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A mouse air pouch model for evaluating the immune response to *Taenia crassiceps* infection

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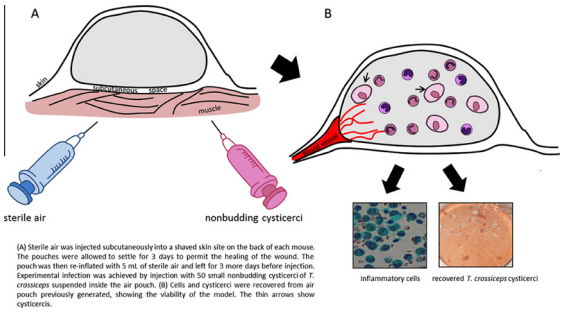
HIGHLIGHTS

- We establish a new experimental model to study *T. crassiceps* infection in mice.
- This model was compared to the previously established intraperitoneal infection model.
- The air pouch provides a convenient cavity that allows studying *T. crassiceps* parasite.

GRAPHICAL ABSTRACT



(A) Sterile air was injected subcutaneously into a shaved skin site on the back of each mouse. The pouches were allowed to settle for 3 days to permit the healing of the wound. The pouch was then re-inflated with 5 mL of sterile air and left for 3 more days before injection. Experimental infection was achieved by injection with 50 small nonbudding cysticerci of *T. crassiceps* suspended inside the air pouch. (B) Cells and cysticerci were recovered from air pouch previously generated, showing the viability of the model. The thin arrows show cysticercis.

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ABSTRACT

The experimental system of *Taenia crassiceps* cysticerci infection in BALB/c mice is considered to be the most representative model of cysticercosis. In our work, mice were sacrificed 7 and 30 days after infection, and pouch fluid was collected to determine the number of accumulated cells and the concentrations of IFNγ, IL-2, IL-4, IL-6, IL-10 and nitric oxide. The injection of 50 nonbudding cysticerci into normal mouse dorsal air pouches induced a high level of IFNγ and nitric oxide production relative to the parasite load. The air pouch provides a convenient cavity that allows studying the cellular immunological aspects of the *T. crassiceps* parasite. The nonbudding cysticerci recovered from the air pouches contained cells that can reconstitute complete cysts in the peritoneal cavity of mice. In conclusion, these results demonstrate that the air pouch model is an alternative tool for the evaluation of the immune characteristics of *T. crassiceps* infection.

1. Introduction

Taenia crassiceps is a tapeworm whose adult form is usually found in the intestines of European and North American red foxes. *T. crassiceps* cysticerci cause a chronic infection in laboratory animals such as mice (Freeman, 1962) and rats (Blair and Campbell, 1976).

Several strains of *T. crassiceps* have been isolated and maintained under laboratory conditions (Freeman, 1962; Freeman et al., 1973). The ORF strain has become the most widely used due to its ability to multiply rapidly in the mouse peritoneal cavity (Freeman, 1962; Sciutto et al., 1991). The antigenic cross-reactivity between *Taenia solium* and *T. crassiceps* cysticerci (Espíndola et al., 2000; Larralde et al., 1989) has enabled the use of *T. crassiceps* antigens in the serodiagnosis of human cysticercosis caused by *T. solium* (Larralde et al., 1989, 1990; Vaz et al., 1997). As *T. solium*

Abbreviations: VF-Tcra, *Taenia crassiceps* vesicular fluid; NO, nitric oxide; IL, interleukin; OD, optical density; i.p., intraperitoneal immunization; s.c., subcutaneous immunization.

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cysticerci are often difficult to obtain, murine cysticercosis caused by *T. crassiceps* has frequently been used as an experimental model (Espíndola et al., 2000; Toenjes and Kuhn, 2003). This worm species can be experimentally maintained in the mouse peritoneal cavity, where cysticerci reproduce by budding (Fragoso et al., 1996; Sciutto et al., 1991). *T. crassiceps* cysticercosis has been used as an experimental model to study the immunological (Espíndola et al., 2000; Robinson et al., 1997; Terrazas et al., 1999; Toenjes et al., 1999) and genetic (Fragoso et al., 1996, 1998; Sciutto et al., 1991) factors involved in resistance to *T. crassiceps* infection.

Resistance and susceptibility to different parasitic diseases have been associated with the predominance of either a Th1- or Th2-type immune response (Mosmann, 1991). Th1 cells produce interleukin-2 (IL-2), interferon-gamma (IFNγ), and tumor necrosis factor-beta (TNF-β) and are involved in cell-mediated immune reactions. Th2 cells secrete mainly IL-4, IL-5, IL-6, IL-10 and IL-13, in addition to mediating B cell activation, antibody production, and regulation of Th1 responses (Basso et al., 2009; D'Elios et al., 2011; Mosmann and Coffman, 1989).

In the early stages of *T. crassiceps* infection of BALB/c mice, a transient increase in Th1-type cytokine production can be detected in the spleen cells of infected animals (Terrazas et al., 1998). By 30 days post-infection, however, the response shifts toward a Th2-type profile (Terrazas et al., 1998). This Th2-type profile has been shown to continue for up to 4 months after infection (Villa and Kuhn, 1996), when the number of larvae inside the body cavity is in the thousands (Peón et al., 2013).

