The Immune Response to Toxocariasis Does Not Modify Susceptibility to Mycobacterium tuberculosis Infection in BALB/c Mice

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Abstract. Mycobacterium tuberculosis and helminth infections coincide geographically and are classically described as TH1 and TH2 pathologies. There is much interest in exploring how concurrent worm infections might alter immune responses to mycobacterial infection. To explore this issue, mice were infected with Toxocara canis and co-infected with M. tuberculosis. Mice infected with M. tuberculosis had high numbers of neutrophils and mononuclear cells within the alveolar spaces, with increased parenchymal interferon (IFN)-γ levels. However, in toxocara-infected mice we detected increased eosinophil numbers in bronchoalveolar lavage fluid (BALF) and increased parenchymal levels of interleukin (IL)-5. In co-infected mice the BALF demonstrated enhanced eosinophil influx with decreased neutrophil and mononuclear cell accumulation. However, co-infected mice had similar mycobacterial proliferation in their lungs accompanied by similar histopathological changes and similar cytokine/nitric oxide production compared with Mycobacterium only—infected mice. Our results suggest that T. canis infection does not necessarily lead to increased susceptibility to pulmonary tuberculosis.

INTRODUCTION

Helminth parasites cause a significant burden of illness worldwide, with estimates of two billion people infected. The largest proportions of human populations infected live in tropical and subtropical regions. Helminth infections are potent inducers of TH2-type responses in both humans and experimental models, which are characterized by eosinophilia, high titers of circulating IgE, enhanced TH2 cytokine profile (e.g., increased secretion of interleukin [IL]-4 and IL-5), and reduced TH1 type cytokines (e.g., interferon [IFN]-γ). Studies conducted in animal models of TH1-inducing pathogens and/or human preclinical tests of certain vaccines revealed that the potency of TH2 polarization induced by helminthic parasites impaired subsequent TH1 immune responses.

To improve therapies and vaccination protocols against tuberculosis, it is important to investigate the influence of coinfection with different worms on the immune response against mycobacterial pathogens. Little is known about the interaction between toxocariasis, caused by the helminths Toxocara canis or Toxocara cati, and tuberculosis along the TH1/TH2 paradigm described above. However, toxocariasis is an important parasitic infection in tropical and subtropical regions that induces robust TH2 responses in humans. We hypothesized that prior T. canis infection would modify the immune response pattern (TH1 versus TH2) and susceptibility to Mycobacterium tuberculosis (Mtbc) infection. We found that infection of BALB/c mice with T. canis elicited a TH2 response, but it did not significantly alter the TH1 immune response (or susceptibility) to subsequent infection with M. tuberculosis.

MATERIALS AND METHODS

Animals. Specific pathogen-free female 6-week-old BALB/c mice were obtained from the animal facilities of Faculdade de Ciências Farmacêuticas—Universidade de São Paulo and bred in a SPF facility. All experiments were approved and conducted in accordance with the guidelines of the Animal Care Committee of the University. Infected animals were kept in biohazard animal room (Laboratory Biosafety Level 3) and housed in cages within a laminar-flow safety enclosure.

Experimental infection. T. canis helminth eggs were obtained by the method of Olson and Schulz, as modified by Faccioli and others. In brief, pregnant female worms were recovered from dogs. The eggs were then rescued from the worm uterus, washed, and sustained in 0.5% formalin at 37°C in shallow dishes, where they were allowed to develop into an infective stage. Before being used, the eggs were thoroughly washed with saline. Infective doses of 500 embryonated eggs in 0.5 mL of saline were prepared. Mice were infected by gastric intubation via a metal cannula. Control group animals received 0.5 mL of saline only.

M. tuberculosis. The H37Rv strain of M. tuberculosis (American Type Culture Collection, Rockville, MD) was grown in 7H9 Middlebrook broth (Difco Laboratories, Detroit, MI) for 7 days. The culture was harvested by centrifugation, and the cell pellet was resuspended in sterile phosphate-buffered saline (PBS) and vigorously agitated. The homogeneous suspension was filtered through 2-μm filters (Millipore, Bedford, MA). Viability of the M. tuberculosis suspension was pretested with fluorescein diacetate (Sigma, St. Louis, MO) and ethidium bromide. An anterior midline
incision was made to expose the trachea. A 30-gauge needle attached to a tuberculin syringe was inserted into the trachea, and intratracheal dispersion was used to introduce 10⁵ viable colony-forming units (CFU) of M. tuberculosis H37Rv in 100 μL of PBS into the lungs. At 30 and 70 days after the M. tuberculosis challenge, mice from all groups were killed. Control mice received intratracheal PBS only. In the co-infected group, infection with intratracheal M. tuberculosis was performed 18 days after the T. canis infection, the time at which the helminth induces a strong TH2 response.¹⁵

