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Basic nutritional investigation

Protein malnutrition impairs the immune control of *Trichinella spiralis* infection

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ABSTRACT

Objectives: We aimed to analyze the effect of a protein-deficient diet on mucosal and systemic immunity during a *Trichinella spiralis* infection.
Methods: Two groups of weaning Wistar rats received a protein-deficient diet (6.5% casein) and the other two groups received a control diet (20% casein). After 10 d, one group of each diet was infected (PD_I and C_I) with muscle larvae (infecting stage). Food intake and body weight were assessed over time. Blood eosinophils counts, antibodies in serum, and tissue extracts were assessed at different days postinfection. Histologic studies were done in the lungs and intestines, and adult worm (AW) fecundity index score and muscle parasite burden were determined.
Results: Food and protein intake were lower in PD_I than in C_I. Body weight was lower in PD_I than in a non-infected protein-deficient diet. Eosinophils counts were lower in PD_I than in C_I. Total and specific antibodies were lower in PD_I than C_I. PD_I had a reduced number of mast and goblet cells in the lungs and intestines compared with C_I. The persistence of AW in the intestines and migrant larvae at the lungs was longer in PD_I than in C_I. The AW fecundity index score was higher in PD_I than in C_I. Finally, PD_I evidenced a higher muscular parasite burden than C_I.
Conclusions: Protein deficiency affects the mucosal and systemic immune response to *Trichinella spiralis* and delays the expulsion and increases the fecundity index score of AW, which leads to a higher parasite burden in the muscles.

Introduction

The immune system is known to be affected by malnutrition, which causes a higher susceptibility to infections [1]. Malnutrition and gastrointestinal nematode infections are chronic diseases that often coexist in individuals from developing countries [2]. Trichinellosis is a foodborne parasitic disease acquired by the ingestion of either raw or undercooked meat that is infected with *Trichinella spiralis* (*T. spiralis*) muscle larvae (ML). In the stomach, ML are released and penetrate the epithelial layer of the gut where they molt into adult worms (AW). After mating, female AW release migrant or newborn larvae (NBL) on days five to six postinfection (p.i.), which go through the bloodstream and lymphatic system and pass through different organs, including the lungs, during their migration to the skeletal muscle [3]. Each larva enters a muscle cell where they develop into ML [4].

The effector immunological response against *T. spiralis* is focused on two parasitic stages. At the gut level, AW are expelled by the combined action of cellular (mast and goblet cells) and humoral responses [5–7], and at the systemic level, NBL are killed by antibody dependent cellular cytotoxicity (ADCC) [8–10]. Recent results from our laboratory have demonstrated an allergic-like inflammatory response in the lung parenchyma with bronchus-associated lymphoid tissue (BALT) hyperplasia before and during NBL passage through this organ [11–12]. In particular, there is an increase of mast cells, eosinophils, and goblet cells. Moreover, the retention and destruction of NBL by ADCC were shown to take

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place in the lungs of infected rats [13]. These responses are globally described as type-2 immune responses and include both innate and adaptive components. Among the latter, the induction of a T helper 2 (Th2) phenotype with the production of interleukin (IL) 4, IL-5, and IL-13 cytokines is of the utmost importance to induce and sustain type-2 responses. Helminth infections induce a Th2 immune response, but protein deficiency promotes higher levels of Th1 cytokines and diminishes Th2 effector cells [14].

Several studies have explored the impact of protein deficiency on the outcome of helminth infections, but to our best knowledge, only two previous studies have evaluated these issues in *T. spiralis* infections [15–16]. Although both studies revealed a link between protein deficiency and delayed worm expulsion or increased numbers of muscle larvae, the immunologic determinants of this impaired infection control were not assessed.

In the present study, we evaluated the parasitologic, histologic, and immunologic consequences of protein deficiency in the context of a *T. spiralis* infection in weaning rats. This study is the first to assess the impact of protein deficiency, not only on intestinal but also on pulmonary *Trichinella*-specific immune response, which was recently shown to be involved in the control of *T. spiralis* infection.

Materials and methods

Animals and infection

Weaning female Wistar rats (21-23 d old) weighing an average of 42.21 ± 1.92 g (initial body weight [BW_i]) were housed individually in screen-bottomed cages and exposed to a 12-h light-dark cycle. Room temperature was kept at 21.0 ± 1.0°C, and animals were provided with water and food ad libitum. The rats were divided into four groups of five animals each. Two groups received a protein-deficient diet (PD), and the others a control diet (C) for growing rats.

After 10 d, one group of rats on each diet was orally infected through a feeding probe with 900 ML of *T. spiralis* per rat (PD_I; C_I) suspended in saline solution. ML were obtained from muscle tissue of infected Swiss mice through the artificial digestion method [17]. The other groups were used as non-infected control groups (PD_{NI}, C_{NI}).

All experimental protocols were approved by the Institutional Committee of Care and Use of Laboratory Animals (CICUAL-FFyB, Res N° 2470/17).

Experimental diets

The PD and C groups received experimental isocaloric diets that provided 6.5% or 20% protein, respectively, and all essential nutrients as recommended by the American Institute of Nutrition [18]. Casein (Friesl and Campina, 89.2%) was incorporated as the only source of protein to provide the required protein concentration. Choline, soy oil, and a vitamin and mineral mix were added, and the mixture was filled up to 1000 g by adding dextrin, as previously reported [19].

Nutritional parameters

Body weight (g) and food intake (g/day) were recorded from the first day of the diet administration. At the end of the experimental period, the diets were withheld for 4 h, the final body weight (BW_f) was determined, and the ponderal growth rate (PGR, expressed as g per 100 g of rat/d) was calculated as follows:

$$PGR = [(BW_f - BW_i)/n\ days]/[(BW_i + BW_f)/2] \times 100 \quad (1)$$

Daily protein and energy intake were measured and expressed as mg/$BW^{0.75}$/d and kcal/$BW^{0.75}$/d, respectively, where $BW^{0.75}$ is the metabolic mass.

Blood samples

Blood samples were obtained from all groups at different p.i. times by tail vein puncture, using heparin as anticoagulant when necessary (eosinophil counts). At day 33 p.i., blood was obtained by cardiac puncture. Serum samples were aliquoted, and kept at −70°C until use.

Blood eosinophils counts

Blood eosinophil counts were determined by counting cells in a Fusch-Rosenthal hemocytometer after staining whole blood with Discombe's solution [20].

