffer in a ratio of 50 mg of tissue per 0·5 ml, homogenized and then centrifuged. Then 20 μl of supernatant was added to each of 2 wells of the ELISA plate, followed by 200 μl of development reagent (4·175 mg o-dianisidine dihydrochloride in 25 ml of potassium phosphate buffer and 12·5 μl of H_2O_2). The absorbance at 450 nm in each well was measured twice using a Beckman spectrophotometer, once immediately after adding the developing reagent and 1 min later in order to record the change of absorbance. One unit of MPO activity was defined as the quantity catalysing the decomposition of 1 μmol of H_2O_2 to water per min at 25 °C (Bradley *et al*. 1982) and the activity was expressed in units per mg of tissue.

Serum cytokines and chemokines

We selected 2 cytokines to measure quantitatively by ELISA: monocyte chemotactic protein-5 (MCP-5) as an inflammatory cytokine recently linked with nematode infections (Sarafi *et al*. 1997) and leptin as a chemokine tied both to food intake (Flier, 1997) and Th1 responses (Lord *et al*. 1998). The mouse MCP-5 immunoassay kit (R&D Systems, Inc. MN, USA, Cat. MCC120) was used according to the manufacturer's instructions. Briefly, 50 μl of standard, control or sample was added to anti-mouse MCP-5 pre-coated ELISA plates that were then incubated for 2 h at room temperature. After 5 washes, wells were incubated for 2 h with 100 μl of mouse MCP-5 conjugate, followed by a 30-min incubation with the substrate 3,3′,5,5′-tetramethylbenzidine (TMB). The reaction was stopped with diluted HCl. The plates were read with an EL 309 Microplate Autoreader (Bio-Tek Instruments, Inc. Winooski, Vermont) at both 450 nm and 540 nm. The reported OD value was obtained by subtracting readings at 540 nm from the readings at 450 nm. Each sample was measured in duplicate.

Serum leptin was measured using a mouse leptin ELISA kit (Linco Research, Missouri, USA, Cat. EZML-82K) following the manufacturer's instructions. Briefly, 10 μl of samples were added to the microtitre plate and then 50 μl of pre-titred anti-rodent leptin serum was added to each well. After incubation for 2 h, followed by 3 washes, 100 μl of pre-titred biotinylated anti-mouse leptin antibody were added to each well and the plate was then incubated for 1 h. Then 100 μl of pre-titred streptavidin-horseradish peroxidase conjugate were added and the plate was incubated for 30 min. Substrate TMB was then added, the plate was incubated for 30 min and the reaction was stopped by addition of 0·3 M HCl. The plates were read with an EL 309 Microplate autoreader at 450 nm and 590 nm. The OD value was calculated by subtracting the readings at 590 nm from the readings at 450 nm. Each sample was measured in duplicate.

In addition, we screened for broader effects of protein deficiency and challenge dose on a wide range of cytokines and chemokines in nematode-infected mice on day 14 pci. An equal amount of serum from each mouse within each subgroup was pooled and diluted 1 : 10 in blocking buffer and tested in duplicate. The pooled sera were assayed, according to the manufacturer's instructions, using a RayBio™ Mouse Cytokine Antibody Array II (RayBiotech Inc., Norcross, Ga, USA). The intensity of each spot was calculated by subtracting the average intensity of the background around each spot from the intensity of the spot itself as read by densitometry (Versa Doc Imaging system, Bio-Rad, USA). The intensity of the positive control was assigned a value of 100, and the intensity of the lightest spot was assigned a value of 0. These were used as reference values to determine the relative intensity for each cytokine, then duplicate values were averaged. If the relative intensity of the sample from mice challenged with 100 larvae was at least 50% lower than the sample from unchallenged mice, we considered the cytokine to be down-regulated whereas if the relative intensity was at least double that of unchallenged mice, we considered it to be up-regulated (Klein *et al*. 2006). Similarly, we compared responses between mice challenged with 200 versus 100 larvae, and between PS and PD mice at each dose of larval challenge.

Statistics

Data on the number of worms, percentage viable adults, percentage recovery of worms and spatial distribution of parasites were analysed using 2×2 ANOVA with main effects of diet (PD *vs* PS) and larval dose (100 *vs* 200) whereas all other data were analysed using 2×3 ANOVA with main effects of diet (PD *vs* PS) and larval dose (0, 100 and 200), with Scheffe's post-hoc test. When there was no

Table 1. Comparison of nutritional indicators and parasite numbers between protein-deficient (PD) and protein-sufficient (PS) mice at days 7 and 14 days post-challenge infection (pci)

(Values are mean$\pm$S.E.M. Means with different superscripts differ significantly between PD and PS mice ($P < 0.05$).)

