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Evidence for Asthma in the Lungs of Mice Inoculated with Different Doses of *Toxocara canis*

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Abstract. *Toxocara canis*, a common roundworm that mainly causes toxocariasis, is a zoonotic parasite found worldwide. Humans, an accidental host, can acquire *T. canis* infection through accidental ingestion of *T. canis*–embryonated egg-contaminated food, water, and soil, and by encapsulated larvae in a paratenic host's viscera or meat. Long-term residence of *T. canis* larvae in a paratenic host's lungs may induce pulmonary inflammation that contributes to lung injury, airway inflammatory hyperresponsiveness, and collagen deposition in mice and clinical patients. This study intended to investigate the relationship between *T. canis* infection and allergic asthma in BALB/c mice inoculated with high, moderate, and low doses of *T. canis* eggs for a 13-week investigation. The airway hyperresponsiveness (AHR) to methacholine, collagen deposition, cytokine levels, and pathological changes in lung tissues was assessed in infected mice at weeks 1, 5, and 13 postinfection. The cell composition in bronchoalveolar lavage fluid of infected mice was assessed at weeks 5 and 13 postinfection. Compared with uninfected control mice, all groups of *T. canis*–infected mice exhibited significant AHR, a dose-dependent increase in eosinophilic infiltration leading to multifocal interstitial and alveolar inflammation with abundant mucus secretion, and collagen deposition in which the lesion size increased with the infective dose. Infected mice groups also showed significant expressions of eotaxin and type 2 T-helper–dominant cytokines such as interleukin (IL)-4, IL-5, and IL-13. Overall, these results suggest that *T. canis* larval invasion of the lungs may potentially cause pulmonary inflammatory injury and could subsequently contribute to the development of allergic manifestations such as asthma.

INTRODUCTION

Toxocariasis is a zoonotic infection mainly caused by *Toxocara canis* which is the parasitic roundworm commonly found in the intestines of dogs and cats to a lesser extent. It is one of the five major indigenous neglected parasitic infections that should be comprehensively controlled in the United States as highlighted by the CDC.[1] It is also a cosmopolitan disease that occurs in various regions worldwide, although more frequently in developing countries.[2]

The adult female worm can produce up to 200,000 unembryonated eggs per day, releasing them into the environment via a dog's feces which can become infectious in soil under appropriate conditions after an incubation period of 2–5 weeks, depending on both temperature and humidity.[3,4] Under suitable conditions, its survivability has remained for as long as 2–4 years or more.[5] Humans may acquire an infection through accidental consumption of embryonated egg-contaminated soils, raw fruits, or vegetables[6,7]; however, to a lesser extent, humans can also become infected by ingesting larvae via raw or undercooked meat or giblets from paratenic hosts, for example, chickens, cows, or ducks.[8–11] When ingested, larvae may emerge from the swallowed eggs, penetrate through the submucosa of the small intestine, and further migrate to various internal organs including the lungs through the circulatory system,[6,7] and the invading larvae retain their current form with no further development.[12] At present, diagnosis predominantly relies on immunological techniques, for example, *T. canis* larval excretory–secretory antigen-based ELISA (TcES-ELISA) or utilization of fractionated TcES antigens in Western blotting.[6,13]

The global toxocariasis seroprevalence was estimated to be 6.3–86.8%, which indicates the profound impact of *Toxocara* on global human health, and this parasitic infection is highly related to various risk factors, including poor personal hygiene, consumption of foods contaminated by eggs or encapsulated larvae of *Toxocara*, and contact with dogs and cats.[14] Common victims of *T. canis* infection are children who may become infected by accidentally ingesting dirt contaminated with dog or cat feces containing contagious *Toxocara* eggs, and clinical symptoms such as wheezing, coughing, and episodic airflow obstruction, associated with hypereosinophilic syndrome, were described and bear a clinical resemblance to pulmonary inflammatory responses observed in asthma patients.[2,15,16]

Allergic asthma is a chronic inflammatory disorder of the airways characterized by increased serum IgE levels after exposure to allergens and by eosinophilic inflammation, mucus hypersecretion, and bronchial hyperreactivity.[17] Asthmatic complaints include shortness of breath, wheezing, and forced inhalation. This disorder is controlled by type 2 T-helper (Th2) cells that secrete interleukin (IL)-4, IL-5, and IL-13, which mediate the synthesis of IgE, recruitment of immune cells, degranulation of eosinophils, and hyperreactivity of the airway.[18,19] Although whether *Toxocara* infection can cause asthma is still under debate from several small-scale epidemiological studies,[20–22] a recent large-scale study using a systematic review and meta-analysis to analyze a total of 17 studies, including 11 studies with a case–control design (1,139 patients and 1,023 controls) and six studies with a cross-sectional design (a total of 5,469 participants, 872 asthmatic and 4,597 non-asthmatic children), found an increased risk of asthma in children with *Toxocara* infection seropositivity (odds ratio, 1.91; 95% CI, 1.47–2.47).[23]

Substantial experimental studies showed that *T. canis* larvae induce a Th2-dominant immunological response, leading

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to the secretion of IL-4, IL-5, and IL-13; subsequent IgE production; and differentiation and activation of eosinophils in mice.[24] A previous study by Pinelli et al.[25] indicated that the presence of *Toxocara* larvae in the lungs of infected BALB/c mice at 60 days postinfection following a single inoculation of a high dose of 1,000 *T. canis*–embryonated eggs could induce persistent pulmonary inflammation, airway hyperreactivity, eosinophilia, and increased IgE production.[25]

In the present study, we explored the relationship between *T. canis* infection and asthmatic hyperreactivity in the lungs of BALB/c mice inoculated with high, moderate, and low doses of *T. canis* eggs for a 13-week investigation. The number and composition of leukocytes in bronchoalveolar lavage fluid (BALF) were counted, and Th2 cytokine levels associated with asthma were determined in lung tissues. Meanwhile, pulmonary inflammation and collagen deposition were evaluated using histological sections stained with hematoxylin and eosin (H&E), periodic acid–Schiff (PAS), and Masson's trichrome (MT) stains.