Air pouches have been used with superb results in the analysis of inflammatory responses induced by a wide range of materials, including bacteria (Castro et al., 1991; Clark and Weinhold, 1979; Tessier et al., 1998), protozoa (Matte and Olivier, 2002; Pacheco and Lenzi, 1997) and other parasites (Daly et al., 1999; Bower et al., 2008), but this model had not been previously tested for *T. crassiceps* infection.

Although substantial progress has been made in understanding the immunological basis for resistance and susceptibility to several helminthic infections using murine models, little is known about the molecular mechanisms that mediate susceptibility to experimental cysticercosis caused by *T. crassiceps* (Alonso-Trujillo et al., 2007). When injected intraperitoneally into susceptible BALB/cAnN mice, complete cysticerci were recovered in a number that is proportional to the quantity of injected cells (Toledo et al., 1997).

To the best of our knowledge, this is the first description of an air pouch *T. crassiceps* model. The cysts were maintained in an air pouch in the back of a mouse for 30 days. The characteristics of recovered cysts were analyzed. When injected intraperitoneally into susceptible BALB/c mice, complete cysticerci were recovered. The recovered VF-Tcra antigens were compared with those recovered from the intraperitoneal model using SDS–PAGE, immunoblot, and ELISA. The findings indicate that the air pouch model is a simple model that can facilitate the identification of immunological mechanisms.

Additionally, in our model, we analyzed the roles of nitric oxide (NO) and the cytokines IFNγ, IL-2, IL-4, IL-6 and IL-10 in determining the outcome of murine cysticercosis caused by the cestode *T. crassiceps*. These findings suggest that macrophage activation and NO production may be important contributors to host resistance to *T. crassiceps* infection as previously described (Alonso-Trujillo et al., 2007).

We conclude our study by reporting several aspects of the immune response against this pathogen that could be useful for understanding the immunological events that occur during the initial stages of experimental cysticercosis. Our results will also be useful for making comparisons with traditional studies based on the classical model that was used for more than 50 years.

2. Materials and methods

2.1. Parasites and infections

Metacestodes of *T. crassiceps* (ORF strain) were harvested under sterile conditions from the peritoneal cavity of female BALB/c mice 1 month after infection. The cysticerci were washed four times in sterile phosphate-buffered saline (PBS; 0.15 M, pH 7.2). Experimental infection was achieved by injection with 50 small (~2 mm in diameter) nonbudding cysticerci of *T. crassiceps* suspended in 0.5 mL of PBS per mouse intraperitoneally or inside the air pouch. Mice were sacrificed 30 days after infection, and the cysts found inside the peritoneal cavity and the air pouch were counted. The variation in the number of parasites within the groups of injected and control mice was attributed to the differences in infectivity of each parasite inoculum. Consequently, each analysis of the immune response per parasite intensity included a group of non-immunized mice as controls to assess the infectivity of each inoculum. Thus, the effects of immunization measured by each experiment were compared with a control. The vesicular fluid of *T. crassiceps* (VF-Tcra) was obtained as described (Vaz et al., 1997). The study was conducted under an institutional committee approved protocol (IAL/CCD/CTC).

2.2. Antigens

Intact *T. crassiceps* parasites were ruptured and centrifuged at 15,000×*g* for 60 min at 4 °C, and the supernatants were sonicated at 20 kHz and 1 mA for four periods of 60 s each on an ice bath. The supernatant obtained after further centrifugation represented VF-Tcra. Phenylmethylsulfonyl fluoride (Sigma Chemical Company, St. Louis, MO.) was added to each antigen extract at a final concentration of 4×10^{-1} mM. This supernatant fraction was used as the antigen for serum antibody detection by enzyme-linked immunosorbent assay (ELISA) and immunoblot (Espíndola et al., 2000).

2.3. Immunization of mice and serum collection

Groups of six to ten BALB/c mice were subcutaneously immunized with two doses of 10 μg of VF-Tcra antigens in saponin (Sigma Chemical Co.) per mouse at a concentration of 10 μg/mouse at intervals of 15 days. The booster consisted of the same immunizing dose with the same adjuvant used before. The animals were bled from the retro-orbital plexus at 15, 30, 45 and 60 days after immunization. The sera were pooled and kept frozen in aliquots at −20 °C. In this study the sera were used within 60 days after immunization.