Parameter	PD Mice	PS Mice
Initial body weight (g)[1]	16.3 ± 0.2	16.6 ± 0.1
Cumulative food intake (g)[1]		
Day 7 pci	104.6 ± 2.1^b	90.8 ± 1.2^a
Day 14 pci	131.4 ± 1.8^b	116.1 ± 1.4^a
Body weight gain (g)[1]		
Day 7 pci	0.6 ± 0.9^a	2.9 ± 0.9^b
Day 14 pci	0.9 ± 1.0^a	2.9 ± 0.6^b
Number of worms on day 7 pci		
Dose of 100	22 ± 2	23 ± 7
Dose of 200	65 ± 29^b	44 ± 19^a
Number of worms on day 14 pci		
Dose of 100	33 ± 5^b	18 ± 6^a
Dose of 200	68 ± 20^b	50 ± 12^a

[1] Values pooled across the 3 challenge infection doses.

significant diet*dose interaction, the data were pooled according to main effects, and re-analysed. Intestinal mucosal leakage was analysed using linear regression with log worm numbers as the independent variable. All analyses were done with SAS 9.01 and the analyses were considered significant at $P < 0.05$.

RESULTS

Initial body weights were similar, but PD mice gained less weight than PS mice, despite higher cumulative food intake (Table 1). Cumulative food intake and weight gain were unaffected by dose of challenge infection (data not shown).

Parasite outcomes

Worm establishment and/or survival over the first week of challenge infection was higher in PD mice challenged with 200 L$_3$ compared with those challenged with 100 L$_3$ (Table 1). Also, as expected based on our previous work (Ing *et al.* 2000), a higher percentage of total worms and viable worms were recovered on day 14 pci in PD mice challenged with both 100 and 200 L$_3$, compared with PS mice (Fig. 1B).

Our hypothesis that worm expulsion would be more rapid in PS mice challenged with 200 compared with 100 L$_3$ was in part supported by the more posteriorad location of worms on day 14 pci in PS mice challenged with 200 L$_3$ (Fig. 1A), but not by the percentage viable adults nor percentage recovery
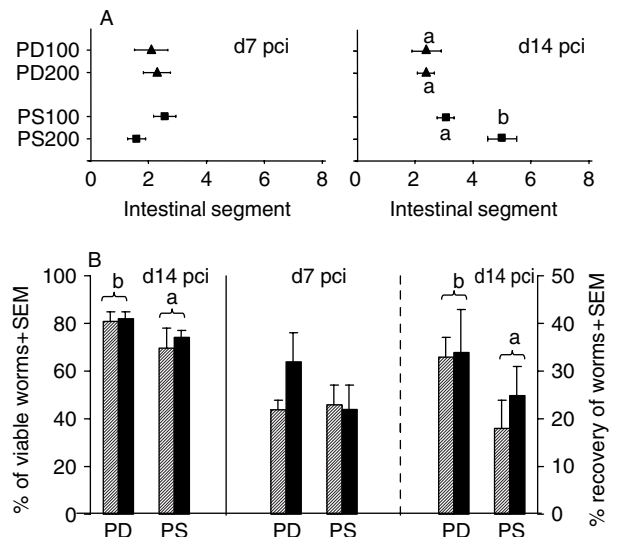

Fig. 1. The influence of dose of infection and diet on *Heligmosomoides bakeri* location in the intestine and survival on day 7 and day 14 post challenge infection (pci). (A) Median location of worms along the intestinal tract (main effect of diet: $F_{1,24} = 21.35$, $P < 0.0001$; main effect of dose: $F_{1,24} = 8.65$, $P = 0.0071$; interaction of diet*dose: $F_{1,24} = 8.65$, $P = 0.0071$); (B) Left axis – percentage viable worms (main effect of diet: $F_{1,24} = 4.63$, $P = 0.04$); right axis – percentage recovery of worms (main effect of diet: $F_{1,24} = 5.05$, $P = 0.03$). Mice were fed either a protein-sufficient (PS, ■) or protein-deficient (PD, ▲) diet and given a challenge infection with either 100 (cross-hatched bars) or 200 (solid bars) larvae. Different letters indicate significant differences within a time-point ($P < 0.05$).

(Fig. 1B). In contrast, we found no evidence for dose-dependent worm expulsion in PD mice. Regardless of challenge dose, parasites at day 14 pci were found in the anterior small intestine (Fig. 1A) and the percentage viable adults or percentage recovery (Fig. 1B) were similar between PD mice challenged with 100 and 200 L$_3$.

