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Protein deficiency during *Trichinella spiralis* infection impairs lung immunity against newborn larvae

Running title: Lung ADCC to *T. spiralis* in malnutrition

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The data that support the findings of this study are available from the corresponding author upon reasonable request.

Abstract

Aims: To analyze the effects of a protein deficient (PD) diet on antibody-dependent cell-mediated cytotoxicity (ADCC) in vitro against newborn larvae (NBL) of *Trichinella spiralis* in the lungs of infected rats.

Methods and results: Two groups of weaning Wistar rats received a PD diet (6.5% casein) and other two received a control diet (C, 20% casein). After ten days, one group of each diet was infected (PD_I and C_I) with muscle larvae. Lung tissue extracts (LTE) and lung cell suspension (LCS) were obtained. PD_I had lower titers of anti-NBL antibodies in LTE than C_I. In ADCC assays using control cells, NBL mortality percentage was lower with LTE from PD_I than LTE from C_I ($P < 0.01$). In assays using control cytotoxic sera, ADCC was exerted by LCS from C_I at all days post-infection (p.i.), but only by LCS from 13 days p.i. from PD_I. ADCC assays combining LTE and LCS from the same group showed a lower response for PD_I than for C_I ($P < 0.0001$). LCS from PD_I contained lower numbers of neutrophils, eosinophils and $Fc\varepsilon RI^+$ cells than C_I.

Conclusion: PD may diminish ADCC activity against *T. spiralis* NBL in lungs through alterations in specific antibodies and effector cells.

Keywords. Protein deficiency; *Trichinella spiralis;* infection; Lung; Newborn larvae; Antibody-dependent cell cytotoxicity.

1. Introduction

Malnutrition is an important cause of diminished immune responses worldwide, predisposing the host to infectious diseases, and even to death in cases of severe malnutrition [1]. The association between undernutrition and gastrointestinal nematode infection has been recognized for many decades: both are chronic diseases that often co-exist in individuals from developing countries [2]. Several studies have shown that when both conditions exist in the same individual, there is a higher parasite burden as the result of an impaired immune response [3-6].

Trichinella spiralis is a worldwide parasitic disease. Besides its epidemiological relevance, it is a good model to study the interactions between malnutrition and parasitic disease. The effector immunological response against *T. spiralis* is focused on the adult worm (AW) at the gut mucosa and the newborn larvae (NBL) at the systemic and lung levels. We have recently shown in a rat model of *T. spiralis* infection that animals receiving a protein deficient diet (PD) have diminished cellular and humoral immune responses against AW with longer persistence of this stage in the intestine, accompanied by a higher fecundity index of female AW [6].

Regarding the NBL, it is known that they are killed by antibody dependent cellular cytotoxicity (ADCC) mechanism [7-9]. In this mechanism specific antibodies bound to the NBL surface are recognized by Fc receptors on the surface of eosinophils, macrophages and neutrophils. As a result, these cells are activated and release molecules that harm and finally kill NBL. Previous publications showed that IgE and its high affinity receptor (FcεRI) have an important role in this mechanism [10-11].

During their migration to reach the muscles NBL circulate through the lungs, which seem to be involved in the retention and destruction of the larvae [12-13]. Of note, lung cells have been shown to kill NBL in the presence of specific antibodies [11, 14].

In a recent work, we analysed the effects of a PD diet during early *T. spiralis* infection on cellular and humoral immune factors [6]. The PD infected (PD_I) group showed reduced counts of goblet and mast cells in lungs as well as diminished levels of total antibodies, suggesting that the pulmonary mucosal response is affected by PD. Moreover, NBL were observed in the lungs from the PD_I group at 33 days post infection (p.i.), which constitutes the first report of this long persistence.

These observations suggest that the allergic-like inflammatory response that usually occurs in the lung parenchyma during *T. spiralis* infection [14] may be affected by PD. In line with these findings, we hypothesized that the lung ADCC response against NBL could also be affected by PD. In the present study we analysed the effects of a PD diet on the ADCC activity of lung cells and antibodies against NBL during early *T. spiralis* infection in rats. We found that PD diminishes the ability of lung effectors to mediate *in vitro* ADCC against NBL of *T. spiralis*.