2.4. Generation of air pouches in mice

Air pouches were formed as previously described (Edwards et al., 1981), with some modifications. Briefly, 5 mL of sterile air was injected subcutaneously into a shaved skin site on the back of each mouse. The pouches were allowed to settle for 3 days to permit the healing of the wound. The pouch was then re-inflated with 5 mL of sterile air and left for 3 more days before injection. The sterile air was obtained in a laminar flow station by filtration through a Millipore filter (0.22 μm) directly into a 10 mL syringe. On day 8, the pouches of the experimental group were filled with 50 nonbudding cysticerci of *T. crassiceps* suspended in 0.5 mL of endotoxin-free saline using individual sterile needles; the control group received only endotoxin-free saline. All air pouch procedures were conducted under light ether anesthesia. Mice were sacrificed by CO_2 asphyxiation, and the pouches were washed with endotoxin-free saline, followed by two separate washes with 2 mL of

endotoxin-free Iscove's medium. The pouch washings were centrifuged at 100×*g* for 10 min before enumeration of the total cells and viable cells using trypan blue exclusion and a hemocytometer. The supernatants were removed, and the cells were resuspended in PBS, stained in Turk's solution (crystal violet 0.01% w/v in acetic acid 3% v/v), and counted. Two hundred thousand cells were centrifuged onto microscope slides at 100×*g* for 5 min using a cytospin centrifuge (Citospin, Incibrás, Brasil). The slides were air dried and then stained by the May-Grünwald method, and 200–400 viable cells per slide were counted. The supernatants of individual mice were snap frozen for future analysis of nitric oxide and IFNγ, IL-2, IL-4, IL6 and IL-10.

2.5. ELISA

ELISA was used to measure the specific IgG antibodies to VF-Tcra antigens from mice. MaxiSorp 96-well plates (Nunc) were coated with antigen using 100 µl (1 µg) of VF-Tcra antigens diluted in 0.02 M carbonate–bicarbonate buffer, pH 9.6, for 18 h in a humidified chamber at 4 °C. The plates were blocked with 5% skim milk (Molico skim milk; Nestlé, Araçatuba, São Paulo, Brazil) in 0.01 M PBS (0.0075 M Na_2HPO_4, 0.025 M NaH_2PO_4, 0.14 M NaCl, pH 7.2). Immune serum and conjugate concentrations were obtained by titration. We diluted control (non-immunized mouse) serum to 1:100 and added peroxidase-labeled mouse anti-IgG (Kirkegaard and Perry, Gaithersburg, MD). The enzymatic reaction was developed with the chromogenic substrate tetramethylbenzidine and hydrogen peroxide (Bio-Rad Laboratories, Inc., Hercules, Calif.) for 20 min in the dark and blocked with 1 N sulfuric acid. Labeling intensities were quantified with a plate reader at 450 nm (Multiskan MCC, Lab Systems and Flow Lab, Finland). The absorbance (optical density [OD]) obtained for each test was subtracted from the control reading (non-immunized mice). All incubations were carried out at 37 °C for 1 h, except for the blocking step, which was carried out for 2 h. Between the sample, conjugate, and substrate incubation steps, the plates were washed in an automatic washer with four cycles of saline solution containing 0.05% Tween. All plates contained a control with VF-Tcra antigens (1 µg). The cutoff OD value was determined based on the analysis of the results of the control group. Samples presenting an OD value equal to or higher than the cutoff OD were considered to be positive. The cut off was chosen using the ROC curve as described by Kollef and Schuster (1994).

2.6. SDS–PAGE and immunoblotting

Antigen extracts were analyzed by electrophoresis (SDS–PAGE) using the method of Laemmli (1970). The samples that tested positive by ELISA were submitted to immunoblotting for analysis of antibody specificity. The VF-Tcra antigen extract (20 µg) of each individual mouse was separated by polyacrylamide gel electrophoresis in the presence of dodecyl sulfate (SDS–PAGE) on a mini gel (13%) and transferred electrophoretically to a 0.2 µm nitrocellulose membrane (Millipore, Bedford, MA, USA). The serum samples were diluted 1:100 in 1% skim milk in phosphate-buffered saline (PBS: 0.01 M; pH 7.2; 0.0075 M Na_2HPO_4, 0.025 M NaH_2PO_4, and 0.14 M NaCl). The immunoenzymatic reaction was carried out as previously described (Espíndola et al., 2000).

2.7. Determination of IFNγ, IL-2, 4, 6, 10 and nitric oxide levels

Exudates were recovered from the air pouches and peritoneal exudate cells were collected from the peritoneal cavities of infected mice or uninfected control mice 7 and 30 days after infection with *T. crassiceps*. IFNγ, IL-2, IL-4, IL-6, and IL-10 production in the air pouch of each individual mouse was measured by capture ELISA using antibody pairs purchased from Pharmingen (San Diego, CA) according to the manufacturer's instructions. The cells (5×10^6 per well with more than 85% viability, as measured by Trypan blue exclusion (Sigma) were stimulated with 2 µg/mL of VF-Tcra or concanavalin A (ConA) (Sigma) in 24-well plates in triplicate; culture medium was used in all experiments as a negative control. The plates were centrifuged, and the supernatant was analyzed for IL-6 (20, 48, and 72 h post-infection), IL-2 (20 h post-infection), IFNγ, IL-4, and IL-10 (24, 48, and 72 h post-infection). The results are expressed as the mean and standard error (SE) for each cytokine (in pg/mL) in three independent experiments. Readings were taken at 450 nm using a microplate spectrophotometer (Multiskan MCC, Lab Systems and Flow Lab, Finland). The level of nitrite production in the air pouch 7 and 4 weeks after infection was determined by the Griess reaction with sodium nitrite as a standard, as previously described (detection limit: 1.56 µM), and was used as an indicator of NO production. For this reaction, 50 µl of supernatant combined with 50 µl of a freshly mixed solution of *N*-[1-naphthyl]-ethylenediamine (1 mg/mL, sulfanilamide (10 mg/mL), and 5% (v/v) phosphoric acid in distilled water was incubated for 10 min in the dark at room temperature. The absorbance was measured at 540 nm.