Gut histopathology

Compared with unchallenged mice, challenge with 100 or 200 L$_3$ induced elevation in numbers of mucosal mast cells (Fig. 2A), eosinophils (Fig. 2C), Goblet cells (Fig. 2E) and Paneth cells (Fig. 2G) in the proximal duodenum, at both day 7 and day 14 pci. Neither diet (PD *vs* PS) nor challenge dose (100 *vs* 200 L$_3$) influenced cell populations in this region. A broadly similar pattern was observed in the distal region of the duodenum (Fig. 2B, D, F, H), although eosinophilia was not detected in challenged PD mice on day 7 pci (Fig. 2D), significant elevation of Goblet cell numbers only occurred in mice challenged with 200 L$_3$ (Fig. 2F), and Paneth cell numbers were unaffected by challenge infection on both day 7 and day 14 pci (Fig. 2H). In the distal duodenum, mucosal mast cells (Fig. 2B) and eosinophils (Fig. 2D) were
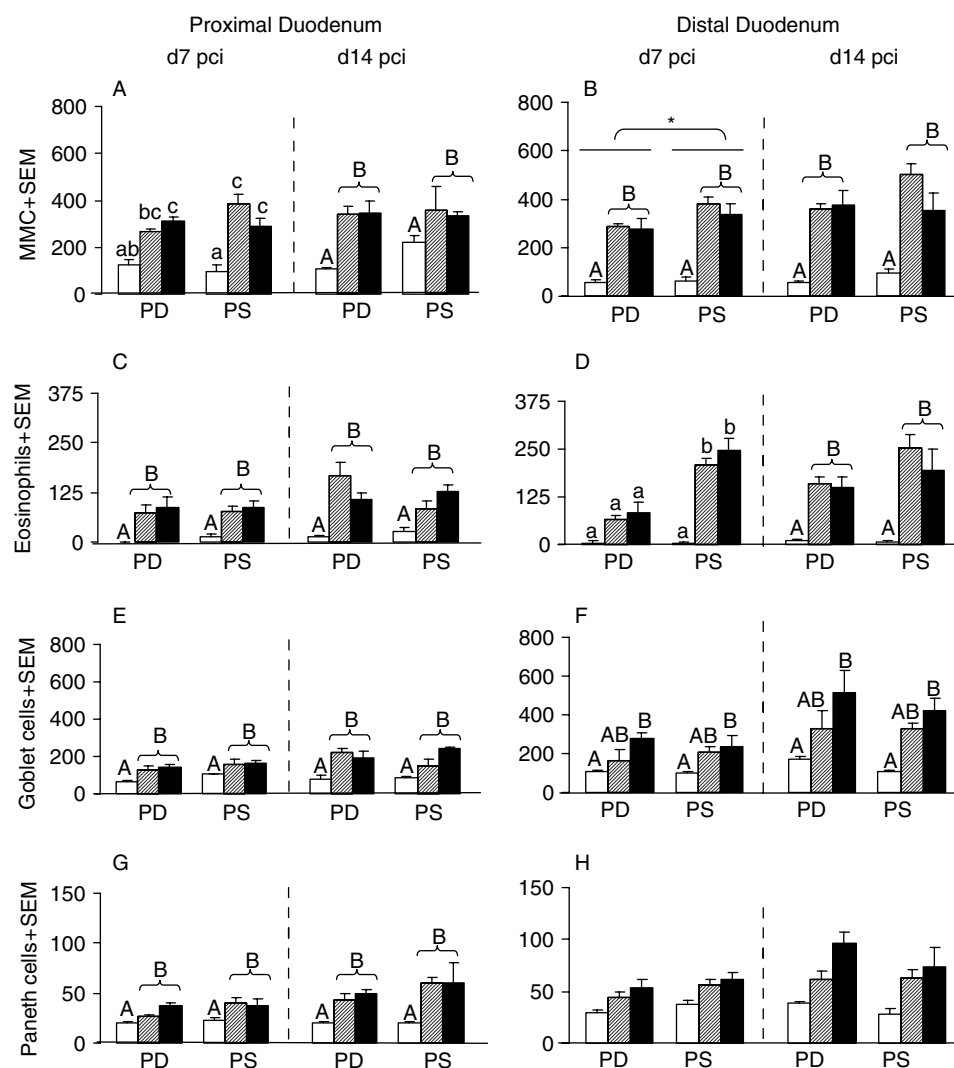
Fig. 2. Histopathology of the proximal (A, C, E, G) and distal (B, D, F, H) duodenum induced by protein deficiency and *Heligmosomoides bakeri* challenge infection on days 7 and 14 post-challenge infection (pci). (A, B) Mucosal mast cells (MMC); (C, D) eosinophils; (E, F) Goblet cells; (G, H) Paneth cells. Cell populations are reported as mean numbers per 10 villus crypt units. PS: protein sufficient; PD: protein deficient; challenge dose of 0 L_3 (open bars), 100 L_3 (cross-hatched bars) or 200 L_3 (solid bars). Different uppercase letters represent significant differences among challenge doses, based on post-hoc analyses following a significant main effect of dose. A significant main effect of diet is represented by a*. Different lowercase letters represent significant differences among dose and diet groups within a time-point, based on Scheffe's post-hoc comparison, if there was a significant interaction. (A) Day 7 pci – main effect of dose: $F_{2,12} = 34 \cdot 88$, $P < 0 \cdot 0001$; interaction of diet*dose: $F_{2,12} = 4 \cdot 42$, $P = 0 \cdot 0364$; day 14 pci – main effect of dose: $F_{2,12} = 8 \cdot 74$, $P = 0 \cdot 0045$; (B) day 7 pci – main effect of diet: $F_{1,12} = 5 \cdot 30$, $P = 0 \cdot 04$; main effect of dose: $F_{2,12} = 8 \cdot 92$, $P = 0 \cdot 0042$; day 14 pci – main effect of dose: $F_{2,12} = 9 \cdot 18$, $P = 0 \cdot 0038$; (C) day 7 pci – main effect of dose: $F_{2,12} = 13 \cdot 82$, $P = 0 \cdot 0008$; day 14 pci – main effect of dose: $F_{2,12} = 12 \cdot 46$, $P = 0 \cdot 0012$; (D) day 7 pci – main effect of dose: $F_{2,12} = 46 \cdot 26$, $P = 0 \cdot 0001$; day14 pci – main effect of dose: $F_{2,12} = 10 \cdot 25$, $P = 0 \cdot 0025$; (E) day 7 pci – main effect of dose: $F_{2,12} = 7 \cdot 79$, $P = 0 \cdot 0068$; main effect of diet; $F_{1,12} = 49 \cdot 06$, $P < 0 \cdot 0001$; interaction diet*dose: $F_{2,12} = 12 \cdot 42$, $P = 0 \cdot 0012$; day14 pci – main effect of dose: $F_{2,12} = 9 \cdot 14$, $P = 0 \cdot 0039$; (F) day 7 pci – main effect of dose: $F_{2,12} = 7 \cdot 13$, $P = 0 \cdot 0091$; day 14 pci – main effect of dose: $F_{2,12} = 8 \cdot 30$, $P = 0 \cdot 0055$; (G) day 7 pci – main effect of dose: $F_{2,12} = 4 \cdot 19$, $P = 0 \cdot 0417$; day 14 pci – main effect of dose: $F_{2,12} = 9 \cdot 37$, $P = 0 \cdot 0035$.

