EFFECT OF HOST IMMUNITY AND CO-INFECTION ON LARVAL TOXOCARIASIS

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Toxocariasis and toxoplasmosis are two of the most common zoonotic parasites of people worldwide, causing ocular and neurological disease. The probability of being infected with both pathogens is increased due to shared modes of transmission. These parasites behave the same way in all paratenic hosts, which include mice and people, and so mice represent an excellent model for investigating human infection. During secondary infection of mice with *Toxocara canis*, many larvae become trapped in the host's liver, preventing onward migration to other body tissues such as the eye. This dissertation aimed to elucidate the immunomodulatory effect of *Toxoplasma gondii* infection on *T. canis* migratory arrest by first identifying the adaptive immune cells and humoral components contributing to trapping and then the changes elicited by co-infection.

In my research, I show that CD4+ cells are critical for directing the generation of antigen-specific antibodies during primary *T. canis* infection, but they play no direct role in larval trapping during secondary infection. Passive immunization using purified IgG from infected mice enables naïve recipients to trap larvae in the liver, suggesting humoral immunity is the key player.

I then explored the effect of concomitant infection with *T. gondii* on *T. canis* migration. Strikingly, I show that mice pre-infected with *T. gondii* trap far fewer larvae than mice infected only with *T. canis*. This corresponds to a partial shift from IgG1 antibody production to the

IgG2a isotype. Reciprocal modulation by *T. canis* of cell-mediated immunity to *T. gondii* is also observed, as evidenced by a reduction in the abundance of activated CD8+ T lymphocytes in co-infected mice compared to *T. gondii* mono-infected mice.

Finally, I examined factors with potential influence on *T. canis* migration. I show that male BALB/c mice have an enhanced ability to trap larvae relative to females. In contrast, no difference in larval distribution or viability is detected in eosinophil-deficient dblGATA1-/- mice on a C57BL/6 background compared to wild type.

This dissertation provides insight into host immunity against larval toxocariasis and the immune cross-regulation induced by two parasites inhabiting the same host.

BIOGRAPHICAL SKETCH

Che Yu (better known as Alice) was born on September 13th, 1982 in Taipei, Taiwan to Hsueh Hsi and Jui Lan Lee. She spent the first few years of her life there, following her dad to his construction sites, visiting frequently with her grandparents, and taking road trips with her parents and two older sisters. In 1989, she and her family immigrated to Toronto, Canada, where her father hoped his daughters would find a better life and be treated as equals to males. Alice grew up playing flute in the school band and being a member of the volleyball team. She was always drawn to science because it explained so much about the world around her, and she had a particular interest in animals because they're so wonderfully adapted to their environment and lifestyle. This led to her studying biology at Queen's University in Kingston, Ontario, before earning her DVM from the Ontario Veterinary College at the University of Guelph in 2006. After a one-year internship in small animal medicine and surgery at a private practice in Los Angeles, Alice took a research position at Cornell University working on a project aimed at saving dogs' lives within the parasiticide industry. There, under the mentorship of Dr. Dwight Bowman, she fell in love with the world of veterinary parasitology and she hasn't looked back since. She decided to stay on at Cornell to pursue a PhD, studying two zoonotic parasites and their immune interactions during concurrent infection. Alice completed a clinical residency in veterinary parasitology through the National Center for Veterinary Parasitology. She became board certified by the American College of Veterinary Microbiology in the specialty of parasitology in 2015. In her spare time, Alice enjoys reading, traveling, spending time with friends, and recreational sports.

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I also want to thank my special committee members, Dr. Avery August, Dr. Kenneth Simpson, and my field-appointed member Dr. Bettina Wagner. They have not only provided intellectual input on my work but given me helpful advice on life, jobs, and science.

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Last but not least, I want to express my appreciation for my family, whose enduring love, support, and confidence in me have brought me to where I am today.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAM – Alternatively activated macrophage

ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

ACK – Ammonium chloride potassium

ADCC – Antibody-dependent cell-mediated cytotoxicity

AIDS – Acquired immunodeficiency syndrome

BSA – Bovine serum albumin

CD – Cluster of differentiation

CDC – Centers for Disease Control and Prevention

cDMEM – Complete Dulbecco's modified Eagle's medium

CO – Co-infection

COK – Co-infection high-dose

DOI – Digital object identifier

ELISA – Enzyme-linked immunosorbent assay

FcγR – Fragment crystallizable gamma receptor

Foxp3 – Forkhead box P3

F/T – Flowthrough

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IFN – Interferon

Ig – Immunoglobulin

IL – Interleukin

IP – Intraperitoneal

IV – Intravenous

MHC – Major histocompatibility complex

N – Normal

NK – Natural killer

OD – Optical density

OT – Ocular toxocariasis

PBS – Phosphate buffered saline

PMA – Phorbol myristate acetate

PO – Per os

SQ – Subcutaneous

Tc – *Toxocara canis*

TCC – *Toxocara canis* control

TES – *Toxocara canis* excretory-secretory antigen

TGC – *Toxoplasma gondii* control

Th1 – T helper type 1

Th2 – T helper type 2

TNFα – Tumor necrosis factor alpha

Treg – T regulatory

USA – United States of America

USDA-ARS – United States Department of Agriculture, Agricultural Research Service

LIST OF SYMBOLS

α – alpha

β – beta

γ – gamma

ε – epsilon

μ – micro

° – degrees

CHAPTER 1

INTRODUCTION: TOXOCARIASIS AND TOXOPLASMOSIS – COMMON NEGLECTED PARASITIC INFECTIONS IN THE USA

Human toxocariasis

Toxocariasis is the infection of people by larvae of the genus *Toxocara*. Canids are the definitive host for *Toxocara canis*, a very common intestinal nematode of dogs. Eggs laid by adult worms are shed in the dog's feces and, depending on environmental conditions, take up to 4 weeks to develop into the infectious third-stage larva within the egg shell.[1] Eggs remain viable for years in the environment.[1] Humans are infected through ingestion of soil or food contaminated with these larvated eggs, or through ingestion of larvae in the tissues of a paratenic host (i.e., an animal in which the parasite undergoes neither development nor sexual reproduction).[2–8] Such hosts include earthworms, rodents, rabbits, birds, cattle, as well as people.[2,8] Once ingested by a vertebrate paratenic host, *T. canis* larvae penetrate the intestinal mucosa and migrate first to the liver and then onward to the lungs and heart, from which they are disseminated systemically to other tissues; predilection sites include the brain and skeletal muscle (Figure 1.1).[9–11] Larvae do not multiply within host tissues, but they remain viable for years (10 years in experimentally infected monkeys) and will complete their development to adulthood if eventually ingested by the definitive host.[12,13]

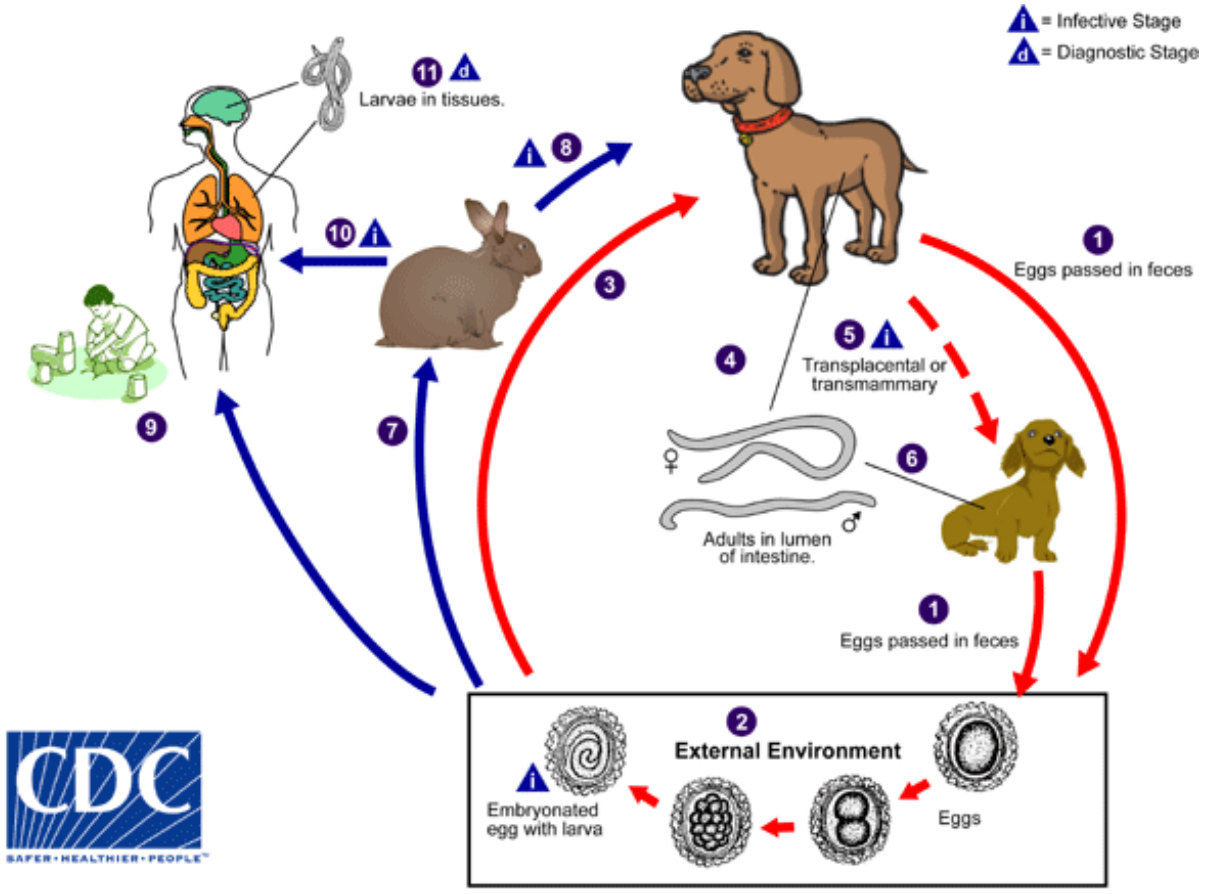
= Infective Stage
= Diagnostic Stage
11
Larvae in tissues.
8
10
1
Eggs passed in feces
3
4
5
Transplacental or transmammary
6
Adults in lumen of intestine.
9
7
1
Eggs passed in feces
2
External Environment
Embryonated egg with larva
Eggs
CDC
SAFER·HEALTHIER·PEOPLE™

Figure 1.1. *T. canis* life cycle. *Toxocara canis* accomplishes its life cycle in dogs, with humans acquiring the infection as accidental hosts. Unembryonated eggs are shed in the feces of the definitive host ❶. Eggs embryonate and become infective in the environment ❷. Following ingestion by dogs ❸, the infective eggs hatch and larvae penetrate the gut wall. In younger dogs, the larvae migrate through the lungs, bronchial tree, and esophagus; adult worms develop and oviposit in the small intestine ❹. In older dogs, patent infections can also occur, but larval encystment in tissues is more common. Encysted stages are reactivated in female dogs during late pregnancy and infect by the transplacental and transmammary routes the puppies ❺, in whose small intestine adult worms become established ❻. Puppies are a major source of environmental egg contamination. *Toxocara canis* can also be transmitted through ingestion of paratenic hosts: eggs ingested by small mammals (e.g. rabbits) hatch and larvae penetrate the gut wall and migrate into various tissues where they encyst ❼. The life cycle is completed when dogs eat these hosts ❽ and the larvae develop into egg-laying adult worms in the small intestine. Humans are accidental hosts who become infected by ingesting infective eggs in contaminated soil ❾ or infected paratenic hosts ❿. After ingestion, the eggs hatch and larvae penetrate the intestinal wall and are carried by the circulation to a wide variety of tissues (liver, heart, lungs, brain, muscle, eyes) ⓫. [Figure and caption from CDC website.]

Recent evidence suggests that toxocariasis is now the most common parasitic helminth infection of people in the United States.[14] The Centers for Disease Control and Prevention (CDC) consider toxocariasis one of the top 5 neglected parasitic infections in the country based on the number of people affected, severity of disease, and opportunity for intervention. Seroprevalence of anti-*Toxocara* antibodies shows that 13.9% of the population, or roughly 45 million Americans, are infected.[15] Prevalence is even higher in middle-income countries such as Indonesia and Brazil, where rates of 36% or more have been reported.[14,16] Larval migration in people has been associated with various clinical syndromes: visceral toxocariasis can cause wheezing, fever, and hepatomegaly; ocular toxocariasis (OT) can lead to permanent vision loss; and covert toxocariasis, the most common manifestation, results in symptoms such as cough, abdominal pain, and headache.[2,7,8,17] Children are most frequently affected due to their higher exposure to contaminated playgrounds or sandboxes and their poor hygienic habits.[2,18] Adults are affected to a lesser extent, with the majority of reports linked to consumption of raw organ meats.[4–7,19] Infection intensity will vary depending on local environmental contamination and dietary habits, but larval burdens in tissues can be significant. A pediatric patient with visceral toxocariasis was found to have 60 *T. canis* larvae per gram of liver tissue and 5 per gram of muscle; the number in the brain was estimated at 3-5 larvae per gram.[20]

The true incidence of clinical toxocariasis is not well known.[21] Large-scale reports are available only for OT, and these are all based on voluntary surveys sent to ophthalmic specialists. One estimate of annual OT cases in Alabama placed the incidence at 1 per 1000 persons.[22] A more recent national survey identified 159 OT patients within a year-long period from 2009 to 2010, with 68 of these being newly diagnosed cases.[18,23] These numbers are suspected to be an underrepresentation of the true burden of toxocariasis in the USA.[18,23]

Globally, from the period of 1990 to 2012, 368 cases of OT were reported.[24] Cases of toxocariasis with central nervous system involvement numbered 86 worldwide during the period of 1985-2014.[24] For the majority of people infected by *T. canis*, no symptoms are apparent.[2] Yet even in clinically unaffected individuals, the presence of these long-lived larvae seems likely to exert some degree of chronic influence on the host's immune system. Because mice and people are both natural paratenic hosts for *T. canis* with similar pathogenesis, the mouse is a good animal model for studying the immune response to chronic *T. canis* infection.[16]

T. canis larvae in the liver

A common occurrence during *T. canis* infection is the development of granulomas in the liver. Granulomas form around larvae, arising initially from infiltrating neutrophils and eosinophils in the first week of infection.[25] Fibrotic encapsulation begins in the third week, and by 5-6 weeks the larva is surrounded by macrophages and eosinophils, all encompassed by a thick collagenous capsule.[16,25] Larvae encapsulated during a primary infection are postulated to be able to escape as larva-free granulomas have been observed; however, larvae encapsulated during a secondary infection appear to be immobilized based on their histological appearance.[26] Additional evidence of migratory arrest comes from examination of larval distribution in the organs after repeat infection. The larval count from livers of mice that were first sensitized by infection with *T. canis* then challenged with a second dose was more than three times higher than the combined count of mice receiving just the sensitizing or challenge dose.[27] Larvae thus "trapped" during a challenge infection are not simply delayed in their migration to other tissues but are in fact retained in the liver until at least 16 weeks post-challenge.[27] This trapping

phenomenon has been observed in several strains of laboratory mice, including the BALB/c strain.[28,29]

A dose-dependent relationship has been observed between the number of *T. canis* eggs administered to a mouse in the sensitizing infection and the number of larvae trapped in the liver upon challenge infection. To induce trapping, a dose of at least 25 eggs must be used for sensitization.[28] The timing of the infections is also critical. Waiting 7 weeks as opposed to a mere 2 weeks before challenging sensitized mice results in three times more larvae being arrested in the liver.[30] There is evidence to suggest that liver trapping confers a protective advantage to the host. Compared with control mice that received a single inoculation with *T. canis*, mice that were doubly infected had markedly more larvae in their livers but no significant rise in larval number in their brains.[26,31] Thus a host's ability to stop larvae from migrating out of the liver and into other tissues may help to prevent physical and immunopathologic damage to important organs such as the brain and eyes.