Detection of total serum immunoglobulins and IgE against muscle larvae excretory–secretory products by ELISA

Serum immunoglobulins (Ig) G, A, and M (IgGAM) and serum IgE specific for *Trichinella* were detected by indirect enzyme-linked immunosorbent assay (ELISA), as described previously [12]. Plates coated with muscle larvae excretory–secretory products (ML-ESP) were incubated with serum samples and, after washing, were incubated with either a biotinylated anti-rat IgGAM serum (Vector Laboratories, Burlingame, CA, USA) followed by a complex of avidin and biotinylated peroxidase (Vector), or a goat anti-rat ε chain serum (Bethyl Laboratories, Montgomery, TX, USA), followed by a horseradish peroxidase-conjugated rabbit anti-goat serum (Bethyl). Color reaction was developed with o-phenylenediamine/H_2O_2 for IgGAM or with tetramethylbenzidine for IgE. The reaction was stopped by adding 4 N H_2SO_4, and the optical density was read in an ELISA reader at 490 nm for IgGAM and 450 nm for IgE.

Histological studies in the lungs and intestines

Rats were euthanized by cardiac puncture under anesthesia to obtain the lungs and intestines. Tissues were perfused by injecting phosphate-buffered saline (PBS) with heparin (10 IU/mL) into the right cardiac ventricle. The organs were removed, and tissue sections were obtained using the Sainte-Marie technique [21]. Tissue sections were stained with Giemsa (general aspects of the tissues), Alcian Blue Safranin (mast cells), and hematoxylin-periodic acid-Schiff (goblet cells).

Goblet cells were counted in 15 villous crypt units (VCU) in the intestine epithelium. In the lungs, goblet cells were counted in the epithelium located close to the BALT. Mast cells were counted in 15 VCU in the gut lamina propria. In the lungs, parenchyma mast cells were counted in 100 randomly selected fields for each sample, employing a grid with a known area (62 500 μm^2). All counts were made at 400 × magnification by two independent observers.

Tissue extracts preparation

Lung and intestine tissue extracts were obtained using the PERFEXT method [22] with slight modifications. Briefly, the rats were euthanized and infused with PBS plus heparin (5000 UI/mL) into the heart. The perfused organs were cut into small pieces, placed in an extraction solution containing CHAPS 90 mM in PBS and protease inhibitors, and frozen at −70°C. After thawing, extraction was performed overnight at 4°C using a homogenizer. After centrifugation, the supernatants were collected, filtered through a 0.22 μm filter, aliquoted, and kept frozen at −70°C until use.

Detection of total and specific antibodies in lung and intestine tissue extracts

Total IgA, IgE, IgG1, and IgG2a were determined with a capture ELISA commercial kit (Bethyl Laboratories). Levels of anti-ML-ESP and anti-AW-ESP IgA, IgE, IgG1, and IgG2a were determined by indirect ELISA, as described previously [12,23].

Fecundity index of female adult worms

To estimate the fecundity index score of female AW, the method described by Marti and Murrell (1986) was employed [24] with slight modifications. After the recovery of the AW from the intestines by Baermann separation, the female AW were identified and counted. Worms were incubated at 37°C in 5% CO_2 in Roswell Park Memorial Institute medium supplemented with antibiotic agents and 5% fetal calf serum. After 3 h, the number of NBL shed by the female worms was counted, and the fecundity index score was calculated as the number of NBL per female AW.

Determination of parasite burden

ML recovered from the carcasses of infected rats through the artificial digestion method [17] at day 33 p.i. were washed, suitably diluted in saline, and mixed 1:1 with agar 1.5%. The mixtures were placed in grooved Petri dishes and allowed to solidify. The ML were counted by two independent observers using an optical microscope.

Statistical analysis

Parasite burden was analyzed using the unpaired *t* test. PGR, food, energy, and protein intake were analyzed using the one-way analysis of variance (ANOVA) test, employing Tukey's multiple comparisons test for post hoc analysis. Body weight, cell count, total and specific isotypes, and fecundity index scores were analyzed using the two-way ANOVA test, employing Tukey's or Sidak's multiple comparisons test for post hoc analysis. The data were analyzed using GraphPad Prism 6 software, and a $P < 0.05$ was considered significant.

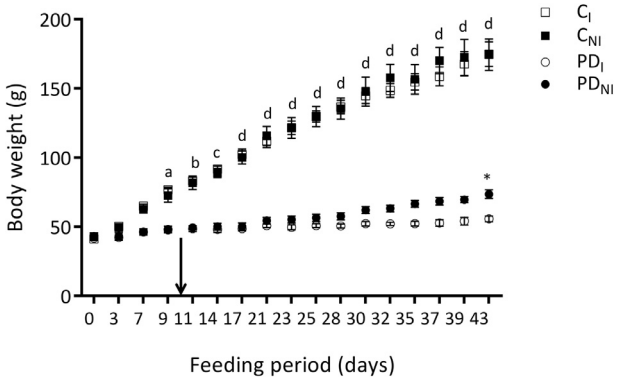

Fig. 1. Body weight during feeding period. Body weight values were registered during all experiments, and the results are expressed as the mean of the body weight/rat (g) ± standard error of mean. The animal groups (n = 5/group) were the controls (C) and those fed the protein-deficient diet (PD), which were infected with *Trichinella spiralis* (C_I and PD_I, respectively) or non-infected (C_{NI} and PD_{NI}, respectively). The arrow indicates the beginning of the infection. Data were analyzed by two-way analysis of variance test ($\alpha = 0.05$) followed by Tukey's multiple comparisons test. The asterisk indicates significant differences between PD_I and PD_{NI} (*$P < 0.05$), and letters significant differences between the control and protein-deficient diet groups ($^aP < 0.05$; $^bP < 0.01$; $^cP < 0.005$; $^dP < 0.0001$).

Results

Nutritional parameters

Body weight was significantly lower in PD_I than in C_I animals before the infection (48.02 ± 2.32 versus 76.68 ± 2.04 g at day nine of the diet; $P < 0.01$; Fig. 1; Table 1). This pattern was observed during all experiments, with the highest difference at the end of the experimental period (day 43 of the diet: 55.67 ± 1.87 versus 174.80 ± 4.47 g; $P < 0.0001$; Fig. 1). In addition, a significant difference was observed between PD_I and PD_{NI} rats at the end of the study (55.67 ± 1.87 versus 73.64 ± 2.62 g; $P < 0.05$; Fig. 1).