less abundant in PD compared with PS mice, but only on day 7 pci.

Although gut MPO was higher in challenged mice than unchallenged mice, indicating higher intestinal inflammation in both regions of the duodenum (Fig. 3A, B), a graded response of MPO to challenge dose was detected only in the distal duodenum on day 7 pci (Fig. 3B). In addition, PD reduced gut MPO in the distal duodenum at both time-points (Fig. 3B).

We had hypothesized that protein deficiency would reduce villus height (Deo *et al.* 1965; Dudley, 1997). However, villi in the proximal duodenum were longer (not shorter) in PD mice than in PS mice

Proximal Duodenum

Distal Duodenum

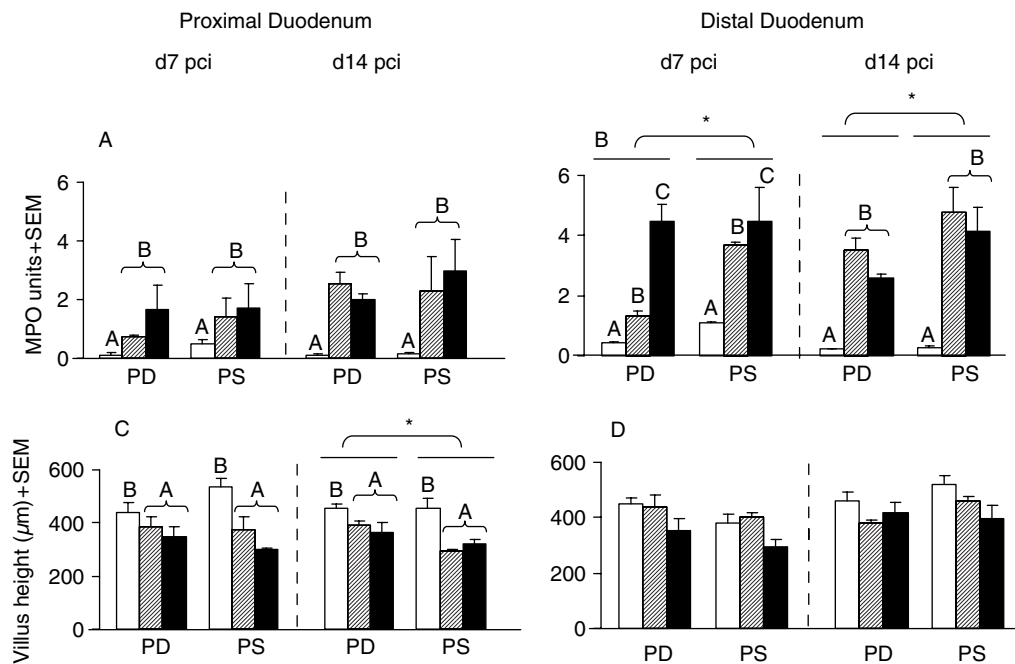d7 pci   d14 pci

d7 pci   d14 pci

Fig. 3. Gut myeloperoxidase (MPO) activity (A, B) and villus height (C, D) of the proximal (A, C) and distal (B, D) duodenum induced by protein deficiency and *Heligmosomoides bakeri* challenge infection on days 7 and 14 post challenge infection (pci). PS: protein sufficient; PD: protein deficient; challenge dose of 0 L_3 (open bars), 100 L_3 (cross-hatched bars) and 200 L_3 (solid bars). Different letters represent significant differences among challenge doses, based on post-hoc analyses following a significant main effect of dose. A significant main effect of diet is represented by a *. (A) Day 7 pci – main effect of dose: $F_{2,12} = 3.99$, $P = 0.047$; day 14 pci – main effect of dose: $F_{2,12} = 7.58$, $P = 0.0074$; (B) day 7 pci – main effect of diet: $F_{1,12} = 5.61$, $P = 0.0355$; main effect of dose: $F_{2,12} = 22.90$, $P < 0.0001$; day 14 pci – main effect of diet: $F_{1,12} = 5.65$, $P = 0.0349$; main effect of dose: $F_{2,12} = 23.42$, $P < 0.0001$; (C) day 7 pci – main effect of dose: $F_{2,12} = 11.07$, $P = 0.0019$; day 14 pci – main effect of diet: $F_{1,12} = 5.39$, $P = 0.0387$; main effect of dose: $F_{2,12} = 12.95$, $P = 0.001$.