2. Materials and methods

2.1. Animals and infection

Weaning female Wistar rats (21-23 days old) weighing an average of 41.27 ± 2.39 g 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*. They were divided into four groups: two groups received a PD diet and the others received a control diet (C) for growing rats. These diets were administered during the whole experiment (before and after infection). PD and C groups received experimental isocaloric diets that provided 6.5% or 20% protein, respectively, and all the essential nutrients as recommended by the American Institute of Nutrition [15]. 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 then filled up to 1000 g by adding dextrin, as previously reported [16].

After ten days, one group of each diet was orally infected, through a feeding probe, with 900 muscle larvae (ML) of *T. spiralis* per rat (PD_I, C_I) suspended in a saline solution. ML were obtained from muscle tissue of infected Swiss mice by the artificial digestion method [17]. The other groups of rats were used as non-infected controls (PD_{NI}, C_{NI}). Animals were euthanized through complete bleeding by cardiac puncture under anesthesia (80 mg/kg ketamine and 10 mg/kg xylazine, i.p.).

All experimental protocols were approved by the Institutional Committee of Care and Use of Laboratory Animals of the Facultad de Farmacia y Bioquímica (CICUAL-FFyB, N° 2470/17), and conducted in compliance with the guidelines established by the National Research Council of Argentina (CONICET).

2.2 Collection of NBL

NBL were obtained as described previously [18]. Briefly, AW were recovered from the intestine of rats 6 days after oral infection with 7000 ML. Worms were cultured in RPMI medium (Gibco, Grand Island, NY, USA) supplemented with antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin, Gibco) and 5% fetal calf serum (FCS) (Natocor, Villa Carlos Paz, Córdoba, Argentina) at 37 °C in a humidified atmosphere with 5% CO_2. NBL were collected 2 h later and used immediately in ADCC assays. Larvae were alive and in good condition as judged by their motility.

2.3. Serum samples

In all cases, rat sera were collected by cardiac puncture and were heat-inactivated at 56 °C for 30 min. Fresh non-infected rat sera (NIRS) obtained from the C_{NI} group were used as a complement source. Rat cytotoxic serum (RCS) was a pool of sera collected at day 45 p.i. from infected rats presenting anti-NBL surface antibodies (Titers: IgA = 256; IgE = 256; IgG1 = 512; IgG2a = 1024). Sera from PD_I and C_I groups were collected at days 6 and 13 p.i. to study specific antibodies and for ADCC assays. Sera from non-infected animals (PD_{NI}, C_{NI}) were used as controls.

2.4. Lung tissue extracts

Lung tissue extracts (LTE) were obtained using the PERFEXT method [19] with slight modifications. LTE contain soluble factors of pulmonary origin, including antibodies [20]. In our study LTE served as a source of locally produced anti-*Trichinella* antibodies for the ADCC assay. Briefly, the rats were bled completely by cardiac puncture and infused with PBS plus heparin (5000 UI/mL) into the heart. The perfused lungs were cut into small pieces, placed in an extraction solution containing CHAPS 90 mM (Research Organics, Cleveland, USA) in PBS and protease inhibitors (EDTA- free Complete, Roche Diagnostics, Mannheim, Germany) at 2 µL/mg of tissue, and frozen at -70 °C. After thawing, the extraction was performed overnight at 4 °C using a homogenizer. After centrifugation, the supernatants were collected, filtered through a 0.22 µm filter (Millipore Co, Bedford, MA, USA), aliquoted and kept frozen at -70 °C until use.

2.5. Cell suspensions

Peritoneal cell suspensions were obtained from C_{NI} rats by peritoneal washes using cold RPMI medium supplemented with antibiotics. Each suspension was collected and washed twice in RPMI by centrifugation. Lung cell suspensions (LCS) were obtained as described previously [11]. Peritoneal and lung cells were counted, and viability was assessed by Trypan Blue dye exclusion using a hemocytometer ensuring viability greater than 95% for all experiments. Finally, cell suspensions were properly resuspended in RPMI medium containing antibiotics and 5% FCS.

2.6. Determination of anti-NBL surface antibodies in LTE and sera

Titration of anti-NBL IgA, IgE, IgG1 and IgG2a in rat LTE and sera were performed by indirect immunofluorescence assay (IFA) on methanol-fixed NBL as described previously [11]. LTE and sera were assayed undiluted and in serial doubling dilutions. Titers were expressed as the inverse of the last dilution yielding a positive result. Samples were considered positive if a fluorescent stain was observed on parasite cuticle using an Olympus BX51 fluorescence microscope (American Inc.). Results were analyzed by two independent observers.