2.8. Statistical analysis

The significance of the results was evaluated by ANOVA with Tukey's posttest. *P* values were considered significant when $p \leqslant 0.05$.

3. Results

3.1. Air pouch and intraperitoneal mouse model

To establish a new experimental model to study *T. crassiceps* infection in mice, animals were sacrificed 30 days after infection with 50 *T. crassiceps* cysticerci inside an air pouch previously raised on the dorsum of anesthetized BALB/c mice (Fig. 1A and B). This model was compared to the previously established intraperitoneal infection model (Fig. 1C). More than 90% of budding *T. crassiceps* cysticerci were recovered. Petri dishes containing cysticerci recovered from air pouch (Fig. 1D and E) or peritoneal washes (F) showed that there was greater recovery of cysticerci with the traditional intraperitoneal model. The number of recovered parasites was significantly greater in intraperitoneal washes (Fig. 1G) than in air pouch washes (Fig. 1G) ($p < 0.05$). Although inoculation with PBS did not induce leukocyte accumulation, inoculation with *T. crassiceps* led to a significant accumulation of leukocytes (51-fold increase over endotoxin-free Iscove's medium) in the air pouch model (compared with a 23-fold increase in intraperitoneal model normalized to parasite numbers, data not shown; $p \leqslant 0.05$).

3.2. Antigenic characterization of cysticerci

Additionally, to compare the two infection models, VF-Tcra antigens isolated from individual mice in the air pouch infection group (Fig. 2A, lanes 1–4) and intraperitoneal infection group (Fig. 2A, lanes 5–8) were separated by electrophoresis in an acrylamide gel (SDS–PAGE). The antigen staining pattern was similar for both air pouch and intraperitoneal antigens. In contrast, immunoblot staining performed with immune serum (developed with anti-IgG antibody) collected 60 days after immunization with VF-Tcra antigens in the air pouch infection model was markedly different than staining with serum of animals immunized with antigens derived from the intraperitoneal infection model (Fig. 2B). Although an 8 kDa antigen in the individually analyzed animals was

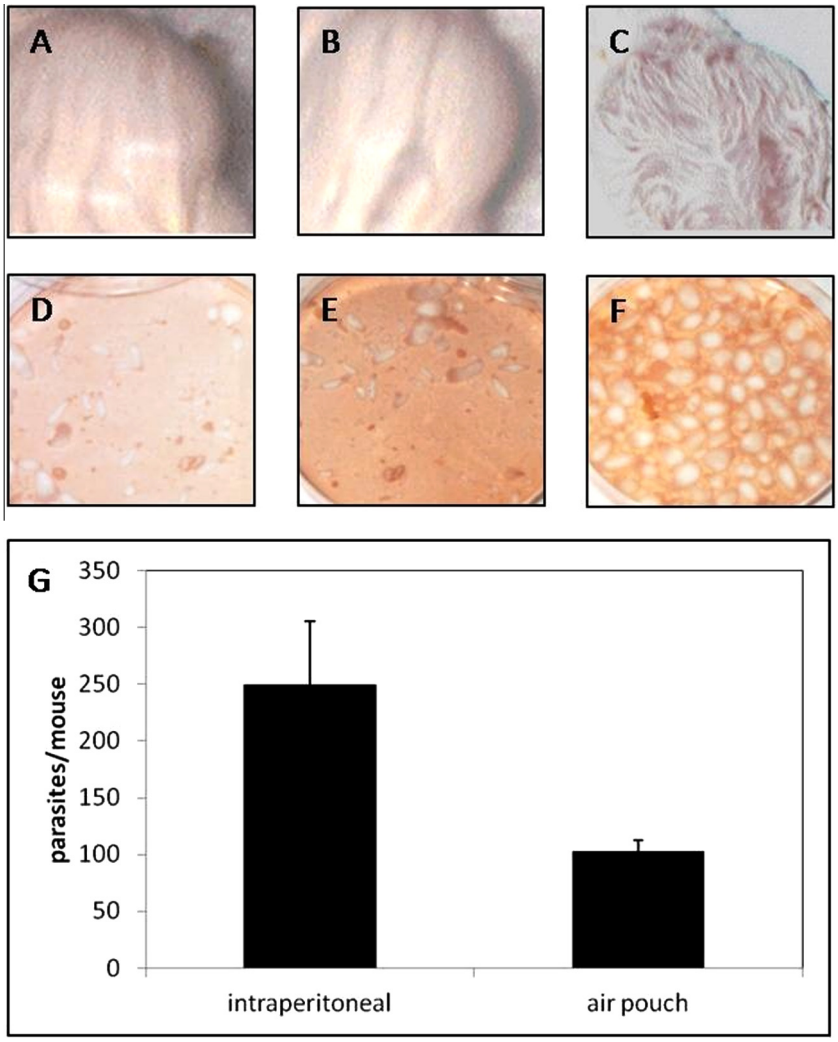

Fig. 1. Mice were infected with 50 *T. crassiceps* cysticerci either inside an air pouch previously raised on the dorsum of anesthetized BALB/c mice (A and B) or intraperitoneally (C). Petri dishes containing cysticerci recovered 30 days after infection from pouch (D and E) or peritoneal washes (F). The recovered parasites were quantified in both intraperitoneal (mice 1–4) and air pouch (mice 5–8) washes (G). The values are displayed as the means ± SD of three independent experiments.