on day 14 pci (Fig. 3C). Interestingly, challenge infection induced a similar degree of villus atrophy on both day 7 and day 14 pci in the proximal (Fig. 3C) duodenum, regardless of challenge dose (100 and 200). However, further along the intestine, there was no evidence of villus atrophy in response to either infection or protein deficiency (Fig. 3D). Crypt depth was not affected by either infection or diet in either region of the duodenum (data not shown). Together, these data demonstrate that only gut MPO was more severe in mice infected with 200 compared with 100 L_3. In addition, the presence of a challenge infection reversed the detrimental effect of protein deficiency on villus height in the proximal duodenum.

Analysis of extravasation units of Evan's Blue in duodenal washes on days 7 and 14 pci revealed no main effect of protein deficiency or of dose of larval challenge (data not shown). However, linear regression showed that fluid leakage on day 14 pci increased with increasing worm burdens in PS mice (Fig. 4), a result consistent with the more posteriorad distribution of worms in the intestines of PS mice infected with 200 compared with 100 L_3. In contrast, fluid leakage decreased with increasing worm burdens in PD mice (Fig. 4).
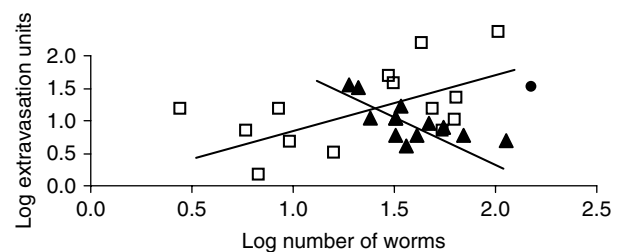

Fig. 4. Relationship between gut permeability as indicated by extravasation units of Evan's Blue in the duodenum and number of *Heligmosomoides bakeri* in protein-sufficient (□) and protein-deficient (▲) mice using data pooled across infection doses of 100 and 200 on day 14 post-challenge infection. Linear regression equation for PS mice: $Y = 0.4249X + 0.5107$, $R^2 = 0.2947$, $P = 0.04$. Linear regression equation for PD mice: $Y = -0.9664X + 2.517$, $R^2 = 0.492$, $P = 0.0075$. The slopes differed significantly ($P = 0.0025$). One outlier in PD mice (filled spot) was excluded from the analyses.

Cytokines

Quantitative comparison of serum leptin revealed an overall diet effect with higher concentrations in PD mice than in PS mice, but no effect of dose of larval challenge (Fig. 5A). On the other hand, serum
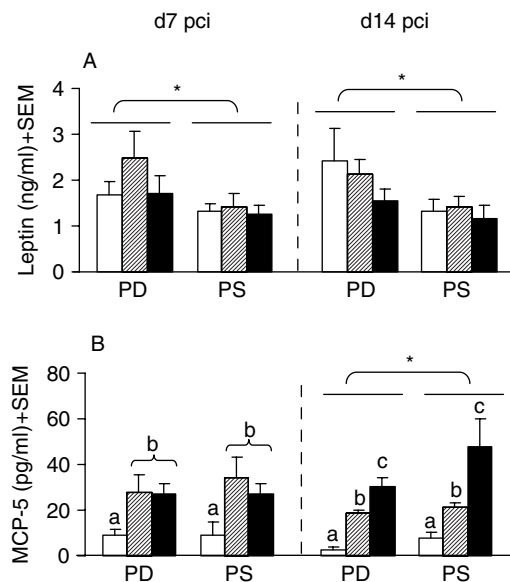
d7 pci   d14 pci

A

B

Fig. 5. Serum concentrations of leptin (A) and monocyte chemoattractant protein 5 (MCP-5) (B) in protein-deficient (PD) and protein-sufficient (PS) mice on days 7 and 14 post-challenge infection (pci) with *Heligmosomoides bakeri*. Challenge dose of 0 L_3 (open bars), 100 L_3 (cross-hatched bars), and 200 L_3 (solid bars). For leptin (A), a significant main effect of diet is represented by a * on day 7 (main effect of diet: $F_{1,54} = 4.75$, $P = 0.03$) and day 14 pci (main effect of diet: $F_{1,54} = 5.57$, $P = 0.02$). For MCP-5 (B), different lowercase letters represent significant differences ($P < 0.05$) among challenge doses within diet groups, based on post-hoc analyses following a significant main effect of dose ($F_{2,54} = 21.61$, $P < 0.0001$). The significant main effect of diet is represented by a * ($F_{1,54} = 4.25$, $P = 0.04$).

MCP-5 was significantly affected by both dose and diet; MCP-5 was higher in challenged mice than unchallenged mice at both time-points in both diet groups (Fig. 5B). On day 14 pci, MCP-5 was further elevated in mice infected with higher numbers of larvae, and levels were higher in PS than in PD mice.

We used a multiple cytokine array to screen pooled serum samples in both experiments. Only 1 cytokine was differentially expressed between unchallenged PD and PS mice; leptin was higher in PD mice than in PS mice (data not shown). In addition, only 3 cytokines were differentially expressed between challenged PD and PS mice challenged with 100 L_3; GCSF was higher in PD mice but values for IL-5 and MCP-5 were reduced in PD mice (data not shown).