PGR was significantly lower in PD_I than in C_I rats (1.53 ± 0.73 versus 6.69 ± 0.57 g/100 g of rat/d; $P < 0.0001$; Table 1) during the feeding period before infection. In addition, the same pattern was observed during the total period (0.63 ± 0.35 versus 2.87 ± 0.40 g/100 g of rat/d; $P < 0.05$). During the infection, PD_I rats showed a lower PGR compared with PD_{NI} rats (0.30 ± 0.46 versus 1.22 ± 0.48 g/100 g of rat/d; $P < 0.01$; Table 1). However, no significant differences in PGR were observed between the C groups throughout the period.

Food intake during the entire study was significantly lower in PD_I than in C_I rats (8.83 ± 1.68 versus 14.25 ± 1.94 g/d; $P < 0.05$; Table 1). Regardless of the infection status, PD groups had lower protein intake than the C groups (PD_I: 31.26 ± 3.28 versus C_I: 84.46 ± 4.30 mg/$BW^{0.75}$/d; $P < 0.005$; Table 1). Finally, there were no differences in energy intake among the groups.

Blood eosinophils count

Compared with their non-infected counterparts, eosinophil counts started to increase at day five p.i. in PD_I animals (91.67 ± 2.08 versus 24.71 ± 3.41 cells/mm^3; $P = 0.0001$; Fig. 2), but this occurred at day three p.i. in C_I animals (83.33 ± 5.51 versus 20.51 ± 2.17 cells/mm^3; $P = 0.0003$).

On day 11 p.i., both infected groups reached the maximum number of eosinophil counts without significant differences between the groups. However, the counts were significantly lower in PD_I than in C_I animals on days 3, 5, 15, and 20 p.i. The largest difference was found on day five p.i. (91.67 ± 2.08 versus 162.50 ± 16.54 cells/mm^3; $P < 0.0001$). At the end of the experiment, eosinophil counts in PD_I animals returned to baseline levels earlier than those in C_I animals.

Determination of IgGAM and IgE against ML-ESP in sera

Optical density values of specific IgGAM and IgE were similar and followed the same kinetics in PD_I and C_I animals. For both groups, the values were positive (above cutoff) from day 11 p.i. and reached the maximum value at day 33 p.i. (Fig. 3).

Table 1
Nutritional parameters evaluated before and during infection with *Trichinella spiralis*

Feeding period (d)	Group	PGR (g/100g rat/d)	BW (g)		Food intake (g/d)	Energy intake (Kcal/BW0.75/d)	Protein intake (mg/BW0.75/d)
			BW_i	BW_f			
Before infection (0-9)	PD_I	1.53 ± 0.73*	41.76 ± 1.87	48.02 ± 2.32‖	6.57 ± 0.64‡	2.39 ± 0.19	33.91 ± 1.95††
	PD_{NI}	1.26 ± 0.94*	43.05 ± 2.31	47.98 ± 1.51‖	6.30 ± 0.88‡	2.36 ± 0.26	28.73 ± 2.10††
	C_I	6.69 ± 0.57†	41.18 ± 1.56	76.68 ± 2.04¶	9.48 ± 1.15§	2.61 ± 0.30	108.80 ± 3.54‡‡
	C_{NI}	6.04 ± 0.95†	42.84 ± 1.97	75.02 ± 2.97¶	9.58 ± 0.77§	2.62 ± 0.20	110.77 ± 3.42‡‡
During infection (10-43)	PD_I	0.30 ± 0.46‡	48.02 ± 2.32‖	55.67 ± 1.87‖	9.33 ± 1.70‖	2.00 ± 0.93	31.23 ± 3.68¶
	PD_{NI}	1.22 ± 0.48§	47.98 ± 1.51‖	73.64 ± 2.62¶	8.75 ± 0.91‖	1.63 ± 0.40	25.36 ± 1.59¶
	C_I	2.21 ± 0.52§	76.68 ± 2.04¶	174.80 ± 4.47**	15.89 ± 2.17¶	1.70 ± 0.66	62.45 ± 4.00**
	C_{NI}	2.25 ± 0.65§	75.02 ± 2.97¶	174.40 ± 5.03**	14.77 ± 1.07¶	1.61 ± 0.20	76.96 ± 1.39**
Total (0-43)	PD_I	0.63 ± 0.35‖	41.76 ± 1.87	55.67 ± 1.87‖	8.83 ± 1.68‖	1.95 ± 0.82	31.26 ± 3.28††
	PD_{NI}	1.22 ± 0.61¶	43.05 ± 2.31	73.64 ± 2.62¶	8.12 ± 0.87‖	1.56 ± 0.40	25.06 ± 1.59††
	C_I	2.87 ± 0.40¶	41.18 ± 1.56	174.80 ± 4.47**	14.25 ±1.94¶	1.71 ± 0.61	84.46 ± 4.30‡‡
	C_{NI}	2.81 ± 0.30¶	42.84 ± 1.97	174.40 ± 5.03**	13.45 ± 0.92‖¶	1.63 ± 0.25	80.28 ± 1.78‡‡

BW, body weight; BW_f, final body weight; BW_i, initial body weight; C_I, control infected; C_{NI}, control non-infected; PD_I, protein-deficient infected; PD_{NI}, protein-deficient non-infected; PGR, ponderal growth rate.
PGR; BW; and food, energy, and protein intake were evaluated during the experimental period of 43 days in rats fed a 20% casein diet (control) or a 6.5% casein diet (protein-deficient diet).
Data are expressed as mean ± standard error of mean (n = 5/group). For PGR and food, energy, and protein intake, a one-way analysis of variance test was performed. For BW, a two-way analysis of variance test was performed. In all cases, a $P < 0.05$ was considered significant, and Tukey's multiple comparisons test for second analysis was used. Comparisons among the four experimental groups were performed for each parameters and each feeding period.
*versus †$P < 0.001$
‡versus §$P < 0.01$
‖versus ¶$P < 0.05$
¶versus **$P < 0.001$
††versus ‡‡$P < 0.005$

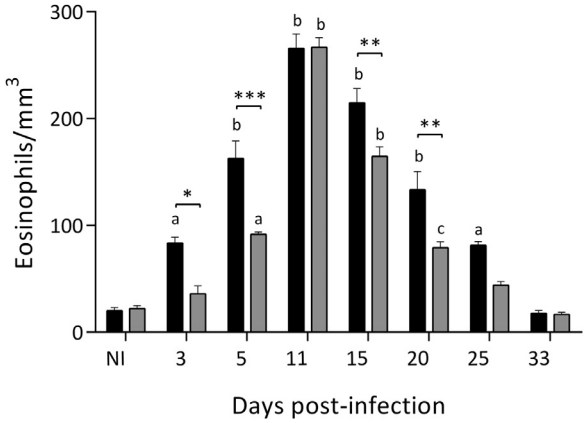

Fig. 2. Blood eosinophil counts during *Trichinella spiralis* infection. Eosinophil cells were counted in blood samples stained with Discombe's solution. The results are expressed as the mean cells/mm^3 ± standard error of mean. The animal groups (n = 3/group) controls (C) and those fed the protein-deficient diet (PD) were infected with *Trichinella spiralis* (C_I and PD_I, respectively) or non-infected (C_{NI} and PD_{NI}, respectively). The data were analyzed by two-way analysis of variance test followed by Tukey's or Sidak's multiple comparisons test ($\alpha = 0.05$). Asterisks indicate significant differences between C_I and PD_I ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.0001$). Letters indicate significant differences between C_I or PD_I with their non-infected controls ($^aP < 0.05$; $^bP < 0.0001$, $^cP < 0.01$).