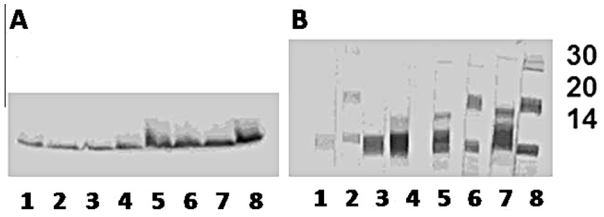

Fig. 2. Electrophoretic separation (SDS–PAGE) and analysis of VF-Tcra antigens isolated of individual mice in the air pouch infection model (lane 1–4) or intraperitoneal infection model (lane 5–8). The proteins were separated in an acrylamide gel and stained with Coomassie blue (A). Immunoblotting of VF-Tcra antigens recognized by IgG antibodies from the serum of mice immunized with air pouch VF-Tcra (1–4) or the VF-Tcra of intraperitoneally infected (5–8) mice 60 days post-immunization (B). Molecular masses (kDa) are shown to the right.

recognized by immune sera from both groups of mice, a greater number of antigens in the range of 14–30 kDa was observed after react with serum derived from animals immunized with VF-Tcra antigens derived from intraperitoneal infection (Fig. 2B, lines 5–8) than after staining with serum derived from animals immunized with air pouch VF-Tcra (Fig. 2B, lines 1–4). This result demonstrates that individual differences between immunoblot staining patterns appear to be more pronounced when animals were immunized with antigens derived from intraperitoneal infection.

3.3. Kinetics of humoral immunity

To follow the kinetics of the humoral immune response elicited by immunization with VF-Tcra antigens from cysts isolated from the air pouch infection group (antigen 1) or intraperitoneal infection group (antigen 2), animals were bled for serum collection at 15, 30, 45 and 60 days post-immunization. An ELISA developed with an anti-IgG antibody was performed to compare both antigens. No significant differences were observed in the absorbance of serum diluted 1/20 at any time point (Fig. 3), indicating that the two antigens were similar in eliciting humoral immunity.

3.4. Quantification of leukocytes in the air pouch and in intraperitoneal T. crassiceps infection models

To quantify and characterize the early leukocyte cell types recruited after *T. crassiceps* infection, nonbudding small cysticerci were injected into air pouches or peritoneal cavities, and cells were recovered from these washes 1 or 4 weeks after infection (Table 1).

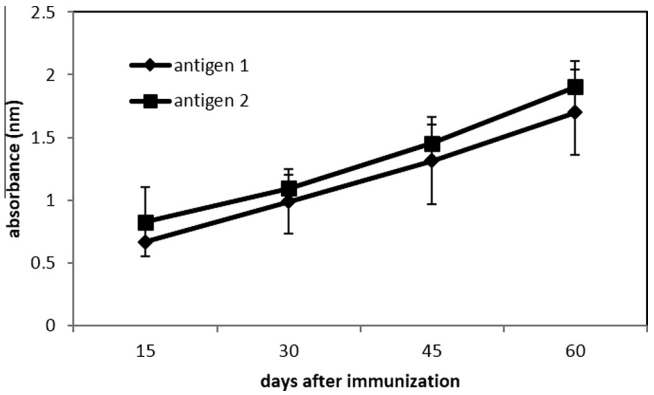

Fig. 3. Humoral immunity kinetics. Enzyme-linked immunosorbent assays for IgG antibodies in serum from 10 experimental immunized mice were performed 15, 30, 45 and 60 days after immunization by intramuscular injection of VF-Tcra isolated from cysts in the air pouch model (antigen 1) or of the VF-Tcra isolated from cysts in the intraperitoneal model (antigen 2). Saponin was used as an adjuvant for both antigens. Normal mouse serum was used as a control.

A greater number of cells, specifically macrophages and neutrophils, was recovered from the air pouch washes than from intraperitoneal washes at both 1 and 4 weeks after infection ($p < 0.05$). Eosinophils and basophils were also quantified.