In contrast to the restricted effect of protein deficiency on just a few cytokines in unchallenged mice, challenge infection of PD mice modified a wide range of cytokines (Table 2). Comparison between unchallenged PD mice and PD mice challenged with 100 L_3 showed that infection up-regulated a very broad range of cytokines, including leptin, GCSF, GM-CSF, eotaxin, IL-9, IL-12P40P70, IL-12P70,

IL-13, IL-17, IFN-γ, KC, MIP-2 and MIP-3β, and down-regulated only 2 chemokines (MCP-1 and VEGF). The effects of challenge infection were more limited in PS mice, where expression of only IL-5, CTACK, MCP-1, TPO and VEGF were down-regulated. Also, IL-4 and IL-13 levels were not detectably elevated in challenged PS mice compared with unchallenged mice, perhaps because of persistence of these cytokines from the primary infection in the unchallenged mice.

Interestingly in both diet groups, higher dose challenge (comparison between doses of 100 and 200 L_3) did not further up-regulate expression of most cytokines (Table 2). Rather, with the exception of VEGF in PS mice, those cytokines responding to dose were down-regulated at the higher infection dose (IL-6 in both PS and PD mice and TIMP-1, GCSF, GM-CSF and IL-17 in PD mice).

DISCUSSION

The immune expulsion of GI nematodes is usually associated with a dominant Th2 immune response (Gause *et al.* 2003). Recently, however, there has been growing interest in other mechanisms that may be involved in expulsion including intestinal inflammation (Lawrence *et al.* 2001), mastocytosis (McDermott *et al.* 2003), increased epithelial cell turnover (Cliffe *et al.* 2005), hyperplasia of Goblet cells (Artis *et al.* 2004), increased mucous production (Khan and Collins, 2004), and elevated gut permeability, ion flux and luminal fluid secretion (Shea-Donohue *et al.* 2001; Madden *et al.* 2002). In *H. bakeri* challenge infection, expulsion of worms is normally completed within 18 to 21 days pci (Ing *et al.* 2000), and does not begin before the fourth-stage larvae have moved from the serosal musculature into the intestinal lumen. This migration to the intestine is delayed compared with a primary infection (Behnke and Parish, 1979) but is usually completed by day 12 pci (Urban *et al.* 1995; Gause *et al.* 2003). We had hypothesized that more rapid expulsion would occur in PS mice challenged with 200 larvae than with 100 larvae, but that a dose-dependent effect would not be evident in PD mice given previous reports that protein deficiency induces immunosuppression in mice and delays expulsion (Ing *et al.* 2000). In addition, we wished to relate the active expulsion to potential inflammatory and histopathological changes in the mouse. Therefore, we examined responses on day 7 pci, when the parasites would still be embedded in the serosal musculature, and day 14 pci when active expulsion of *H. bakeri* has been reported (Urban *et al.* 1995; Ing *et al.* 2000) in Balb/c mice.

Although our data on percentage worm recovery on day 14 pci did not support the hypothesis of dose-dependent expulsion in PS mice, *H. bakeri* were located more distally along the intestine in the mice

Table 2. Effect of challenge dose on cytokine expression in protein-sufficient (PS) and protein-deficient mice (PD) challenged with *Heligmosomoides bakeri*

(Cytokines were measured using a RayBio^{TM} Mouse Cytokine Antibody Array, on day 14 post-challenge infection. ↑: increase of at least double in first of the two comparison groups; ↓: decrease of at least 50% in first of the two comparison groups.)

Diet	PS		PD	
Comparison	100 *vs* 0 L$_3$	200 *vs* 100 L$_3$	100 *vs* 0 L$_3$	200 *vs* 100 L$_3$
IL-5[1]	↓			
CTACK	↓			
MCP-1	↓		↓	
TPO	↓			
VEGF	↓	↑	↓	
IL-6		↓		↓
TIMP-1				↓
Leptin			↑	
GCSF			↑	↓
GM-CSF			↑	↓
Eotaxin			↑	
IL-9			↑	
IL-12p40p70			↑	
IL-12p70			↑	
IL-13			↑	
IL-17			↑	↓
IFN-γ			↑	
KC			↑	
MIP-2			↑	
MIP-3β			↑	

[1] IL: interleukin; CTACK: cutaneous T cell-attracting chemokine; MCP-1: monocyte chemotactic protein 1; TPO: thrombopoietin; VEGF: vascular endothelial growth factor; TIMP-1: tissue inhibitor of metalloproteinases-1; GCSF: granulocyte-colony stimulating factor; GM-CSF: granulocyte-macrophage colony stimulating factor; INF: interferon; KC: keratinocyte-derived chemokine; MIP-2: macrophage inflammatory protein 2; MIP-3β: macrophage inflammatory protein 3β.

challenged with 200 compared with 100 larvae, and were thus displaced from the preferred location in the proximal duodenum (Bansemir and Sukhdeo, 1996). This may indicate that the more distally located adults in PS200 mice were in the process of being expelled, and this is consistent with the reduced viability of adult worms in PS mice compared with PD mice. Nevertheless, we cannot discount the possibility that at least some of these worms might have remained in the mice for several more days. In PD mice, our data are entirely consistent with the hypothesis that dose-dependent expulsion would not be evident in PD mice.