Histological analysis of the intestines and lungs

The gut villi of rats fed the protein-deficient diet (PD_I, PD_{NI}) showed a decrease in size (Figs. 4C and G), and during the infection period, the PD_I and C_I groups showed an inflammatory process at the intestine (Figs. 4E and G) and the BALT (Figs. 4F and H).

The intestinal and pulmonary numbers of goblet cells increased later in PD_I than in C_I animals. Compared with the non-infected control group, the number of intestinal goblet cells increased from day six p.i. in C_I (21.81 ± 1.70 versus 11.20 ± 0.92 cells/VCU; $P < 0.005$; Fig. 5A) and from day nine p.i. in PD_I (18.80 ± 1.53 versus 9.77 ± 1.26 cells/VCU; $P < 0.01$). Compared with the non-infected controls, the number of goblet cells in the lung parenchyma increased from day three p.i. in C_I (7.68 ± 0.76 versus 1.00 ± 1.00 cells/100 lung cells; $P < 0.005$; Fig. 5B) and from day nine p.i. in PD_I (9.00 ± 1.00 versus 0.33 ± 0.76 cells/100 lung cells; $P < 0.001$).

The number of mast cells in the lungs and intestines also increased with a delay in PD_I compared with C_I. The number of intestinal mast cells increased from day six p.i. in C_I (35.63 ± 2.84 versus 5.6 ± 1.46 cells/VCU; $P < 0.001$; Fig. 5C) and from day nine p.i. in PD_I (26.47 ± 2.73 versus 4.07 ± 0.90 cells/VCU; $P < 0.01$). In the lungs, the number of mast cells increased from day three p.i. in C_I (12.25 ± 1.28 versus 5.41 ± 1.16 cells/mm^2; $P < 0.01$; Fig. 5D) and from day nine p.i. in PD_I (11.19 ± 1.41 versus 4.08 ± 1.31 cells/mm^2; $P < 0.01$).

Although AW are usually expelled from the gut at day 13 to 15 p.i. in the rat model, they were found in the intestine of PD_I rats as long as day 33 p.i. (Figs. 4G and I). Also, at day 33 p.i., NBL were found in lungs of PD_I rats (Fig. 4J), but not in those of the C_I controls.

Detection of total and specific isotypes in lung and intestine tissue extracts

In intestine tissue extracts, total IgE levels were lower in PD_I than in C_I (day nine p.i. 31.22 ± 5.08 versus 183.05 ± 11.19 ng/mL; $P < 0.01$; Fig. 6). Total IgA decreased in C_I as of day three p.i. but maintained its level in PD_I during the entire follow-up period. Total IgG1 increased over time in both C_I and PD_I. Regarding anti-ML-ESP antibodies, IgE, IgG1, and IgG2a were increased in the C_I group as of day nine p.i., but the PD_I group showed a non-significant increase. In the case of specific antibodies against AW-ESP, all isotypes were present in the C_I group, but in PD_I, only IgG2a had a significant increase.

In lung tissue extracts (Fig. 7), all isotypes of total antibodies were reduced in PD_I compared with C_I (IgE: day nine p.i. 76.58 ± 6.07 versus 217.83 ± 12.55 ng/mL; $P < 0.05$; IgA: day nine p.i. 384.02 ± 9.55 versus 909.14 ± 17.99 μg/mL; $P < 0.05$; IgG1: day 13 p.i. 3226.45 ± 30. 95 versus 5679.25 ± 61.55 ng/mL; IgG2a: day three p.i. 766.05 ± 15.83 versus 2271.60 ± 24.52 ng/mL; $P < 0.01$). Specific IgA and IgG1 were present in both groups as of day 13 p.i.

Fecundity index scores of female adult worms

In all p.i. days studied, the parasite fecundity index score was higher in PD_I than in C_I (Fig. 8), but this difference was significant as of day nine p.i. (30.58 ± 3.39 versus 14.32 ± 2.95, NBL/female AW; $P < 0.05$). At day 20 p.i., a time point at which normally AW have been completely expelled in well-nourished rats, not only were AW still present in the PD_I group, but still releasing NBL (14.05 ± 2.30 versus 0 ± 0, NBL/female AW; $P < 0.05$).

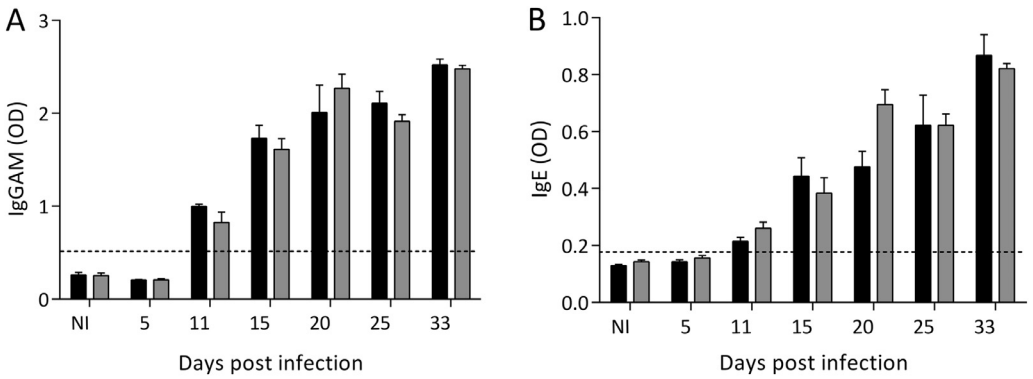

Fig. 3. Kinetics of total immunoglobulins (Ig) G, A, and M (IgGAM) and IgE anti-muscle larvae excretory–secretory products in sera samples by enzyme-linked immunosorbent assay during *Trichinella spiralis* infection. IgGAM and IgE were detected by enzyme-linked immunosorbent assay, and the results are expressed as mean optical density values ± standard error of mean (n = 3/group; cuoff: IgE = 0.176; IgGAM = 0.573). The animal groups were C and PD infected with *T. spiralis* (C_I and PD_I, respectively) or non-infected (C_{NI} and PD_{NI}, respectively). The dotted line indicates the cutoff.