Several studies have investigated the host immune response to *T. canis* but without conclusive results in terms of its role in liver trapping. Passive transfer of 1.5 mL of serum from donor mice that had received two doses of *T. canis* resulted in liver trapping in the naïve recipient mice during a primary challenge.[30] In contrast, mice immunized with serum from naïve donors or donors infected only once with *T. canis* had significantly fewer larvae in the liver.[30] A similar pattern was seen in a separate experiment, though the difference was not significant – perhaps due to the lower volume of serum transferred (0.125 mL/15g body weight).[32] In the latter experiment, mesenteric lymph node cells were also adoptively transferred, either alone or in combination with serum. In both of these cases, the number of larvae in the liver was decreased compared to recipient mice given cells +/- serum from uninfected donors. This may

indicate that cell-mediated and humoral immunity act antagonistically against *Toxocara* larvae in certain tissues. The mitogenic response of splenocytes from infected mice has also been examined. Unstimulated splenocytes from infected mice spontaneously incorporated more tritiated thymidine in culture than did those of uninfected mice.[31,33] Splenocytes harvested in the first 2-3 weeks post-infection showed an initial depression in blastogenic response when stimulated with either concanavalin A or *Toxocara* larval antigen, but rebounded at later time points, especially after secondary infection.[31] To determine whether T lymphocytes induced larval trapping in the liver, Sugane and Oshima infected athymic nude mice (BALB/c-nu/nu) with two doses of *T. canis*, and used BALB/c-nu/+ mice as controls.[26] Athymic mice trapped only 10-20% of the number of larvae that the control mice did. This difference was proposed to be the result of greater eosinophilic infiltrates surrounding the larvae in BALB/c-nu/+ mice, with the theory that the eosinophils hindered larval movement and enhanced granuloma formation. Parsons and colleagues invalidated this theory by treating mice with anti-IL-5 to deplete their eosinophils, and found that trapping was unaffected by this treatment.[34]

Human toxoplasmosis

Toxoplasmosis is the infection of people with the asexually replicative stages of *Toxoplasma gondii.* Cats are the definitive host for this obligate intracellular protozoan parasite. Oocysts are shed in the feces of cats and quickly sporulate to become infectious to the next host. These environmentally resistant stages can remain viable for over 18 months.[35,36] As with *T. canis*, people principally acquire *T. gondii* by ingesting soil, food, or water contaminated by oocysts from cat feces, or by ingesting tissue cysts in undercooked meat from a paratenic host (which for *T. gondii* includes most species of mammals along with birds).[37] Another important

route of transfer is vertical transmission from a naïve mother to her fetus; less common modes of transmission are blood transfusion and organ transplant from infected donors (Figure 1.2).[37,38] In North America, oocyst ingestion is the predominant source of infection for pregnant women and in various outbreaks (one linked to a water reservoir in British Columbia, one at an Atlanta riding stable).[39] Like toxocariasis, toxoplasmosis is one of the most common zoonotic infections in people and is included in CDC's list of top 5 neglected parasitic infections in the USA.

For immunocompetent people, the rapid replication of invading tachyzoites of *T. gondii* – and the associated inflammatory response – causes a transient flu-like illness with fever, malaise, and lymphadenopathy; as the immune system brings infection under control, tachyzoites convert into slow-replicating bradyzoites (contained in tissue cysts) and the person becomes asymptomatic.[37,38] However, in immunocompromised populations, e.g., people taking immunosuppressive drugs or those with AIDS, toxoplasmic encephalitis from uncontrolled tachyzoite replication in the brain can prove fatal.[37] Congenital toxoplasmosis can also result in severe ocular or neurological disease, such as retinochoroiditis and hydrocephalus. Three major *Toxoplasma* lineages are recognized, with type I organisms being much more pathogenic in the mouse model than either type II or III.[40] Human toxoplasmosis cases in North America and Europe are mainly attributed to infection with type II strains, whereas in South America, Africa, and Asia, atypical strains predominate.[40,41]

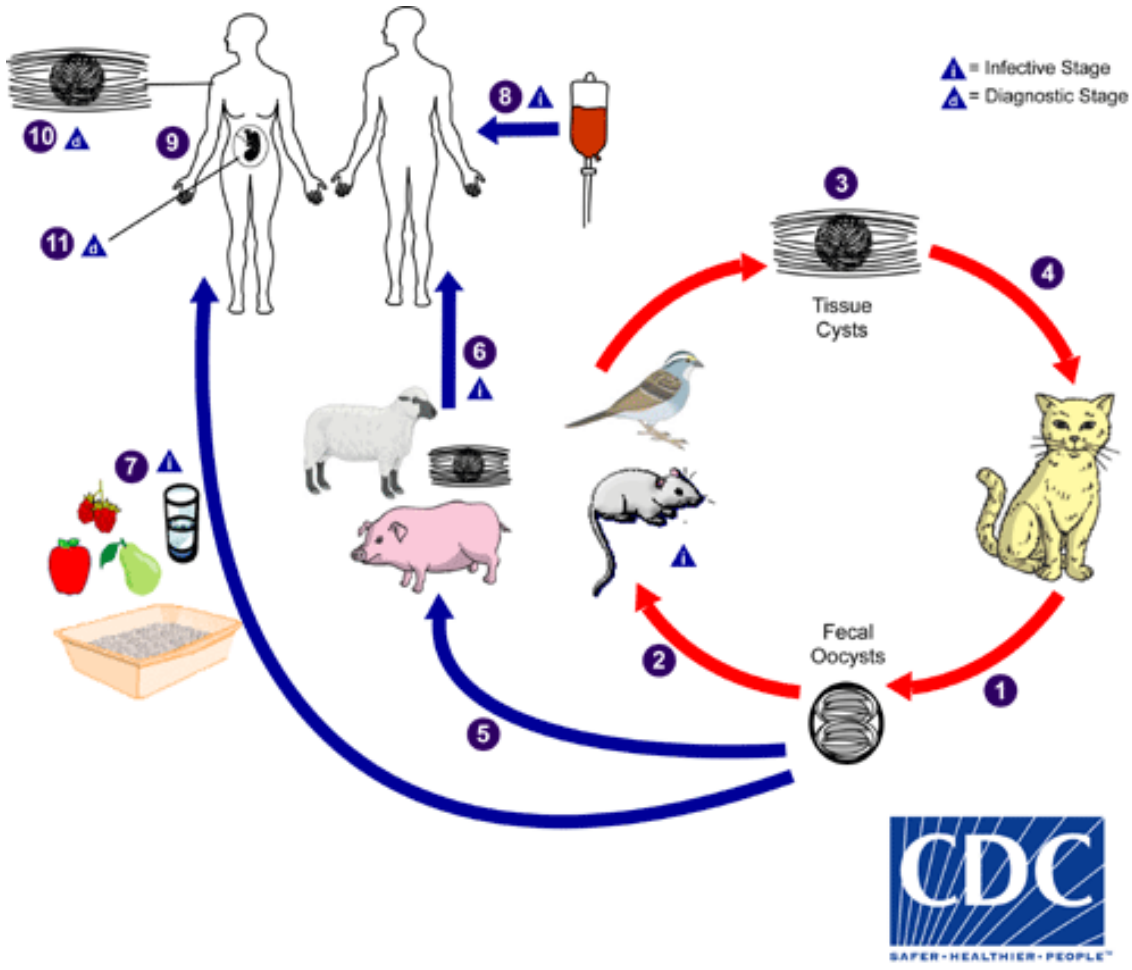
= Infective Stage
= Diagnostic Stage
Tissue Cysts
Fecal Oocysts
CDC
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Figure 1.2. *T. gondii* life cycle. The only known definitive hosts for *Toxoplasma gondii* are members of family Felidae (domestic cats and their relatives). Unsporulated oocysts are shed in the cat's feces ❶. Although oocysts are usually only shed for 1-2 weeks, large numbers may be shed. Oocysts take 1-5 days to sporulate in the environment and become infective. Intermediate hosts in nature (including birds and rodents) become infected after ingesting soil, water or plant material contaminated with oocysts ❷. Oocysts transform into tachyzoites shortly after ingestion. These tachyzoites localize in neural and muscle tissue and develop into tissue cyst bradyzoites ❸. Cats become infected after consuming intermediate hosts harboring tissue cysts ❹. Cats may also become infected directly by ingestion of sporulated oocysts. Animals bred for human consumption and wild game may also become infected with tissue cysts after ingestion of sporulated oocysts in the environment ❺. Humans can become infected by any of several routes: eating undercooked meat of animals harboring tissue cysts ❻, consuming food or water contaminated with cat feces or by contaminated environmental samples (such as fecal-contaminated soil or changing the litter box of a pet cat) ❼, blood transfusion or organ transplantation ❽, transplacentally from mother to fetus ❾. In the human host, the parasites form tissue cysts, most commonly in skeletal muscle, myocardium, brain, and eyes; these cysts may remain throughout the life of the host. Diagnosis is usually achieved by serology, although tissue cysts may be observed in stained biopsy specimens ❿. Diagnosis of congenital infections can be achieved by detecting *T. gondii* DNA in amniotic fluid using molecular methods such as PCR ⓫. [Figure and caption from CDC website.]

Due to ongoing efforts to raise public awareness of proper cooking temperatures and to eliminate *T. gondii*-infected meat from the food supply, the national seroprevalence of anti-*Toxoplasma* antibodies in the United States has been steadily decreasing over the past two decades.[42] Amongst people aged 12-49 who were born in the USA, prevalence has dropped from 14.1% in the period of 1988-1994 down to 9.0% in 1999-2004, and the latest data from 2009-2010 show 6.7% prevalence (equivalent to 22 million people).[42] However, this number may rise again given the popularity of the organic and free range movement and the substantial presence of tissue cysts in pigs, chickens, and small ruminants raised in this manner.[43,44] Globally, it is estimated that 25-30% of the human population is infected by *T. gondii*.[45] Prevalence varies greatly between geographic locations – ranging from 10 to 80% – depending on factors such as climate, sanitation, dietary habits, and socio-economic status.[46]

Host immune response to infection

Parasitic helminths such as *Toxocara* induce a T helper type 2 (Th2)-dominated host immune response. Early in infection, epithelial cells produce IL-25 and IL-33 (alarmin cytokines), which then stimulate innate lymphoid cells to generate the type 2 cytokines IL-5 and IL-13.[47] It is hypothesized that IL-13 alone may be sufficient to drive naïve T cell differentiation toward a Th2 phenotype via the IL-4 receptor. However, classical MHCII antigen presentation by dendritic cells also occurs. Humoral immunity is initiated with the help of IL-4-producing T follicular helper cells in the draining lymph nodes.[47] The resultant Th2 response is characterized by production of IL-4, IL-5, IL-10 and IL-13, eosinophilia, and elevated serum IgE levels.[48] Serum IgE and IgG1 levels appear to increase in a dose- and time-dependent fashion after *T.*

canis infection, while IL-4, IL-5 and IL-10 expression peak within the first two weeks.[48] Production of IL-12 and TNF-α by macrophages is suppressed by *T. canis* infection.[49]

Toxoplasma infection, on the other hand, induces a prototypical T helper type 1 (Th1) response in the host, with heavy reliance on cell-mediated immunity for protection against this intracellular organism. Tachyzoites disseminate widely throughout the body and are recognized by toll-like receptors on dendritic cells and macrophages, which produce IL-12. IL-12 pushes T cells toward a Th1 phenotype and also drives natural killer (NK) cells to produce IFN-γ. The IFN-γ produced by Th1 cells and NK cells is essential for controlling *T. gondii* infection because it induces production of reactive oxygen and nitrogen intermediates by macrophages to allow for rapid microbe destruction.[50,51] IFN-γ also activates indoleamine 2,3-dioxygenase, which degrades tryptophan, an amino acid needed for *T. gondii* replication.[51] Although the function of Th1 cells is mainly pro-inflammatory in nature, it is also known to produce the anti-inflammatory cytokine IL-10 as a self-regulating mechanism to prevent excessive immunopathology.[52]

Toxoplasma and *Toxocara* co-infection

A national serological survey conducted in the USA revealed that a person who is infected with either *T. canis* or *T. gondii* is twice as likely to be infected with both.[53] Given that the two parasites elicit dichotomous Th2 and Th1 responses in the host, it is interesting to consider what effect co-infection may have on host defenses. It is well known that there is cross-talk between different arms of the immune system, including mutual suppression of the activities of Th1 and Th2 cells. This dynamic interplay has important consequences for the host, whether beneficial or detrimental. For example, *T. gondii* pre-infection has been shown to block the

development of allergic airway disease in a murine model.[54] This situation demonstrates how immune interactions can be helpful in controlling disease. In contrast, pre-infection of mice with the fur mite *Myocoptes musculinus* followed by infection with *T. gondii* results in mortality due to deficient IFN-γ production.[55] Clearly, two concurrent disorders or pathogens that induce opposing immune responses can strongly impact each other's disease progression. Another way to control T helper responses is through suppression by T regulatory (Treg) cells. Though a reduction in Treg numbers has been observed during acute toxoplasmosis, the remaining Treg cells exhibit an enhanced suppressive capacity.[56,57] In a co-infection scenario with *T. gondii* and *T. canis*, this Treg-mediated downregulation of T helper cell activation may well interfere with the host's ability to mount an effective response to *T. canis*. Such immune interactions may explain why only a subset of people infected with *Toxocara* experience clinical symptoms.