Our infected mice fed the protein-deficient diet had elevated food intake, and lower body weights. PD mice are known to increase their total food intake as a physiological response to low dietary protein (Swick and Gribskov, 1983; Colombo *et al*. 1992; Deschepper and de Groote, 1995; Du *et al*. 2000) or to increase their food intake relative to body weight (Rothwell and Stock, 1987; Zhao *et al*. 1996) in order to meet their needs for protein, nitrogen and amino acids (Webster, 1993). However, despite increased food intake, low protein intake is unlikely to be sufficient to maintain lean body mass; instead the relative propotion of body fat increases (Meyer, 1958; Swick and Gribskov, 1983; Du *et al*. 2000). Although we did not measure body fat directly, we measured leptin, a chemokine that is predominantly released by adipocytes and is typically used as an index of body fat (Flier, 1997; Singhal *et al*. 2002). Leptin was elevated in our PD mice, an observation consistent with deposition of body fat driven by increased intake of a PD diet observed in uninfected rats (Du *et al*. 2000).

One of the more surprising results from this study was the up-regulation in infected PD mice of a wide range of cytokines, predominantly pro-inflammatory cytokines and chemokines associated with a Th1 response that is known to promote *H. bakeri* survival (Ing *et al*. 2000; Gause *et al*. 2003). Researchers have reported a variety of adaptations to a protein-deficient diet associated with conserving protein, including reduced whole body protein synthesis and breakdown (Golden *et al*. 1977; Wykes *et al*. 1996), urea secretion and increased urea recycling (Badaloo

et al. 1999). The concurrent influence of infection has been reported to increase demand for protein, thus accelerating protein turnover and inducing a negative protein balance (Berclaz *et al.* 1996). Neither the conservation of protein nor the increased protein demand applies to all proteins, however (Golden, 1982; Jahoor *et al.* 1996), and we found several examples where infection elevated protein levels in PD mice but not in PS mice. Elevated IL-12 p70 promotes a Th1 immune response (Trinchieri, 1998), elevated MIP-2 mediates mast cell-dependent inflammation (Biedermann *et al.* 2000), and elevated IL-17 induces production of GM-CSF (Cai *et al.* 1998) which has been postulated as a potential mediator of worm expulsion (Shea-Donohue *et al.* 2001). Others, such as IL-9 (Khan *et al.* 2003; Leech and Grencis, 2006) and eotaxin (Mir *et al.* 2006), are associated with generalized intestinal inflammation. However, we are most intrigued by the possible interrelationships between protein deficiency, leptin, IFN-γ, MPO and MCP-5.

Leptin is now considered as a pro-inflammatory Th1 cytokine as it upregulates IFN-γ production along with a variety of other Th1 cytokines (Faggioni *et al.* 2001; Busso *et al.* 2002; Fenton *et al.* 2007). Leptin is believed to promote T cell activity at least *in vitro*, through leptin receptor signalling on naïve T cells (Lord *et al.* 1998). Moreover a shift to a predominantly Th1 response is observed when leptin is added to T cells from leptin deficient mice (Lord *et al.* 1998). Although we have shown that *H. bakeri* normally induces a strong Th2 response with low levels of IFN-γ in well-nourished hosts, this cytokine profile is disturbed in PD mice, such that PD *H. bakeri*-infected mice have elevated IFN-γ as reported by Ing *et al.* (2000) and seen in our multiple cytokine arrays. Thus, based on the enhanced, generalized Th1 response seen in cytokine profiles of challenged PD mice in the present study, we suggest that elevated leptin resulting from increased consumption of a PD diet may promote a Th1 environment with elevated levels of IFN-γ that in turn suppress the Th2 responses necessary for parasite expulsion, leading to prolonged parasite survival in the PD host.

From our data, it is also possible to suggest that the leptin-induced IFN-γ rich environment interacts with epithelial cell renewal in the villus to promote villus growth and parasite survival. Villus height is a critical factor for this nematode, as the parasites preferentially move to the region of the intestine with the longest villi, they maintain their position in the intestine by twisting around the villi, and the villi also provide a food resource for the parasite (Bansemir and Sukhdeo, 1996). Moreover, recent data have shown that intestinal epithelial cell turnover mediates nematode worm expulsion and is up-regulated by IL-13 and down-regulated by an IFN-γ induced chemokine (Cliffe *et al.* 2005). In our

mice, villi in the proximal small intestine were longer in challenged PD mice than in challenged PS mice, and parasite survival was better in mice with minimal villus atrophy in the duodenum, namely in PD mice. It has been reported that villus growth is induced by IFN-γ which promotes epithelial cell proliferation (Dignassn, 2001). We have previously shown that elevated IFN-γ is associated with prolonged nematode survival in PD mice (Ing *et al.* 2000), and propose that the up-regulation of IFN-γ in infected PD mice may facilitate epithelial cell proliferation, slow the rate of epithelial cell turnover and thus minimize the villus atrophy normally induced by protein deficiency in the absence of infection (Deo *et al.* 1965; Dudley, 1997). Thus the prolonged parasite survival in PD mice may be, in part, a result of the relatively normal villus length in these mice. In contrast, the Th2 environment of the PS infected mouse provides a microenvironment rich in IL-13 (Zhao *et al.* 2003) which induces villus atrophy (Zou *et al.* 1998) that impairs the ability of the worms to maintain their position and to feed.

