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Persistent airway hyper-responsiveness and inflammation in *Toxocara canis*-infected BALB/c mice

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Summary

Background Infection with *Toxocara canis*, the roundworm of dogs, has been associated with asthmatic manifestations. Clinical symptoms such as wheezing, coughing and episodic airflow obstruction have been described for patients infected with this helminth.

Objective In order to characterize the effect of *T. canis* infection on the lungs, we monitored immune responses, pulmonary pathology and lung function over a period of 60 days in BALB/c mice.

Methods Infection was performed by a single oral administration of 1000 *T. canis* embryonated eggs. Airway responsiveness was measured in conscious, unrestrained mice at 7, 14, 30 and 60 days post-infection (p.i.).

Results Infection of mice resulted in airway hyper-responsiveness (AHR) that persisted up to 30 days p.i. Pulmonary inflammation as well as increased levels of IgE and eosinophils in bronchoalveolar lavage (BAL) persisted up to 60 days p.i. Cytokine analysis in BAL indicated increased levels of IL-5 at day 7 and 14 p.i., whereas the levels of IL-2, IFN-γ, IL-4 and IL-10 did not differ from those of uninfected controls. *Toxocara*-specific stimulation of spleen cells using recombinant TES-70 protein resulted in the induction of IL-5 at day 7 and 14 p.i. and IL-10 at day 14 p.i. Production of all other cytokines did not differ from that of uninfected controls. Evaluation of larval burden revealed that *T. canis* was still present in the lungs of infected mice at 60 days p.i.

Conclusion The presence of *Toxocara* larva in the lungs at 60 days p.i. following a single infection could explain the persistent pulmonary inflammation, airway hyper-reactivity, eosinophilia and increased IgE production observed in *T. canis*-infected BALB/c mice.

Keywords airways, cytokines, hyper-reactivity, inflammation, mice, *Toxocara canis*
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Introduction

Toxocara canis, the roundworm of dogs, is known to infect humans and cause the syndrome Visceral Larva Migrans (VLM). VLM is mainly a disease of young children and its symptoms include fever, respiratory distress and hepatosplenomegaly. Other symptoms include an eosinophilic pneumonitis (Loeffler's pneumonia) that bears a clinical resemblance to the pulmonary inflammatory responses observed in asthmatic patients [1–3]. Infection is acquired after ingestion of embryonated *T. canis* eggs that are present in soil contaminated with dog feces. After the eggs hatch the larvae penetrate the small intestine and migrate via blood and lymph vessels throughout the body [1]. In paratenic hosts such as humans and mice the worm does not develop to the adult stage but persists in the larval stage in tissues for many years [4]. *T.canis* is widely distributed throughout the world where dogs and also wild canids such as foxes and wolves are infected; however, the recorded seroprevalence varies between countries and even within countries. In Ireland, *Toxocara* seroprevalence in schoolchildren is high (31%) and rises with age [5]. In the state of Connecticut, USA, seroprevalence varies from 6.1% in New Haven to 27.9% in Bridgeport [6]. The common immunological features of infection with this nematode include eosinophilia and increased serum IgE levels [7].

Studies using murine models have shown that after a single infection, *T. canis* larvae invade the lung and other organs within 24–72 h, and eventually accumulate in the brain [8, 9]. During their migration, larvae cause tissue damage and provoke distinctive immune reactions. The cells infiltrating the lungs of infected mice include neutrophils, eosinophils and lymphocytes [8–10]. Previous studies on larval migration have shown that after 1 week of infection *T. canis* larvae are

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infrequently observed in the lungs [11]. However, it is possible that some larvae continue to transit the lungs at later stages of infection, which may explain the pulmonary inflammation that persists for so long in infected mice.

We have previously shown that infection of BALB/c mice with *T. canis* results at 7 days p.i. in pulmonary inflammation and increased total IgE concentration in serum [12]. To evaluate the effect of infection on the lungs in time, we monitored the airway reactivity of BALB/c mice infected with *T. canis* as well as histopathology of the lungs and immune responses.

Materials and methods

Parasites and experimental infection

T. canis adult worms were collected from the feces of naturally infected dogs, after routine deworming using an antihelmintic treatment. Eggs were collected from the uteri of female worms and were allowed to embryonate in 0.1 M H_2SO_4 in the dark at room temperature for 4–6 weeks. Embryonated eggs were stored in 0.1 M H_2SO_4 at 4 °C until use.