BALB/c mouse model of human infection

The mouse model of visceral toxocariasis closely resembles that of people.[33] The BALB/c strain in particular is used for studies on cerebral toxocariasis due to their tendency to accumulate large numbers of larvae in the brain and the associated cognitive dysfunction.[8,58] BALB/c are also resistant to *Toxoplasma* and will develop a latent chronic infection just as people do.[59] Because mice and humans are both natural paratenic hosts for *T. canis* and *T. gondii* with similar routes of transmission and clinical manifestations, murine co-infection is thus an excellent research model for human disease.

For this research, the oral route of infection was chosen in order to simulate the most common mode of natural *T. gondii* transmission. Oocysts are used for inoculation rather than cysts – as is more typically done in *T. gondii* immunology research – because oocysts account

for the majority of infections in North America.[42] The focus here has been placed on the chronic stage of infection in order to determine the long-term effects of these persistent parasites on the host.

Overview of dissertation research

The aim of this dissertation was to determine immune factors contributing to *Toxocara canis* migration and arrest during secondary infection and during co-infection with *Toxoplasma gondii*. Chapter 2 assesses the roles of cell-mediated and humoral immunity in *T. canis* liver trapping. I hypothesized that hepatic trapping of larvae during secondary infection is mediated by adaptive cellular immunity. Specifically, that Th2 cells would be present in greater numbers in the liver of mice after repeat infection, causing accelerated granuloma formation and preventing larvae from migrating out of the liver. Flow cytometry analysis revealed that CD4+ cells predominate in the liver of mice during secondary infection, with the largest increase in numbers occurring in the Th2 subset. However, CD4+ cell depletion experiments showed that the absence of these cells at the time of trapping did not reduce the number of larvae arrested in the liver, therefore I had to accept the null hypothesis that Th2 cells are not directly required for the trapping process. Based on this knowledge, I formed a new hypothesis that *T. canis*-specific antibodies are necessary and sufficient to induce larval trapping in previously unexposed mice. I further hypothesized that the antibody subclass responsible is IgG1, and that Th2 cells are needed during primary infection in order to generate this isotype. Indeed, CD4+ cell depletion at the time of sensitizing infection dramatically decreased *T. canis*-specific IgG1 antibody levels and abrogated hepatic trapping. Moreover, passive transfer of IgG antibodies from infected to naïve mice enabled recipients to trap larvae upon first encounter with *T. canis*.

In Chapter 3, alterations in the host response to *T. canis* migration are investigated in the context of co-infection with *T. gondii*. I hypothesized that concurrent infection with *T. gondii* would modify the immune response to *T. canis* such that larval trapping during secondary infection would be impaired. Specifically, the strong Th1 environment induced by pre-existing *T. gondii* infection would suppress the generation of *T. canis*-specific Th2 cells and skew antibody production away from the IgG1 isotype, resulting in loss of liver trapping. In fact, co-infected mice did have lower levels of anti-*T. canis* IgG1 antibodies and correspondingly fewer larvae in their livers. They also possessed an increased abundance of IgG2a antibodies, pointing to a shift toward a Th1 phenotype. An ancillary hypothesis was that this Th1 immune bias would persist in chronic toxoplasmosis, and therefore liver trapping would still be abolished if mice were infected with *T. canis* several weeks after *T. gondii* infection. This was shown to be true in mice chronically infected with a high dose of *T. gondii* oocysts.

Chapter 4 explores multiple variables that may affect *T. canis* migratory behavior in order to identify potential future avenues of investigation. The intent was to determine if factors such as host gender, location of immunologic priming, and presence or absence of eosinophils would hinder or enhance larval migration. Female gender and an intravenous route of sensitization appeared to decrease hepatic trapping, while eosinophils had no effect on *T. canis* migration or viability. A previously unanswered question was whether larval arrest in the liver would result in greater numbers of larvae migrating into the brain and eventually the eye. I hypothesized that larval counts in the brain would be higher in co-infected mice due to their inability to trap larvae in the liver. In actuality, there was no difference in the brains of co-infected and *T. canis* mono-infected mice when the BALB/c strain was used. However, for the C57BL/6 strain, a trend toward higher larval numbers was seen in the brains of co-infected mice.

T. canis and *T. gondii* are both long-lived parasites that likely dwell within their host for its lifetime. A better understanding of the immune basis of hepatic larval trapping may help to prevent ocular toxocariasis in people. Moreover, insight into the cross-regulatory changes that come with concomitant infection would allow for development of strategies to counter undesired skewing of immune responses in the arena of vaccine design.

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CHAPTER 2

IGG1 ANTIBODIES MEDIATE *TOXOCARA CANIS* LARVAL TRAPPING IN THE MURINE LIVER UPON CHALLENGE INFECTION

Abstract

Toxocariasis is the most common nematode infection of people in the United States and is highly prevalent worldwide. Migration of *Toxocara* larvae within people can cause clinical disease affecting the viscera or the eye. In mice, repeat infections with *Toxocara canis* results in the trapping of a portion of the larvae in the liver, which is postulated to help protect sensitive organs like the eye from migration-associated damage. Elucidating the immunologic basis of larval trapping could further our understanding of ocular toxocariasis and inform efforts to develop preventive measures. To this end, the cellular and humoral response to *T. canis* larvae within the liver were investigated. Hepatic T cells were characterized by flow cytometry, showing a predominant CD4+ population with a greater influx of T helper type 2 cells in the livers of twice-infected mice compared to controls. The importance of CD4+ T cells in effecting trapping was assessed by in vivo depletion of CD4+ cells. Mice depleted at the time of secondary infection had a similar number of larvae in their livers as mock-depleted mice. In contrast, mice depleted during the primary infection failed to trap larvae to any significant degree. Measurement of *T. canis*-specific antibodies by ELISA demonstrated a positive relationship between IgG1 levels and hepatic larval counts. Passive immunization of mice with IgG antibodies from infected mice enabled naïve recipient mice to trap larvae upon first exposure to *T. canis*. Altogether, these results suggest that CD4+ T cells – in particular the Th2 subset – is necessary at the time of primary *T. canis* infection to generate strong humoral immunity, which then mediates migratory arrest of larvae in the liver during subsequent infections.

Introduction

Toxocariasis is now the most common parasitic helminth infection of people in the United States, affecting 14% of the population or roughly 45 million people.[1,2] Globally, prevalence tends to be low in developed countries but can range from 30-93% in developing countries.[3] People are infected by *Toxocara canis*, a nematode of dogs and wild canids, when they ingest soil, food, or water contaminated by larvated eggs, or if they ingest undercooked tissues from an infected paratenic host such as cattle and poultry.[4–7] In paratenic hosts, which include people, the larvae undergo a characteristic liver-to-lung migration before disseminating systemically to preferred sites such as the brain and cranial skeletal muscles.[8–10] Larvae remain alive and infectious in host tissues for several years.[11,12]

Clinical disease due to larval migration and the inflammatory response to larval death has been grouped into categories encompassing visceral toxocariasis, ocular toxocariasis (OT), and covert toxocariasis, each with a differing set of associated symptoms.[5,7,13] Diagnosis is based on a combination of clinical presentation, peripheral eosinophilia, seroreactivity to *T. canis* antigens, as well as molecular and histologic identification of larvae if biopsies are available.[3] Interestingly, OT patients tend to have low *T. canis* antibody titers compared to patients with visceral toxocariasis.[3] It is thus the general consensus that ocular invasion results from infection with a small number of larvae that does not elicit a strong host immune response. Results from studies of *T. canis* larval migration through the livers of mice lend weight to this theory. It has been established that previous exposure to *T. canis* changes larval distribution within host organs upon a secondary infection. Whereas normally larvae enter and exit the liver within a few days of oral infection, in mice that have already been sensitized to *T. canis* by a prior infection, a large number of larvae from the secondary infection are apparently "trapped" within the liver.[14] They

remain there for at least 16 weeks post-challenge, so it is not simply a matter of delayed migration that prevents them from moving on.[14] Thus mice that are adequately primed against *T. canis* can mount an immune response to contain a portion of the larvae within the liver, while larvae infecting a naïve mouse will rapidly leave the liver and travel onward to the lungs, brain, and perhaps eventually to the eye. The protection afforded by hepatic larval trapping has been shown experimentally, where mice inoculated twice with *T. canis* had many more larvae in their livers than mice infected a single time, but no substantial increase in the number of larvae was observed in their brains.[15,16]

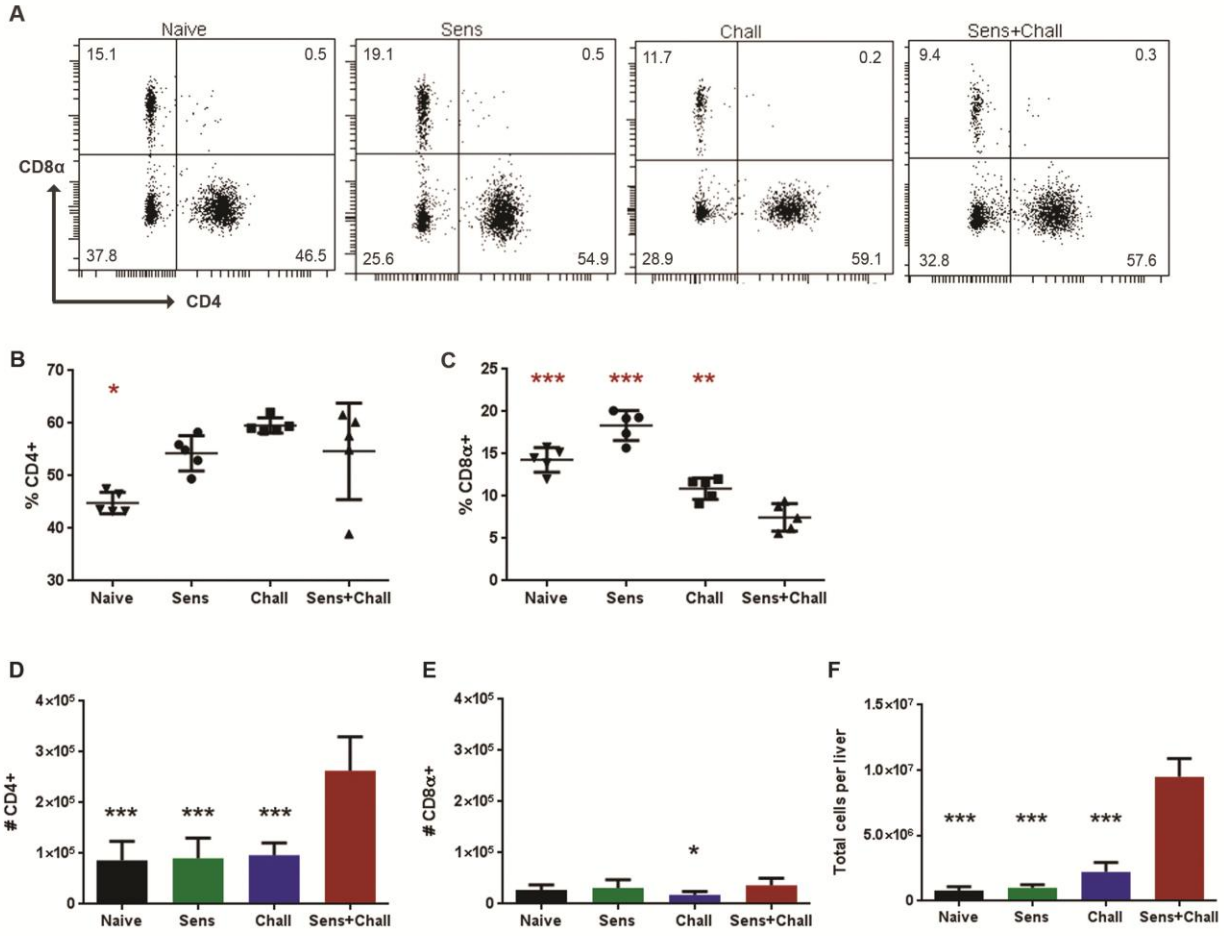
The immunologic mechanism of larval trapping has been studied by various investigators but this knowledge is not yet complete. Passive immunization using serum from infected donor mice enabled naïve recipients to trap larvae in their livers during a primary infection.[17] The specific serum component responsible for this effect was not identified. Cell-mediated immunity also appeared to be important as athymic nude mice (BALB/c-nu/nu) trapped just one-fifth the number of larvae that control mice did.[15] Again, the T cell subset participating in this response was not identified. It was proposed that eosinophils may be involved in hepatic trapping due to the high frequency with which they are found surrounding larvae in histological sections, yet this was proven not to be the case as eosinophil depletion through anti-IL-5 therapy had no effect on trapping.[18]

Given these gaps in knowledge, the aims of this study were to assess the relative importance of T lymphocytes and serum antibodies in effecting hepatic trapping of *T. canis* larvae, and to determine the particular cell populations or antibody isotypes contributing to this response. By better understanding this immune-mediated mechanism of larval migratory arrest, strategies for preventing ocular complications of toxocariasis can be developed.