3.5. IFNγ, IL-2, -4, -6, -10 and nitric oxide

Interleukin levels and nitric oxide production were also measured in both the air pouch and intraperitoneal infection models. For cytokine analysis, cells from air pouch or peritoneal exudates were harvested 1 and 4 weeks post-infection and stimulated with cyst antigens for different lengths of time (20, 48 and/or 72 h, depending on cytokine analyzed). IFNγ (Fig. 4A) and IL-2 (Fig. 4B) were produced in similar levels independent of the infection model. There was also no significant difference between IFNγ and IL-2 production when the cells were harvested at either 1 or 4 weeks post-infection (Fig. 4). IL-4 production was higher in cells harvested from the air pouches than in cells from the peritoneum (Fig. 4C) at 4 weeks post-infection when cells were stimulated for 48 h ($p < 0.05$) and at 1 and 4 weeks post-infection when cells were stimulated for 72 h ($p < 0.01$). IL-6 production (Fig. 4D) was also higher in cells from air pouches than in cells from the peritoneum when cells were harvested 1 week post-infection and stimulated for 48 or 72 h with antigen ($p < 0.05$) and in cells harvested 4 weeks post-infection with 48 ($p < 0.01$) or 72 h ($p < 0.05$) of stimulation with antigen. After 48 h of antigen stimulation, the IL-10 production (Fig. 4E) was higher for cells harvested from air pouches only when cells were harvested 4 weeks after infection ($p < 0.05$). On the other hand, when the cells were stimulated by antigen for 72 h, IL-10 production was higher in the air pouch model than in the intraperitoneal model when the cells were harvested 1 ($p < 0.01$) or 4 weeks after infection ($p < 0.05$).

Table 1
The percentages of macrophages, eosinophils, basophils, and neutrophils in air pouch exudates and peritoneal exudates at 1 and 4 weeks post-infection with nonbudding cysticerci. The results represent the means of five animals for each group.

Cell types (%)	1 Week after infection		4 Weeks after infection	
	Peritoneal	Air pouch	Peritoneal	Air pouch
Macrophages	37	63	68	79
Eosinophils	1	4	4	8
Basophils	5	8	6	7
Neutrophils	42	72	58	105

The nitric oxide (NO) concentration in the fluid of the air pouches and peritoneum was also measured. For this purpose, exudate was collected 1 and 4 weeks post-infection, and uninfected mice injected with saline were used as controls. Infection induced NO production 1 and 4 weeks post-infection ($p < 0.01$). However, significant differences in NO production between the air pouch and intraperitoneal infection model were observed only at 4 weeks after infection ($p < 0.01$) (Fig. 5).

The morphology of the cells in the preparation was also similar between the two models. However, more studies using specific markers for cellular differentiation are necessary.

4. Discussion

An excellent model system for the study of cysticercosis consists of the intraperitoneal infection of BALB/c mice with cysticerci of *T. crassiceps*. Rodents are the natural intermediate host for this parasite, whereas the ultimate hosts are canines. The cysts multiply in the peritoneal cavity of the mouse by budding asexually in a seemingly uncontrolled manner, causing the BALB/c mice to be extremely susceptible to infection. Analysis of the ensuing immune response during infection has shown that it is a mixed Th1/Th2 phenotype (Gottstein et al., 1987; Peón et al., 2013) that is ineffective in controlling parasite growth. Over the last 50 years, experimental results using larval and adult stages of *T. crassiceps* have yielded much information on the morphology, infectivity, proliferation dynamics, host immune response, endocrinological responses and vaccine design, all of which have contributed to our knowledge of cestode biology (Willms and Zurabian, 2010).

In this work, we present, for the first time, a new model to study *T. crassiceps* infection, in which air pouches previously generated in the dorsum of mice are infected with *T. crassiceps* cysts. The data presented here indicate that *T. crassiceps* induces inflammatory cell recruitment into the murine air pouch. Cell recruitment is a vital event in inflammation. The cell number and composition in the initial stages after stimuli greatly influence future responses and the development of adaptive immune responses. Initial cell recruitment is also important in leishmaniasis (Kaye, 1987). Neutrophil recruitment to the infection site is an essential step in controlling *T. crassiceps* infections because these cells are able to phagocytose parasites and produce free radicals (Faurschou and Borregaard, 2003). The data presented in this study indicate that *T. crassiceps* cysticerci induce increased macrophage recruitment into the murine air pouch.

Several studies have described the use of the mouse air pouch model as a very consistent and straightforward model to investigate the inflammatory response, creating an ideal environment for the collection and phenotypic analysis of cells migrating into the pouch space (Forget et al., 2005; Matte and Olivier 2002; Tessier et al., 1998). The murine subcutaneous air pouch has also been used as a model of leukocyte extravasation in response to inflammatory agents. This model allows the collection and phenotypic analysis of cells migrating into the pouch space. In this study, we took advantage of this model to evaluate the exponential growth of *T. crassiceps* and to study the aspects of the immune response at 1 and 4 weeks after infection.

In the search for a simple model for the study of the immunological factors of experimental murine cysticercosis, we have been able to isolate nonbudding cysts of *T. crassiceps* from the air pouch model 30 days after infection. The most remarkable aspect of the finding of cyst regeneration after 30 days is the implication that isolated cells retain the capacity to form fully differentiated cysts (Toledo et al., 1997).