Specified pathogen-free female BALB/c mice (5–6 weeks old) were obtained from Harlan Netherlands BV (Horst, The Netherlands). Mice were housed in Macrolon III cages with food and water *ad libitum*. Infection was performed by oral administration of 1000 embryonated eggs per mouse in 0.3 mL sterile phosphate-buffered saline (PBS). The egg suspension was administered orally to the mice by using a syringe fitted with a blunt needle. Uninfected mice receiving only 0.3 mL PBS orally were also included in this study. Mice were separated in five groups (7, 14, 30, 60 days infected and uninfected controls) consisting of six animals each unless indicated otherwise. At autopsy animals were anaesthetized by intraperitoneal injection of a mixture of Ketalar (Park and Davis, Hoofddrop, The Netherlands) and Rompun (Bayer, Leverkusen, Germany). All experiments were performed according to international and institutional guidelines for animal care.

Determination of airway responsiveness

Airway responsiveness was measured in conscious, unrestrained mice at different time-points after infection using whole-body plethysmography (BUXCO, EMKA Technologies, Paris, France) as described by Hamelmann et al. [13]. Briefly, mice were placed in individual chambers, and the baseline readings were taken and averaged for 3 min. Animals were then exposed to aerosolized PBS or increasing concentrations of methacholine (Sigma, St Louis, MO, USA) from 2 to 15 mg/mL during 3 min. After nebulization the readings were taken and averaged for 3 min. Increases in airway reactivity to the bronchoconstrictor methacholine were expressed as P_{enh} values, which describe the fold increase for each methacholine concentration compared with the P_{enh} values after PBS challenge.

Antigens and culture supernatants

The recombinant TES-70 protein used in this study is a secreted and surface protein of *T. canis* larvae also designated as C-type lectin-4 (CTL-4) [14]. Recombinant TES-70 was expressed in the eukaryotic InsectSelect system (Invitrogen, Paisley, UK). Full-length TES-70 cDNA (including the predicted endogenous signal sequence), encoding a 288-amino acid protein, was sub-cloned into the InsectSelect vector, pIB/V5-His (www.invitrogen.com), and High Five insect cells (Invitrogen) transfected following the manufacturer's protocol. Cell lines expressing recombinant TES-70 were maintained, for up to 35 passages, in Express Five serum-free media containing 10 μg/mL Blasticidin S. Cell culture supernatants were purified by His-tag affinity columns (HiTrap Chelating HP, Chalfont St Giles, UK), eluted in an imidazole concentration gradient, and dialysed into sterile PBS. Aliquots were either lyophilized or stored at −70 °C.

At different time-points after infection spleens were collected and single cell suspension were made. Cells (1.5×10^5 cells/well) were cultured in round bottom 96-well plates (Greiner, Frickenhausen, Germany) in 75 μL RPMI 1640 medium (Gibco, Breda, The Netherlands) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU of penicillin per 100 mL, 100 mg/mL streptomycin (Gibco™) and 50 mM β-mercaptoethanol (Sigma). Cells were cultured for 120 h in the presence of 2 μg/mL TES-70 or medium only in a total volume of 150 μL. Culture supernatants were then collected for cytokine analysis.

Cytokine analysis

The levels of IL-2, IFN-γ, IL-4, IL-5 and IL-10 were measured either in bronchoalveolar lavage (BAL) fluids or in culture supernatants from stimulated spleen cells by capture ELISA using 96-well Nunc-Immuno™ Plate MaxiSorp™ Surface (Nunc, Albertslund, Denmark). IL-10 was measured according to the manufacturer's recommendations using the Mouse IL-10 ELISA kit BD OptEIA™ (Pharmingen, San Diego, CA, USA). For the measurement of IL-2, IFN-γ, IL-4 and IL-5, the 96-well plates were coated overnight at 4 °C with a rat monoclonal antibody specific for the different murine cytokines: IL-2, clone JES6-1A12; IFN-γ, clone R4-6A2; IL-4, clone 11B11; IL-5 TRFK-5 (Pharmingen). After blocking with BSA 1% in PBS—0, 1% Tween 20 for 1 h at 37 °C and washing with PBS/Tween20, standards and samples were added to the wells in twofold serial dilutions and incubated for 2 h at 37 °C. After the plates were washed, biotinylated rat anti-mouse cytokine antibody was added, IL-2 clone JES6-5H4, IFN-γ clone XMG1.2, IL-4 BVD6-24G2, IL-5 clone TRFK-4 (Pharmingen), and incubated for 1 h at room temperature. The wells were again washed and the streptavidin/avidin-horseradish peroxidase conjugate was added for 1 h at room temperature. After washing, tetramethyl-benzidine (TMB) (Sigma) substrate was added for 10 min at room temperature, and to stop the reaction 2 M H_2SO_4 solution was added. The absorbance was measured at 450 nm. A standard titration curve was obtained by serial dilutions starting from a known concentration of the murine recombinant cytokine: IL-2, IFN-γ, IL-4 and IL-5 (Pharmingen). The detection limits of the assays were for IL-2 and IL-4, 15 pg/mL, and for IFN-γ IL-5 and IL-10, 30 pg/mL.