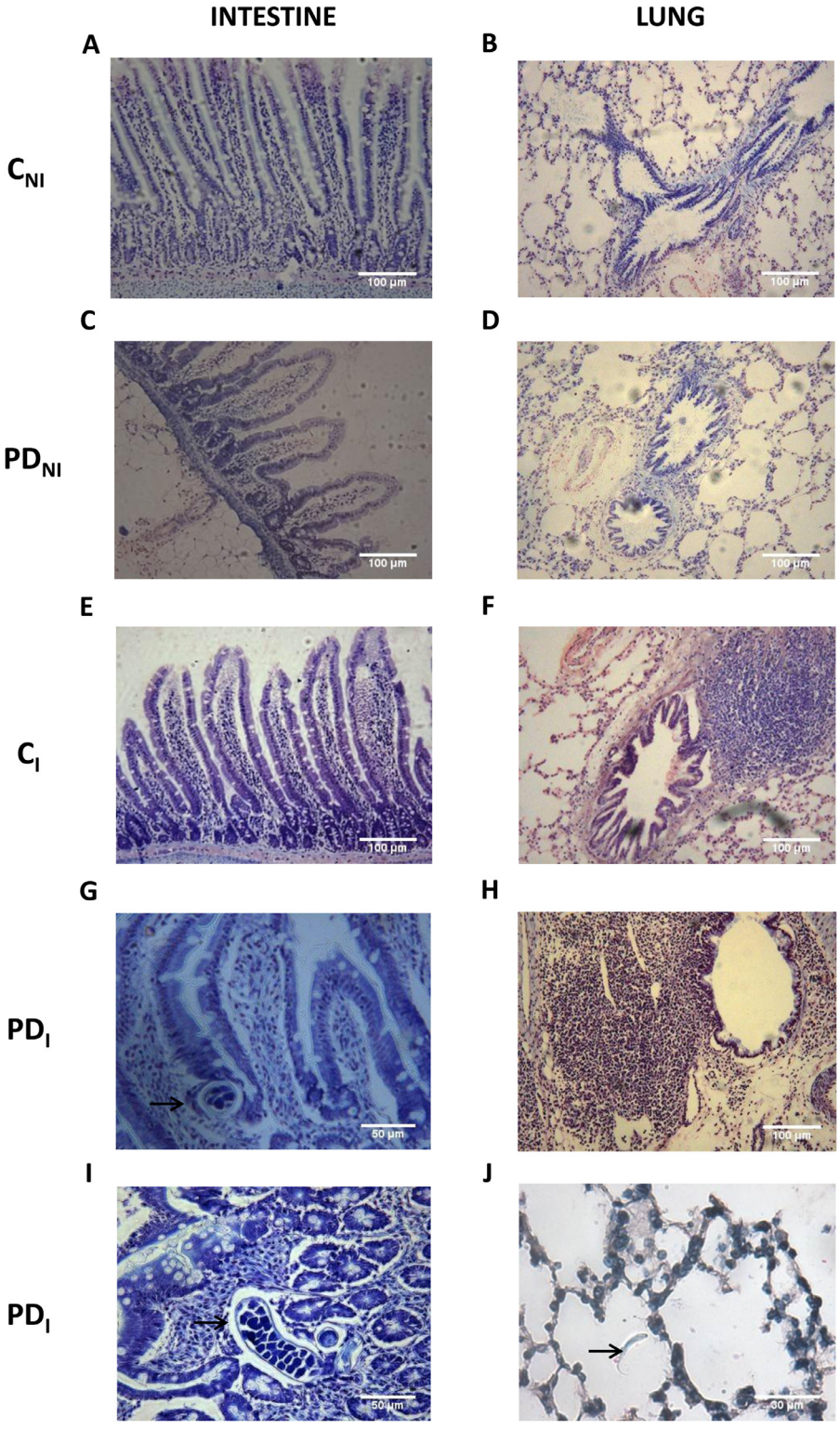

Fig. 4. Histopathology of the gut and lungs during late *Trichinella spiralis* infection. At day 33 p.i., histologic sections of the gut (A, C, E, G, I) and lungs (B, D, F, H, J) were stained with Giemsa. The animal groups were the controls (A, B, E, F) and those fed the protein-deficient diet (C, D, G, H, I, J). Animals were infected with *Trichinella spiralis* (E, F, G, H, I, J), and non-infected animals were used as controls (A, B, C, D). The arrows show the presence of adult worms (G, I) and newborn larvae (J).

Parasite burden

At 33 days p.i., the parasite burden in the muscles was 10 times higher in PD_I than in C_I animals (10 069.99 ± 3077.16 versus 1048.53 ± 206.85 ML/g; $P < 0.01$; Fig. 9). Physical deterioration was observed in PD_I rats, which was the only group that recorded two deaths (on days 25 and 32 p.i.). In the case of C_I, no physical deterioration was observed.