MATERIALS AND METHODS

Mice. Male BALB/c mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and maintained in the Animal Center of Taipei Medical University. All mice were used at 4–5 weeks of age when they weighed 20–22 g. Food and water were provided ad libitum. Animal care and handling protocols were approved by the Animal Committee, Taipei Medical University (LAC-2016-0426). All animal experiments were carried out under institutional policies and guidelines for the care and use of laboratory animals, and all efforts were made to minimize animal suffering.

Source of *T. canis*–embryonated eggs. The method for preparing embryonated *T. canis* eggs was described in a previous study.[26] In brief, adult *T. canis* female worms were collected from puppies treated with antihelminthic drugs. After confirmation by morphological identification under a microscope that only eggs of *T. canis* were present, eggs were collected from the uteri of female adult worms. About a 1-cm-long section of the uterus near the vulva was collected and shaken in 2% formalin at room temperature to prepare a *T. canis* egg suspension. Once the eggs had fully embryonated, they were further stored at 4°C until use.

Mice inoculation. On day 0, 72 mice were divided into three infected groups including low-, moderate-, and high-dose groups, and each mouse was infected with 0.2 mL of saline containing 10 (low-dose), 100 (moderate-dose), and 1,000 (high-dose) embryonated *T. canis* eggs through oral intubation. The control group consisted of 24 uninfected mice that were further divided into three subgroups with eight mice per group and were used at the experimental times of 1, 5, and 13 weeks postinfection (wpi).

Determination of the airway hyperresponsiveness (AHR). Airway hyperresponsiveness is a broad term that reflects the degree of airway narrowing in response to certain bronchodilator concentrations. Airway hyperresponsiveness was assessed by measuring the functional change from the baseline with a directly inhaled smooth muscle spasm agonist (e.g., methacholine [MCh]).[27] Airway hyperresponsiveness is a highly standardized, objective, and reproducible outcome measure,[28] which also is a method for measuring airway function.[29]

In brief, at 1, 5, and 13 wpi, airway function was measured by detecting changes in lung resistance in response to increasing doses of aerosolized MCh (acetyl-β-methyl-choline chloride, Sigma-Aldrich, St. Louis, MO) in anesthetized mice in each infected group. Mice were anesthetized, tracheostomized, and mechanically ventilated at a rate of 150 breaths/minutes, with a tidal volume of 0.3 mL and a positive end-expiratory pressure of 3–4 cm H_2O with a computer-controlled small-animal ventilator (model 683, Harvard Rodent Ventilator, Mount Holly, NJ). PE-50 tubing was inserted into the esophagus to the level of the thorax and coupled with a pressure transducer. The flow was measured by electronic differentiation of the volume signal, and changes in pressure, flow, and volume were recorded. Pulmonary resistance was calculated using software (model PNM-PCT100 W, LDS PONEMAH Physiology Platform, LDS Gould, Champaign, IL). Methacholine aerosol was generated with an inline nebulizer and directly administrated through the ventilator. The resistance of the orotracheal tube was subtracted from all airway resistance measurements. Data are expressed as pulmonary resistance (R_L).[30]

Analysis of the cellular composition of BALF. After measuring pulmonary function parameters, mice were sacrificed, and their tracheas were immediately lavaged three times via a tracheal cannula with 1 mL of Hank's balanced salt solution (HBSS) that was free of ionized calcium and magnesium. The lavage fluid was cooled on ice and centrifuged (400 × *g*) at 4°C for 10 minutes. After washing, cell pellets were resuspended in 1 mL HBSS. After staining with Türk solution, total cell counts per lung were calculated under light microscopy using a Burker–Türk chamber (BR71952, Sigma-Aldrich). The cytocentrifuged preparation of BALF cells was fixed and stained with Liu's staining solution (Chi I Pao, Taipei, Taiwan). At least 200 cells of the total number of cells in the BALF were counted with a standard hemocytometer, and macrophages, eosinophils, neutrophils, and lymphocytes were differentiated based on the standard morphological criteria. (changes correct?)

Pulmonary pathology and airway remodeling assessment. For the pathological and collagen deposition study, the lungs of each mouse were immediately removed and fixed in a 10% neutral-buffered formalin solution (pH 7.2) for 12–24 hours. Representative pieces of tissue were trimmed and embedded in paraffin. Serial sections at 5-μm thickness were cut and stained with H&E and PAS stains to examine the inflammation location, the degree of inflammatory injury and mucus secretion, and the intensity and type of infiltrate using light microscopy. Collagen deposition was measured and calculated by MT staining.[31] At least 5–10 fields under ×100 and ×400 magnification were randomly selected and examined.[32]

Larval recovery assessment from *T. canis*–infected mice lungs. To confirm whether or not *T. canis* larvae had invaded mice lungs, a previously described larval recovery method was used.[26] In brief, lung tissues from each *T. canis*–infected mouse were cut into small pieces and individually digested in 25–30 mL of an artificial acidic pepsin/HCl solution (pH 1–2, Sigma-Aldrich) in a modified Baermann apparatus at 37°C for at least 2 hours. Subsequently, the filtered solution was poured into a petri dish, and numbers of *T. canis* larvae were counted with an inverted microscope (Olympus, Tokyo, Japan) at ×100 magnification.