Analysis of bronchoalveolar lavage cells

To obtain the BAL a cannula was inserted into the trachea of control and infected mice. Lung lavage was performed three

times with 1 mL PBS solution (pH 7.2, 37 °C). BAL was centrifuged at 400 **g**, 4 °C, 10 min and the supernatant stored at −80 °C for cytokine determination. The cell pellet was resuspended in 1 mL PBS and total cell number was performed by mixing 0.5 mL of the cell suspension with 9.5 mL isoton and using a cell counter (Beckman Coulter BV, Mijdrecht, The Netherlands). From the cell suspension cytospins were made and differential cell counts were performed after the preparations were stained with May-Grunwald/Giemsa. At least 400 cells/slide were counted by light microscopy. The amount of eosinophils in BAL is expressed as the percentage of total cell number.

Histology

Preparation of lung sections for histological analysis was performed as previously reported [12]. Briefly, after lung lavage, the lungs were fixed with formalin and embedded in paraplast (Kendall, Tyco Healthcare, Boston, MA, US). Transverse sections of 5 μm sections were stained with haematoxylin–eosin (HE) and with Periodic Acid Schiff (PAS) for mucus staining. Histological changes (peribronchiolitis, perivascular infiltrate, hypertrophy of goblet cells, alveolitis and eosinophil influx) were semi-quantitatively and blindly scored from absent (0), minimal (1), slight (2), moderate (3), marked (4) to severe (5). Scoring values for the various histological parameters are illustrated in Fig. 5.

Total IgE

Total serum IgE levels were measured in BAL by using a capture ELISA (OptEIA^{TM} Set) according to the manufacturers recommendations (Pharmingen). Briefly, plates were coated overnight at 4 °C with the Capture Antibody (anti-mouse IgE) diluted in Coating Buffer. After blocking each well, standard samples and controls were added. A working detector solution (biotinylated anti-mouse IgE antibody and peroxidase-conjugated streptavidin) was then added. Finally, colour development was performed using TMB as substrate, and the absorbance read at 450 nm. Total IgE concentration was calculated from the standard titration curve using the purified mouse IgE provided by the OptEIA^{TM} Set. The detection limit of the total IgE assay is 1.6 ng/mL.

Larval counts

The total number of larvae recovered from the whole brain and lungs of individual mice was calculated according to the Baermann procedure. Briefly, the right and the left lung were removed and macerated in 1% pepsin (Merck, Frankfurter, Germany). Brains were macerated in 0.85% saline solution. Macerated tissues were then transferred to tubes. Each tube contained individual macerated organs that were placed above a double layer of sterile gauze brought in contact with 1% pepsin in the case of lungs or saline for brains. Tubes were place in a water bath set to 37 °C and were left for 24 h then centrifuged and finally 6% formalin was added to the pellets. Larval counts were performed by microscopical examination of fixed samples. This procedure works on the basis that any live larvae present in the tissue sample will migrate through the gauze towards the warm enzyme/saline solution.

Statistical analysis

The statistical differences between groups were determined using ANOVA. The histological parameters were statistically tested with the Wilcoxon test. A probability value $P<0.05$ was considered statistically significant.

Results

Monitoring airway responsiveness in Toxocara*-infected BALB/c mice*

The effect of *T. canis* infection on airway function was monitored in conscious, unrestrained mice. After mice were exposed to increasing doses of methacholine, the airway responsiveness, expressed as P_{enh}, was measured. At 7, 14 and 30 days p.i. airway responsiveness was significantly higher for *T. canis*-infected mice compared with the non-infected controls (Fig. 1). No significant difference was measured among the dose–response curves to methacholine of infected mice at these time-points after infection. In contrast, at 60 days p.i. the airway response of *T. canis*-infected mice dropped to control levels.

