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Evidence for eosinophil recruitment, leukotriene B_4 production and mast cell hyperplasia following *Toxocara canis* infection in rats

D. Carlos[1], E.R. Machado[1], L. De Paula[1], A. Sá-Nunes[4], C.A. Sorgi[1], M.C. Jamur[2], C. Oliver[2], W.T. Lima[3] and L.H. Faccioli[1]

[1]Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil
[2]Departamento de Biologia Molecular e Celular, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil
[3]Departamento de Farmacologia, [4]Departamento de Imunologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, SP, Brasil

Abstract

It is well known that eosinophilia is a key pathogenetic component of toxocariasis. The objective of the present study was to determine if there is an association between peritoneal and blood eosinophil influx, mast cell hyperplasia and leukotriene B_4 (LTB_4) production after *Toxocara canis* infection. Oral inoculation of 56-day-old Wistar rats (N = 5-7 per group) with 1000 embryonated eggs containing third-stage (L3) *T. canis* larvae led to a robust accumulation of total leukocytes in blood beginning on day 3 and peaking on day 18, mainly characterized by eosinophils and accompanied by higher serum LTB_4 levels. At that time, we also noted increased eosinophil numbers in the peritoneal cavity. In addition, we observed increased peritoneal mast cell number in the peritoneal cavity, which correlated with the time course of eosinophilia during toxocariasis. We also demonstrated that mast cell hyperplasia in the intestines and lungs began soon after the *T. canis* larvae migrated to these compartments, reaching maximal levels on day 24, which correlated with the complete elimination of the parasite. Therefore, mast cells appear to be involved in peritoneal and blood eosinophil infiltration through an LTB_4-dependent mechanism following *T. canis* infection in rats. Our data also demonstrate a tight association between larval migratory stages and intestinal and pulmonary mast cell hyperplasia in the toxocariasis model.

Key words: Mast cells; Leukotriene B_4; Eosinophils; Eosinophil peroxidase; *Toxocara canis*; Rat

Introduction

Intestinal nematodes cause some of the most prevalent parasitic infections in humans. *Toxocara canis* is an intestinal parasite of dogs, and is the etiologic agent of visceral larva migrans syndrome (VLMS). In non-compatible hosts such as rodents and humans, VLMS results from the ingestion of *T. canis*-embryonated eggs that eclode in the small intestine. After ingestion, the larvae migrate to other tissues, inducing inflammation in reaction to the excretory/secretory products produced by the larvae (1). In general, helminthic parasites infect vertebrate hosts and typically promote a Th2-type inflammatory response that is marked by eosinophilia, high levels of immunoglobulin E (IgE) and mast cell hyperplasia (2). Studies from our laboratory have shown an accumulation of eosinophils and increased total serum IgE during the course of *T. canis* infection in guinea pigs (3) and Wistar rats (4). Despite the presence of elevated levels of circulating IgE during eosinophilic inflammation (5,6) and the capacity of mast cell secretory products to promote the traffic of leukocytes to the challenge sites (7,8), the relative importance of the IgE-mast cell system for the maintenance of the chronic inflammatory response to helminthic infections is controversial.

Mast cells are effector cells that are prominent at the interfaces between the immune system and the external environment, such as the skin and the gastrointestinal and respiratory tracts. When IgE bound to its high-affinity receptor, FcεRI, is cross-linked with multivalent antigens, the activation of mast cells is initiated, resulting in the release of

Correspondence: L.H. Faccioli, Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, FCFRP, USP, Av. do Café, s/n, 14040-903 Ribeirão Preto, SP, Brasil. Fax: +55-16-3602-4725. E-mail: faccioli@fcfrp.usp.br

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a variety of preformed mediators, including β-hexosaminidase and tumor necrosis factor-alpha (TNF-α); in addition, *de novo* synthesis of proinflammatory mediators such as prostaglandin, leukotrienes (LTs) and cytokines occurs (9,10). LTs are generated by the metabolism of arachidonic acid through the 5-lipoxygenase pathway (11). 5-LO expression is generally restricted to myeloid cells, in particular neutrophils, eosinophils, monocytes, macrophages, and mast cells (12,13). Leukotriene B_4 (LTB_4) was originally discovered as an arachidonate metabolite that stimulates neutrophils (14), and has been described to be a chemoattractant for eosinophils (15,16). It has been shown that IgE-driven inflammation may lead to eosinophil accumulation through a mechanism dependent on eotaxin, platelet-activating factor and LTs (17). Moreover, it has been shown that eosinophil migration to the rat peritoneal cavity is mediated by mast cells, which release chemotactic factors such as LTB_4 after stimulation (18).