Western blotting analysis of Th2 cytokine expressions. The remaining lung tissues from the same experimental group were pooled together, further homogenized, and lysed in radioimmunoprecipitation assay buffer containing 1% of a protease inhibitor cocktail (Sigma-Aldrich, Darmstadt, Germany) at 4°C for at least 1 hour; protein supernatants were then harvested by centrifugation at 13,000 rpm and 4°C for 10 minutes. Thereafter, protein concentration was calculated using the Bradford method with a Bio-Rad protein assay kit (Life Sciences, Taipei, Taiwan). Subsequently, 50 μg of each protein sample was boiled for 5 minutes to denature the protein, then further separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and finally transferred onto a 0.45-μm pore size polyvinylidene difluoride membrane for 2 hours. Membranes were blocked with 5% bovine serum albumin in tris-buffered saline with 0.3% Tween 20 (TBST) for 2 hours. Primary antibodies, including a mouse anti-β-actin monoclonal antibody (1:6,000, cat. no. A2228, Sigma-Aldrich), rabbit anti-IL-4 polyclonal antibody (pAb) (1:800, cat. no. SG24221744, Invitrogen, Carlsbad, CA), rabbit anti-IL-5 pAb (1:800, cat. no. 821800603, GeneTex, Irvine, CA), rabbit anti-IL-13 pAb (1:800, cat. no. GR161337-6, Abcam, Cambridge, MA), and rabbit anti-eotaxin pAb (1:800, cat. no. 821802424, GeneTex), were added for hybridization at 4°C overnight. After washing with TBST several times, membranes were further incubated with secondary antibodies of horseradish peroxidase–conjugated IgG, including rabbit anti-mouse IgG (cat. no. A9044, Sigma-Aldrich) and goat anti-rabbit IgG (cat. no. A0545, Sigma-Aldrich), at 1:5,000 dilutions. Immunoreactions were detected with a Western Lightning ECL Pro kit (PerkinElmer, Waltham, MA), and the densities of immunoreactive bands were measured using a UVP Biospectrum AC System (UVP, Upland, CA) in the Core Facility Center of Taipei Medical University. Reactive bands of β-actin, IL-4, IL-5, IL-13, and eotaxin were detected at 42, 14, 17, 16, and 10 kDa, respectively.

Statistical analysis. All data are presented as the mean value ± standard error of the mean (SEM). The statistical difference in the AHR assay, leukocyte enumeration in BALF, number of recovered larvae, the pathology and quantity of collagen deposition, and expressions of various cytokines between the experimental and uninfected groups were assessed by a one-way analysis of variance followed by Tukey's multiple comparison test using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA). Significant differences between the control and the infected groups were evaluated by an unpaired *t*-test. *P*-values of < 0.05 were considered statistically significant.

RESULTS

***Toxocara canis* infection induced severe AHR and airway inflammation.** To assess the effects of *T. canis* infection in mice lungs on allergic asthma, both the AHR and accumulation of inflammatory cells in BALF were investigated. After 1, 5, and 13 wpi, the airway responsiveness to aerosolized MCh of each group of mice was measured. The AHR of uninfected mice was consistent throughout the experiment; by contrast, *T. canis*–infected mice showed significantly enhanced airway responsiveness to MCh inhalation. Compared with results at 1 wpi, AHR had significantly increased at 5 wpi and remained stably high at 13 wpi (Figure 1A). Throughout the entire experiment, the moderate-dose and high-dose groups showed a similar significant increase in AHR compared with low-dose infection; noteworthy, at 13 wpi, the AHR of high-dose–infected mice partially showed significantly higher severity than that in the other two groups at 1 and 5 wpi, respectively (Figure 1A). Furthermore, in the BALF analysis, the number of leukocytes of infected mice in the chronic phase (13 wpi) had slightly increased beyond that in the early phase (5 wpi). However, numbers of inflammatory cells, including eosinophils, neutrophils, and lymphocytes, at 5 wpi were significantly higher than those found at 13 wpi. By contrast, macrophages showed an increase at 13 wpi. Not only total cells but also the various types of cells in the uninfected group remained significantly lower than those in the infected mouse groups ($P < 0.0001$) (Figure 1B).

***Toxocara canis* larvae were found in the lungs of infected mice in the early phase.** Results of the larval recovery study from the lungs are shown in Table 1. No larvae were found within any dose-infected mouse group at 13 wpi, whereas only one larva was found in each of two mice receiving the high-dose infection at 5 wpi. The high-dose–infected mice showed a greater number of mice lungs harboring larvae and also more larvae invading the lungs of infected mice than the low- and moderate-dose infection groups of mice in the very early stage (at 1 wpi).

***Toxocara canis*–infected mice exhibited airway inflammation.** Hematoxylin and eosin staining of pulmonary sections from infected mice with low-, moderate-, and high-dose inoculation revealed widespread disruption of the epithelial layer; epithelial cells showed irregular shapes, and some had been shed into the airway lumen. These infected groups also exhibited severe inflammatory cell infiltration around the peribronchiolar and perivascular regions, leading to peribronchiolitis and perivasculitis at 1, 5, and 13 wpi. In addition, an excessively thick layer of vascular cells and epithelial basement membrane was detected (Figure 2).

***Toxocara canis* infection enhanced the hypersecretion of lung mucus.** Although the lung tissue structure in control mice was preserved, prominent increases in goblet cell hyperplasia and mucus secretion from hyperplastic goblet cells using PAS staining were observed in infected mice (Figure 3A). High-dose infection produced increased inflammatory cells and mucus production at 5 and 13 wpi, but not at 1 wpi, at which time, moderate-dose infection showed the highest mucus secretion level. Hypersecretion of lung mucus in the infected groups had considerably increased by 1 wpi, and then decreased with time postinfection at 5 and 13 wpi (Figure 3B).