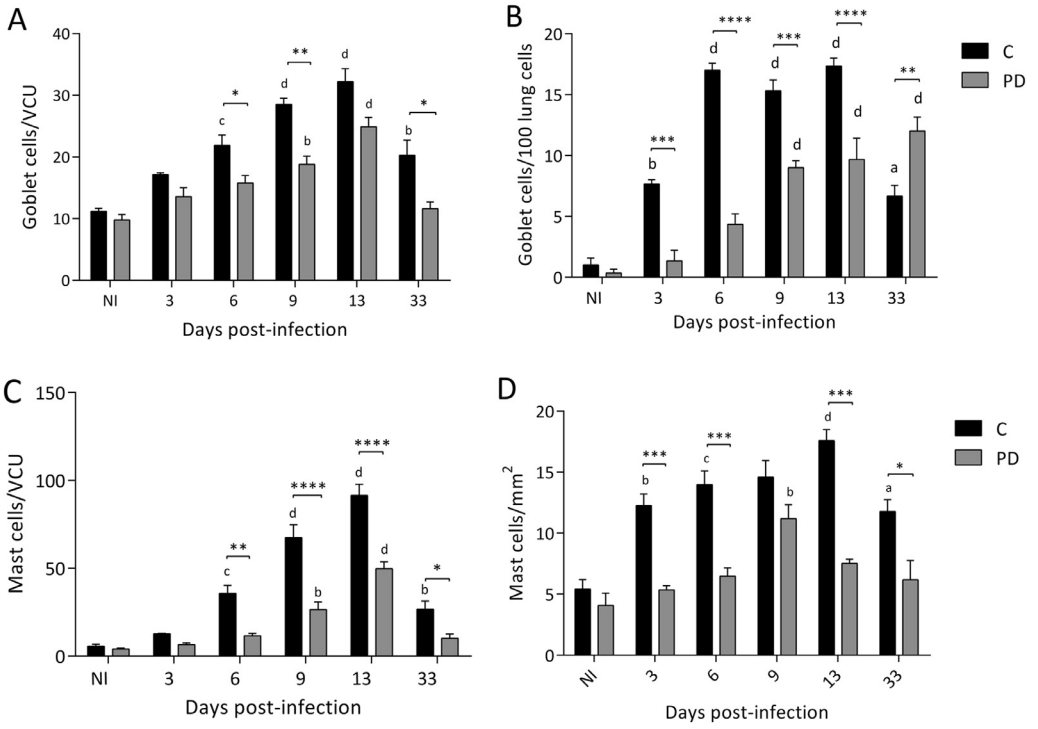

Fig. 5. Kinetics of the appearance of goblet and mast cells in the small intestine and lungs during *Trichinella spiralis* infection. In the gut, the results are expressed as mean cells/villous crypt units ± standard error of the mean (A, C). In the lungs, the results are expressed as mean cells/100 lung cells ± standard error of mean for goblet cells and mean cells/mm^2 of the lungs ± S.E.M for the mast cells (B, D). The animal groups (n = 3/group) were the controls (C) and those fed the protein-deficient diet (PD), which were then infected with *Trichinella spiralis* (C_I and PD_I, respectively) or non-infected (C_{NI} and PD_{NI}, respectively). Data were analyzed by two-way analysis of variance test, followed by Sidak's multiple comparisons test ($\alpha = 0.05$). The asterisks indicate significant differences between PD_I and C_I ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$; $^{****}P < 0.001$), and letters indicate significant differences between PD_I or C_I with their non-infected controls ($^aP < 0.05$; $^bP < 0.01$; $^cP < 0.005$; $^dP < 0.001$).

Discussion

Although previous studies have analyzed the consequences of PD on the course of *T. spiralis* infection (in terms of worm expulsion or larval load in muscles) [15–16], the effects of PD on cellular and humoral immune factors involved in the control of such infections have not been assessed. In this study we analyzed the parasitologic, histologic, and immunologic consequences of PD at both the gut and lung levels in *T. spiralis*-infected rats.

The administration of the PD diet provoked a severe delay in the growth of the animals, which was observed from the first days of treatment before the infection. However, the lower body weight and PGR of the PD_I compared with the PD_{NI} group at the end of the study confirmed the negative effect of the combination of malnutrition and infection. As in our previous studies, no deaths were recorded among uninfected rats that were fed the protein-deficient diet [19]; however, the addition of *T. spiralis* infection (PD_I group) provoked a higher morbidity and led to death in two cases. No deaths were reported by Gbakima [16] in protein-deficient mice infected with *T. spiralis,* but the low protein diet was begun later than in the present study (2 wk after weaning).

Previous studies have shown that a low protein concentration and a low quality of dietary protein impair mucosal immunity [25]. In the present study, the PD_I group not only showed a decrease in gut villi size and a lower number of mast and goblet cells during the first month p.i., but also AW were found at day 33 p.i., when in the rat model they are normally expelled on days 13 to 14 p.i. [26], which indicates that the parasite rejection is delayed in these animals. Our results are in line those of previous studies on *T. spiralis* and other helminth infections in adult mice with protein malnutrition [16,27–29].

Regarding the humoral response in the gut mucosa, the PD_I group showed lower levels of total and specific antibodies, which is probably due to a decreased protein synthesis as a consequence of the lower protein intake. This phenomenon has been also observed in malnourished mice with schistosomiasis [30]. Altogether, these results indicate a diminished mucosal immune response in PD_I animals at both the cellular and humoral levels, which would facilitate the persistence of the AW in the gut.

Specific IgE and IgA in the intestinal mucosa are known to reduce the fecundity of the AW; thus, limiting the production of NBL and the subsequent invasion of the skeletal muscle [31]. In this work, the levels of specific IgA and IgE were lower in the PD_I group, which could reduce the detrimental effect of these antibodies on AW fecundity and allow for a higher number of NBL to birth and reach the systemic circulation. The higher fecundity index score in the PD_I group is in line with the results of specific antibodies. In addition, in the PD_I group, NBL production was prolonged until at least day 20 p.i., but this phenomenon normally finishes on day 13 to 14 p.i. in well-nourished animals [26]. To the best of our knowledge, this is the first study to evaluate the fecundity index score of PD animals with *T. spiralis*.

NBL migrates through the lymph and blood system of the host and is the second target of the immune response. In the murine model, there is a systemic response that is characterized by eosinophilia and an increase of specific Ig levels [32]. In this work, PD_I animals had decreased eosinophil counts, but serum levels of anti–ML-ESP IgGAM and IgE were not affected. These results suggest that at a systemic level, the cellular immune response may be