Cytokines in bronchoalveolar lavage and in supernatants derived from stimulated spleen cells

Cytokine levels were measured in BAL at different time-points after infection. *T. canis*-infection resulted in significantly increased IL-5 levels at days 7 and 14 after infection (Fig. 2). The levels of IL-2, IFN-γ, IL-4 and IL-10 were either not significantly different at any time-point after infection compared with the uninfected mice or were below the detection limit of the assay.

The levels of these cytokines were also measured in supernatant derived from spleen cells that were stimulated with the *T. canis* recombinant TES-70 protein. Results indicate an increase in IL-5 at days 7 and 14 p.i. and of IL-10 at day 14 p.i. (Figs 3a and b). The levels of IL-4, IFN-γ and IL-2 were not different from those of the non-infected controls (data not shown).

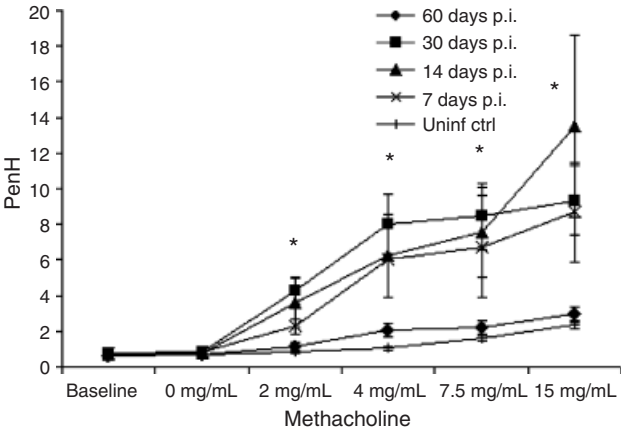

Fig. 1. Airway responsiveness after exposure to 0, 2, 4, 7.5, 15 mg/mL methalcholine of control uninfected (uninf. ctrl) mice and *Toxocara canis*-infected mice at different time-points post-infection (p.i.). Significant differences are shown as $^*P<0.05$ compared with uninfected mice.

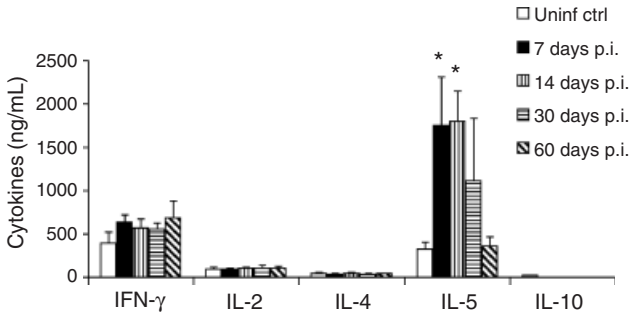

Fig. 2. Cytokines in bronchoalveolar lavage from uninfected control mice (uninf ctrl) and *Toxocara canis*-infected mice at different time-points post-infection (p.i.). Significant differences are shown as $^*P<0.05$ compared with uninfected mice.