***Toxocara canis* infection produced collagen deposition.** As to collagen deposition in infected lung sections, MT staining showed a diffuse blue-stained collagen band surrounding the airways beneath the epithelial layer. *Toxocara canis*–infected mice developed significantly increased areas of peribronchial trichrome staining compared with the uninfected group. In the airway of moderate- and high-dose mice, the band of collagen appeared more intense and was also thicker than that of low-dose mice (Figure 4A). Dose- and time-dependent airway remodeling occurred during the entire experiment, except in the moderate group at 13 wpi, which

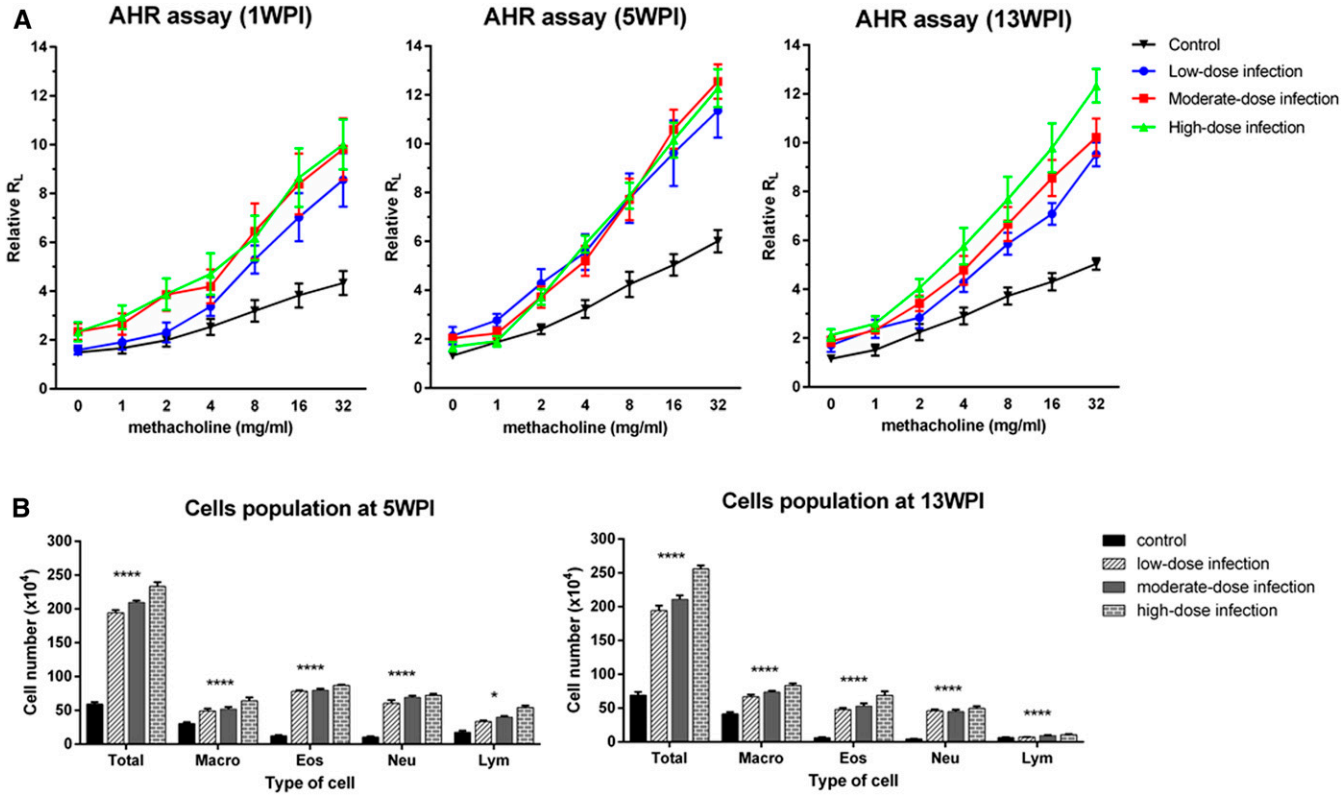

FIGURE 1. *Toxocara canis* infection of mice induced the development of airway hyperresponsiveness and abundant infiltrating cells. (**A**) Airway resistance was measured in response to increasing concentrations of methacholine (0–32 mg/mL) by invasive-body plethysmography. (**B**) Cellular composition of the bronchoalveolar lavage fluid (BALF) of mice. After measuring pulmonary function parameters, each group of mice was sacrificed, and cells in the BALF were collected, counted, and classified as Eos = eosinophils; Lym = lymphocytes; Macro = macrophages; and Neu = neutrophils. Results are expressed as the mean ± standard error of the mean ($n = 8$ mice in the control group and $n = 8$ mice in all three experimental groups) of pulmonary resistance (R_L) after methacholine challenge and cell numbers in the BALF. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and ***** $P < 0.0001$ with a one-way analysis of variance for pulmonary resistance (R_L) and (*t*-test) vs. the control group for cell populations. This figure appears in color at www.ajtmh.org.

showed a slight decrease in the area of peribronchial collagen (Figure 4B).

Toxocara canis infection induced significant IL-4, IL-5, IL-13, and eotaxin expressions in the lungs of low-, moderate-, and high-dose–infected mice.

Expression levels of Th2 cytokines (IL-4, IL-5, and IL-13) and eotaxin, a chemokine that recruits eosinophils, increased in all infected groups; by contrast, control mice showed significantly lower levels (Figure 5A). Expressions of eotaxin and IL-5 were significantly induced during infection, which possibly subsequently recruited more eosinophils, whereas enhanced IL-4 may have offered a potent stimulant for high IgE production and enhanced IL-13 expression, which can promote AHR. Eotaxin and IL-5 levels significantly increased in the early phase (at 1 wpi), but had decreased by 5 wpi; thereafter, they were slightly higher in the chronic phase (at 13 wpi). Noteworthy, we observed enhancement of IL-4 and IL-13 expressions in infected mice over the time postinfection with the highest level at 13 wpi (Figure 5B).

TABLE 1

Toxocara canis larvae recovered from mouse lungs with low-, moderate-, and high-dose infections at 1, 5, and 13 weeks postinoculation ($n = 8$ mice in the control group and $n = 8$ mice in all three experimental groups)

Time postinfection	Control		Low-dose infection*		Moderate-dose infection†		High-dose infection‡	
	MPL	TLR	MPL	TLR	MPL	TLR	MPL	TLR
Week 1	0	0	1	1	2	10	6	38
Week 5	0	0	0	0	0	0	2	2
Week 13	0	0	0	0	0	0	0	0

MPL = number of mice positive for larvae; TLR = total number of larvae recovered.
* Inoculated with 10 embryonated eggs.
† Inoculated with 100 embryonated eggs.
‡ Inoculated with 1,000 embryonated eggs.