Bronchoalveolar lavage fluid (BALF). On days 30 and 70 following M. tuberculosis infection, animals were euthanized with sodium pentobarbital. The anterior chest cavity of each animal was carefully opened, and the trachea was exposed and catheterized. The catheter was tied in place, and sterile PBS was infused in three 1-mL aliquots. Lavage fluid was recovered and placed on ice. Total cell counts were immediately performed in a Neubauer Chamber (Boeco, Hamburg, Germany). Differential counts were obtained using Rosenthal-stained cytospin preparations.¹⁶ Lavages were performed prior to removal of lungs for microbiological and histologic analyses (below), and spleens were removed aseptically from the same mice.

Determination of M. tuberculosis CFU in lungs. Recovery of M. tuberculosis was performed as described previously. Briefly, the number of live bacteria recovered from the lungs was determined as CFU by plating 10-fold serial dilutions of homogenized tissue on Middlebrook 7H11 agar (Difco) and counting colonies after 28 days at 37°C (expressed as log₂₁₀ CFU/g of lung tissue).

Measurement of cytokines and nitrite in lung tissues. For cytokine measurements, lungs were removed on days 30 and 70 post-M. tuberculosis infection. Tissue was homogenized in 2 mL of RPMI 1640 and centrifuged at 450g, and the supernatant was stored at −70°C until assayed. Commercially available enzyme-linked immunosorbent assay (ELISA) antibodies were used to measure IL-5, IL-10, IL-12, and IFN-γ (OptEIA, BD-Pharmingen, San Diego, CA). The plates were coated with 100 μL/well of the capture antibody (1–4 μg/mL) diluted in coating buffer (0.1 M sodium carbonate, pH 9.5) and incubated overnight at 4°C. Plates were then washed 5 times with 300 μL/well of wash buffer (PBS with 0.05% Tween-20), and nonspecific binding was blocked by addition of 200 μL/well assay diluent (PBS with 10% fetal bovine serum, pH 7.0) and incubated at room temperature (RT) for 1 hr. After the plate was washed as described above, 100 μL of each standard, sample, and control was added to appropriate wells followed by a 2-hr incubation at RT. The plates were again washed as above, and 100 μL of working detector solution [biotinylated detection antibody (0.5–2.0 μg/mL) + streptavidin-conjugated horseradish peroxidase reagent] was added to each well with a 1-hr incubation at RT. After 7 total washes, we added 100 μL of substrate solution (TMB substrate reagent set) to each well and incubated the plates for 30 min at RT in the dark. The reaction was stopped by addition of 50 μL of stop solution (2 N H₂SO₄), and the optical density was measured at 450 nm within 30 min.

Nitric oxide (NO) production was assessed by measuring the amount of nitrite in lung homogenates by the Greiss method.¹⁸ Data are presented as micromoles of NO₃⁻.

Spleen cell cultures and cytokine determination. Spleens from mice killed 30 or 70 days post-M. tuberculosis infection were aseptically removed and minced, and the released cells were washed three times in RPMI 1640 (Gibco BRL, Grand Island, NY). Cells were suspended at 5 × 10⁶ cells per mL in RPMI supplemented with 10% fetal bovine serum (Gibco BRL), penicillin (100 U/mL, Gibco BRL), and streptomycin (100 μg/mL, Gibco BRL) and dispensed into 96-well flat-bottom microtiter plates in a volume of 0.1 mL. Concanaavalin A (2 μg/mL, Sigma) and 10 μg/mL of total proteins isolated from T. canis or heat-killed M. tuberculosis 10⁵ bacilli/mL were added to wells (0.1 mL) in triplicate and maintained for 48 hr at 37°C. Heat-killed preparations of M. tuberculosis strain H37Rv (HK-Mtb) were obtained by killing the bacilli at 80°C for 2 hr. Commercially available ELISA antibodies were used to measure IFN-γ and IL-5 (OptEIA, BD-Pharmingen) on supernatants of cultured cells as above.