Results

To assess the population of adaptive immune cells responding to *T. canis* hepatic migration through the liver during repeat infection, flow cytometry was performed on single cell suspensions prepared from the livers of mice. Groups that received any type of *T. canis* infection – whether a single sensitizing dose several weeks prior to euthanasia, a single "challenge" dose 7 days prior, or both infections combined – had a higher percentage of CD4+ T cells within their livers compared to the baseline levels found in naïve mice (Figure 2.1 A and B). However, livers of twice-infected mice experienced a greater overall influx of cells as determined by the total number of mononuclear cells recovered after collagenase digestion and density gradient exclusion of hepatocytes (Figure 2.1 F). As a result, the total number of CD4+ cells in the liver of these mice was 2.5x higher than in all other groups (Figure 2.1 D). In contrast, though the percentage of CD8+ T cells was lower in twice-infected mice than in singly or non-infected individuals (Figure 2.1 A and C), the absolute number was essentially unchanged (Figure 2.1 E).

Within the CD3ε+ CD4+ T cell population, no difference was noted in the percentage of IFN-γ-producing Th1 cells except for a lower value in naïve mice (Figure 2.2 A and D). IL-4-producing Th2 cells made up a significantly larger percentage of the cells in the liver of twice-infected mice than in the 3 other groups (Figure 2.2 B and E). With respect to Foxp3+ Treg cells, repeated infection resulted in an equivalent or lower proportion of these cells compared to single or no infection (Figure 2.2 C and F). Repeated infection was associated with higher total numbers of all three T cell subsets relative to the other groups (Figure 2.2 G-I), with the largest difference observed in the Th2 population. Taken together, these data suggest that CD4+ T lymphocytes – and Th2 cells in particular – are heavily recruited to the liver upon secondary infection with *T. canis*.


A
Naive
Sens
Chall
Sens+Chall
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0.5
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46.5
19.1
0.5
25.6
54.9
11.7
0.2
28.9
59.1
9.4
0.3
32.8
57.6
CD8α
CD4
B
% CD4+
C
% CD8α+
D
CD4+
E
CD8α+
F
Total cells per liver
Naive
Sens
Chall
Sens+Chall

Figure 2.1. Repeated infection with *T. canis* induces influx of CD4+ T cells into the liver. Mice were inoculated with a sensitizing and challenge dose of *T. canis* eggs (Sens+Chall), a sensitizing dose only (Sens), a challenge dose only (Chall), or not at all (Naïve). Single cell suspensions were prepared from the liver and stained for flow cytometric analysis of CD3ε, CD4, and CD8α expression. Plots from representative mice (A) showing percentage of CD3ε+ cells expressing CD4 and CD8α. Percentage (B-C) and total number (D-E) of CD4+ and CD8α+ T cells in the livers of twice-infected, singly infected, and naïve mice. Total number of mononuclear cells isolated from the livers of each group (F). Each dot represents an individual mouse (n = 5 per group). Graphs display means and 95% confidence intervals. The Sens+Chall group was set as the control to which other groups were compared. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

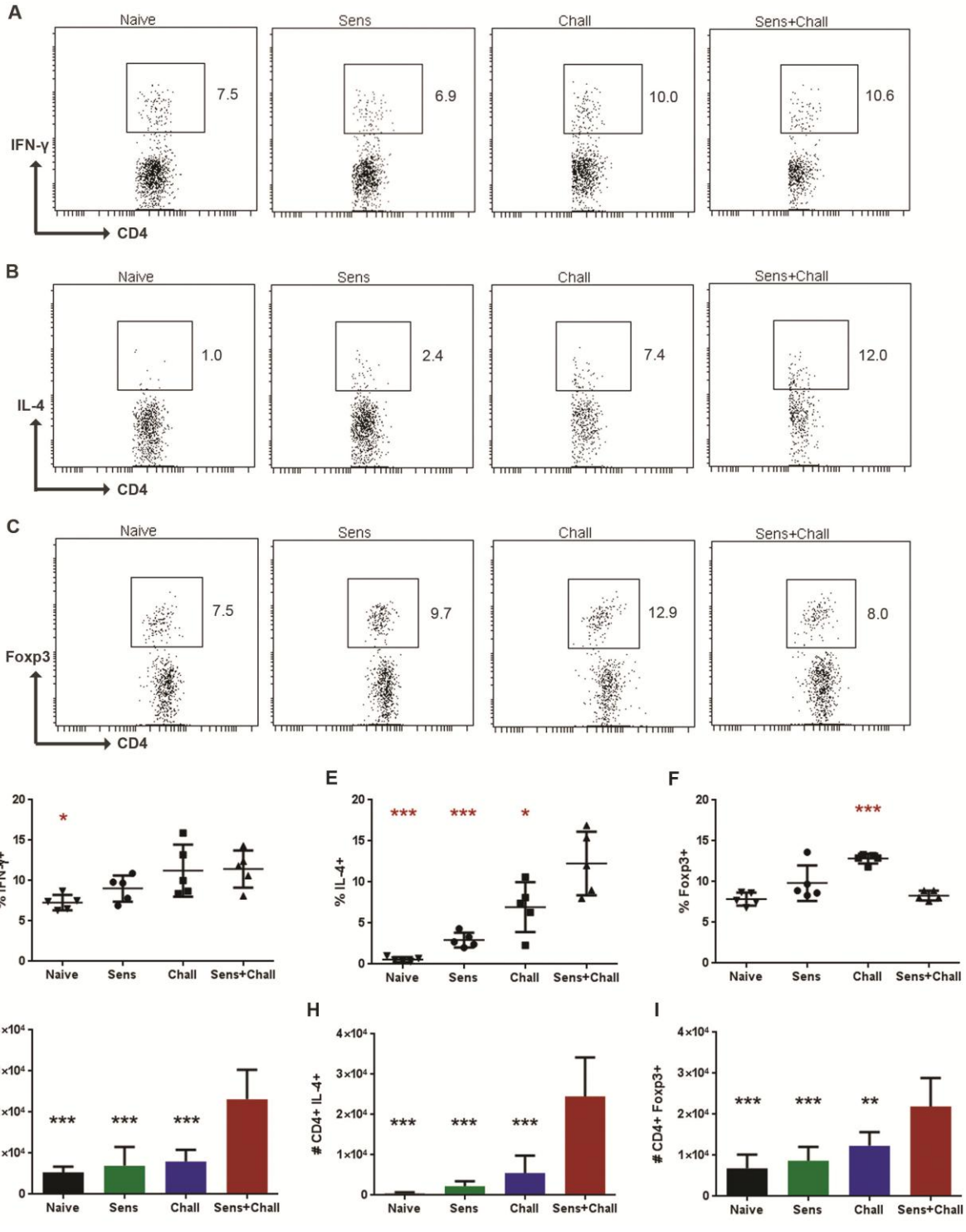
A
Naive
Sens
Chall
Sens+Chall
7.5
6.9
10.0
10.6
IFN-γ
CD4
B
Naive
Sens
Chall
Sens+Chall
1.0
2.4
7.4
12.0
IL-4
CD4
C
Naive
Sens
Chall
Sens+Chall
7.5
9.7
12.9
8.0
Foxp3
CD4
D
% IFN-γ+
E
% IL-4+
F
% Foxp3+
G
CD4+ IFN-γ+
H
CD4+ IL-4+
I
CD4+ Foxp3+
Naive
Sens
Chall
Sens+Chall

Figure 2.2. Of the CD4+ T cell subsets examined within the liver, Th2 cells undergo the greatest expansion after a repeat *T. canis* infection. Mice were inoculated with a sensitizing and challenge dose of *T. canis* eggs (Sens+Chall), a sensitizing dose only (Sens), a challenge dose only (Chall), or not at all (Naïve). Mononuclear cells were isolated from the liver by Histopaque-1077 density gradient centrifugation. (A-B, D-E, G-H) Cells were cultured for 4 hours in the presence of brefeldin A, PMA, and ionomycin. Cells were then stained for viability, CD3ε, CD4, CD8α, and for intracellular IFN-γ and IL-4. (C, F, I) Uncultured cells were stained for CD3ε, CD4, CD8α, and the transcription factor Foxp3. Plots from representative mice (A-C) showing percentage of CD3ε+ CD4+ cells belonging to the IFN-γ+ Th1 subset, IL-4+ Th2 subset, and Foxp3+ Treg subset. Percentage (D-F) and total number (G-I) of Th1, Th2, and Treg cells in the livers of twice-infected, singly infected, and naïve mice. Each dot represents an individual mouse (n = 5 per group). Graphs display means and 95% confidence intervals. The Sens+Chall group was set as the control to which other groups were compared. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

In order to assess whether the CD4+ T cells present in the liver at the time of secondary infection are important for trapping *T. canis* larvae in vivo, cell depletion experiments were carried out. Administration of 3 doses of anti-CD4 antibodies to mice just before and just after a challenge infection successfully ablated CD4+ cells as evidenced by lack of CD4 expression in flow cytometric analysis of splenocytes (Figure 2.3 A). Yet there was surprisingly no effect on the number of larvae present in the liver (Figure 2.3 B). Given that serum transfer on its own is known to enable recipients to trap larvae in the liver, the level of serum IgG specific for *T. canis* excretory-secretory (TES) antigen was evaluated in these mice using an indirect ELISA. CD4-depleted mice had relatively less *T. canis*-specific IgG but the difference was not dramatic (Figure 2.3 C).

Utilizing a modified study design, the impact of the timing of cell depletion was addressed. Anti-CD4 antibodies were injected just once before the first infection (designated Dep1 group), once before the secondary infection (Dep2), or at both times plus a booster in between infections (Dep1+2). Control mice were mock depleted with an irrelevant isotype antibody (Mock). The lowered dosage provided excellent depletion in the Dep1 and Dep1+2 groups after the first infection, as assessed by CD4 expression in peripheral blood cells (Figure 2.4 A). Depletion was not as complete in the splenocytes of the Dep2 group after the second infection (Figure 2.4 A), however, an average of 82% CD4+ cell ablation was achieved. The Dep1+2 group remained well depleted at 99.8%. Though trapping fewer larvae than the Mock group, Dep2 mice still had many larvae in their livers, similar to the previous experiment (Figure 2.4 B). Both the Dep1 and Dep1+2 groups had a minimal number of hepatic larvae (Figure 2.4 B), indicating that CD4+ cells exert their influence on larval arrest at the time of first encounter with *T. canis*, not during the trapping stage itself. Examining the anti-TES antibodies showed

that the IgG1 subclass, associated with a Th2 type response, was most abundant in mice that trapped larvae and scarce to absent in mice that did not (Figure 2.4 C). The IgG2a subclass, linked to Th1 type responses, showed no such pattern (Figure 2.4 D). Interestingly, the non-trapping groups, Dep1 and Dep1+2, generated IgM antibodies (Figure 2.4 E) yet produced little IgG1. This points to a possible defect in antibody class switching, implicating that the main contribution of CD4+ T cells toward *T. canis* retention in the liver may be through regulation of the antibody isotype produced after primary infection. The strong correlation existing between serum IgG1 levels and the number of larvae in the liver ($r = 0.90$, $p < 0.001$) further supports this hypothesis (Figure 2.4 F). Results of in vivo cell depletion demonstrate that CD4+ T cells do not directly cause larval migratory arrest but instead guide the humoral response against *T. canis*.

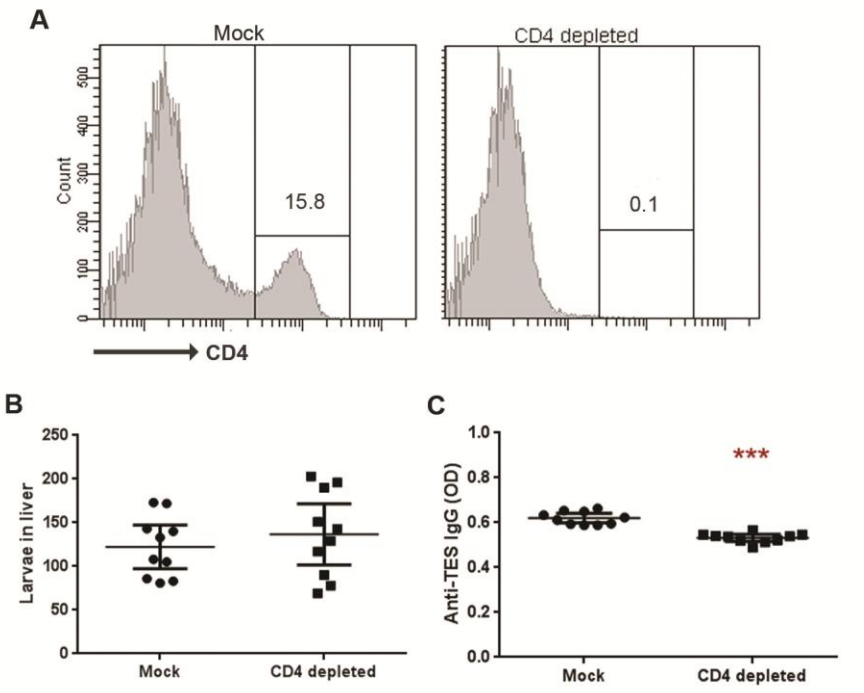

Figure 2.3. Antibody-mediated CD4+ cell depletion at the time of challenge infection does not impede hepatic trapping of *T. canis* larvae. Mice were inoculated 4 weeks apart with two doses of *T. canis* and were depleted of CD4+ cells or mock depleted at the time of challenge infection. Displayed are representative histograms of CD4 expression in splenocytes from mice injected with anti-CD4 or isotype control antibodies (A). Larvae in the liver were liberated by acid-pepsin digestion and counted under a light microscope (B). Serum IgG antibodies specific for *T. canis* antigen (TES) were measured by ELISA (C). Group means (n = 10) and 95% confidence intervals are shown. Each dot represents a single mouse. ***$p < 0.001$.