DISCUSSION

The Th2 immune response is a common immunological feature in both allergic asthma and toxocariasis, specifically characterized by the high production of IgE and eosinophils. However, the role of parasitic infections in the development of asthma is not well understood.[33] In the present study, we clarified that *T. canis* infection is capable of inducing asthma in mice, and these data may provide important clues on helminth–allergy associations.

Asthma is characterized by airway inflammation and remodeling, thus leading to AHR.[34] In the present study, *T. canis* larvae were used as allergic stimuli to determine whether *T. canis* infection can modulate and increase the allergic inflammatory response as assessed by different doses of infection after different infection times in BALB/c mice. Our

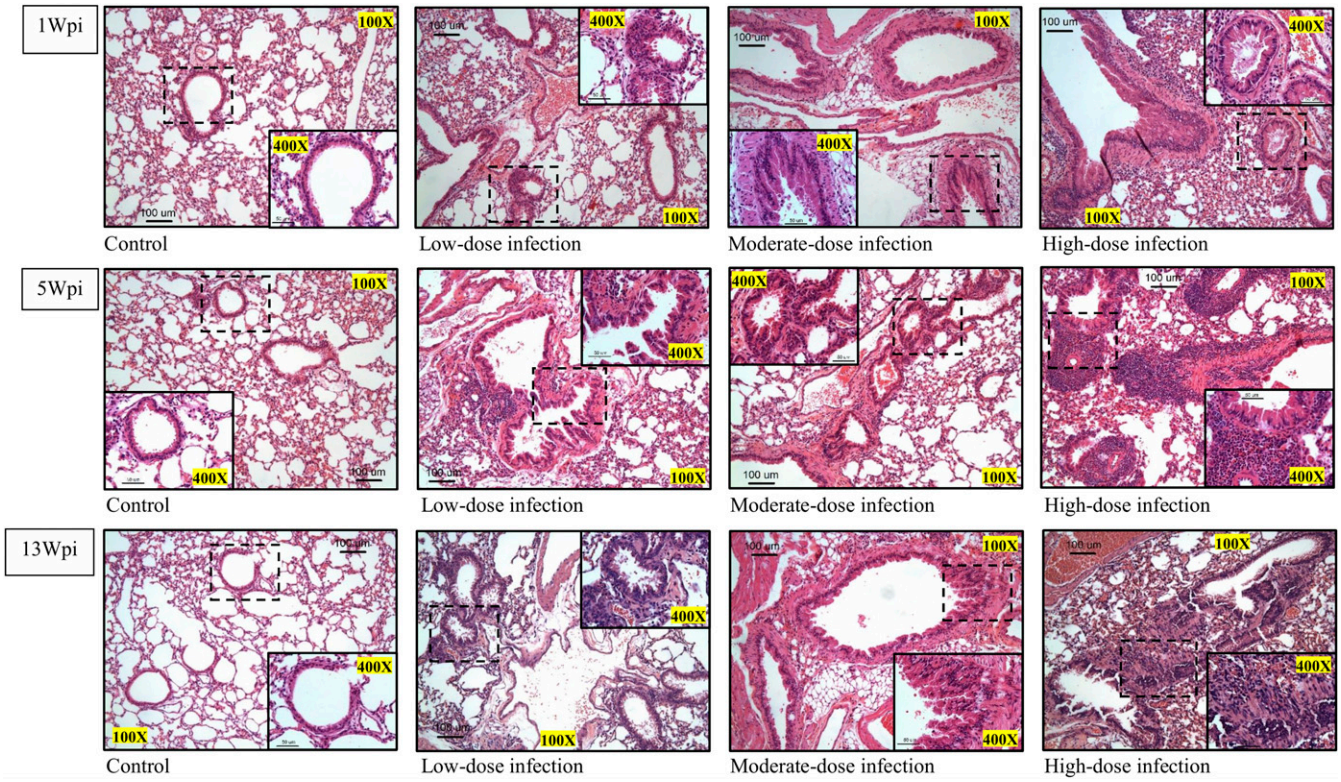

FIGURE 2. *Toxocara canis* infection increased inflammatory cell infiltration around the airway wall at 1, 5, and 13 weeks postinfection, and the thickness of the layers, destruction of cell networks, and inflammatory cell composition (including neutrophils, macrophages, eosinophils, and lymphocytes) which induced airway narrowing were evaluated. Sections were stained with hematoxylin and eosin for the morphological analysis. Tissues were examined by light microscopy (scale bars: ×100 magnification, 100 μm; ×400 magnification, 50 μm). This figure appears in color at www.ajtmh.org.

study found an intensification of the dose-dependent AHR which significantly increased with time (Supplemental Figure S1). Significant differences were observed at the 4 mg/mL dose at 1 wpi (Supplemental Figure S1A) and in all doses of MCh at 5 and 13 wpi (Supplemental Figure S1B and C), suggesting which dosage of MCh is adequate to be used for AHR assays in murine models of helminthic diseases. More importantly, AHR was detected to have increased with time from 5 to 13 wpi in *T. canis*–infected mice with different doses of infection in the present study. In a study using tracheal contractility assays in mice and guinea pigs, those researchers observed tracheal hyperreactivity within 24 hours after *T. canis* infection followed by a hyporeactive state that persisted for more than 4 wpi.[35] Another study using unconscious ventilated rats showed that infection with *T. canis* led to AHR at 2 wpi which coincided with the peak of eosinophil lung infiltration.[36]