A previous study showed clearly that the eosinophil response induced by *T. canis* infection was significantly lower in mast cell-deficient W/W^v mice when compared to normal littermates, suggesting that mast cells play a pivotal role in blood eosinophilia during infection with *T. canis* (19). The present study was undertaken to evaluate the kinetics of peripheral blood and tissue eosinophil recruitment, eosinophil peroxidase (EPO) activity, and the levels of LTB_4 in the serum of *T. canis*-infected rats. We also determined if changes in peritoneal mast cell number correlated with eosinophilia and increased LTB_4 release. In addition, parasitological parameters, such as the number of *T. canis* larvae in the lungs and intestine as well as the inflammatory consequences of larval migration in intestinal and lung mast cell hyperplasia, were also assessed following infection of Wistar rats with *T. canis*.

Material and Methods

Animals

Female Wistar rats (200-250 g) were obtained from the animal facility of the School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Brazil. They were maintained under standard laboratory conditions, and all experiments were approved and conducted in accordance with the guidelines of the Animal Care Committee of the University of São Paulo.

Infection of animals

T. canis eggs were obtained according to the method of Olson and Schulz (20), modified by Faccioli et al. (3). Briefly, female worms were recovered from young dogs and the eggs were removed from the uteri, washed and allowed to develop to the infective stage (L3) in shallow dishes containing 0.5% formalin at 37°C. Before use, the eggs were thoroughly washed with saline. Infective doses of 1000 embryonated eggs were prepared in 1 mL saline. Rats were infected with the embryonated eggs by gastric intubation using a metal cannula. Controls received 1 mL saline without eggs.

Collection of peritoneal cavity fluid, blood and serum

At 3, 6, 18, or 24 days after infection, the animals were euthanized with a lethal dose of 2.5% tribromoethanol *ip* (Acros Organics, USA), and blood samples were obtained by cardiac puncture. Peritoneal cells were obtained by injecting rats with 5 mL PBS containing 0.5% sodium citrate. The peritoneal wash was gently collected with a Pasteur pipette after laparotomy and placed in plastic tubes. Total blood or peritoneal cavity fluid cell counts were performed immediately using a Neubauer chamber. Differential counts were obtained using Rosenfeld-stained cytospin preparations. After blood coagulation, sera were collected and stored at -20°C.

Detection of LTB_4 in the sera

Quantification of serum LTB_4 was performed by enzyme immunoassay (Cayman Chemical, USA) according to the method of Pradelles et al. (21). Supernatant dilutions were incubated with conjugated eicosanoid-acetylcholinesterase and with antiserum in 96-well plates precoated with anti-rabbit immunoglobulin G antibodies. After overnight incubation at 4°C, plates were washed and enzyme substrate (Ellman's reagent) was added for 60 to 120 min at 25°C. Sample absorbance was determined at 412 nm in a microplate reader, and concentrations of eicosanoids were calculated based on a standard curve.

Histological examination

For histological examination of the intestine (ileum) and lungs, fragments of tissue were fixed in buffered formalin, dehydrated, and embedded in paraffin. Tissues were sectioned and stained with 0.1% Toluidine blue, pH 2.8, for 20 min. For staining of mucosal mast cells, the ileum was fixed in Carnoy's fixative and stained with 1% Alcian blue at pH 2.5 for 30 min. All sections were analyzed, and mast cell numbers were determined using an Olympus BX50 microscope equipped with a Nikon DXM1200 digital camera and the Image-Pro Plus software (Media Cybernetics, USA). A counting field was superimposed on the image obtained with a 40X objective. Each counting point corresponds to a field area of 5000 μm^2 on the section.