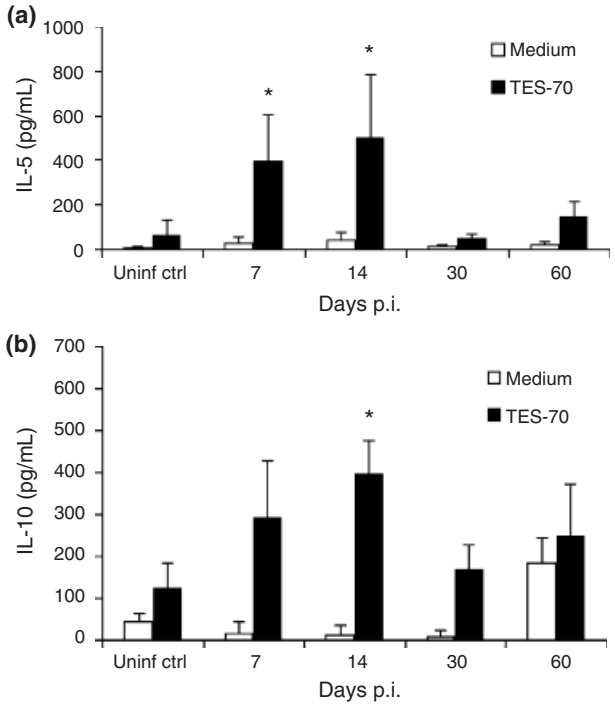

Fig. 3. Cytokine production by spleen cells after stimulation with the recombinant TES-70 *Toxocara canis* (*T. canis*) protein. IL-5 (a) and IL-10 (b) production by spleen cells from uninfected control (uninf ctrl) and *T. canis*-infected mice at different time-points post-infection (p.i.). Significant differences are shown as $^*P<0.05$ compared with uninfected mice.

Eosinophils and IgE in bronchoalveolar lavage

The percentage of eosinophils in BAL is shown in Fig. 4a. *T. canis* infection of BALB/c mice resulted in accumulation of eosinophils in BAL. Eosinophils represent at 14 days p.i. 70% of the total cells present. Increased percentage of eosinophils persisted up to 60 days p.i.

It has been well established that infection of mice with this nematode results in increased levels of total and parasite-specific IgE in serum [15]. In this study we measured total IgE levels in BAL derived from infected and uninfected mice. The concentration of total IgE in BAL derived from *T. canis* infected and uninfected mice is shown in Fig. 4b. Infection resulted in a significant increase in total IgE at 14, 30 and 60 days p.i.

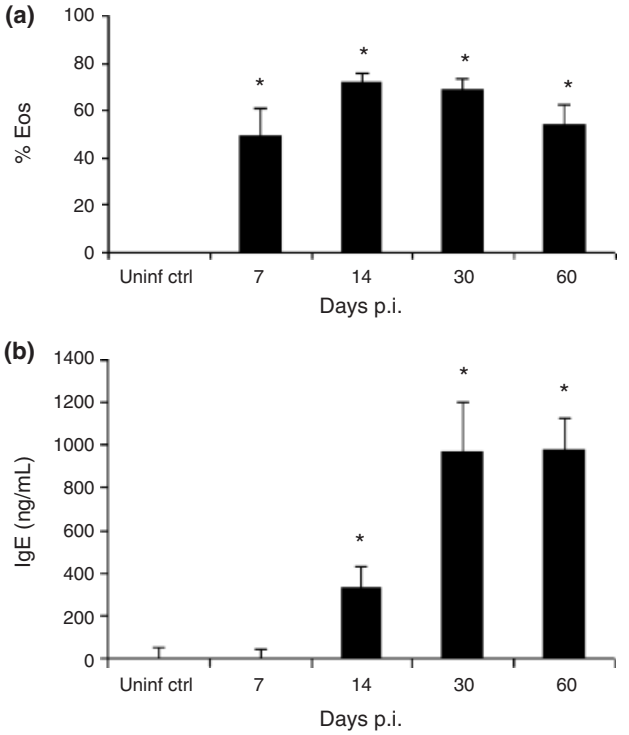

Fig. 4. Eosinophils (a) and total IgE (b) in bronchoalveolar lavage of uninfected control (uninf ctrl) and *Toxocara canis*-infected mice at different time-points post-infection (p.i.). Significant differences are shown as $^*P<0.05$ compared with uninfected mice.

Histology

Histological changes and scores given to lungs of *T. canis* infected and uninfected controls at different times after infection are shown in Table 1. Moderate-to-marked perivascular infiltration mainly consisting of eosinophils and lymphocytes was observed in lungs of infected mice at all time-points after infection (Figs 5b and c). Peribronchiolitis with the same pattern of inflammatory cells was also present but to a lesser extent. In addition, foci with interstitial pneumonia with slight-to-moderate diffuse alveolitis containing a large number of macrophages, eosinophils and microhemorrhages were observed. Marked-to-severe hypertrophy of the bronchiolar goblet cells was seen with PAS-positive staining indicating mucus production (not shown). After infection no significant difference was noted among the histological parameters at day 7 compared with day 60, except for perivascular infiltrate. These results indicate an ongoing inflammatory response that is still present at day 60 p.i. Uninfected controls did not show any pulmonary changes in the course of this study.