On the other hand, airway inflammation is initiated and propagated by the penetration of inflammatory cells and various inflammatory mediators, such as cytokines, chemokines, and lipid mediators.[37] We found that inoculation with *T. canis* promoted allergic inflammation as evidenced by a strong induction in the number of inflammatory cells, particularly of eosinophils, which had been recruited to the airways by 5 wpi; however, at 13 wpi, the number had slightly declined while remaining meaningfully high. In helminthic diseases, moderate-to-severe eosinophilia occurs as a pathophysiologic response to parasitic infection, and toxocariasis is one of the main causes of eosinophilia[38]; eosinophilia also occurs in a subgroup with the phenotype of "severe eosinophilic asthma." In this study, an increase in the number of eosinophils in the *T. canis*–infected group may reflect increased excitation induced by antigens excreted from *T. canis* larvae.[24] Although we unfortunately lost cells at 1 wpi so that inflammatory cells could not be counted at that time, a significant number of eosinophils were still present in BALF at 5 and 13 wpi. Their presence was correspondingly ascribed to *T. canis* larvae in the lungs 1 week after infection, and they remained detectable at 5 wpi but had disappeared by 13 wpi. Several studies revealed that eosinophils are recruited from the circulation to inflammatory tissues in response to allergic stimuli, and contribute to the pathogenesis of allergic asthma including tissue damage and AHR.[39] In the present study, despite the eosinophil number being relatively reduced at 13 wpi, AHR had still increased in the infected group, indicating that airway inflammation caused by *T. canis* infection was able to increase pulmonary resistance, decrease dynamic compliance, and cause perivascular/peribronchial edema as evidenced by the AHR study and pathological findings from H&E–stained lung sections. Nevertheless, some studies also indicated that such phenomena were independent of the presence of eosinophils; in addition, a lessening of tracheal spasms was associated with airway eosinophilia.[40]

Previous studies indicated that *Toxocara* infection may induce a Th2 response in allergic patients with enhanced expressions of Th2 cytokines, such as IL-4, IL-13, and IL-5.[41] These cytokines are responsible for IgE production and mucus production, which are, respectively, responsible for the

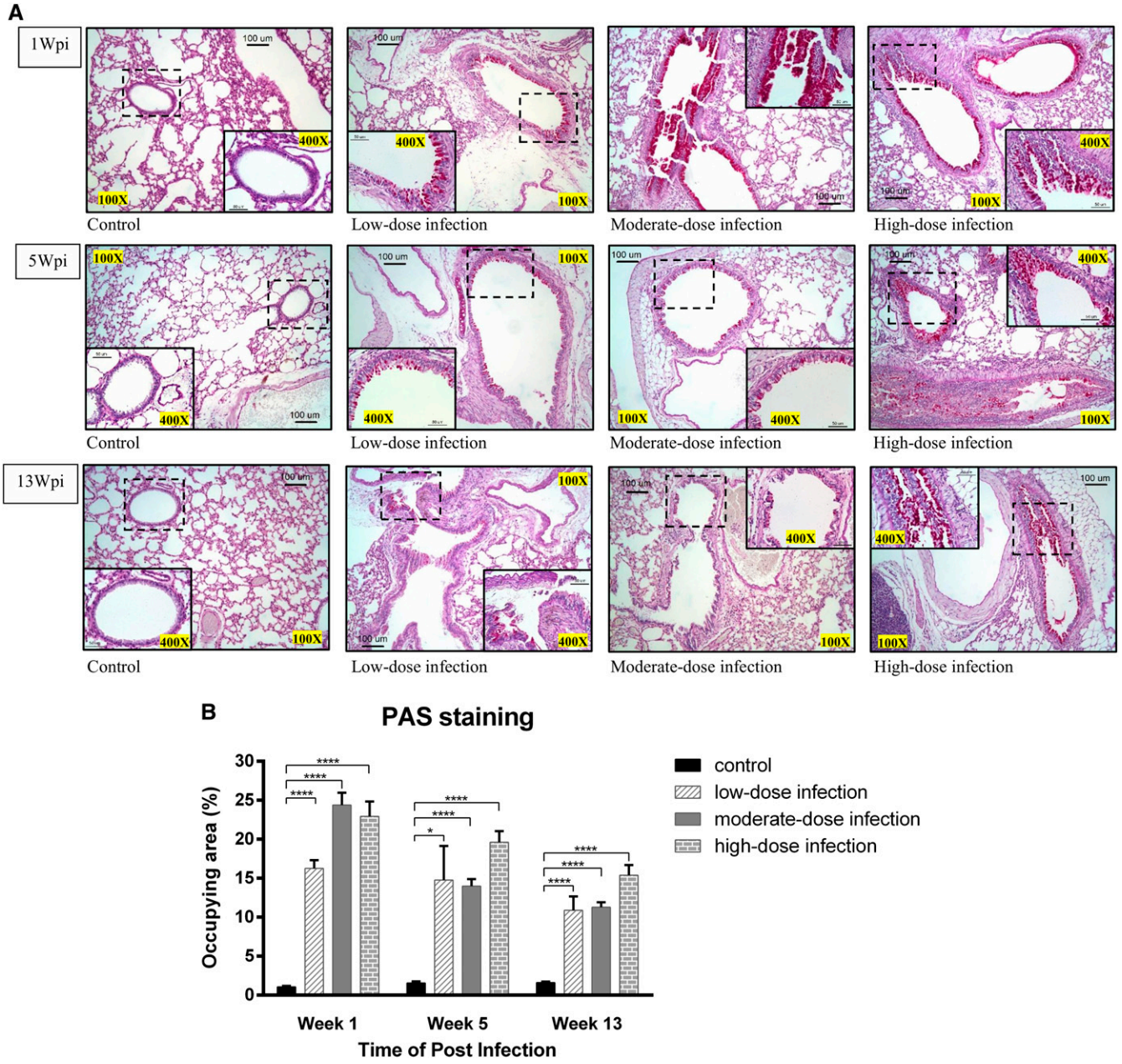

FIGURE 3. *Toxocara canis* infection enhanced mucus production in the lungs. (**A**) Sections were stained with periodic acid–Schiff (PAS) stain for the morphological analysis. Epithelial cells were replaced by blue/purple-stained goblet cells. Tissues were examined by light microscopy (scale bars: ×100 magnification, 100 μm; ×400 magnification, 50 μm). (**B**) Quantification of PAS staining. Results are expressed as the mean ± standard error of the mean ($n = 8$ mice in the control group and $n = 8$ mice in all three experimental groups) of the percentage of the area occupied by mucus production. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ (*t*-test) vs. the control group. This figure appears in color at www.ajtmh.org.