Eosinophil peroxidase activity

EPO activity in the peritoneal cavity fluid was determined with a colorimetric assay as described by Strath and colleagues (22) with slight modifications as follows. In brief, the cell suspensions were recovered and centrifuged, and the cells were exposed to Tris-NH_4Cl buffer (0.16 M NH_4Cl and 0.17 M Tris-HCl; final solution was 9 parts NH_4Cl stock solution and 1 part Tris-solution, pH 7.2) to lyse the erythrocytes. The cells were then washed once with PBS, and the cell number was adjusted to 10^6 cells/mL. A total of 100 µL was transferred to a 96-well microplate in duplicate. The

microplates were then centrifuged at 200 *g* at 4°C for 10 min. The supernatants were carefully removed, 100 µL substrate solution (2.4 mM o-phenylenediamine dihydrochloride in 50 mM Tris-HCl, pH 8.0, with 6.6 mM H_2O_2) was added to each well, and the plates were incubated at room temperature for 15 min. This substrate is specific for EPO and does not recognize myeloperoxidase (23). The reaction was stopped by addition of 50 µL 4 M H_2SO_4, and the absorbance of the samples was determined at 490 nm.

Recovery of larvae

Rats were orally infected with 1000 embryonated *T. canis* eggs. On days 1, 3, 6, 18, and 24 after inoculation, 3 rats were euthanized, and larvae were recovered from the lungs and small intestine by digesting the tissue in 0.5% HCl for 24 h at 37°C. The sedimental liquid was poured into a tube and centrifuged for 2 min at 400 *g*. After centrifugation, 2 mL of the sediment was collected and mixed thoroughly, and 100 µL of the samples was observed with a light microscope to count the number of larvae (24).

Statistical analyses

Each experiment was performed twice. Data are reported as means ± SEM. Statistical differences were analyzed by the Student *t*-test and the level of significance was set at $P < 0.05$.

Results

Mast cell and eosinophil accumulation after *T. canis* infection

During the course of *T. canis* infection, an intense inflammatory reaction characterized by an increase in the total number of leukocytes in the blood and peritoneal cavity was observed (Figure 1A and B). Also, we found increased numbers of blood eosinophils in *T. canis*-infected rats from day 3 to day 18, with decreasing numbers thereafter (Figure 1C). A massive infiltration of eosinophils into the peritoneal cavity was first noted at 6 days and peaked within 18 days of infection. This correlated with higher EPO activity in the peritoneal cavity (Figure 2A and B). In infected rats, the peritoneal mast cell number was significantly higher than in control rats from day 3 to day 24 after infection (Figure 2C). In fact, eosinophil recruitment into the peritoneal cavity occurred in parallel to increased peritoneal mast cell accumulation in this compartment (Figure 2A and C). These data show that eosinophil extravasation from the blood into the peritoneal cavity is related to alterations in mast cell numbers during toxocariasis.

Increased LTB_4 levels in serum after *T. canis* infection

The production of LTB_4 was assessed in the serum of *T. canis*-infected rats and -uninfected rats that received only saline (control). Levels of LTB_4 in the serum from *T. canis*-infected rats were significantly higher on the 3rd, 18th, and 24th days after infection compared to control rats. Although not expected, LTB_4 values for controls almost tripled at day 24 (Figure 3). These results indicate that release of the LTB_4 chemotactic factor correlates with mast cell accumulation and appears to contribute, at least in part, to eosinophil recruitment following *T. canis* infection.