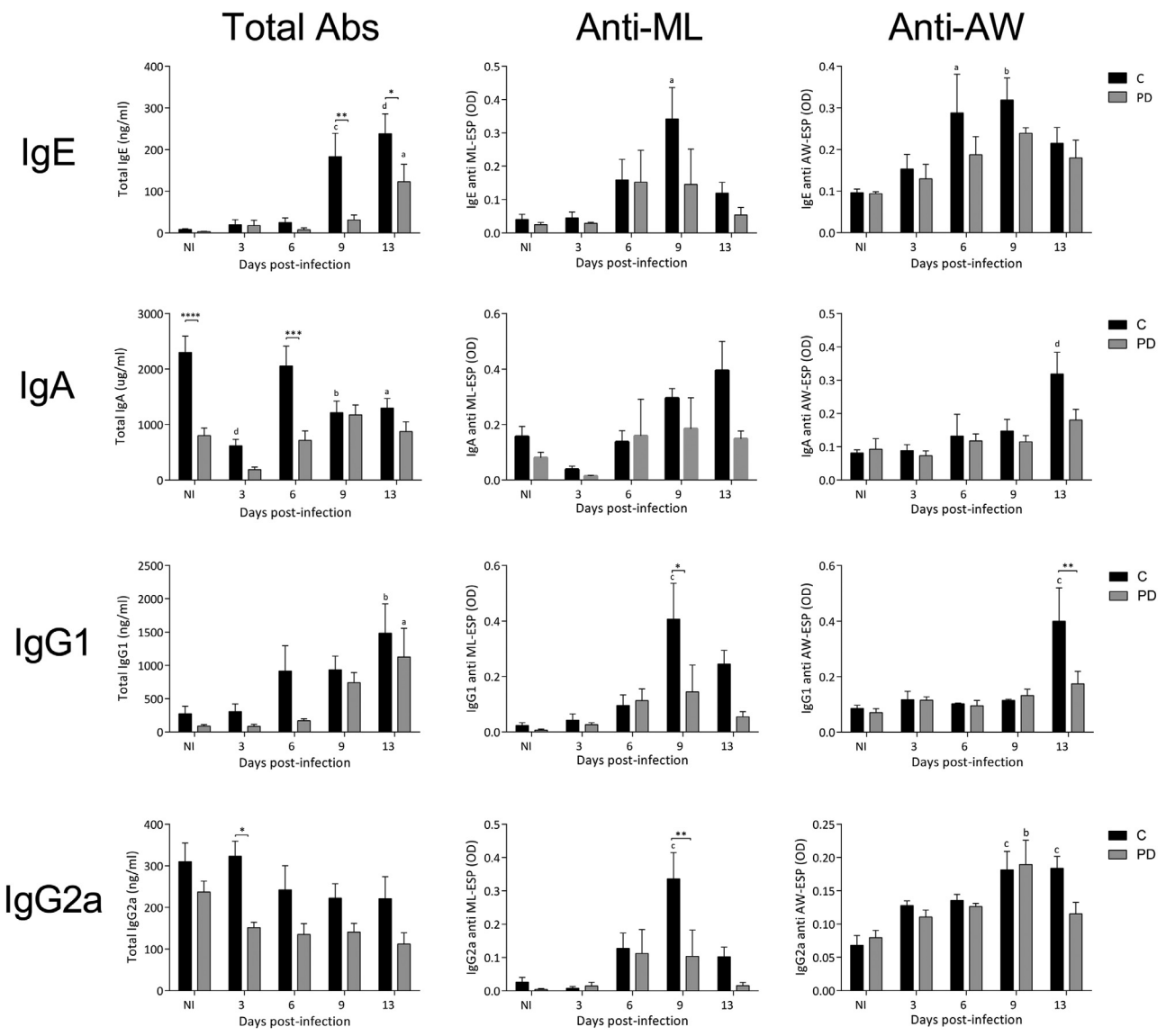

Fig. 6. Kinetics of total, antimuscle larvae and anti-adult worm excretory–secretory products immunoglobulins (Igs) in intestine tissue extracts during *Trichinella spiralis* infection. Total Ig concentrations were determined by enzyme-linked immunosorbent assay and expressed as concentration ± standard error of mean. Specific Igs were determined by enzyme-linked immunosorbent assay and expressed as their optic density values ± standard error of mean. The animal groups (n = 5/group) were controls (C) and those fed the protein-deficient diet (PD), which were infected with *Trichinella spiralis* (C_I and PD_I, respectively) or non-infected (C_{NI} and PD_{NI}, respectively). The data were analyzed by two-way analysis of variance test followed by Tukey's or Sidak's multiple comparisons test ($\alpha = 0.05$). The asterisks indicate significant differences between PD and C ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.001$), and letters significant differences between PD_I or C_I with their non-infected controls ($^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.005$; $^{d}P < 0.0001$).

more affected than the humoral response. In contrast with what we found in our study, Boulay et al. [33] reported that during *Heligmosomoides polygyrus* infections, blood eosinophilia and serum levels of Igs were decreased in PD animals.

We have previously shown that during NBL migration, an allergic-like inflammatory response occurs in the lung parenchyma that includes mucosal mastocytosis and goblet cells hyperplasia [12–13]. This pulmonary response generates a suitable scenario for the attack of NBL by immunological effectors. The present study is the first to assess the effect of PD on parasitologic, histologic, and immunologic parameters in the lungs of *T. spiralis*-infected animals. The PD_I group had reduced mast and goblet cell counts in the lungs, which suggests that the pulmonary mucosal cellular response is affected by protein deficiency. However, the local humoral response did not seem to be affected because the levels of specific antibodies in lung extracts did not differ between the C_I and PD_I groups. Nevertheless, NBL were observed as long as 33 days p.i. in the lungs of the PD_I group, which is the first time that this long persistence is reported. This suggests that the lung cellular immune response has a key role in the control of NBL migration.

Overall, these results suggest that the higher parasite burden found in the PD_I group may be associated with the longer persistence of AW in the intestines and higher fecundity index scores of female AW, which are consequences of the lower humoral and cellular immune response. The reduced number of blood eosinophils in the PD_I group may also compromise the systemic immunological effector mechanisms against NBL and contribute to the higher parasite burden in the muscles. Further studies will be required to determine whether the ADCC mechanism against NBL is affected by protein deficiency.