Preparation of T. canis total antigen. To prepare a total protein lysate from T. canis eggs, a suspension of larvae eggs obtained as described above was washed five times with sterile PBS.¹⁹ The suspension was sonicated by a sonic dismem-
Toxocariasis does not interfere with experimental tuberculosis

**Figure 1.** Number of colony forming units (CFU) of *M. tuberculosis* in the lungs. Co-infection with *T. canis* was performed 18 days before intratracheal inoculation with *M. tuberculosis* and the numbers of bacilli in the lungs were determined at 30 (○) and 70 days (■) post-bacterial challenge. Bars represent the arithmetic means.

Figure 2 represents lung histology at 30 days post-*M. tuberculosis* infection. Histologic sections of H&E-stained lungs from co-infected and *M. tuberculosis*-infected mice were characterized predominantly by the presence of macrophages and lymphocytes accumulated mainly in perivascular and peribronchial areas (Figure 2A). By ZN staining for acid-fast bacilli, there were no obvious differences in the bacillary loads between lung sections of co-infected and *M. tuberculosis*-infected mice (Figure 2B).

**Previous *T. canis* infection modifies leukocyte recruitment induced by *M. tuberculosis*.** Intratracheal inoculation of *M. tuberculosis* induced significant neutrophil and mononuclear leukocyte accumulation in the lungs at 30 and 70 days post-infection, as judged by BALF, while *T. canis* infection induced predominantly eosinophils. No such cellular accumulation occurred in the control-uninfected group (Figure 3). Also, we compared the cellular accumulation between co-infected mice and animals only infected with *M. tuberculosis*. Co-infection elicited significantly fewer neutrophils and mononuclear cells at both 30 and 70 days after infection compared with singular *M. tuberculosis* inoculation. However, at these time points, eosinophil numbers in the bronchoalveolar space of co-infected animals were significantly greater than those of *M. tuberculosis*-infected animals and were similar to the eosinophil counts observed in *T. canis*-infected mice (Figure 3).

**T. canis infection does not alter cytokine or nitric oxide production induced in lung cells by *M. tuberculosis*.** Because proinflammatory and TH1 cytokines play important roles in the immune response against *M. tuberculosis*, we sought to investigate whether co-infection could alter cytokine production in the lung parenchyma. At 30 and 70 days post-*M. tuberculosis* infection, lung cells were obtained from animals that were either co-infected or singly infected (*M. tuberculosis* or *T. canis* only). In *M. tuberculosis*-infected animals, IL-12 and IFN-γ levels in the lungs increased at the two analyzed periods, with highest levels being observed at day 30. In the co-infected animals, the levels of IL-12 and IFN-γ were similar to *M. tuberculosis*-infected animals (Figure 4A and B) at both time points. No such increases in IL-12 or IFN-γ levels were observed in *T. canis*-infected mice above the control group. Levels of IL-10 were similar in *T. canis* and co-infected animals and greater than PBS-inoculated animals, whereas IL-5 increased only at day 30 in the *T. canis*-infected group (Figure 4C and D).

As nitric oxide (NO\(^{-}\)) is an important microbicidal factor, we analyzed its production within the lung parenchyma. *M. tuberculosis*-infected mice produced increased levels of NO\(^{-}\) compared with cells from control animals. Cells obtained from co-infected animals showed similar NO\(^{-}\) production compared with *M. tuberculosis*-infected animals, and the lung cells from *T. canis*-infected mice did not produce NO\(^{-}\) above the levels found in control mice inoculated with PBS (Figure 4E).

**Immune responses to *M. tuberculosis* in the spleen are not switched by co-infection.** We were interested in characterizing the TH1 versus TH2 immune responses to either *M. tuberculosis* or *T. canis* by isolated splenocytes, and testing the hypothesis that prior *T. canis* infection per se can modulate the TH profile in response to *M. tuberculosis* antigens. Therefore, spleen cells were collected from either Mtb-infected or co-infected mice and then stimulated in vitro with either heat-killed *M. tuberculosis* (HK-Mtb), or total proteins from infective eggs of *T. canis* containing L3 larvae (TP-Tc), or the nonspecific stimulus concanavalin-A (Con-A). We observed similar immune response profiles at days 30 and 70 post-infection, and Figure 5 represents the data from day 30. Spleen cells from *M. tuberculosis*-infected mice stimulated in vitro with HK-Mtb or Con-A produced high levels of IFN-γ, indicative of a TH1 response. These cells produced lower amounts of IFN-γ when stimulated in vitro with TP-Tc, when compared with other stimuli. Cells from *T. canis*-infected mice did not produce IFN-γ after in vitro stimulation. Cells from co-infected mice produced significantly more IFN-γ compared with cells from uninfected animals when stimulated with HK-Mtb or Con-A, but this increase was not as robust as we observed in splenocytes from *Mycobacterium*--only infected mice. We also observed that spleen cells from *T. canis*-infected and co-infected mice both produced high levels of IL-5 when stimulated with TP-Tc or Con-A, suggesting a TH2 immune profile (Figure 5).