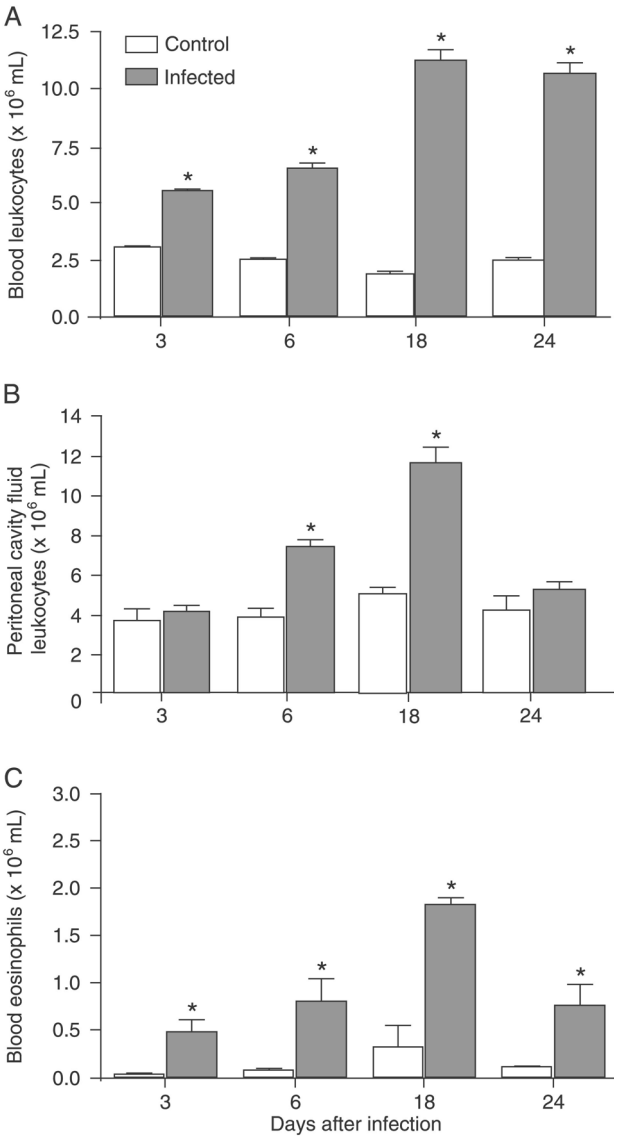

Figure 1. Kinetics of inflammatory cell increase following *Toxocara canis* infection. The numbers of blood leukocytes (A), peritoneal cavity fluid leukocytes (B) and blood eosinophils (C) were assessed. Samples were collected 3, 6, 18, and 24 days after *po* inoculation of 1000 infective *T. canis* eggs (filled bars). Control uninfected rats received only 1 mL saline *po* (open bars). Data are reported as means ± SEM of two independent experiments (N = 8). *P < 0.05 *vs* control group (Student *t*-test).

Mast cell hyperplasia in the small intestine and lungs following *T. canis* infection

The major site of mast cell accumulation during infection was the small intestine, where mast cell counts per field increased 3-fold compared to uninfected rats on the 1st day after infection. Quantification of mast cell numbers established that maximal accumulation occurred on the 24th day after infection, when a 915-fold increase over uninfected controls was observed (Table 1 and Figure 4B-F). A slight but significant increase of the mast cell population was observed on the 1st and 3rd days after infection in the lung. On the 6th and 18th days, there was a marked increase in the number of mast cells in the lungs of infected rats. A maximum was reached on the 24th day, when mast cell counts per field increased 5-fold compared to uninfected controls (Table 2 and Figure 4H-L).