The cysticerci of *T. crassiceps* recovered from the air pouch are able to multiply in the peritoneal cavity of BALB/c mice. The

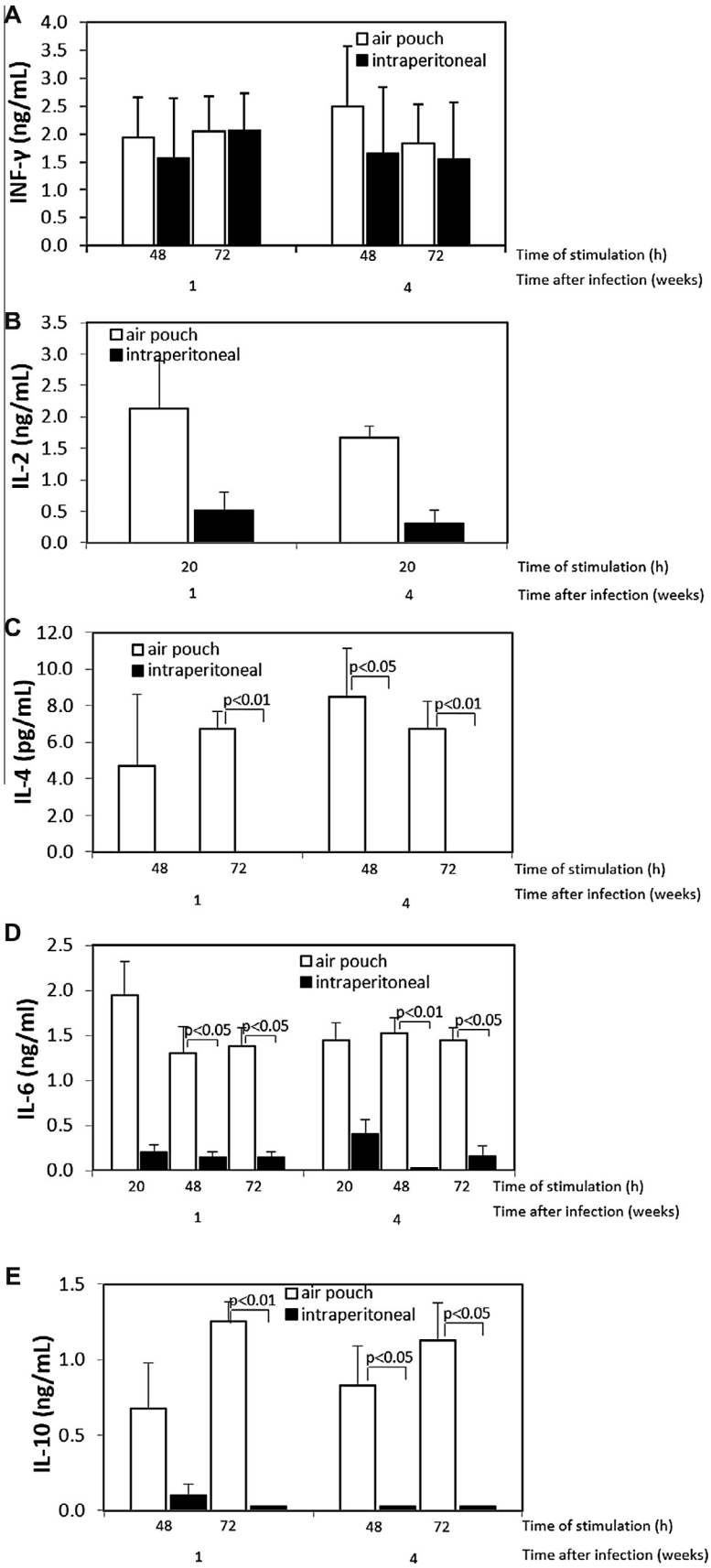

Fig. 4. Production of interleukins in *T. crassiceps* infection models. The levels of IFNγ (A), IL-2 (B), IL-4 (C), IL-6 (D) and IL-10 (E) in the air pouch model and intraperitoneal cavity were determined after 20, 48 or 72 h of antigen stimulation in cells harvested 1 or 4 weeks after infection. Data are presented as the means ± SE of 4 animals. The *p* values are indicated in the figure.