development of AHR and eosinophil accumulation.[42,43] (changes correct?) IL-4 activates B cells by promoting immunoglobulin switching toward facilitating IgE production, which is involved in asthma development by binding to mast cells, basophils, and eosinophils, causing them to release inflammatory mediators.[44] (changes correct?) Our results showed a significant increase in IL-4 production in lung tissues with all doses of infection, and the highest level was observed at 13 wpi. Simultaneously, a similar phenomenon of an increase in IL-13 production was observed. In particular, IL-13 is a Th2 cytokine that increases epithelial permeability and mucus production, thereby promoting the development of AHR.[45] Remarkably, in the present study, the high-dose group reached the highest level of IL-13 expression at 13 wpi. Merging this finding with the AHR assay, the AHR of infected mice increased with the time of infection. Furthermore, in the lungs, endothelial cells, smooth muscle cells, epithelial cells, alveolar macrophages (AMs), and eosinophils can produce eotaxin. Among these inflammatory cells, eosinophils highly express CC chemokine receptor 3, which can bind to eotaxin, a more-potent chemokine than the others in inducing eosinophilia and leukotriene C4 release.[46] On the other hand, IL-5 is another key Th2-related cytokine that promotes maturation and differentiation of eosinophils in the bone marrow,

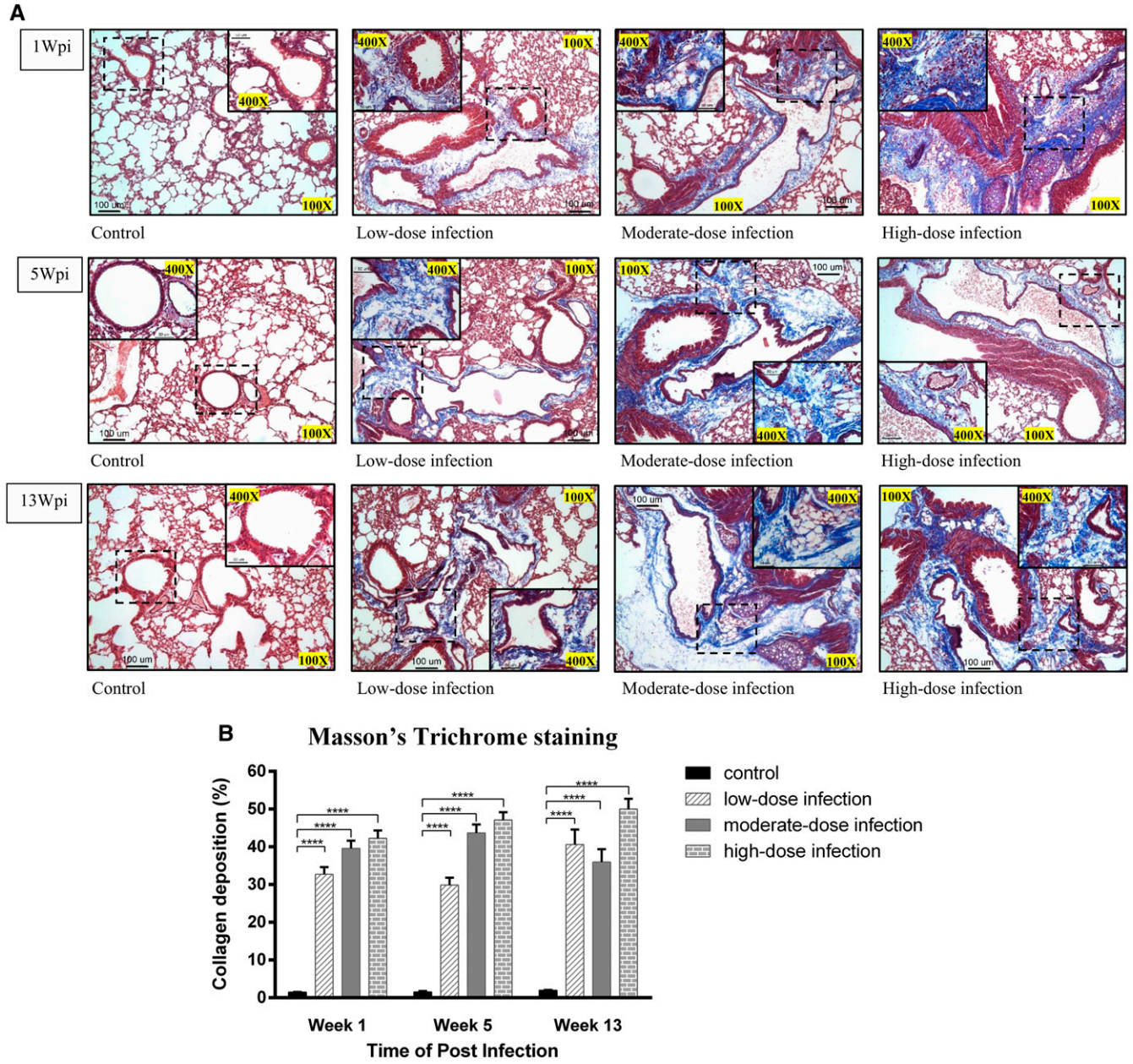

FIGURE 4. *Toxocara canis* infection caused collagen deposition in mouse lung tissues. (**A**) Sections were stained with Masson's trichrome (MT) stain for the morphological analysis. In MT staining, blue staining of subepithelial collagen was observed. Tissues were examined by light microscopy (scale bars: ×100 magnification, 100 μm; ×400 magnification, 50 μm). (**B**) Quantification of MT staining. Results are expressed as the mean ± standard error of the mean ($n = 8$ mice in the control group and $n = 8$ mice in all three experimental groups) of the percentage collagen deposition. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ (*t*-test) vs. the control group. This figure appears in color at www.ajtmh.org.