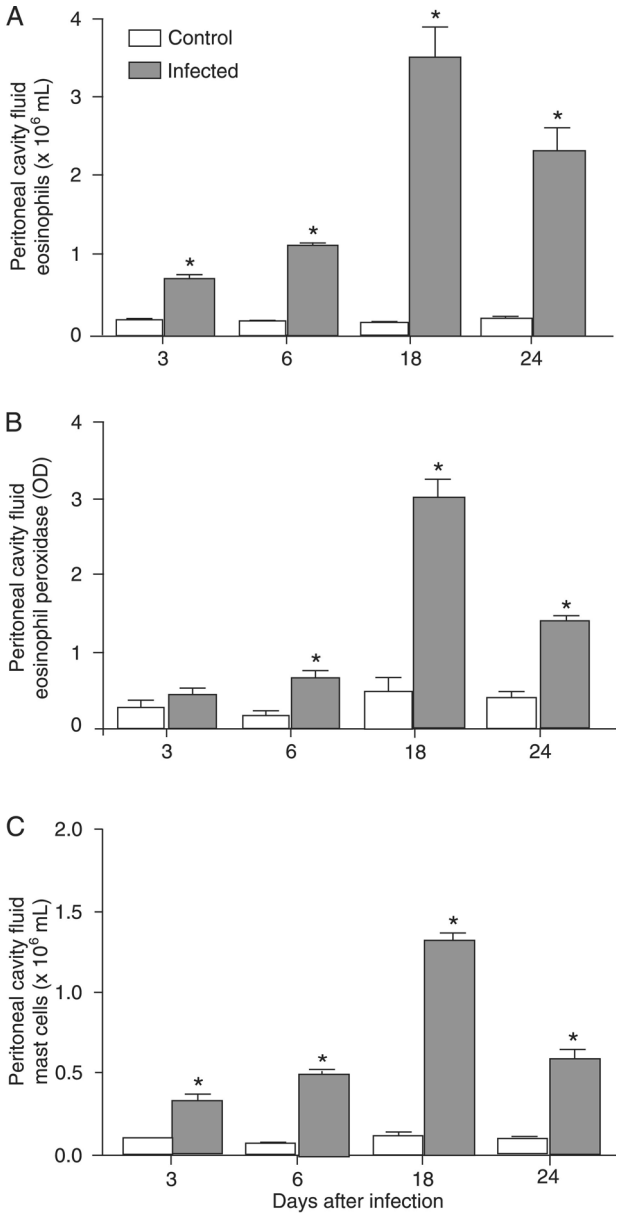

Figure 2. Kinetics of inflammatory cell increase following *Toxocara canis* infection. The number of peritoneal cavity fluid eosinophils (A), peritoneal cavity fluid eosinophil peroxidase (B) and peritoneal cavity fluid mast cells (C) were assessed. Samples were collected 3, 6, 18, and 24 days after *po* inoculation of 1000 infective *T. canis* eggs (filled bars). Control uninfected rats received only 1 mL saline *po* (open bars). Data are reported as means ± SEM of two independent experiments (N = 8). *P < 0.05 *vs* control group (Student *t*-test).

Larval counts in the lungs and small intestines of rats infected with *T. canis*

In order to determine whether migration of the larvae

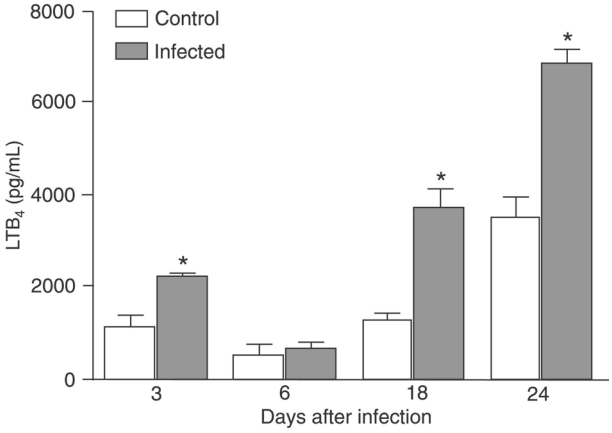

Figure 3. Kinetics of leukotriene B4 (LTB_4) release into the blood induced by *Toxocara canis* infection. Enzyme immunoassay of serum leukotriene concentration was performed 3, 6, 18, and 24 days after *po* inoculation of 1000 *T. canis* eggs (filled bars). Control uninfected rats received only 1 mL saline *po* (open bars). Data are reported as means ± SEM of at least 4 animals. *P < 0.05 *vs* control group (Student *t*-test).

Table 1. Differences in mast cell numbers in the intestinal mucosa from uninfected and *Toxocara canis*-infected rats.