**DISCUSSION**

Toxocariasis is an important helminth pathogen in developing countries\(^{20,21}\) including Brazil, where the prevalence rates reach ~40%.\(^{22,23}\) Tuberculosis remains one of the leading causes of morbidity and mortality in many settings, particularly in the world’s poorest countries. It is estimated that nearly 1.7 million people die each year from tuberculosis, and in 2004 approximately 8.9 million people developed the disease.\(^{24}\) Because mycobacterial and helminth pathogens are coendemic and tend to induce opposing immune responses, we investigated the pattern of inflammatory and immune response in a model of *T. canis* and *M. tuberculosis* co-infection. In this report, we studied the effect of previous *T. canis* infection on the susceptibility of BALB/c mice to subsequent *M. tuberculosis* infection, by determining the bacterial load, the degree of lung pathology, and the in vivo and in vitro...
TH1/TH2-type responses of splenic lymphocytes (IFN-γ and IL-5 synthesis, respectively) to mycobacteria and *T. canis* egg antigens.

Our study was limited by the use of a single, BALB/c, murine strain. We have chosen this strain because it is relatively resistant to lethality from *M. tuberculosis*,\(^\text{25,26}\) facilitating long-term studies of systemic and tissue immune responses. Because BALB/c mice are prone to developing TH2-type immune responses to infection,\(^\text{27}\) it is possible that the use of other mouse strains might yield different results than the present report. Furthermore, for our *in vivo* infections we used a single inoculum size for both *T. canis* and *M. tuberculosis*. We selected these infecting doses based on previously published protocols\(^\text{14,28}\) with the intention of causing sublethal infections. Whether different inoculum sizes would yield contrary results remains uncertain.

We believe that the present study for the first time demonstrates that mice infected with *T. canis* are not more susceptible to subsequent *M. tuberculosis* infection. Co-infection did not increase bacterial propagation in vivo and was associated with the development of IFN-γ producing TH1 cells, similar to mice infected only with *M. tuberculosis*. Our data agree with literature showing that the rodent hookworm *Nippostrongylus brasiliensis* did not lead to enhanced susceptibility to mycobacterial infection.\(^\text{29}\) However, Elias and colleagues\(^\text{30}\) demonstrated that *Schistosoma mansoni* infection altered the immune response pattern to *M. tuberculosis*, leading to an increased bacterial burden during infection. These experimental differences highlight the complex interactions among various parasitic organisms, mycobacteria, and infected hosts.

Although *T. canis* infection did not modify host susceptibility to tuberculosis infection, we postulated that immune responses characteristic of tuberculosis infection might be al-
Infection lead to increased neutrophil and this lung compartment was chosen for as-

Moreover, in vitro and co-infected animals (Figure 2).

Our results demonstrated that co-
fected mice, in comparison with mice with only M. tuberculosis (Figure 4). The importance of NO−3, IL-12, and IFN-γ has been well documented in murine tuberculosis.32–34 IFN-γ is a central factor in the activation of anticytobacterially activities of macrophages and is considered crucial for protection against tuberculosis.35 Nitric oxide is an effective host-defense mechanism against many microbial pathogens and plays an essential role in the killing of M. tuberculosis by mononuclear phagocytes.36 As there is evidence that leuko-
cytes present in the lung parenchyma might be the source of such mediators,37 this lung compartment was chosen for assessing immune responses during infection, to complement our studies of cellular BALF components. Additionally, Franke-Ullmann and others38 have shown that interstitial macrophages are more efficient in releasing immunoregulatory cytokines such as IL-1 and IL-6, expressing MHC class II molecules, and are more effective in functioning as accessory cells for mitogen-stimulated lymphocyte proliferation compared with alveolar macrophages. Therefore, we suggest that cells from either Mycobacterium-infected mice or co-infected mice are equipped to cooperate with interstitial lymphocytes in inducing a specific TH1-immune reaction, independent of the presence of T. canis.