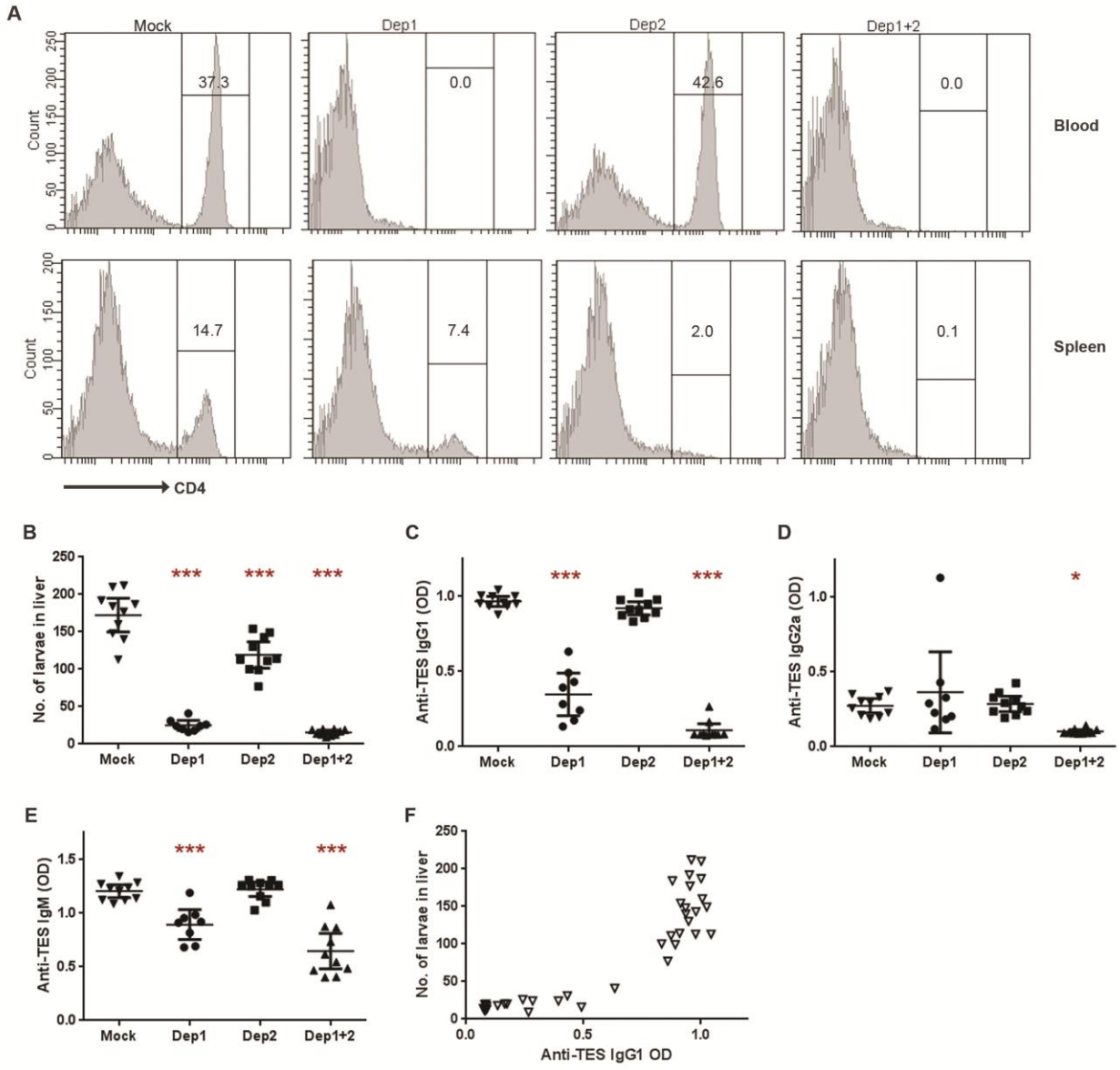
A
Mock
Dep1
Dep2
Dep1+2
37.3
0.0
42.6
0.0
Blood
14.7
7.4
2.0
0.1
Spleen
Count
CD4
B
No. of larvae in liver
C
Anti-TES IgG1 (OD)
D
Anti-TES IgG2a (OD)
E
Anti-TES IgM (OD)
F
No. of larvae in liver
Anti-TES IgG1 OD

Figure 2.4. CD4+ cell depletion at the time of sensitizing infection abrogates hepatic trapping of *T. canis* and reduces parasite-specific IgG1 production. Mice were inoculated 4 weeks apart with two doses of *T. canis*. CD4+ cell-depleting antibodies were administered prior to the first infection (Dep1), the second infection (Dep2), or at both time points as well as midway through the experiment (Dep1+2). Control mice (Mock) were injected with an isotype antibody. Shown are representative histograms of CD4 expression in peripheral blood cells and in splenocytes after the sensitizing infection and at euthanasia, respectively (A). Larvae in the liver were recovered by acid-pepsin digestion and quantified (B). Serum IgG1 (C), IgG2a (D), and IgM (E) specific for TES were measured by ELISA. The relationship between IgG1 levels and liver trapping is displayed ($r = 0.90$, $p < 0.001$) (F). Group means ($n = 8$-10) and 95% confidence intervals are shown. Each dot represents a single mouse. The Mock group was set as the control to which other groups were compared. $^{*}p < 0.05$, $^{***}p < 0.001$.

To provide further confirmation of the crucial role of IgG antibodies in effecting hepatic larval trapping, naïve mice were passively immunized with serum from *T. canis*-infected mice. Donors were dosed twice with *T. canis* eggs, with varying time intervals between the two infections. Using the typical period of 4 weeks that had been applied in previous experiments resulted in modest trapping in recipients compared to control mice given normal serum (Figure 2.5 A). This was reflected in the corresponding IgG measurements (Figure 2.5 B). Waiting for a longer period of 26 weeks between infections of donor mice increased the number of hepatic larvae found in recipients (Figure 2.5 C) as well as the amount of *T. canis*-specific IgG being transferred (Figure 2.5 D).

Next, purified IgG from infected mice was injected intraperitoneally into naïve recipients to rule out the effect of other antibody isotypes or serum proteins on trapping. Because a 26-week inter-infection interval was impractical, a shorter period of 4-10 weeks was used for donor mice. IgG was extracted from serum through protein A affinity chromatography. The flowthrough solution was kept and used as a non-IgG antibody control. Both fractions were concentrated by centrifugal filtration back to the original sample volume to mimic concentrations found in whole serum. Equal volumes of IgG, flowthrough, whole serum from infected mice, and whole serum from naïve mice were transferred into recipients, which were inoculated the following day with *T. canis* eggs. Liver larval counts from the *T. canis* serum and IgG groups were similar and higher than those from the flowthrough or normal serum groups (Figure 2.6A). Again, the pattern was clearly mirrored by IgG1 levels in the recipient mice (Figure 2.6 B). IgG2a antibodies were detected to a higher degree in *T. canis* serum and IgG groups but the optical density (OD) was still low overall (Figure 2.6 C). These experiments show that IgG alone is sufficient to mediate hepatic larval trapping, and this effect is likely due to the IgG1 subclass.

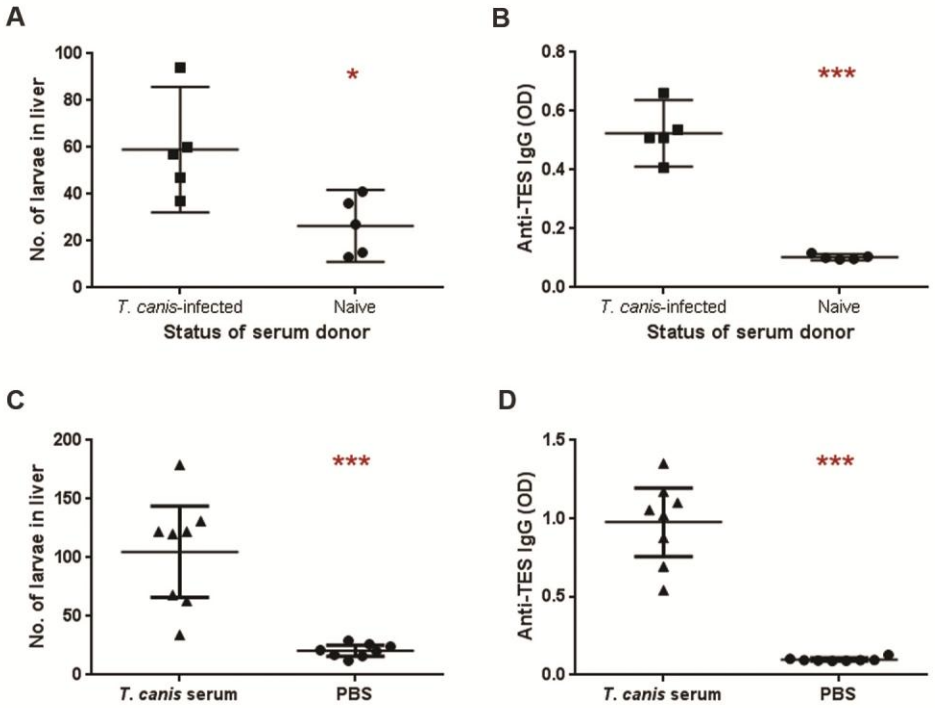

Figure 2.5. Passive immunization enables naïve mice to trap larvae, and this ability is enhanced when serum donors are more chronically infected. Donor mice were infected twice with *T. canis* separated by an interval of 4 weeks or 26 weeks. Naïve recipient mice were intraperitoneally injected with 0.8 mL of serum from infected mice, naïve mice, or PBS (n = 5-8 mice per group). They were inoculated the following day with *T. canis* eggs. Livers were digested in acid-pepsin to count larvae, and the abundance of serum IgG in the recipient mice was quantified using ELISA. Hepatic larval counts and IgG levels are shown for mice whose donors experienced 4 weeks of wait time in between infections (A-B) vs. 26 weeks (C-D). Graphs display means with 95% confidence intervals, and individual dots represent individual mice. *$p < 0.05$, ***$p < 0.001$.

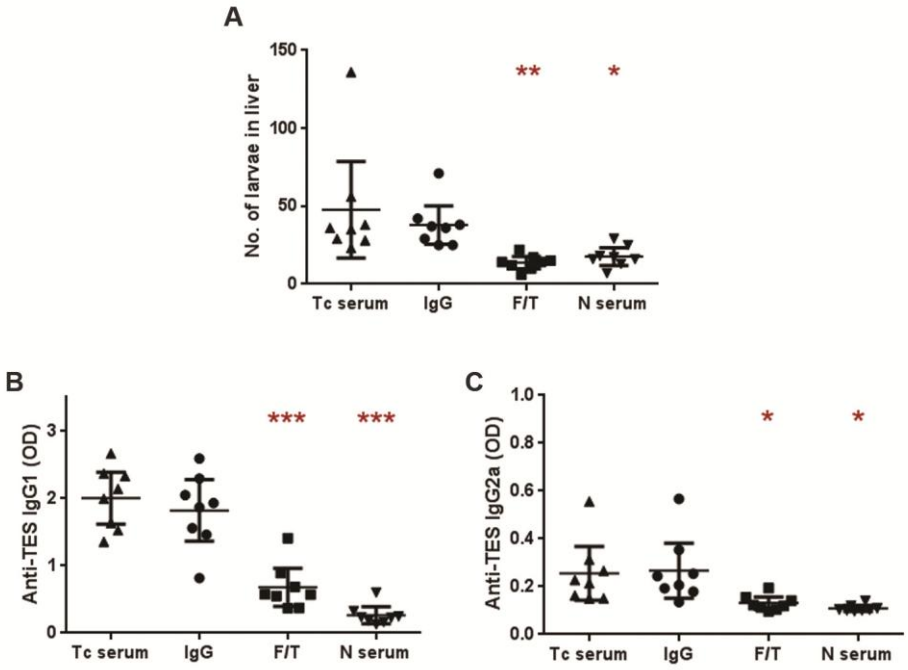

Figure 2.6. IgG, and not other antibody subclasses or serum proteins, confers trapping ability on serum recipients. IgG was affinity purified from the serum of twice-infected donor mice. Recipient mice (n = 8 per group) were injected IP with 0.8 mL of purified IgG fraction (IgG), non-IgG flowthrough from the chromatography column (F/T), whole serum from infected donors (Tc serum), or normal serum from naïve donors (N serum). The number of larvae found in the livers of recipient mice (A) as well as their corresponding serum IgG1 (B) and IgG2a (C) levels are shown. The Tc serum group was set as the control to which other groups were compared. Graphs display means with 95% confidence intervals, and individual dots represent individual mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Discussion

The finding of a high ratio of CD4+ to CD8+ T cells in the liver of *T. canis*-infected mice is in line with observations made by Othman et al. based on immunohistochemical staining.[19] Analysis of transcription factor and cytokine expression of these CD4+ T lymphocytes by flow cytometry further allowed differentiation of the cell subsets found in the liver. An increase in the total numbers of Th1, Th2, and Treg cells was noted in twice-infected mice compared to once-infected or naïve mice. Given that CD4+ cells are not required for *T. canis* hepatic trapping to occur, it is doubtful their presence here is related to this process. The expansion of Th1 and Th2 populations in the liver of re-infected mice may simply reflect the granulomatous reaction incited by invading larvae, a response that is mixed Th1/Th2 in nature.[3] Inflammation and granuloma formation are more pronounced during repeat infections, which would explain the greater influx of these cells into the liver of twice-infected mice.[19] Treg cells have been reported to infiltrate the liver of *T. canis*-infected mice by five weeks post-infection, with the numbers increasing markedly by 16 weeks.[20] In previously sensitized mice, infiltration is much faster and becomes obvious by 2 weeks post-infection.[20] The current study demonstrates that Treg recruitment occurs as early as 1 week following challenge infection, likely for the purpose of dampening the enhanced inflammatory response in this organ.