Toxocara canis *larvae in lungs at 60 days p.i.*

To determine larval distribution in BALB/c mice 60 days after infection, lung and brain tissues were examined for the presence of *T. canis* larvae. Results indicate that four out of the six infected mice contained living larvae in lung tissues (Table 2). The number of larvae per lung ranged from 1 to 4. In the brain the larval accumulation ranged from 94 to 176. No larvae were found in lungs and brains from uninfected controls (data not shown).

Table 1. Summary of pathological changes in the lung of *T. canis*-infected and uninfected BALB/c mice at 7, 14, 30 and 60 days p.i.

	Days p.i. 1000 *T. canis* eggs				Days p.i. Uninfected controls			
	7	14	30	60	7	14	30	60
Peribronchiolitis	2.3	2.0	2.3	2.0	0	0	0	0
Perivascular infiltrate	4.0	4.0	3.5	3.0*	0	0	0	0
Hypertrophy goblet cells	4.3	4.0	3.0	3.3	0	0	0	0
Alveolitis	2.5	3.0	1.8	2.0	0	0	0	0
Eosinophils influx	4.0	3.8	3.0	2.5	0	0	0	0

Data are mean of histological scores of four mice/group.
*Significantly different ($P<0.05$) from mice at 7 days p.i. Histopathological lesions were scored: absent (0), minimal (1), slight (2), moderate (3), marked (4), severe (5).
T. canis, *Toxocara canis*; p.i., post-infection.

Discussion

T. canis larvae migrate to the lungs of infected hosts causing tissue damage and pulmonary inflammation. In the present study we measured airway reactivity as well as immune responses and lung pathology at different time-points after *T. canis*-infection of BALB/c mice.

Airway reactivity was measured after conscious unrestrained mice were exposed to increasing concentrations of the bronchoconstrictive agent methacholine. To our knowledge this is the first study in which airway reactivity of *T. canis*-infected BALB/c mice has been measured in a non-invasive way. In a previous study using tracheal contractility assays in mice and guinea-pigs the authors observed tracheal hyperreactivity within 24 h after *T. canis* infection followed by a hyporeactive state that persisted for more than 4 weeks p.i [16]. In another study using unconscious ventilated rats, Yoshikawa et al. [17] showed that infection with *T. canis* led to airway hyper-responsiveness (AHR) at 14 days p.i. which coincided with the peak of eosinophil lung infiltration. Our results indicate that AHR could be measured as soon as 7 days p.i and that it persisted up to 30 days p.i.

Recently, Adler et al. have argued that unrestrained plethysmography (UP) may not be appropriate for AHR measures, because UP includes general responses largely reflecting reflex control of breathing processes rather than the lung mechanics. Furthermore, the degree to which reflex responses influence UP was found by these authors to vary according to the strain of mouse [18]. Our results, however, indicate (1) a significant difference between *T. canis* infected and un-infected control mice, (2) the increased P_{enh} measured after infection is dose-dependent: infection with 10 and 100 embryonated eggs are not sufficient to measure AHR using UP parameters whereas infection with 500 (data not shown) and 1000 embryonated eggs is effective; (3) increased P_{enh} after infection follows a specific kinetics: absent at 4 days p.i. (data not shown), increasing at 7 days p.i., remaining high up to 30 days p.i. and declining back to background levels at day 60 p.i.; and finally (4) we have measured AHR using UP parameters in both BALB/c and C57BL/6 *T. canis*-infected as compared with uninfected mice (data not shown). These findings could not be explained only as a reflection of reflex control of breathing processes and are therefore most probably indicative of the effect of infection on lung mechanics.