enhances eosinophil recruitment, and activates and produces cytotoxic proteins for tissue destruction in the airway.[47] Numerous studies confirmed that IL-13 is the dominant profibrotic mediator in several experimental models of fibrosis including fibrosis formation in the *Clonorchis sinensis*–infected mouse liver.[48–50] In the present study, we found that *T. canis* larval invasion of the lungs may induce airway inflammation, and concomitant collagen deposition and hypertrophy of mucous cells; simultaneously, eotaxin, IL-5, and IL-13 expressions were remarkably enhanced in the mouse lungs throughout the entire experiment, although a slight decrease was observed at 5 wpi. Therefore, we speculated that eosinophil accumulation and enhancement of cytokines may play important roles in contributing to the significant thickening of the mucosal smooth muscle layers of the airways. These cells secrete oxygen radicals that may contribute to airway and vascular damage,[36] thus facilitating asthma development. Moreover, mucus is produced by goblet cells in the airways, and mucus hypersecretion is a major cause of airway obstruction in asthma leading to coughing and wheezing. We found that *T. canis* infection may cause marked mucus hypersecretion with dose dependence and that goblet cell hyperplasia was found at 1 wpi and persisted until 5 wpi, and then had lessened by 13 wpi. Meanwhile, a significant dose-dependent increase in collagen deposition was also observed. Goblet cell hyperplasia and deposition of

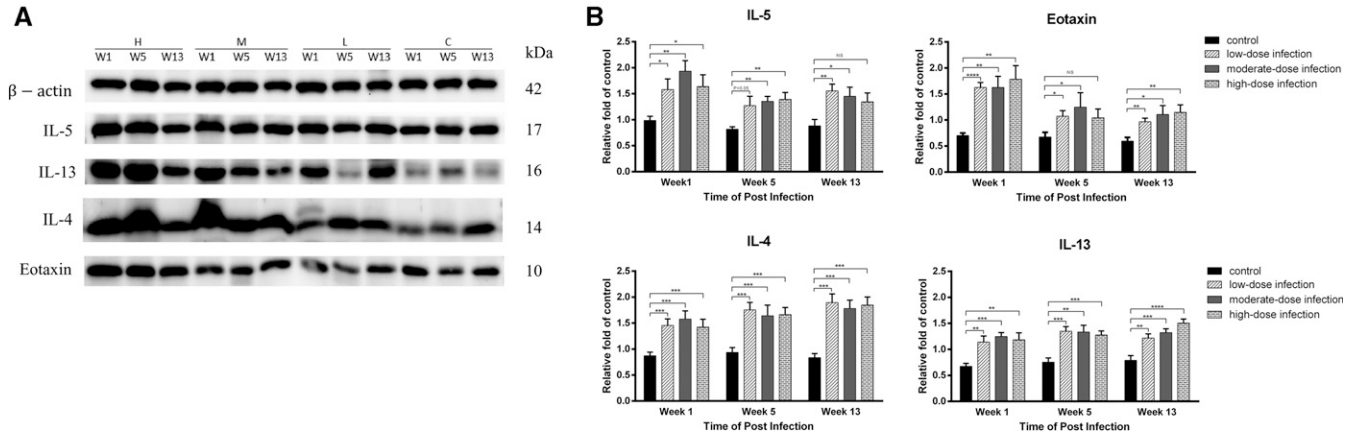

FIGURE 5. Type 2 T-helper-type cytokine expressions, including interleukin (IL)-4, IL-5, IL-13, and eotaxin, increased in *Toxocara canis*–infected mice inoculated with low-, moderate-, and high-dose infections at 1, 5, and 13 weeks postinfection. (**A**) Type 2 T-helper-type cytokine accumulation in lung tissues was analyzed by Western blotting. (**B**) Quantification of cytokine accumulation. Results are expressed as the mean ± standard error of the mean (n = 8 mice in the control group and n = 8 mice in all three experimental groups) of relative multiples of the control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ (t-test) vs. the control group. NS = no significant difference.

extracellular matrix, such as collagen, in the subepithelial layer resulted in airway thickening, which is a characteristic of airway remodeling and which can cause irreversible airflow limitations and an increase in AHR that may develop into asthma.[51,52]

Remarkably, the high number of macrophages probably represented signs of severe AHR emergence at 13 wpi. Three types of macrophages have been identified in lung tissues, including bronchial macrophages, AMs, and interstitial macrophages. Alveolar macrophages, one of the prominent immune system cells found in the airways, are involved in the development and progression of asthma.[53] Two such subpopulations are currently known as M1 and M2 subsets. M1 cells are major macrophages in nonallergic asthma, and exhibit high levels of pro-inflammatory cytokines, whereas M2 cells predominate in allergic asthma and exhibit high levels of Th2-type cytokines.[54] The M2a subtype is induced by IL-4 and IL-13, facilitates the packaging and killing of parasites, and worsens AHR and inflammation in asthmatic mice.[55] However, it is unclear which type of macrophage is predominately involved in this event, which needs further clarification. Notably, pulmonary fibrosis (PF) and asthma are two different respiratory diseases, and they often share the same symptoms, such as difficulty breathing and shortness of breath. Asthma does not cause PF, but PF can be difficult to identify and is sometimes misdiagnosed. Asthma is a bronchial disease that tightens and restricts bronchial muscles, leading to shortness of breath, whereas PF is a disease of tissues inside the lungs and is characterized by scarring.[55] Therefore, it is worth exploring the effects of *T. canis* infection in promoting PF induced by Th2 responses in future studies.

Taken together, in our study, the four main processes responsible for the clinical features of asthma were observed in *T. canis*–infected mice, including airway inflammation, mucus hypersecretion, airway remodeling, and AHR. The migration of larvae through different organs, including the lungs, resulted in airway inflammation and AHR. Murine models have proven to be very valuable for investigating possible factors that contribute to the observed association between these disorders.

In conclusion, *T. canis* infection led to an exacerbation of dose-dependent allergic airway inflammation, airway remodeling, and AHR due to a dominant Th2 type of immune response, depending on the time of inoculation. Our study provides a novel suggestion of disease mechanisms and a correlation between chronic *T. canis* infection and asthma progression.

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