2.7. Leukocytes in lung cell suspensions

The percentage of total leukocytes ($CD45^+$ cells) in LCS was determined by flow cytometry as described in the next section. To evaluate specific leukocyte populations, LCS from all groups were concentrated by cytocentrifugation at 400 rpm for 10 min (Shandom Cytospin III, USA) and stained with Giemsa (Merck, Darmstadt, Germany). Absolute numbers of neutrophils, lymphocytes, macrophages and eosinophils per 200 total lung cells were determined using an Olympus BX51 optical microscope (American Inc.). Additionally, the percentage of each cell type within the total leukocyte population (leukocyte formula) was calculated.

2.8. FcεRI expression in lung cell suspensions

The FcεRI expression was examined by flow cytometry. Five hundred thousand lung cells were incubated with the working dilution of purified mouse anti-rat high affinity IgE receptor (BD Biosciences, San Diego, CA, USA) or purified mouse IgG1, κ isotype control (BD Biosciences) in a final volume of 100 µl at 4 °C for 30 min in PBS containing 3 % FCS and 5 mM EDTA. After performing the corresponding washes, cells were incubated under the conditions mentioned previously with the working dilution of R-PE-conjugated Goat F(ab′)2 anti-mouse IgG (H+L) (Caltag Laboratories, Burlingame, CA, USA). After performing the corresponding washes, cells were incubated under the conditions mentioned previously with the working dilution of APC/Cy7-conjugated anti-rat CD45 antibody (BioLegend). Samples were processed on a FACSAria II flow cytometer (BD Biosciences), and the Flowing Software 2.5. version was used for data analysis. Doublets were excluded from the analysis using a FSC-A *vs.* FSC-H plot. SSC-H *vs*. FSC-H plots were used to remove debris and to select the cluster corresponding to the LCS. Data were expressed as mean ± S.E.M. of $CD45^+$ $Fc\varepsilon RI^+$ cells percentage obtained from four independent experiments.

2.9. In vitro ADCC assay

To study the role of LTE and sera antibodies in ADCC, approximately 50 NBL in supplemented RPMI media were placed into each well of a flat-bottomed microwell module (Nunc, Roskilde, Denmark). After that, 20 µl of LTE or sera from each group were added, followed by the addition of peritoneal cells from C_{NI} rats (8000 leukocytes/NBL).

In order to study the cytotoxic properties of lung cells, the NBL suspension was placed into each well with RCS or NIRS (20 µl), followed by the addition of LCS (6000 leukocytes/NBL) from each group.

Finally, to study the combined cytotoxic effect of lung cells and LTE antibodies, NBL were incubated with LTE (20 µl) and LCS (6000 leukocytes/NBL) from each group.

In all cases, fresh non-infected serum was added as a source of complement and larvae were counted before the LCS were added. Reactions were maintained at 37 °C for 20 h in a humidified atmosphere with 5% CO_2. NBL death was assessed using direct microscopy observation by two independent observers in a blind fashion and the mortality percentage was calculated as follows:

$$\% \text{ NBL mortality} = [(NBL_i - NBL_f)/NBL_i] \times 100$$

where NBL_i and NBL_f are the numbers of living NBL, judged by their motility, counted at the beginning and at the end of the reaction, respectively. Results were expressed as the mean ± S.E.M. of NBL mortality percentages from five independent experiments performed in duplicate.

2.10. Statistical analysis

All the statistical analyses were carried out using the statistical program R (version 4.0.2) [21] and the level of significance used was 0.05. In general, data were analysed by two-way ANOVA followed by Tukey's multiple comparisons test (*nlme* and *multcomp* packages, respectively). In order to test normality distribution of the data, graphical analysis was done by a QQ-plot graph and analytical analysis was done using Shapiro-Wilk test (*nlme* package). To test homocedasticity, Levene test was employed (*car* package). Data from the IFA assays were analyzed employing the Scheirer–Ray–Hare test followed by Dunn test (*rcompanion* and *FSA* packages, respectively) [22]. All data were graphed using GraphPad Prism version 8.0.1 for Windows, GraphPad Software, La Jolla California USA, (www.graphpad.com).