Days after infection	Mast cell numbers	
	Uninfected	Infected
1	34.7 ± 4.2	93.3 ± 0.6*
3	47.3 ± 9.3	175.3 ± 12.5*
6	70.7 ± 2.3	333.7 ± 20.7*
18	76.3 ± 5.5	532.7 ± 18.6*
24	76.3 ± 5.5	775.0 ± 33.0*

Data are reported as means ± SEM for 5-7 rats in each group. The mast cell count was performed 1, 3, 6, 18, and 24 days after infection. *P < 0.05 compared to the uninfected group (Student *t*-test).

was associated with mast cell hyperplasia, we quantified the number of larvae recovered from the small intestine and lungs 1, 3, 6, 18, and 24 days after inoculation. The greatest number of larvae was seen 1 day after infection in the small intestine. After the first day, the number of larvae in this compartment was drastically decreased. In the lungs, the number of larvae recovered increased only slightly on days 3, 6, and 18 after infection (Figure 5).

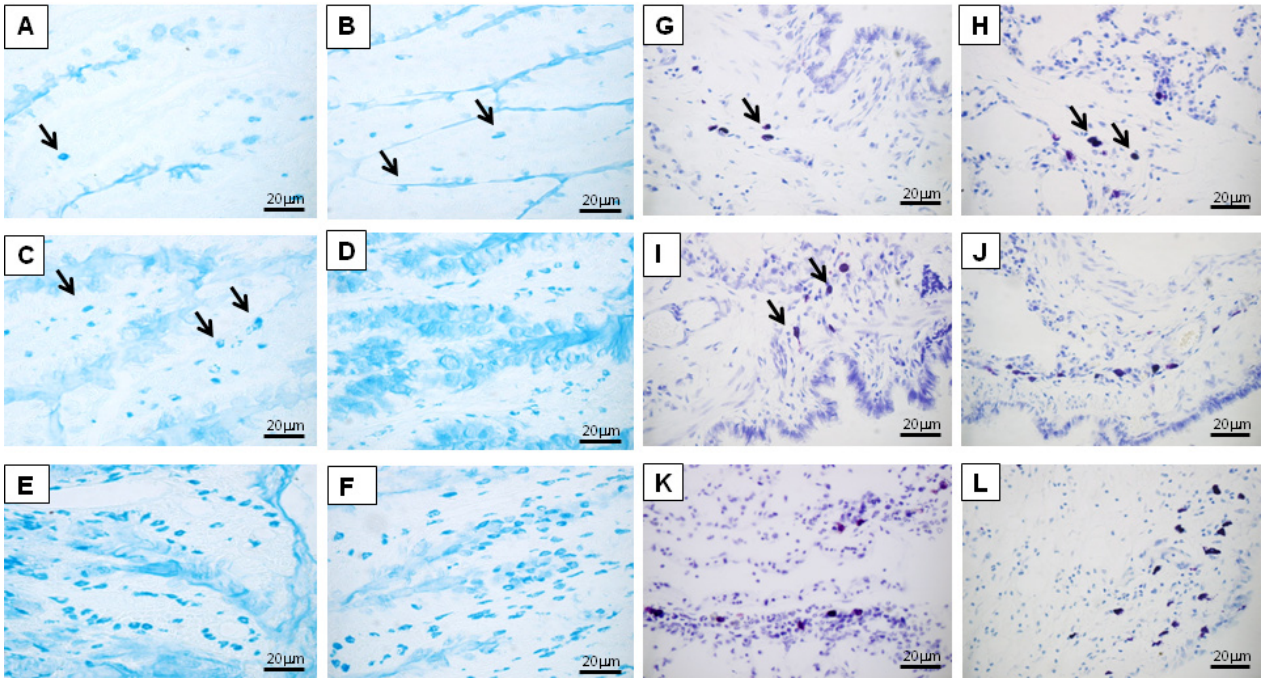

Figure 4. Light microscopy analysis of mast cell hyperplasia in the intestine or lung 1 (B, H), 3 (C, I), 6 (D, J), 18 (E, K), and 24 (F, L) days after infection, respectively. The analysis was conducted on Alcian blue- or Toluidine blue-stained tissue sections from the ileum and lung of *Toxocara canis*-infected rats or uninfected rats (A and G). Mast cells were readily detected in the intestinal mucosa and pulmonary parenchyma after 1 day and progressively increased until day 24. Mast cells are indicated by arrows.