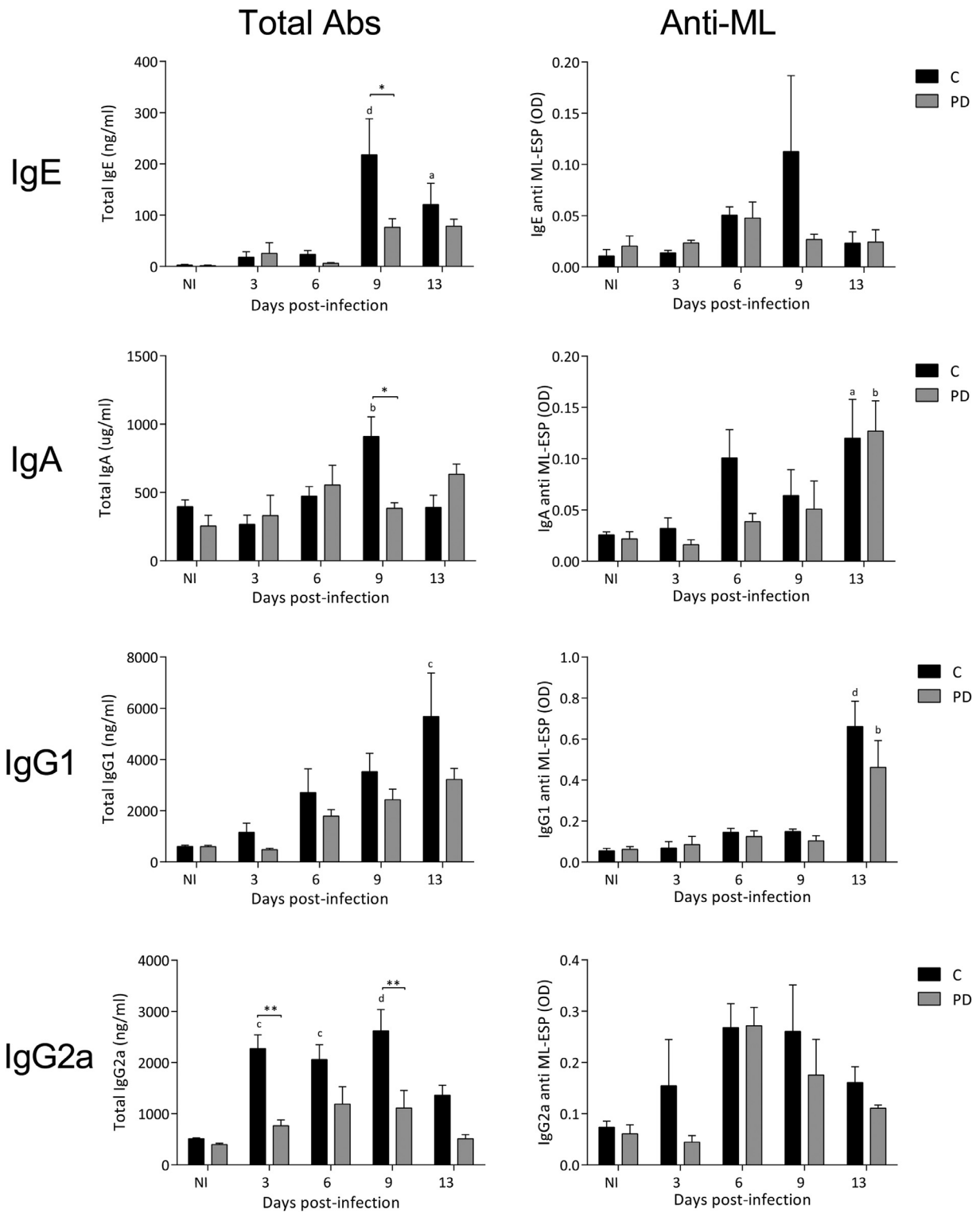

Fig. 7. Kinetics of total and antimuscle larvae excretory–secretory product immunoglobulins (Igs) in lung tissue extracts during *Trichinella spiralis* infection. Total Igs concentrations were determined by enzyme-linked immunosorbent assay and expressed as concentration ± standard error of mean. Specific Igs levels are determined by enzyme-linked immunosorbent assay and expressed as their optic density values ± standard error of mean. The animal groups (n = 5/group) were controls (C) and those fed the protein-deficient diet (PD), which were infected with *Trichinella spiralis* (C_I and PD_I, respectively) or non-infected (C_{NI} and PD_{NI}, respectively). The data were analyzed by two-way analysis of variance test followed by Tukey's or Sidak's multiple comparisons test ($\alpha = 0.05$). The asterisks indicate significant differences between PD and C ($^{*}P < 0.01$; $^{**}P < 0.005$), and letters significant differences between PD_I or C_I with their non-infected controls ($^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.005$; $^{d}P < 0.0001$).

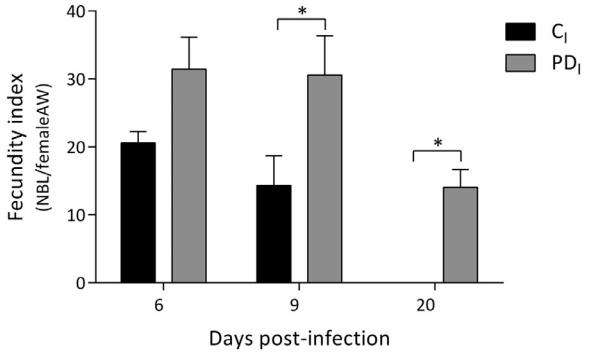

Fig. 8. Fecundity index scores of female adult worms during acute phase of *Trichinella spiralis*. After recovery of female adult worms (AW) were identified and counted. AW were incubated for 3 h, and the number of newborn larvae (NBL) shed by female AW were counted using an inverted microscope. The fecundity index score was calculated as the number of NBL/female AW ± standard error of mean. The animal groups (n = 4/group) were rat controls (C) and those fed the protein-deficient diet (PD), which were infected with *Trichinella spiralis* (C_I and PD_I, respectively). The data were expressed as mean number of NBL/female AW ± standard error of mean, and analyzed by two-way analysis of variance, followed by Sidak's multiple comparisons test ($\alpha = 0.05$; n = 4/group). An asterisk indicates significant differences between the PD_I and C_I groups ($^*P < 0.05$).

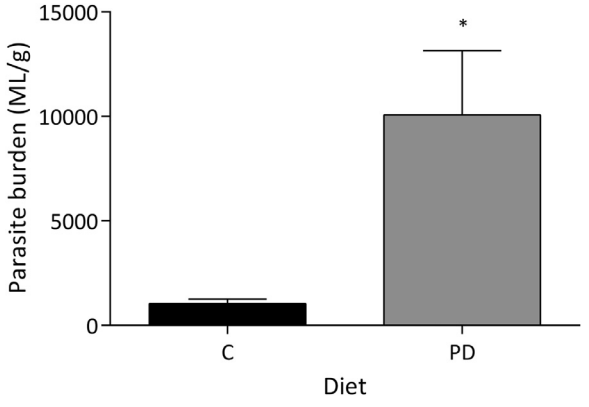

Fig. 9. Parasite burden of animals at day 33 postinfection. The parasite burden of the animals was determined by artificial digestion, and the results are expressed as the mean of the muscle larvae/g rat ± standard error of mean. The animal groups (n = 5/group) were rat controls (C) and those fed the protein-deficient diet (PD), which were infected with *Trichinella spiralis*. The data were analyzed using the unpaired *t* test ($\alpha = 0.05$), and the asterisk indicates significant differences ($^*P < 0.01$).

Conclusions

Protein deficiency diminishes the intestinal, pulmonary, and systemic immune responses to *T. spiralis*, which delays the expulsion and increases the fecundity index scores of AW. These alterations are associated with a higher parasite burden in the muscles.

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