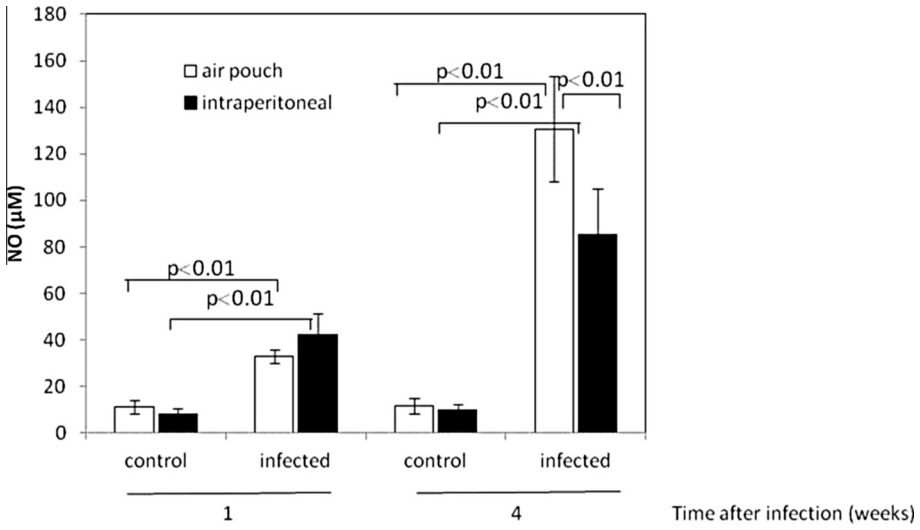

Fig. 5. Nitric oxide (NO) concentration in the fluid from the air pouches and peritoneum of BALB/c mice. The fluid was collected 1 or 4 weeks after infection with 50 *T. crassiceps* cysticerci. A normal mouse injected with saline was used as a control. The values are presented as the means ± SD of four samples. The *p* values are given in the figure.

regeneration of budding cysts after injection of 50 nonbudding cysts into the mouse peritoneal cavity suggests that cell proliferation is also notable. Budding cysts from both infection models, air pouch and peritoneum, were isolated, and the soluble extracts were analyzed by SDS–PAGE and immunoblot. Additionally, these soluble extracts were used as antigens to immunize mice, and the humoral immunity was evaluated by ELISA.

In the traditional experimental model of murine cysticercosis, the infection of inbred mice with *T. crassiceps* induces a strong Th2-like response, similar to that observed after an infection with helminthes, such as *Nippostrongylus brasiliensis* or *Trichuris muris* (Finkelman et al., 1997). Although it is widely accepted that a Th2-like response mediates protective immunity against most helminthes (Urban Jr. et al., 1995), its role in mediating protection against murine cysticercosis is unclear (Toenjes et al., 1999).

Previous studies have found that although *T. crassiceps*-infected mice develop a Th1-like response during the early phase of infection (Morales-Montor et al., 2008), they eventually develop a Th2 response associated with an increase in parasite load (Terrazas et al., 1998). Furthermore, one study found that the administration of IFNγ-neutralizing Abs to *T. crassiceps*-infected mice during the early phase of infection rendered them more susceptible to cysticercosis (Terrazas et al., 1999). These findings suggest that whereas a Th2-type response may be involved in mediating susceptibility, a Th1-type response may play a role in the development of protective immunity against cysticercosis.

Recent evidence indicates that in experimentally infected mice there is a Th1-type immune response concomitant with limited parasite growth at early stages of infection. Later, the immune response progressively polarizes toward a Th2-type as the number of parasites significantly increases. At this late stage of infection, there is a reduced cellular immune response to *T. crassiceps*-soluble antigens, accompanied by an elevated production of IL-10, IL-6, and IL-4 as well as anticysticercal antibodies of the IgG1 and IgG2b isotypes (Terrazas et al., 1999). Interestingly, we observed a smaller number of parasites recovered from the air pouch exudate than for the peritoneal exudate. This reduced parasite proliferation was not correlated to IFNγ or IL-2 production because the production of these cytokines was similar in both models. In contrast, the IL-4, IL-6 and IL-10 levels were increased in cells from the air pouch compared with cells from the peritoneum. More studies are necessary to determine the percentage of living or dead cysts in this new model proposed for studying the immune response.

It is now generally accepted that NO and related nitrogen oxides produced by activated macrophages have an important role in the killing of different pathogens. NO can be induced by cytokines such as IFNγ (Ding et al., 1988). From an evolutionary point of view, NO formation may have originated as a first-line of defense for metazoan cells against intracellular pathogens. As described, NO is an important mediator of homeostasis, and changes in its generation or actions can contribute to host defense or damage under pathological conditions. Insights into the effects of NO have been important not only to our understanding of immune responses but also to the development of new tools for research and treatment of various diseases (Pavanelli et al., 2010). Although NO is produced in response to infection in both the air pouch and intraperitoneal infection models, it seems that it is closely related to infection control because the production was higher in the air pouch model 4 weeks after infection.

5. Conclusion

Our air pouch model developed in this study is a feasible tool for evaluating the immunological aspects of *T. crassiceps* infection and is alternative to the traditional intraperitoneal model of infection. It confines the development of larvae to the site of infection, as does the traditional model, while enabling the observation of developing cysts for up to 30 days. Further investigations are necessary to explore the biological factors involved in the host–parasite interaction, as well as the kinetics of humoral and cellular immune responses. Our results clearly demonstrate that *T. crassiceps* infection rapidly mobilizes leukocytes (predominantly neutrophils) from the peripheral blood into subcutaneous tissues; the recruitment of neutrophils is accompanied by the accumulation of Th1 and Th2 type cytokines and nitric oxide production at the site of administration. The massive infiltration of polymorphonuclear leukocytes (PMNs) into the air pouch soon after infection suggests that PMNs could participate in reducing the parasite load and controlling the spread of *T. crassiceps* infection. However, direct evidence for the participation of PMNs in host defense against *T. crassiceps* infection is lacking, as are other aspects of this model, but this model allows one to perform investigations like those already described in the literature.

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