3. Results

3.1. Determination of antibodies against NBL surface antigens in LTE and sera

The levels of anti-NBL antibodies in LTE increased during the infection in animals fed the control diet (C_I) and those fed the protein deficient diet (PD_I) but tended to be lower in the later group (Figure 1). At day 6 p.i., specific IgG1 and IgG2a were detected in some rats from the C_I group whereas no antibodies were detected in the PD_I group. At day 13 p.i., C_I and PD_I had a positive reaction for IgE, IgG1 and IgG2a, although a tendency to lower titers was observed in the PD_I group for all isotypes. It is worth noting that specific IgA was not detected in LTE from any group during the whole follow-up.

In the case of sera, all isotypes of anti-NBL surface antibodies were found in both groups since day 13 p.i. (Figure 1). As in the case of LTE, titers tended to be higher in the C_I group than in the PD_I group.

3.2. Role of LTE and serum antibodies and lung cells in ADCC assays

In these *in vitro* assays, the cytotoxic effect (ADCC) of rat lung cells and LTE antibodies on NBL was analyzed both separately and together at different days p.i. First, we analyzed if anti-NBL antibodies present in LTE were able to exert ADCC in the presence of peritoneal leukocytes from well-nourished rats used as control effector cells (Figure 2A). The NBL mortality percentage attained with LTE obtained from the C_I group at day 6 p.i. was significantly higher than that produced by LTE from the corresponding non-infected (NI) controls (C_{NI}) (16.73 ± 1.28 *vs.* 7.72 ± 0.82 % NBL mortality, $P < 0.01$), and increased further at day 13 p.i. (31.05 ± 2.27 % NBL mortality). In the PD_I group, in contrast, only the LTE obtained at day 13 p.i. induced an NBL mortality significantly higher than the corresponding NI controls (PD_{NI}) (22.49 ± 2.63 *vs.* 7.59 ± 0.73 % NBL mortality, $P < 0.0001$). At day 13 p.i. the mortality percentage attained with LTE from the PD_I group was significantly lower than that reached with LTE from C_I (22.49 ± 2.63 *vs.* 31.05 ± 2.27 % NBL mortality, $P < 0.05$).

Then, the cytotoxic capacity of LCS from C and PD groups was evaluated in the presence of normal serum (NIRS) or cytotoxic serum (RCS) (Figure 2B). In the case of the C_I group, LCS obtained at any day p.i. exerted a significantly higher NBL mortality percentage in the presence of RCS than in the presence of NIRS. However, in the case of PD_I group a significant difference in NBL mortality between RCS and NIRS was observed only when LCS from day 13 p.i. were used (31.67 ± 4.05 *vs.* 7.59 ± 2.51 % NBL mortality, $P < 0.01$). At all time points, the mortality percentages obtained using RCS were higher for LCS from the C group than for LCS from the PD group, although the differences did not reach statistical significance.

Finally, both the LTE and the LCS from either C or PD groups were used together in ADCC assays in order to evaluate the combined effect of both factors against NBL (Figure 2C). The mortality percentages obtained with LTE and LCS from the PD_I group did not differ from those obtained in the PD_{NI} group at any time p.i. In contrast, the mortality percentages obtained with LTE and LCS from the C_I group were significantly higher than those of the C_{NI} group at both 6 and 13 days p.i. At day 13 p.i., the mortality percentage obtained with LTE and LCS from the C_I group was significantly higher than that obtained with their PD_I counterparts (45.80 ± 6.88 *vs.* 16.76 ± 2.68 % NBL mortality, $P < 0.0001$).

As shown above, the nutritional status of the animals affected the ability of LTE to mediate ADCC against NBL, with a diminished activity of LTE from PD_I as compared to C_I. To analyze whether protein deficiency also affects the ability of systemic antibodies to mediate ADCC, cytotoxicity assays were performed using sera from C_I or PD_I animals in the presence of peritoneal leukocytes from well-nourished rats used as control effector cells (Figure 2D). ADCC activity was detected for sera from day 13 p.i. for both groups, as the NBL mortality percentage attained was significantly higher than that produced by sera from the corresponding non-infected controls (PD_I: 21.00 ± 0.89 *vs*. PD_{NI}: 6.61 ± 1.86 % NBL mortality, $P < 0.01$; C_I: 22.00 ± 1.76 *vs*. C_{NI}: 7.06 ± 1.59 % NBL mortality, $P < 0.05$). No differences were found in % NBL mortality between PD_I and C_I groups.