Table 2. Differences in mast cell numbers in the pulmonary parenchyma from uninfected and *Toxocara canis*-infected rats.

Days after infection	Mast cell numbers	
	Uninfected	Infected
1	46.7 ± 8.1	61.3 ± 2.3*
3	36.0 ± 4.0	65.3 ± 5.0*
6	31.3 ± 2.3	114.7 ± 7.6*
18	32.0 ± 6.9	130.0 ± 19.1*
24	39.3 ± 6.2	217.3 ± 18.0*

Data are reported as means ± SEM for 5-7 rats in each group. The mast cell count was performed 1, 3, 6, 18, and 24 days after infection. *$P < 0.05$ compared to the uninfected group (Student *t*-test).

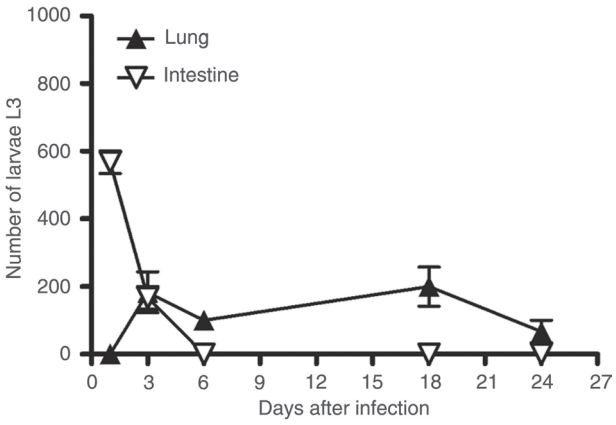

Figure 5. Kinetics of *Toxocara canis*-migrating larvae recovered from lung and intestine. Larvae were recovered from the lungs (triangles) and small intestines (inverted triangles) of rats inoculated with 1000 infective *T. canis* eggs. Larval number was counted in a pool of 4 animals killed at 1, 3, 6, 18, and 24 days after infection. Data are reported as means ± SEM.

Discussion

To gain insight into the mechanisms underlying increased eosinophilia during infection with *T. canis*, we compared some features of the inflammatory response in infected Wistar rats. Three major effects were observed: 1) increased peripheral blood and tissue eosinophil influx and EPO activity, 2) significant peritoneal mast cell accumulation and LTB_4 production in the serum following *T. canis* infection, and 3) mast cell hyperplasia in the lungs and intestinal tissues probably induced by the passage of larvae into these compartments.

Increased eosinophilia was observed in the blood and peritoneal cavities of rats infected with *T. canis*. The peritoneal eosinophil accumulation reached a peak on day 18 of infection and occurred in parallel to a marked increase in the number of mast cells in the peritoneal cavity. Consistent with these data, previous findings from our laboratory demonstrated elevated serum IgE levels on the 18th day after infection (4). We also evaluated EPO activity, a specific marker for eosinophils, and detected higher amounts of EPO in the peritoneal exudates of infected rats compared to uninfected rats. Our results also showed that mast cell accumulation in the peritoneal cavity correlates with blood eosinophil mobilization and extravasation to the peritoneal cavity during toxocariasis. In this context, a study by Nawa et al. (19) reported that production of eosinophils and their release from bone marrow into peripheral blood was impaired in mast cell-deficient W/W^v mice compared to their normal littermates after *T. canis* infection. For instance, the results of the present study agree with previous investigations that have shown that IgE-dependent mast cell-mediated mechanisms play a crucial role in eosinophilia and in the immunological control induced in rats by other nematodes, such as *Angiostrongylus cantonensis* (25), *Strongyloides venezuelensis* (26), *Nippostrongylus brasiliensis* (27), and *Trichinella spiralis* (28).