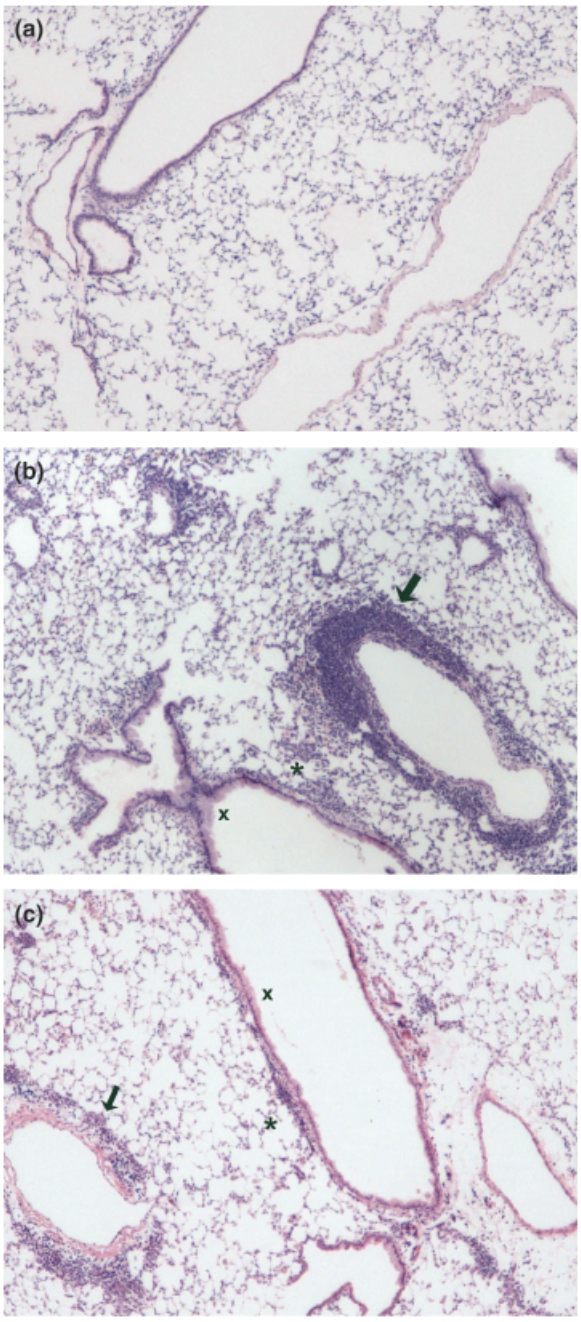

Fig. 5. Photomicrograph of lung sections taken from uninfected and *Toxocara canis* (*T. canis*)-infected BALB/c mice. (a) Lung from an uninfected mouse shows an artery (right) and bronchiole (left) without any sign of inflammation. The alveoli are clean and separated from each other by thin alveolar septa. Haematoxylin–Eosin (HE), × 160. (b) Lung from a mouse 14 days after infection with 1000 *T. canis* embryonated eggs. A severe perivascular inflammation (score 5, arrow) and a slight peribronchiolitis (score 2, *) are shown. The hypertrophy of the bronchiolar epithelium is moderate (score 3, x). Moderate alveolitis (score 3) is diffusely observed. HE, × 160. (c) Lung from a mouse 60 days after infection with 1000 *T. canis* embryonated eggs. A moderate perivascular infiltrate (score 3, arrow) and a slight peribronchiolitis (score 2, *) accompany a slight-to-moderate hypertrophy (score 2, x) of the bronchiolar epithelium. HE, × 160.

Parallel to this finding we observed that infection of BALB/c mice with this nematode resulted in pulmonary inflammation,

Table 2. Number of *T. canis* larvae recovered from brain and lungs at 60 days p.i.

Mouse No.	Number of *T. canis* larvae		
	Brain	Right lung	Left lung
1	132	1	1
2	164	3	2
3	176	0	0
4	94	1	3
5	111	0	0
6	141	1	4

T. canis, *Toxocara canis*; p.i., post-infection.

with eosinophil infiltration that persisted up to 60 days p.i. Others have also reported on pulmonary inflammation of *T. canis* infected mice that persisted up to 3 months p.i. [16]. Infiltration of eosinophils into the airways is believed to play an important role in AHR in bronchial asthma [17]. However, we show that AHR disappeared at 60 days p.i.; meanwhile pulmonary inflammation with infiltration of eosinophils was still present. The number of eosinophils in lung tissue and BAL declined at 60 days compared with 14 days p.i. but was significantly higher than that of uninfected controls. In a study using IL-5-deficient mice infected with the nematode *Nippostrongylus brasiliensis,* eosinophilic infiltration was abrogated; meanwhile the AHR was unimpaired. The authors concluded that eosinophil accumulation is not responsible for the induction of airway reactivity following infection with this nematode [19]. Whether in *T. canis* infected mice the induction of airway responsiveness depends on threshold levels of eosinophil accumulation or whether it depends on the presence of a certain number of parasites in the lung still remains to be investigated.