3.3. Leukocyte populations in LCS

Considering the different ADCC performance of LCS from C and PD groups, we decided to analyze the leukocyte populations in these suspensions. When we analyze the percentage of leukocytes ($CD45^+$ cells) from LCS, we found a significant increase in C_I group since day 6 p.i. (as compared to the corresponding non-infected animals), whereas in the PD_I group only a non-significant increase was found at day 13 p.i. (Figure 3A). Results also showed an increase in the absolute numbers of neutrophils and eosinophils at day 6 p.i. in the C_I group as compared to the C_{NI} group, with a further increase at day 13 p.i. (Figures 3D and 3E). In C_I these increases were at the expense of a decrease in lymphocytes (Figure 3B). In the PD_I group, in contrast, only macrophages showed a small but significant increase in the lungs during the infection (Figure 3C). PD_I animals had lower counts of neutrophils and eosinophils than C_I at both p.i. times (Figures 3D and 3E). The percentage of each cell type within the total leukocyte population was calculated (Figures 3F to 3I). The percentages of neutrophils and eosinophils increased in the C_I group at both p.i. times as compared to the C_{NI} group, whereas such increase was not observed in the PD_I group (Figure 3H and 3I). The percentage of lymphocytes decreased in both C_I and PD_I at 13 days p.i., as compared to their respective NI controls (Figure 3F). No differences were found in the percentage of macrophages overtime in any group (Figure 3G). Altogether, these data demonstrate that both the percentage and the total counts of neutrophils and eosinophils increased along the infection in the C_I group whereas none of these parameters changed in the PD_I group.

3.4. FcεRI expression in LCS

Considering the important role of FcεRI in ADCC, we decided to evaluate its expression by flow cytometry within the leukocyte population ($CD45^+$ cells) in LCS from all the experimental groups (Figure 4). This analysis revealed an increase in the percentage of $CD45^+$ $FcεRI^+$ cells in LCS of C_I group at day 13 p.i. compared to C_{NI} (8.30 ± 1.16 *vs*. 4.27 ± 0.65 % $FcεRI^+$ $CD45^+$ cells, $P < 0.05$). In contrast, the percentage of leukocytes expressing FcεRI did not increase significantly in the PD_I group during the follow-up (Figure 4).

4. Discussion

It is known that a deficiency of dietary protein impairs mucosal immunity and impacts negatively on type 2 immune responses necessary to control helminth infections [2]. The aim of this work was to test whether a PD diet affects the helminthotoxicity of the two immune players (antibodies and cells) of ADCC against NBL in rat lungs during early *T. spiralis* infection. In particular, days 6 and 13 p.i. were evaluated in ADCC assays. It is important to mention that during this period the NBL are released by pregnant female AW in the intestine, circulate systemically, and may be killed by ADCC. Day 6 p.i. is the moment of maximal circulation of NBL through the lungs [23]. Day 13 p.i. marks the end of NBL circulation due to the expulsion of AW from the intestine.

When the role of anti-NBL antibodies from LTE was evaluated, we found that the mortality percentage by ADCC increased significantly later and was significantly lower in PD_I than in C_I, which may be explained by the lower levels and the later appearance of these antibodies in LTE from the PD_I group (Figure 1A). For both time points, the LTE levels of specific anti-NBL surface antibodies of the IgG1, IgG2a and IgE isotypes were lower in the PD_I group than in the C_I group. Of note, a similar difference was observed for the total levels of these isotypes in LTE from these same experimental groups in a previous study (Vila et al 2019).

Regarding the role of lung cells in the ADCC mechanism, two findings were of interest. The first is associated with a quantitative aspect: in the PD_I group the LCS from day 13 p.i., but not that from day 6 p.i., was able to exert ADCC against NBL, whereas in the C_I group the suspensions from both days p.i. could exert this cytotoxic mechanism (Figure 1B). This difference may be linked to the lower counts and percentages of neutrophils and eosinophils found in the PD_I group at day 6 p.i. (Figure 3), given the importance of these cells in ADCC [24].