Despite the expansion of Th2 cell populations in the liver during repeat infection, it was shown that CD4+ T cells do not directly participate in larval trapping. Mice that were CD4-depleted at the time of challenge had just as many larvae in their livers and produced similar amounts of IgG antibody as mock-depleted mice. However, the absence of CD4+ cells at the time of sensitization had a distinctly detrimental effect on trapping that was associated with a corresponding paucity of IgG1 antibodies against *T. canis*. Interestingly, Dep1 and Dep1+2 mice

still generated some IgM antibodies even without T helper cells to activate B cells. Dep 1 mice were only depleted of CD4+ cells during primary infection, so the gradual return of T helper cells over time could account for B cell activation and class switching to produce a measurable amount of IgG1 antibody. The Dep1+2 group, on the other hand, was depleted of CD4+ cells throughout the experiment. IgM production in this case may represent a T cell-independent mechanism that relied on B cell receptor crosslinking by carbohydrate moieties of TES to induce activation.[21] Regardless, no IgG1 was produced by this group, suggesting an inability to class switch due to the absence of T helper cells.

Transfer of serum from infected mice to naïve mice endowed recipients with the ability to trap *T. canis* larvae in the liver. Separation of the IgG fraction from the rest of the serum components prior to passive immunization of mice allowed for confirmation that this isotype is indeed the one responsible for mediating migratory arrest. Given the relative abundance of IgG1 and the low OD values for IgG2a in passively immunized mice, it seems probable that IgG1 is the key player in inducing trapping. The exact mechanism by which this happens is still unknown. One possibility is that the antibodies may bind to chemosensory receptors called amphids on the larvae, blocking environmental cues and thus stopping them from following their normal migratory route. This has been reported in a plant parasitic nematode of the *Meloidogyne* genus, which experienced impairment of its root-finding ability when its amphids were bound up by antibodies.[22] Another option is antibody binding of innate cells through Fc receptors causing reduced larval motility. This has been observed in *T. canis* larvae incubated with immune serum and activated macrophages in vitro.[23] The same phenomenon was demonstrated in vivo with the nematode *Heligmosomoides polygyrus*, with the IgG1 and IgG3 isotypes inducing alternatively activated macrophages (AAM).[24] The resulting Arginase-1 products, L-ornithine and

polyamines, were shown to immobilize larvae via presumably detrimental effects on larval metabolism.[24] AAM and Arginase-1 have also been implicated in the trapping of skin-penetrating *Nippostrongylus brasiliensis* larvae, with the instigator in this case being IgE-primed basophils.[25] A similar antibody-mediated mechanism could well be at work in *T. canis* hepatic trapping.

One potential limitation of this study is the use of BALB/c strain mice. Mice and people are both natural paratenic hosts for *T. canis* and share a similar pathogenesis, so the mouse is a good animal model for studying host immune response to *T. canis* infection.[3] However, the BALB/c strain has a well-recognized, inherent bias toward a Th2-dominated immune phenotype, and the predominance of IgG1 antibodies made against *T. canis* in this study could be a reflection of this predisposition. It is possible, therefore, that the results reported herein would be different if another mouse strain was utilized. This problem could be addressed by repeating this work in Th1-biased C57BL/6 mice, by using an outbred strain of mouse such as Swiss Webster, or by administering a Th1-inducing molecule such as CpG oligodeoxynucleotides to BALB/c mice to see if any of the findings consequently change.

Ocular toxocariasis patients tend to have low *Toxocara* titers, so it is postulated that OT occurs when a person who has not been previously sensitized is infected with small numbers of larvae that escape the notice of the host's immune system.[3,26] Results of the present study provide some support for this hypothesis. Hepatic larval trapping appears to be dependent on serum antibody levels, and low levels would permit more larvae to pass beyond the liver, increasing the likelihood of ocular invasion. Ocular larval counts were not performed in this case, so the theory cannot be fully validated, but the logic seems sound. Clearly, not all inoculated larvae were trapped in these experiments. Therefore prior sensitization would not

provide complete protection against OT if natural exposures to *T. canis* were as high as the doses used herein. However, if OT truly is the result of exposure to small numbers of larvae at a time, then a protective immune response in the liver may well be sufficient to trap all incoming larvae.

The present study extended our understanding of the cellular and humoral contributions to hepatic trapping of *T. canis* larvae in the liver. CD4+ T cells were shown to be indirectly responsible for trapping through promotion of class switching to the IgG1 isotype during primary infection. *T. canis*-specific IgG1 antibodies were strongly correlated to hepatic larval counts, and passive immunization of mice with anti-*T. canis* IgG antibodies conferred trapping ability to naïve recipient mice.

Future work should focus on definitively confirming IgG1 as the antibody isotype causing trapping. This can be investigated using IgG1 or FcγR knockout mice. The exact mechanism of antibody-mediated trapping must also be explored. One method to address the theory of chemosensory deprivation is to examine larval response to a salt gradient after incubation with immune serum, as nematode larvae almost invariably migrate toward areas of high salt concentration.[27] Another avenue to pursue would be the use of clodronate liposome depletion of phagocytic cells, or macrophage-specific FcγR knockout mice, to check for macrophage participation in the trapping process.

Methods

Mice and inoculations

Mice were female BALB/c strain acquired from Charles River Laboratories (Wilmington, MA), except for one experiment utilizing BALB/cJ mice from The Jackson Laboratory (Bar Harbor, ME). Naïve mice serving as donors of normal serum were 10 to 48 weeks old. Mice utilized for *T. canis* infections were 8 to 25 weeks old at the time of initial infection.

T. canis eggs were collected from dissection of the uteri of adult female worms or from the feces of infected dogs. Eggs were cleaned by agitation in 0.6% sodium hypochlorite for 10 minutes and washed four times with water. Eggs were cultured in 0.1N sulfuric acid in vented flasks at 28°C for 4-5 weeks, then stored at 4°C until use. For inoculation, eggs were resuspended in water and aliquots were taken to determine the concentration of larvated eggs. Sensitizing and challenge doses consisted of 100-200 and 500 *T. canis* eggs, respectively, in a final volume of 100 μL. Doses were delivered by gavage.

Hepatic cell population analysis

Four groups of mice were assessed: 1) mice given a sensitizing and challenge dose of *T. canis* 4 weeks apart, followed by euthanasia a week later; 2) mice given the sensitizing dose only; 3) mice given the challenge dose only; and 4) uninfected mice. At euthanasia, the caudal vena cava was severed and the liver was perfused with 5 mL of ice-cold phosphate-buffered saline (PBS) until it became pale in color. After removal of the gallbladder, the liver was diced and pushed through a sterile, 800 μm stainless steel mesh. Liver tissue was resuspended in complete Dulbecco's modified Eagle's medium (cDMEM) containing 10% bovine growth serum (Hyclone), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 30 mM HEPES, 100

U/mL penicillin, 100 μg/mL streptomycin (all from Life Technologies), and 0.05 mM β-mercaptoethanol (Sigma). Collagenase IV was added at a concentration of 550 U/mL (Gibco). The suspension was incubated on a shaker at 37°C for 30 minutes, passed through a 70 μm cell strainer, then centrifuged and washed twice in PBS at 400 x g for 5 minutes. Lymphocytes were isolated by centrifugation with Histopaque-1077 (Sigma), washed with PBS, and resuspended in cDMEM.

For flow cytometry, samples were divided into two. One aliquot was used for surface staining and Foxp3 detection. Lymphocytes were stained with viability dye (eBioscience) as per manufacturer instructions, then incubated for 20 minutes with anti-CD3ε (BD Biosciences), anti-CD4 (BioLegend), and anti-CD8α (eBioscience). After washing twice with ice-cold FACS buffer (1% bovine serum albumin (BSA) and 0.1% sodium azide in PBS), the cells were fixed, permeabilized, and stained using an anti-Foxp3 staining set (eBioscience). The second aliquot was used for intracellular cytokine detection. Lymphocytes were incubated for 4 hours at 37°C with 3 μg/mL brefeldin A (BD Biosciences), 10 ng/mL PMA (Sigma), and 1 μg/mL ionomycin (Sigma). They were stained with viability dye followed by surface staining and fixation/permeabilization as described above. Antibodies used for intracellular cytokines were anti-IFN-γ and anti-IL-4 (both from BioLegend). Flow cytometry was performed on a FACS Canto II (BD Biosciences), and data were analyzed using FACS Diva software (BD Biosciences).

CD4+ T cell depletion

All mice were given a sensitizing and challenge dose of *T. canis* eggs 4 weeks apart, with euthanasia occurring 1 week after challenge. In the first experiment, mice were injected

intraperitoneally (IP) with 0.2 mg of anti-CD4 or isotype antibody (GK1.5 and LTF-2 clones, Bio X Cell, West Lebanon, NH) on the day prior to challenge and on days 1 and 4 post-challenge. In the second experiment, injections were reduced to a single 0.2 mg dose on the day prior to *T. canis* inoculation. The four groups received CD4-depleting antibodies at different times: during the sensitizing infection only (Dep1 group), during challenge only (Dep2), during both sensitization and challenge (Dep1+2), or not at all (Ctrl). The Dep1+2 group was administered an additional 0.1 mg of anti-CD4 antibody midway through the experiment to ensure sustained depletion of target cells. Any group not scheduled to receive the depleting antibody at each injection time point was given isotype antibody instead.

To assess adequacy of CD4+ cell depletion, spleens were collected at euthanasia and examined by flow cytometry. Spleens were macerated and strained through a 70 μm nylon mesh, pelleted by centrifugation, and subjected to ACK lysis buffer (Lonza) for 2 minutes. After washing with PBS, the cells were again passed through a 70 μm mesh. Incubation with anti-CD4 (BioLegend) for 20 minutes at 4°C was followed by two washes with FACS buffer. Flow cytometry was performed on a FACS Canto II (BD Biosciences), and data were analyzed using FACS Diva software (BD Biosciences). During the second experiment, blood was also sampled at 2, 14, and 24 days after the sensitizing infection to verify depletion status. The staining protocol for heparinized blood was similar to that described for splenocytes but with a second lysis step added (5 minutes each time).

Passive immunization with whole serum or purified fraction

Donor mice were chronically infected (4-26 weeks) with *T. canis* prior to the challenge inoculation. Mice were euthanized 2-6 weeks post-challenge and blood was aseptically collected

by cardiac puncture. Normal serum was acquired from naïve mice as a control. IgG was purified by affinity chromatography using the Econo-Pac protein A kit (Bio-Rad Laboratories, Hercules, CA). Completeness of IgG extraction was verified with the Easy-Titer mouse IgG assay kit (Thermo Scientific). The IgG and flowthrough were concentrated by centrifugal filtration with a 30 kDa exclusion limit (EMD Millipore, Billerica, MA) and resuspended in PBS. Whole serum or purified fractions were injected into naïve recipient mice (0.8 mL IP per mouse) one day prior to oral infection with 500 *T. canis* eggs. Euthanasia was carried out a week later.

Larval recovery from the liver

Livers were diced, homogenized in a blender, and shaken in a 0.7% pepsin + 0.7% HCl solution for 1-2 hours at 37°C. After washing with PBS, the solution was neutralized using 1M NaOH. Gradient centrifugation with Histopaque-1077 (Sigma) at 1000 x g for 25 minutes was used to separate larvae from liver sediment. Larvae were transferred to a gridded Sedgwick slide (Wildlife Supply Company, Yulee, FL) for quantification by light microscopy.

Serum antibody measurement

Serum was collected by cardiac puncture after mice were euthanized. *T. canis*-specific antibodies were measured using an indirect ELISA according to the method of Bowman et al. with some modifications.[28] High-binding 96-well plates (Immulon 2HB, Thermo Scientific) were coated with 10 μg/mL of larval *T. canis* excretory-secretory antigen (TES) in 0.1 M carbonate buffer pH 9.6. Serum dilutions ranged from 1:200 to 1:16,000 depending on the experiment and the antibody isotype being measured. Horseradish peroxidase-conjugated antibodies consisted of rabbit anti-mouse IgG, goat anti-mouse IgG1, goat anti-mouse IgG2a, or rabbit anti-mouse IgM

(Jackson ImmunoResearch, West Grove, PA) diluted 1:5000 in PBS. Absorbance at 405 nm was determined by an automated spectrophotometer (model ELx800, Bio-Tek Instruments, Winooski, VT).

Production of larval TES

Excretory-secretory antigen produced by *T. canis* larvae was collected using a modified version of an established protocol.[28] Larvae were cultured at 37°C in RPMI-1640 with glutamine (Lonza, Walkersville, MD) supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 250 ng/mL of amphotericin B (Gibco). Every two weeks, the culture medium was removed and stored at -80°C and fresh medium was added. After several months the frozen medium was thawed, and the TES contained therein was concentrated by ultrafiltration with a 3,000 kDa exclusion limit (EMD Millipore, Billerica, MA) then resuspended in PBS. The final concentration of TES was measured using a micro-volume spectrophotometer (Quawell Technology, San Jose, CA). TES was frozen at -80°C until use.

Statistical analysis

Differences between two groups were analyzed using unpaired t-tests. For 3 or more groups, one-way ANOVA with Dunnett's post-test was performed. Pearson correlation coefficient was used to evaluate relationships. Significance was set at a p-value of < 0.05. Data were analyzed using Prism software (GraphPad Software Inc., La Jolla, CA).

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CHAPTER 3

PRIOR INFECTION WITH *TOXOPLASMA GONDII* SKEWS HUMORAL RESPONSE TO *TOXOCARA CANIS* AND ABROGATES LARVAL TRAPPING

Abstract

The apicomplexan parasite *Toxoplasma gondii* induces a strong T helper (Th) type 1 immune response in its host. In contrast, helminths such as *Toxocara canis* promote a characteristic Th2 type response. These two zoonotic parasites are highly prevalent on a global scale, and the odds of dual infection are higher due to shared routes of transmission. Because Th1 and Th2 cells mutually suppress each other's function, it is probable that co-infection with *T. gondii* and *T. canis* could alter host immunity to one or both parasites. Previous work in mice showed that host trapping of *T. canis* larvae in the liver during secondary infection was associated with high levels of *T. canis*-specific IgG1 antibodies. The present study was carried out to assess whether prior infection with *T. gondii* would affect the host response to *T. canis* and thus interfere with liver trapping. Mice were infected with *T. gondii* (type II, ME49) oocysts and then inoculated twice with *T. canis* eggs. Mice infected acutely with *T. gondii* or chronically with a high dose had significantly fewer larvae in their livers than *T. canis* mono-infected mice. This corresponded to lower levels of *T. canis*-specific IgG1and higher levels of IgG2a in co-infected mice, suggesting that *T. gondii* shifted host antibody production away from one toward the other. Analysis of hepatic infiltrates showed a reduction in activated CD8+ T lymphocytes in co-infected mice compared to *T. gondii* mono-infected mice, pointing to *T. canis*-induced suppression of cell-mediated immunity to *T. gondii*. Given that these two parasites likely persist for the lifetime of the host, this evidence of cross-regulation may have important implications for an infected host's response to vaccination and other immunological challenges throughout life.