It is not clear why eosinophil infiltration persists in *T. canis*-infected mice after the decrease in the number of inciting larvae, but the involvement of various eosinophil chemotactic factors has been suggested (29). A recent report established a tight correlation between tissue lesions caused by larval migration and plasma cytokine production. Moreover, the authors mentioned eotaxin and RANTES as potential factors responsible for the marked eosinophilic response that is a hallmark of *T. canis* infection (30). In fact, our group described an increased concentration of eotaxin in lung homogenates of *T. canis*-infected mice, which also correlated with eosinophil recruitment to this organ (31). Although the *in vivo* factors responsible for eosinophil accumulation at inflammatory sites of parasitic infections are known to be produced by mast cells, only a single study has reported that cultured mast cells obtained from mice infected by *T. canis* released eosinophil chemotactic factors after stimulation with calcium ionophore A23187 or IgE-antigen. These investigators also suggested that these factors might be arachidonic acid metabolites (32). In agreement with the results of these studies, treatment of *T. canis*-infected rats with a specific LTB_4 receptor antagonist significantly reduced eosinophil chemotactic activity in bronchoalveolar fluid, suggesting that LTB_4 also contributes to the accumulation of eosinophils in the lungs (33). In another study, treatment of mice infected with the nematode *S. venezuelensis* with the leukotriene inhibitor MK886 significantly inhibited the recruitment of eosinophils in the bronchoalveolar space, peritoneal cavity and blood (34). To determine the *in vivo* relevance of mast cell-derived LTB_4 in eosinophilia, we examined the release of this mediator in rats following *T. canis* infection. We observed increased levels of LTB_4 in the serum of infected rats compared to control, with the maximal response occurring 24 days after infection. These data suggest that LTB_4 released by mast cells is a potent inflammatory mediator probably involved in eosinophil accumulation in blood and maybe in the peritoneal cavity in this experimental model. Another study also demonstrated a role for endogenous stem cell factor production in mediating eosinophil recruitment in an allergic pleurisy model in mice (35). Moreover, the authors also showed that the effects of stem cell factor were dependent on the release of LTB_4, which was most likely produced by mast cells.

The biological cycle of *T. canis* has common features with many nematodes; the larvae penetrate the small intestine, enter the circulation and are free to migrate to the liver, lungs and brain (36). Excretory/secretory products released by the larvae interact with molecules expressed on the immune system cells, control their activity and induce inflammation in several tissues (37). We observed that most of the *T. canis* larvae penetrated the intestinal mucosa within 24 h and migrated to the lungs after 72 h of the L3 larvae inoculation. In fact, these larvae are metabolically active and inflammatory mediators are secreted due to activation of the host's immune cells by larval products, resulting in an increased influx of leukocytes (38). In our study, mast cell hyperplasia was already greater in the small intestine after 1 day of infection, which coincided with the presence of the *T. canis* larvae in this organ. In addition, we also showed that in *T. canis*-infected rats, L3 larvae were most prevalent in the lungs on the 3rd day, although a second cycle of larval invasion could be detected on the 18th day of infection. Prominent mast cell recruitment is a common feature of infections by nematodes in most host species. The timing and degree of mast cell hyperplasia can vary between species and with the intensity of infection. Infection with the nematode *Trichinella spiralis* is associated with early and intense recruitment of intestinal mast cells (39). However, with other parasites, such as *Schistosoma mansoni*, chronic infection causes only a slight increase in mast cell numbers in the lungs and small intestine of rats (40).

The results of the present study suggest that mast cells play an essential role in peritoneal and blood eosinophil

infiltration and in the release of LTB_4 during infection with *T. canis* in rats. In parallel, mast cell numbers were histologically assessed in the lung and intestine after staining with Toluidine and Alcian blue, respectively. An important correlation was established between mast cell hyperplasia and the time course of larval migration into the small intestine and lungs. These data showed that infection with *T. canis* in rats can be considered an alternative model to delineate the crucial features of the immune response evoked by *T. canis.* In addition, this model may contribute to our understanding of the interplay between the activation and accumulation of mast cells, LTB_4 production and eosinophil recruitment.

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