To perform in vitro analyses of immune responses, we stimulated spleen cells with whole T. canis eggs as well as mycobacterial lysates. This approach, in contrast to utilizing recombinant or highly purified proteins/antigens, mimics in vivo infection and allows for a broad array of mycobacterial lysates to stimulate cells of the innate and adaptive immune system, including superantigens, Toll-like receptor ligands, and nonprotein signature molecules.3,29 The cell-wall component lipoarabinomannan from M. tuberculosis, for example, is involved in the inhibition of phagosome maturation, apoptosis, and IFN-γ signaling in macrophages and IL-12 secretion of dendritic cells.40 Moreover, complex carbohydrates present in worm antigens are potent inducers of TH2 responses by binding to cell-surface receptors, such as DCSIGN, L-SIGN, the mannose receptor, macrogalactose binding lectin, and other lectins, which might contribute to the induction of TH2-associated adaptive responses.41

Our in vitro data, obtained by using spleen cells collected 30 (and 70) days after M. tuberculosis infection and stimulated with or without whole immunogens, support our in vivo results obtained in lung tissue at 30 and 70 days post-infection (Figure 5). The M. tuberculosis infection stimulated an im-

Figure 3. Alveolar leukocyte numbers during early (C) and late (D) phases of infection by M. tuberculosis. BALF cells were obtained from mice after intratracheal injection of PBS; oral infection with T. canis (Tc), and intratracheal infection with M. tuberculosis (Mtb) or both (Tc/Mtb). (A) Mononuclear cells; (B) neutrophils; (C) eosino-

phils. Cells were enumerated and identified after Rosenfeld staining. *PBS vs. other groups; "Tc vs. other groups; "Mtb vs. other groups. P < 0.05.
mune response in the lungs and spleen toward a TH1 profile, as indicated by our finding that cells from Mtb-only infected mice produced higher levels of IFN-γ and lower levels of IL-5 in response to HK-Mtb (or Con-A) observed in splenocytes obtained from co-infected mice was significantly greater than splenocytes from un-infected mice. Although this was qualitatively similar to our observation in cells from Mtb-only infected animals, the absolute increase in IFN-γ was lower in co-infected than Mtb-only infected splenocytes (Figure 5A). These data suggest that prior T. canis infection can partially suppress TH1 responses during subsequent Mtb infection, but this influence was insufficient to alter the pathologic course of the second infection. Furthermore, cells from T. canis or co-infected mice responded in vitro to specific T. canis antigens, as judged by similarly high levels of IL-5 produced. These results indicate that subsequent M. tuberculosis infection (after 18 days) did not interfere the pre-established TH2 response in the spleen. Moreover, TH2 cells present in the lung of co-infected animals were unable to deviate the TH1 immune response against Mtb infection. Our data, in agreement with that of Erb and others,29 show that prior sensitization of the immune system by helminths does not necessarily influence subsequent responses to unrelated pathogens, as was demonstrated by other authors.42–47 It has recently become apparent that regulatory T cells (Treg) might be important in directing immunologic responses to pathogens,48,49 and Oldenhove and others50 clearly demonstrated that enhanced Treg function associated with helminth infection may suppress TH1 responses directed against unrelated antigens and/or pathogens. It seems that uncontrolled TH2 immune responses induced by certain helminthic infections provide a propitious microenvironment for enhanced mycobacterial growth, which was not observed in our model. We suggest that the immune response induced by toxocarasis, at least in the period analyzed by us, modifies the homeostasis of the immune system but not to such an extent that it impairs the host’s ability to mount an effective TH1 response against M. tuberculosis. Taken together, we propose that the impact of helminthiasis on the host response to intracellular pathogens is not yet clear and is dependent on the nature of the parasite. It is essential to be evaluating the reasons that some helminth infections modify the immune response to tuberculosis while others do not, which might clarify the regulatory mechanisms involved during concurrent infections.
infection increases susceptibility to M. tuberculosis infection among aboriginal populations. 

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Figure 5. Cytokine levels in the supernatant of spleen cells harvested 30 days after infection with M. tuberculosis. Mice were subjected to either intratracheal PBS injection; oral infection with T. canis (Tc); intratracheal infection with M. tuberculosis (Mtbo); or both (Tc+Mtbo). The cells were stimulated in culture with total Tc antigens (V) or heat-killed TB (U) or IFN-γ (A) and IL-5 (B) levels of culture supernatant were determined by ELISA. Bar graphs represent the average. *PBS vs. other groups; **Tc vs. other groups. P < 0.05.

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