Previous studies have shown that larval recovery from the heart and lungs decreases over time after infection [11]. These authors showed that *T. canis* larvae were recovered from the heart and lung region at 7, 14, 28 and 56 days p.i. with maximal recovery at 7 days p.i. Results from this and other studies [8] show that *T. canis* larvae migrate and accumulate in the brain. We also observed accumulation of larvae in the brain and we found that at 60 days p.i. living *T. canis* larvae were still present in lungs of infected mice. It is important to bear in mind that the degree of tissue damage depends not only on living *T. canis* larvae but also on the presence of dead larvae [1]. Recently, we measured *Toxocara*-DNA in lungs of experimentally infected mice. Results indicate that *Toxocara*-DNA is present in lungs of all the infected mice (five mice per group) at 7, 14, 30 and 60 days p.i. (manuscript in preparation). The detected *Toxocara*-DNA is presumed to derive from living and dead larvae. The possible role of parasite antigen derived from dead larvae on the maintenance of pulmonary inflammation in *T. canis*-infected mice requires further investigation.

Compared with uninfected mice, parasite-specific stimulation using the recombinant TES-70 protein resulted in induction of IL-5 at days 7 and 14 p.i and IL-10 at day 14 p.i. TES-70 is a surface and secreted *T. canis* protein that has been characterized as a C-type lectin [14]. In its native form, TES-70 has been shown to bind to the surface of mammalian cells in a Ca^{2+}-dependent manner and has been suggested to play a role in host–parasite interactions [14]. Our results show that the recombinant TES-70 protein stimulates spleen cells of infected mice to produce *in vitro* T-helper type 2 (Th2) cytokines. In BAL only IL-5 was significantly higher in infected compared with non-infected mice. Similar to what was measured in supernatants derived from TES-70-stimulated spleen cells, IL-5 in BAL was significantly higher at days 7 and 14 p.i. Whether TES-70 plays a role in the induction of IL-5 in the lungs still remains to be investigated.

The role of IL-5 during *Toxocara* infection is not clear. IL-5 does not seem to play an important role in protective immunity, since previous studies have shown that the number of larvae in tissue does not change in IL-5 transgenic compared with non-transgenic mice [20]. Other studies have shown that mice that are genetically deficient in IL-5 carry similar numbers of larvae compared with those of wild-type mice [21]. Furthermore, treatment of mice with anti-IL5 suppresses both blood and tissue eosinophilia but does not influence the number of larvae recovered from tissue [22]. The increased production of IL-5 and eosinophilia observed in *Toxocara*-infected animals may play an important role in pathology. Decreased lung damage has been reported in *T. canis*-infected mice that are genetically deficient in IL-5 and have a lower number of eosinophils, suggesting a role for IL-5 and eosinophils in pathology [21].

In addition to IL-5 we observed that parasite-specific stimulation of spleen cells derived from infected animals resulted in a significant induction of IL-10 at day 14 p.i. Increased production of anti-inflammatory cytokines such as IL-10 has been suggested as an explanation for the inverse association observed between helminth infection and allergic manifestations [23]. *T. canis*-infected mice fail to produce IL-10 in a parasite-specific manner during the chronic stage of infection. Furthermore, no IL-10 was detected in BAL throughout this experiment. Whether failure to maintain the production of IL-10 is the explanation for the persistent pulmonary inflammation observed in *T. canis*-infected mice requires further investigation.

Although in this study we could not measure increased IL-4 production in BAL or in supernatants of TES-70-stimulated spleen cells after infection, the production of this cytokine by lung cells derived from *T. canis*-infected mice has been previously reported [24]. Additional experiments including measurement of cytokines produced by lung cells after stimulation with *T. canis* antigens are required in order to monitor the production of different regulatory cytokines after infection.

Recently, much attention has been given to the inverse association between many infections, including those caused by parasites and allergic disorders [23, 25, 26]. Whether infection with *T. canis* augments or protects from developing allergic manifestations still remains to be further investigated. Among the many variables that must be considered when investigating the effect of worm infections on allergic manifestations are the intensity of worm burden, the parasite species in question, whether the infection is acute or chronic and the frequency of exposure.

In conclusion, our results show that a single infection of BALB/c mice with *T. canis* results in AHR that persists up to 30 days p.i. In addition, infection leads to pulmonary inflammation, increased IgE levels and eosinophilia that

persist up to 60 days p.i. Finally, we observed that at 60 days p.i. living larvae were still present in lungs of infected mice and were probably responsible for the immunopathological changes observed. Further research will focus on the role of different parasite antigens in the induction and maintenance of the observed changes after *T. canis* infection.

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