A further link with leptin is seen in our data on gut MPO, which was lower in PD mice and also showed a dose-dependent response to larval challenge on day 7 pci in the distal small intestine. Interestingly, this response was evident during the phase when larvae are embedded in the gut tissues, but not when they had emerged into the gut lumen. Gut MPO is commonly observed in intestinal inflammation in *Trichinella spiralis* (Khan *et al.* 2002; Venkova and Greenwood-van Meerveld, 2006), a nematode that lives within the tissues of the intestine, and both MPO (Gay *et al.* 2000) and MCP-5 (Sarafi *et al.* 1997) are elevated during infections with the lumen-dwelling phase of *Nippostrongylus brasiliensis*. Cakir *et al.* (2004) reported that exogenous leptin induced less gut damage in a model of intestinal colitis and also reversed the elevated MPO levels. This is consistent with the possibility that elevated leptin in PD mice established a Th1 environment where inflammation was reduced and indicators of intestinal inflammation, such as gut MPO, were also reduced.

Of all the indicators measured in this study, MCP-5 was the most consistent with worm expulsion data, as it responded to both diet and challenge dose on day 14 pci, and to challenge infection on day 7 pci. MCP-5 is expressed in the lung during *N. brasilienses* infection (Sarafi *et al.* 1997), it is up-regulated in the small intestine by IL-4 (Ruth *et al.* 2000) and IL-13 (Cho *et al.* 2006), and it is involved in inflammation through induction of monocyte migration and activation of macrophages (Sarafi *et al.* 1997). The role of MCP-5 in *H. bakeri* infection has not previously been investigated, but we suggest it may be critical through its effect on alternatively activated macrophages. It is believed that delayed larval development and stunting of parasites (Cypess *et al.* 1988) involves alternatively

activated macrophages that accumulate around the larvae (Morimoto *et al.* 2004), as *in vivo* depletion of these macrophages delayed worm expulsion (Anthony *et al.* 2006). If MCP-5 is important in recruitment of these cells to the site of the larvae, then the reduced serum levels of MCP-5 in PD mice on day 7 pci and in PD mice infected with lower doses of *H. bakeri* on day 14 pci may provide a partial explanation for prolonged parasite survival in these mice. In contrast, in PS immunocompetent mice, the elevated IL-4 and IL-13 induced by *H. bakeri* (Zhao *et al.* 2003) would promote MCP-5 expression leading to infiltration of alternatively activated macrophages that would damage the developing parasites. To our knowledge, our study is the first to examine MCP-5 during *H. bakeri* infection and also the first to report that protein deficiency suppresses serum levels of MCP-5. This would need to be confirmed by studies that associate tissue levels of MCP-5 to recruitment of alternatively activated macrophages around the larval parasite.

It has been assumed that intestinal inflammation results from infiltration of mucosal mast cells, eosinophils, Goblet cells, and Paneth cells into the villi of the infected host (Fakae *et al.* 2000; Shea-Donohue *et al.* 2001). The elevated d14 pci cellular infiltration observed in our infected PS mice, compared with unchallenged PS mice, and the reduced eosinophil and mucosal mast cell populations in the distal small intestine of PD mice are consistent with this hypothesis. However, none of these cell populations differed between PS mice challenged with 100 and 200 larvae, and thus it is unlikely that these cells were directly responsible for the apparently more rapid expulsion in PS mice challenged with 200 L_3.

In summary, we have previously assumed that impaired expulsion in PD mice was due to suppression of Th2 immune effectors, and that the elevation of IFN-γ was a secondary consequence (Ing *et al.* 2000). It is difficult to know, however, whether protein deficiency down-regulates Th2 responses with the down-stream consequence of up-regulated Th1 responses, or whether protein deficiency up-regulates Th1 responses leading, in turn, to down-regulated Th2 responses. Our current data are consistent with the latter. We have shown that infected PD mice increase their food intake which leads to elevated leptin concentrations, as reported by others (Du *et al.* 2000) and observed in this study. Leptin upregulates IFN-γ (Faggioni *et al.* 2001) and thus establishes a pro-inflammatory Th1 environment in the PD mice. Our multiple cytokine arrays and our previous work (Ing *et al.* 2000) both demonstrate elevated IFN-γ in PD infected mice. The reduced serum concentration of MCP-5 observed in this study may reduce infiltration of macrophages around the larval parasites, as shown in other systems (Sarafi *et al.* 1997), thus permitting

normal larval development. Additionally, the elevated levels of IFN-γ in the PD mice could account for the reduced villus atrophy in the duodenum of the PD mice, which would in turn provide a more permissive luminal environment for the adult worms. Thus, larvae would be able to develop normally in PD mice, adult worms would be able to remain twisted around the villi, intestinal inflammation would be reduced, and worm survival would be prolonged. We suggest that the roles of leptin as an initiator and MCP-5 as an effector of prolonged parasite survival in PD mice be explored further.

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