The second finding is of qualitative nature, as it relates to the ability of cells to mediate ADCC. When the ability of LCS to exert ADCC (%M with RCS vs. NIRS) is evaluated for each group, it can be observed that cells from the C_{NI} group exerted ADCC whereas those from PD_{NI} or those obtained from PD_I at day 6 p.i. did not. However, the counts and percentages of effector cells did not differ significantly between these three groups (Figure 3), suggesting that the lung cells from the PD group may have a diminished functionality as compared to those from the C group. One cause of diminished activity of the cells involved in ADCC could be a reduced expression of the high affinity IgE receptor, FcεRI. To test this hypothesis, we measured the expression of this receptor in LCS. The percentage of the cells expressing FcεRI receptor did not increase in the PD_I group during the infection, whereas it increased in the C_I group at day 13 p.i. The lower number of cells expressing this receptor in PD_I could contribute to the tendency to lower ADCC activity in this group as compared to C_I. However, no differences in FcεRI expression were found between C_{NI}, PD_{NI} and PD_I at 6 days p.i. that could explain why the lung cells from the first group exerted ADCC while those from the other two groups did not.

Regarding the results mentioned above, other mechanisms that may affect the cellular activity in PD animals should be considered. Garcia *et al*. (1986) studied blood and peritoneal neutrophils in PD rats, and they observed a reduced peroxidase activity with a consequent reduction in bactericidal capacity [25]. A study by Moriguchi *et al*. (1983) found that alveolar macrophages from PD rats conserved their phagocytic activity but failed to respond to cytokines [26]. Finally, de la Fuente *et al*. (1992) found a reduced production of superoxide anion in peritoneal macrophages from mice receiving a PD diet [27]. The reduced effector activities of macrophages and neutrophils from PD animals reported in previous studies suggests that the ability of these cells to mediate ADCC may be also compromised beyond the expression of the FcεRI receptor.

Finally, in order to have an approximation to the effect of PD on the ADCC response *in vivo,* where both the humoral and cellular effectors would be affected by the nutritional status of the host, we evaluated the cytotoxic effect exerted by the combination of LTE (anti-NBL lung antibodies) and LCS of each group. When LCS and LTE from the PD_I group were combined no ADCC effect was observed at any time p.i., whereas a significant and increasing ADCC activity was observed for combined samples from the C_I group. This decreased ADCC activity in the PD_I group was observed in spite of the significant ADCC activity displayed by lung antibodies (LTE) or LCS from PD_I at day 13 p.i. (as compared to PD_{NI}) when they were evaluated in combination with control cells or control antibodies, respectively (Figure 1A and 1B). Therefore, while lung antibodies or cells from PD_I animals display some ADCC activity when they are combined with partners (cells or antibodies) from normal animals, their ability to mediate cytotoxicity is lost when they are combined with partners from protein deficient animals.

Globally, this study shows that the ADCC activity against the NBL of *T. spiralis* is drastically diminished during the first 13 days p.i. in rats receiving a PD diet. Of note, our previous study using the same model of *T. spiralis* infection revealed that animals from the PD_I group harbor in their gut viable female AW which release more NBL at longer times p.i. compared to C_I group [6]. These NBL would reach the systemic circulation and pass through the lungs but, as shown in the present study, the pulmonary immune response would not be enough for retention and death of the NBL, allowing them to reach muscle cells and to transform intro muscle larvae.

To the best of our knowledge, this is the first study to evaluate the effect of protein deficiency on the ADCC mechanism during a helminthic infection. The reduced ADCC activity of both lung antibodies and lung cells from PD_I animals could contribute, together with the diminished mucosal immunity against AW, to the higher parasite burden in muscles previously reported in this malnourished group [6]. Further studies will be required to determine which specific ADCC-related activities of effector cells are affected by protein deficiency.

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Authors' contributions

CCV: Conceptualization, Investigation, Formal analysis, Visualization, Writing original draft. **MPS:** Conceptualization, Investigation, Formal analysis, Visualization, Writing original draft. **TL:** Investigation, Formal analysis, Visualization, Writing original draft. **GHF:** Investigation, Formal analysis, Writing original draft. **MD:** Investigation, Formal analysis, Writing original draft. **MAC:** Investigation, Formal analysis. **ANP:** Conceptualization, Methodology, Writing original draft. **PCB:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Writing original draft.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure legends

Figure 1 Titers of anti-NBL surface antibodies. The results of specific IgE (**A**, **B**), IgA (**C**, **D**), IgG1 (**E**, **F**) and IgG2a (**G**, **H**) present in LTE (**A**, **C**, **E**, **G**) or sera (**B**, **D**, **F**, **H**) were determined by IFA. Results are expressed as titers. Experimental groups (n = 5/group) were rats fed the control diet (C) or the protein-deficient diet (PD), which were infected or not (NI) with *T. spiralis*. Infected animals were examined at 6 and 13 days post-infection (p.i.). Asterisk indicates significant differences (* $P < 0.05$).