Introduction

The Centers for Disease Control and Prevention include toxocariasis and toxoplasmosis on their list of top 5 neglected parasitic infections in the USA. These are two of the most common zoonotic parasites of people, affecting millions of individuals, particularly in poor communities.[1–3] Co-infection with *Toxocara canis* and *Toxoplasma gondii* is likely to occur due to their shared routes of transmission, including contaminated food, water, or soil, and use of paratenic hosts in their life cycles.[4,2] Evaluation of seroprevalence of antibodies against these two organisms in the USA indicates that a person who has one of these infections is twice as likely to also have the other.[4]

Previous work from our lab has shown that trapping of *T. canis* larvae in the liver of mice during secondary infection is strongly associated with *T. canis*-specific serum IgG1 antibodies (see Chapter 2, Figure 2.4 B-C and F, Figure 2.6 A-B). This is a T helper type 2 (Th2)-dominated response characteristic of many helminth infections. It is thought that by hindering larval exit from the liver, invasion of the brain – and subsequently the eyes – can be prevented. However, if the host were to experience other immunological stimuli that alter this response, then the potential protective benefit would be lost. The likelihood of human co-infection with *T. canis* and *T. gondii* thus brings up the interesting question of how a host's immune system would handle co-infection by two parasites that induce dichotomous Th2 and Th1 responses.

Cross-talk between the different arms of the adaptive immune system is well established, and one of the best studied is the mutually suppressive effect of Th1 and Th2 cells on each other's development and function. Many researchers have investigated such interactions in the context of concomitant infection or immune-mediated disease. For example, pigs infected with the nematode *Ascaris suum* exhibited poor seroconversion after vaccination against the

bacterium *Mycoplasma hyopneumoniae*.[5] Mice infected with the fluke *Schistosoma mansoni* prior to *T. gondii* infection experienced high mortality due to deficient production of IFN-γ and nitric oxide.[6] Conversely, infection with *T. gondii* followed by the nematode *Nippostrongylus brasiliensis* resulted in reduced IL-4 and IL-5 produced in response to the latter.[7] When *Fasciola hepatica* fluke infection was preceded by *T. gondii*, the immune response was shifted dramatically: no *F. hepatica*-specific IgG1 antibodies were generated, splenocytes failed to produce IL-4 and IL-5, and macrophages were no longer alternatively activated.[8] Depending on the pathogens involved and the order of infection, the change in immune response varies widely.

Unlike some of the disease models listed above, which study non-murine pathogens using a murine host, the present study investigated co-infection with *T. canis* and *T. gondii* in a natural paratenic host, the mouse, with a focus on the hepatic larval trapping process. Since people are also natural paratenic hosts for both of these parasites and experience similar pathogenesis, the mouse is an excellent model for human co-infection. Two different time points were considered: acute *T. gondii* infection and chronic. The acute phase of murine toxoplasmosis covers the 3- to 4-week period after oocyst ingestion, when tachyzoites are still replicating and active inflammation persists.[9] Mice are often clinically ill during this time and may die from the disease. Around 3 weeks post-infection, surviving mice begin to return to clinical health and transition into the chronic, latent phase of toxoplasmosis.[9] Immunocompetent hosts keep the infection under control with continual production of IFN-γ by Th1 and CD8+ T cells.[10] This is the phase of most relevance to *T. gondii*-infected people since the majority of them have passed beyond the acute stage and into a chronic carrier state.

Assessing the alterations in hepatic larval trapping and the immune response during acute and chronic co-infection would shine a light on how immunologically active the latent phase of

toxoplasmosis is. It could also provide clues as to whether co-infection raises the risk of ocular toxocariasis. Finally, it may help inform vaccine design to ensure the desired response is elicited even in the face of pre-existing infections that inappropriately bias the host immune response.

Results

To evaluate the effect of a pre-existing *T. gondii* infection on the host immune response to *T. canis* and the associated larval trapping in the liver, co-infection studies were performed. In the first set of experiments, designated acute co-infection, mice were inoculated with the sensitizing dose of *T. canis* eggs just two weeks after *T. gondii* infection – at a time when active replication of tachyzoites was still ongoing. The challenge dose of *T. canis* was delivered four weeks later. Intriguingly, co-infected mice (CO) had three times fewer larvae present in their livers compared to the *T. canis*-only control (TCC) group (Figure 3.1 A). As previously demonstrated in Chapter 2, the extent of larval trapping was positively associated with the abundance of anti-*T. canis* IgG1 antibodies in the serum (Figure 3.1 B). Past investigators have observed a lymphocytic and eosinophilic inflammation in the submucosa and lamina propria of the small intestine in response to *T. canis* larval invasion, with development of an encapsulating granuloma as early as 7 days after infection.[11,12] To rule out the possibility that *T. gondii* infection enhanced inflammation in the gastrointestinal tract and thereby blocked larvae from reaching the liver, the small and large intestine of TCC and CO mice were also processed for larval quantification. No significant difference was detected in the number of larvae found in the intestines of the two groups (Figure 3.1 C).

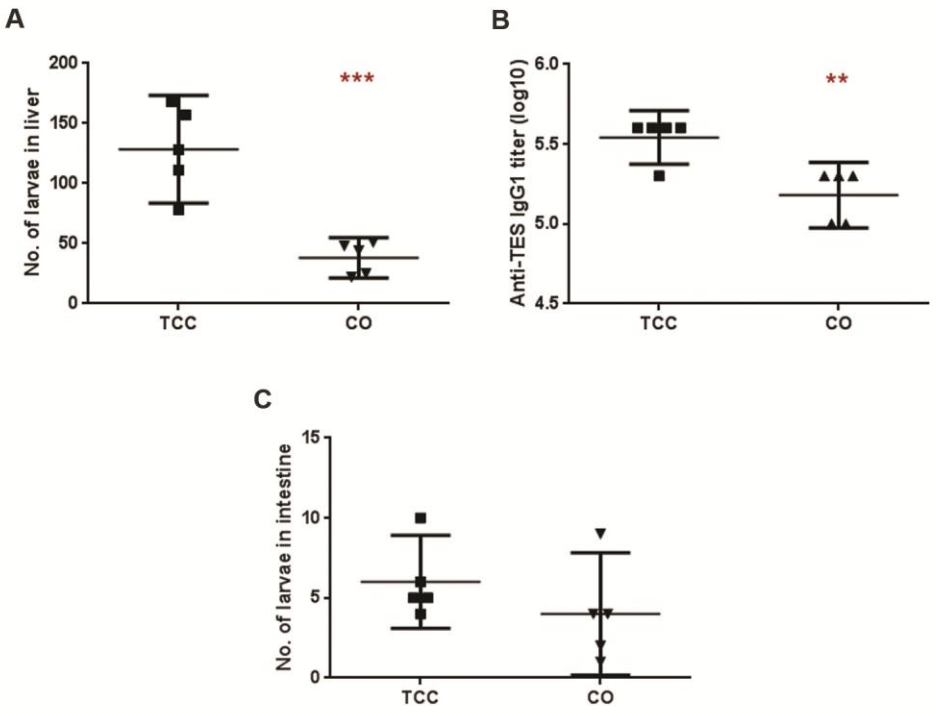


Figure 3.1. Acutely co-infected mice lose the ability to trap *T. canis* larvae in the liver. The co-infected (CO) and *T. gondii* control (TGC) groups were inoculated with *T. gondii* oocysts. Two weeks later, CO and *T. canis* control (TCC) mice were given a sensitizing dose of *T. canis* eggs followed by a challenge dose four weeks after that. Larvae in the liver (A) and intestines (C) were recovered by acid-pepsin digestion. Titer of *T. canis* excretory-secretory (TES)-specific IgG1 antibodies was determined by ELISA (B). Group means (n = 5) and 95% confidence intervals are shown. Each dot represents a single mouse. For Dunnett's post-test, all groups were compared to the reference group TCC. ** $p < 0.01$, *** $p < 0.001$.

Flow cytometry was performed to assess changes in immune cell populations within the livers of CO mice versus TCC and *T. gondii* control (TGC) mice. CO and TGC groups had a lower proportion of CD4+ T cells (Figure 3.2 A-B), but no difference was evident when evaluating the absolute number of CD4+ T cells (Figure 3.2 D). Not surprisingly, a marked rise in CD8α+ T cell numbers was observed in CO mice compared to TCC mice (Figure 3.2 A, C, and E), most likely reflecting an expansion of *T. gondii*-specific cells. Within the CD4+ T cell population, Th1 numbers in the CO group were elevated but not significantly different from TCC group ($p = 0.058$) (Figure 3.3 A, D, and G). Aside from a very low Th2 cell count in the TGC group, no differences were found in the Th2 or Treg subsets between the three groups (Figure 3.3 B-C, E-F, and H-I). Interestingly, examination for IFN-γ production in CD8α+ T cells revealed a population of cytotoxic effector T lymphocytes in the CO group that was intermediate in size between TCC and TGC (Figure 3.4 A, C, and E). This finding supports a downregulatory effect imposed by *T. canis* on the host response to the concurrent *T. gondii* infection. Also noteworthy is the decreased number of CD11b+ phagocytes in the liver of CO mice relative to TCC mice (Figure 3.4 B, D, and F). Alternatively activated macrophages (AAM) recruited to the site of tissue invasion of certain parasitic larvae have been shown to inhibit larval motility via Arginase-1 production of L-ornithine and polyamines.[13,14] A similar mechanism may be at work here in the hepatic trapping of *T. canis* larvae, and *T. gondii* may abrogate that response by interfering with the activation and subsequent recruitment of AAMs to the liver during co-infection.

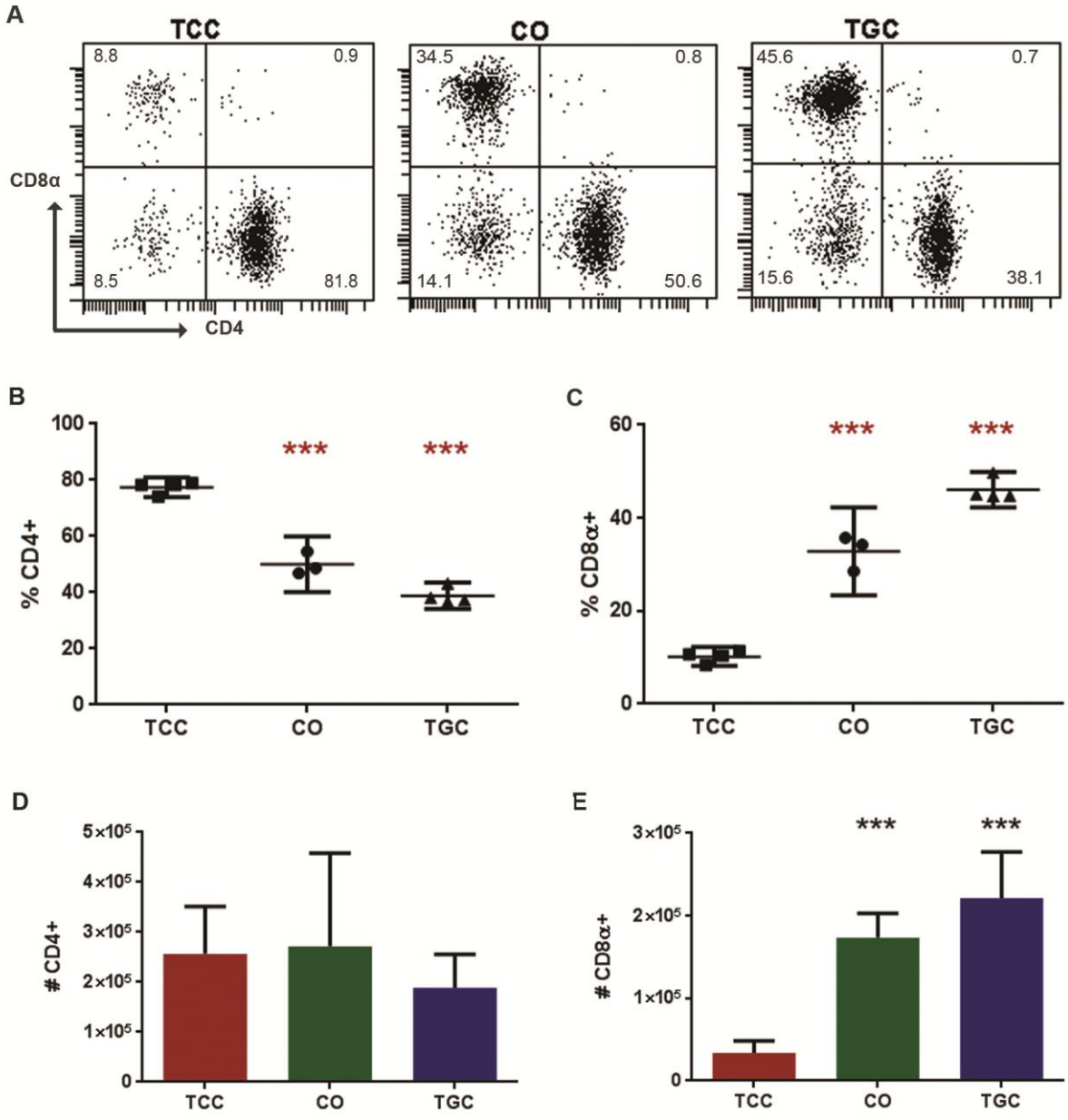
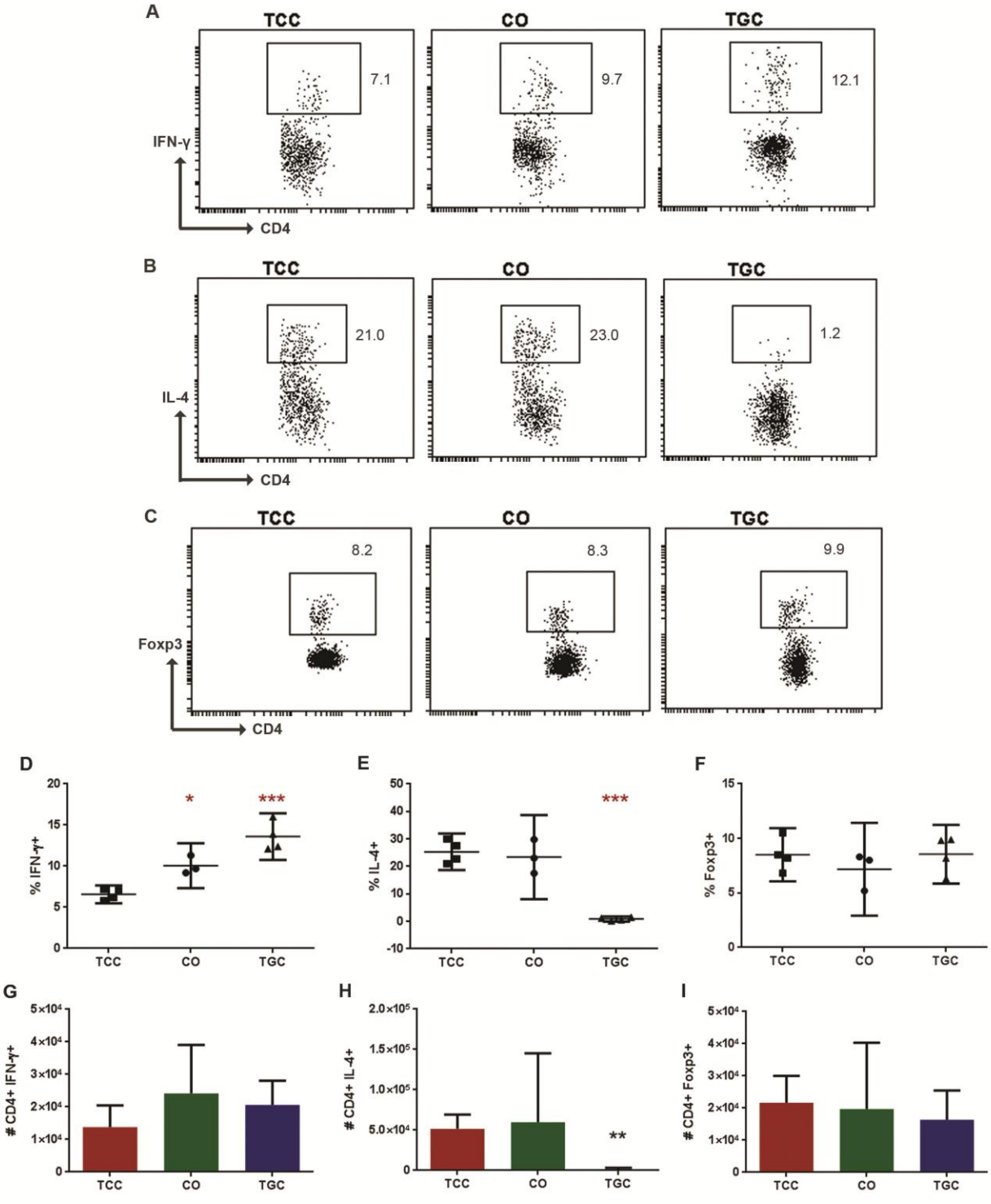