Figure 2 Cytotoxic activity of lung cell suspensions, LTE and sera. ADCC assays were performed using peritoneal cells from normal animals combined with LTE (**A**) or rat sera (**D**), or using lung cell suspensions combined with the corresponding LTE (**C**) or with reference cytotoxic serum (RCS) or sera from non-infected rats (NIRS) (**B**). Results are expressed as the mean ± S.E.M. of mortality percentages of NBL. Experimental groups (n = 5/group) were those described in Figure 1. Results were obtained from at least five independent experiments performed by duplicate. Asterisks indicates significant differences between infected C and PD and their non-infected controls (* $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$). The number sign (#) indicates significant differences between C at day 13 p.i. with C at day 6 p.i. and PD at day 13 p.i. ($P < 0.05$).

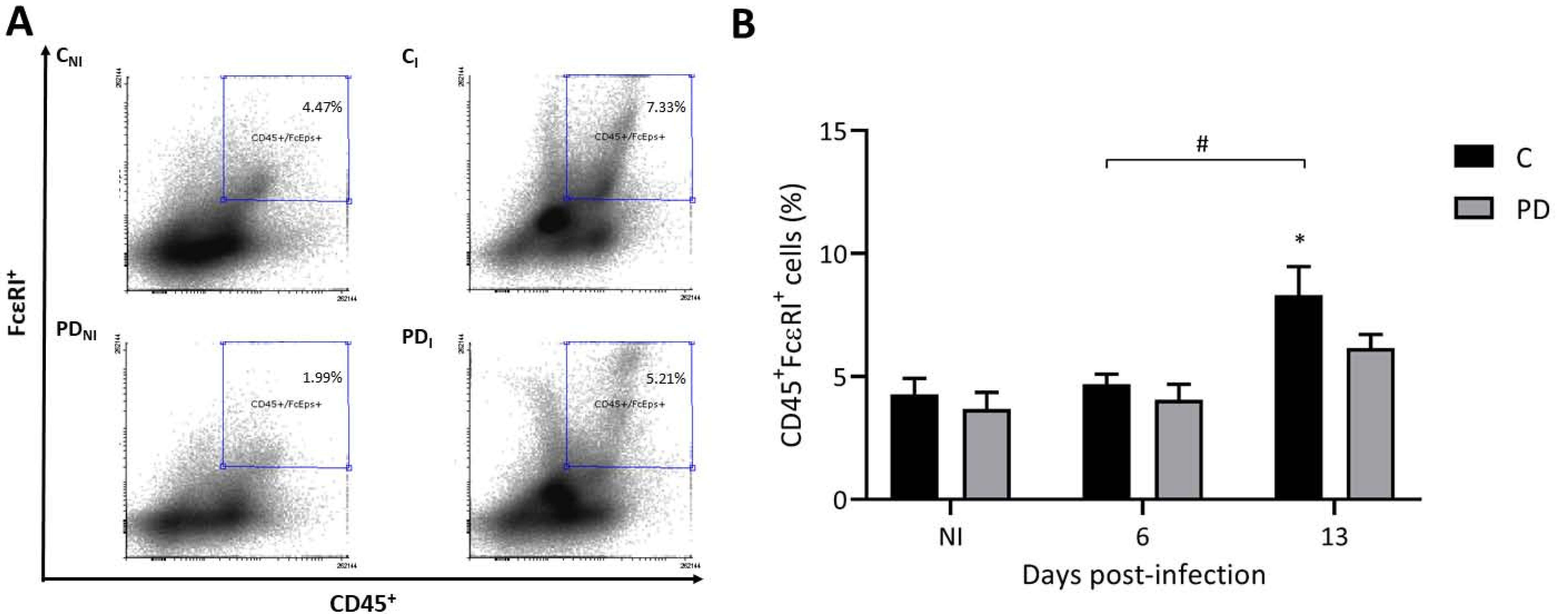
Figure 3 Leukocytes in lung cell suspensions. **A)** Percentage of CD45$^+$ cells as measured by flow cytometry. Total counts of lymphocytes (**B**), macrophages (**C**), neutrophils (**D**), and eosinophils (**E**) per 200 lung cells was determined by microscopical examination after Giemsa staining. The percentage of each cell type within the leukocyte population is shown in panels F to I. Results are expressed as the mean count or percentage ± S.E.M. Experimental groups (n = 5/group) were those described in Figure 1. Results were obtained from five independent experiments. Asterisks indicates significant differences (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$ **** $P < 0.0001$).

Figure 4 Surface expression of CD45 and FcεRI in lung cells suspensions. **(A)** Density plots of a representative experiment showing CD45 and FcεRI expression in cells obtained at 13 days p.i. (**B)** Percentage of CD45$^+$ FcεRI$^+$ cells. Experimental groups (n = 4/group) were those described in Figure 1. Results are expressed as the mean of the percentages of positive events of CD45$^+$ FcεRI$^+$ in the lung cell suspension ± S.E.M. The asterisk (*) indicates a significant difference between infected and non-infected C animals ($P < 0.05$). The number sign (#) indicates significant differences between day 6 and 13 p.i. in the infected C group ($P < 0.05$).


Lung tissue extracts (LTE)
Sera
A
IgE anti-NBL (titer)
8
6
4
2
0
NI
6
13
Days post-infection
*
B
IgE anti-NBL (titer)
100
80
60
40
20
0
NI
6
13
Days post-infection
C
PD
C
IgA anti-NBL (titer)
8
6
4
2
0
NI
6
13
Days post-infection
D
IgA anti-NBL (titer)
50
40
30
20
10
0
NI
6
13
Days post-infection
C
PD
E
IgG1 anti-NBL (titer)
8
6
4
2
0
NI
6
13
Days post-infection
F
IgG1 anti-NBL (titer)
200
150
100
50
0
NI
6
13
Days post-infection
C
PD
G
IgG2a anti-NBL (titer)
8
6
4
2
0
NI
6
13
Days post-infection
H
IgG2a anti-NBL (titer)
100
80
60
40
20
0
NI
6
13
Days post-infection
C
PD


A
100
80
60
40
20
0
% NBL mortality
C
PD
#
#
**
****
****
NI
6
13
Days post-infection
B
100
80
60
40
20
0
% NBL mortality
NIRS
RCS
**
****
****
**
NI
6
13
NI
6
13
C
PD
Days post-infection
C
100
80
60
40
20
0
% NBL mortality
C
PD
#
#
*
****
NI
6
13
Days post-infection
D
100
80
60
40
20
0
% NBL mortality
C
PD
*
**
NI
6
13
Days post-infection


A
% CD45⁺
0
20
40
60
80
100
NI
6
13
Days post-infection
*
**
C
PD
B
Lymphocytes per 200 total lung cells
0
50
100
150
NI
6
13
Days post-infection
*
*
*
C
Macrophages per 200 total lung cells
0
50
100
150
NI
6
13
Days post-infection
*
C
PD
D
Neutrophils per 200 total lung cells
0
20
40
60
80
100
NI
6
13
Days post-infection
***
**
*
**
E
Eosinophils per 200 total lung cells
0
20
40
60
80
100
NI
6
13
Days post-infection
**
***
***
**
C
PD
F
Lymphocytes (%)
0
20
40
60
80
100
NI
6
13
Days post-infection
*
*
**
G
Macrophages (%)
0
20
40
60
80
100
NI
6
13
Days post-infection
C
PD
H
Neutrophils (%)
0
20
40
60
80
100
NI
6
13
Days post-infection
**
**
*
**
I
Eosinophils (%)
0
20
40
60
80
100
NI
6
13
Days post-infection
**
***
**
**
C
PD


A
C_{NI}
C_I
PD_{NI}
PD_I
4.47%
7.33%
1.99%
5.21%
CD45+/FcEps+
262144
FcεRI$^+$
CD45$^+$
B
CD45$^+$FcεRI$^+$ cells (%)
15
10
5
0
#
*
C
PD
NI
6
13
Days post-infection