A
TCC
CO
TGC
8.8
0.9
8.5
81.8
34.5
0.8
14.1
50.6
45.6
0.7
15.6
38.1
CD8α
CD4
B
% CD4+
100
80
60
40
20
0
***
***
TCC
CO
TGC
C
% CD8α+
60
40
20
0
***
***
TCC
CO
TGC
D
CD4+
5×10⁵
4×10⁵
3×10⁵
2×10⁵
1×10⁵
0
TCC
CO
TGC
E
CD8α+
3×10⁵
2×10⁵
1×10⁵
0
***
***
TCC
CO
TGC

Figure 3.2. Mono- or co-infected mice with acute toxoplasmosis have greater expansion of CD8α+ T cells in the liver than *T. canis* mono-infected mice. Single cell suspensions were prepared from the livers of *T. canis* control (TCC), co-infected (CO), and *T. gondii* control (TGC) mice and stained for flow cytometric analysis of CD3ε, CD4, and CD8α expression. Plots from representative mice (A) showing percentage of CD3ε+ cells expressing CD4 and CD8α. Percentage (B-C) and total number (D-E) of CD4+ and CD8α+ T cells in the livers of mono- and co-infected mice. Each dot represents an individual mouse (n = 3-4 per group). Graphs display means and 95% confidence intervals. For Dunnett's post-test, all groups were compared to TCC. *** $p < 0.001$.


A
TCC
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TGC
7.1
9.7
12.1
IFN-γ
CD4
B
TCC
CO
TGC
21.0
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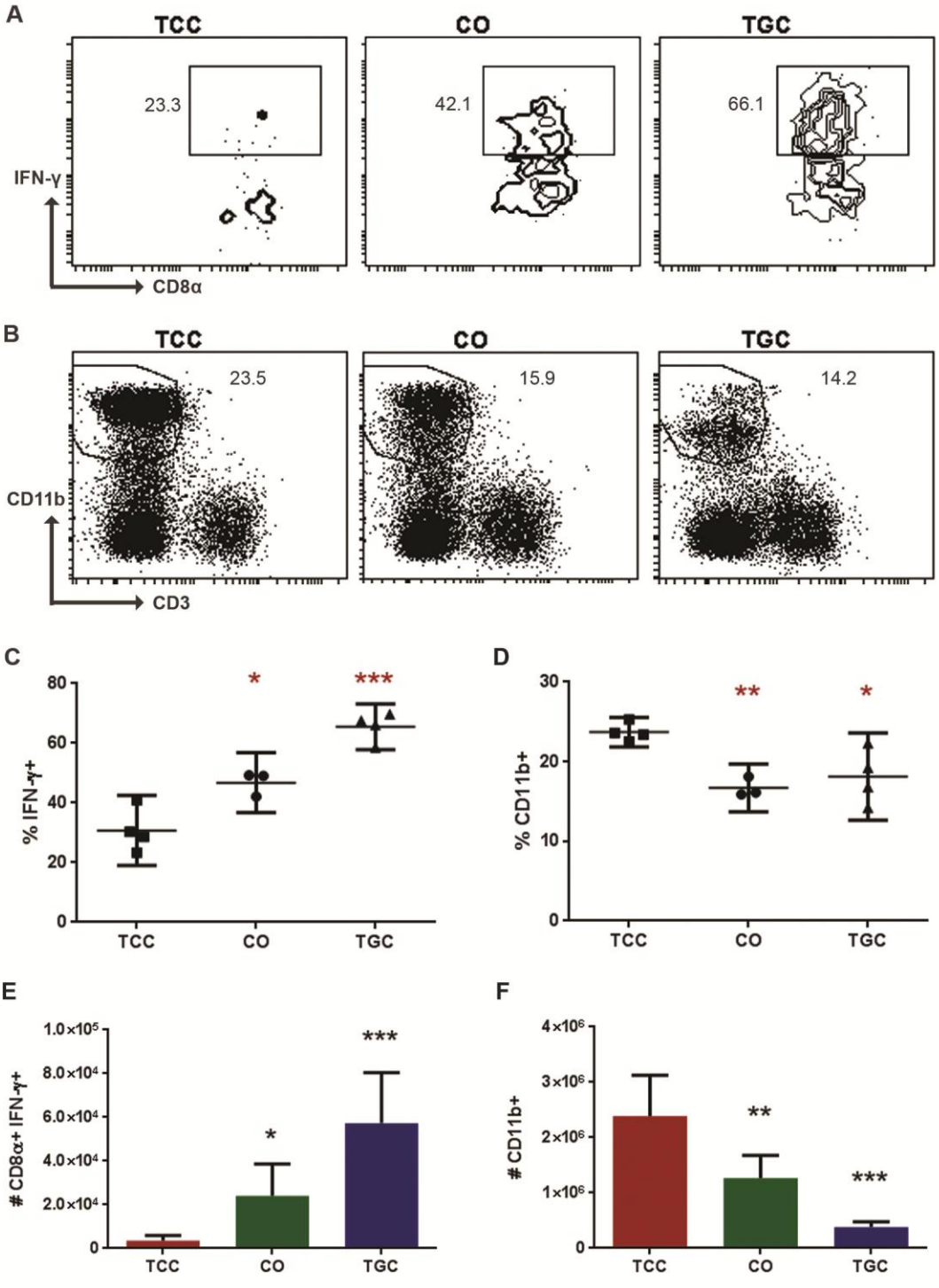
IL-4
CD4
C
TCC
CO
TGC
8.2
8.3
9.9
Foxp3
CD4
D
% IFN-γ+
*
***
TCC
CO
TGC
E
% IL-4+
***
TCC
CO
TGC
F
% Foxp3+
TCC
CO
TGC
G
CD4+ IFN-γ+
TCC
CO
TGC
H
CD4+ IL-4+
**
TCC
CO
TGC
I
CD4+ Foxp3+
TCC
CO
TGC

Figure 3.3. T helper 1 cell numbers are non-significantly increased in co-infected mice compared to *T. canis* controls. Mononuclear cells were isolated from the livers of *T. canis* control (TCC), co-infected (CO), and *T. gondii* control (TGC) mice by Histopaque-1077 density gradient centrifugation. (A-B, D-E, G-H) Cells were cultured for 4 hours in the presence of brefeldin A, PMA, and ionomycin. Cells were then stained for viability, CD3ε, CD4, CD8α, and for intracellular IFN-γ and IL-4. (C, F, I) Uncultured cells were stained for CD3ε, CD4, CD8α, and the transcription factor Foxp3. Plots from representative mice (A-C) showing percentage of CD3ε+ CD4+ cells belonging to the IFN-γ+ Th1 subset, IL-4+ Th2 subset, and Foxp3+ Treg subset. Percentage (D-F) and total number (G-I) of Th1, Th2, and Treg cells in the livers of mono- and co-infected mice. Each dot represents an individual mouse (n = 3-4 per group). Graphs display means and 95% confidence intervals. For Dunnett's post-test, all groups were compared to TCC. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.


A
TCC
CO
TGC
23.3
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66.1
IFN-γ
CD8α
B
TCC
CO
TGC
23.5
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CD11b
CD3
C
% IFN-γ+
80
60
40
20
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TCC
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D
% CD11b+
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CD8α+ IFN-γ+
1.0×10⁵
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CD11b+
4×10⁶
3×10⁶
2×10⁶
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TCC
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Figure 3.4. Acutely co-infected mice exhibit an intermediate level of cytotoxic T lymphocyte activation and macrophage recruitment to the liver compared to mono-infected controls. Mononuclear cells were isolated from the livers of *T. canis* control (TCC), co-infected (CO), and *T. gondii* control (TGC). (A, C, E,) Cells were cultured for 4 hours in the presence of brefeldin A, PMA, and ionomycin, then stained for viability, CD3ε, CD4, CD8α, and IFN-γ. (B, D, F) Uncultured cells were stained for CD3ε and CD11b. Plots from representative mice showing percentage of CD3ε+ CD8α+ cells with IFN-γ expression (A) and percentage of CD3ε- CD11b+ cells (B). Percentage (C-D) and total number (E-F) of IFN-γ+ cytotoxic T lymphocytes and CD11b+ macrophages in the livers of mono- and co-infected mice. Each dot represents an individual mouse (n = 3-4 per group). Graphs display means and 95% confidence intervals. For Dunnett's post-test, all groups were compared to TCC. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The majority of people infected with *T. gondii* are beyond the active stage of replication and are instead chronic carriers of tissue cysts. Therefore to determine whether the above-noted alterations in *T. canis* larval trapping and hepatic immune cell populations holds true during long-term *T. gondii* infection, chronic co-infection studies were carried out. In this case, *T. canis* inoculation was delayed for eight to ten weeks after *T. gondii* inoculation to ensure the latter transitioned naturally into the latent phase of infection. Two co-infection groups were included, one given a standard dose of 100 *T. gondii* oocysts (CO) as used in the acute co-infection experiments, and the other given a high dose of 1000 oocysts (COK).

A downward trend in hepatic larval counts was seen in co-infected mice, with a significant difference from TCC mice evident in the COK group (Figure 3.5 A). The same pattern was observed in the serum IgG1 titers against *T. canis* (Figure 3.5 B). Clearly, there is a dose dependence to the degree of influence *T. gondii* infection exerts on *T. canis* larval migratory arrest in the liver. With chronicity of toxoplasmosis, a higher initial *T. gondii* dose is required for the effect on trapping to become apparent. Nevertheless, the alteration is appreciable.

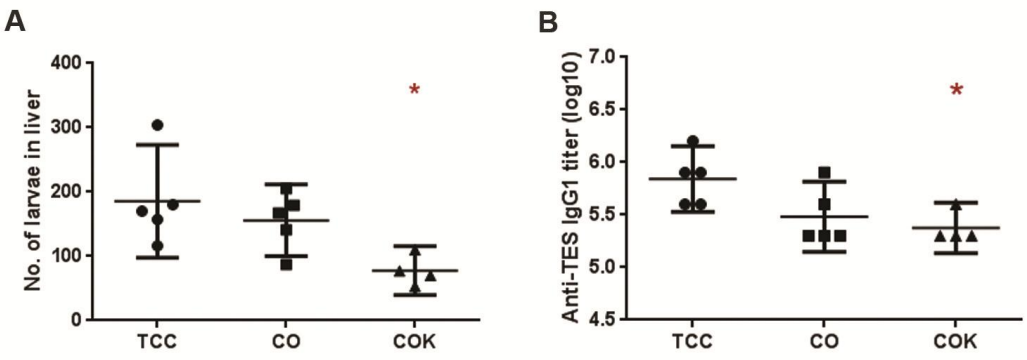
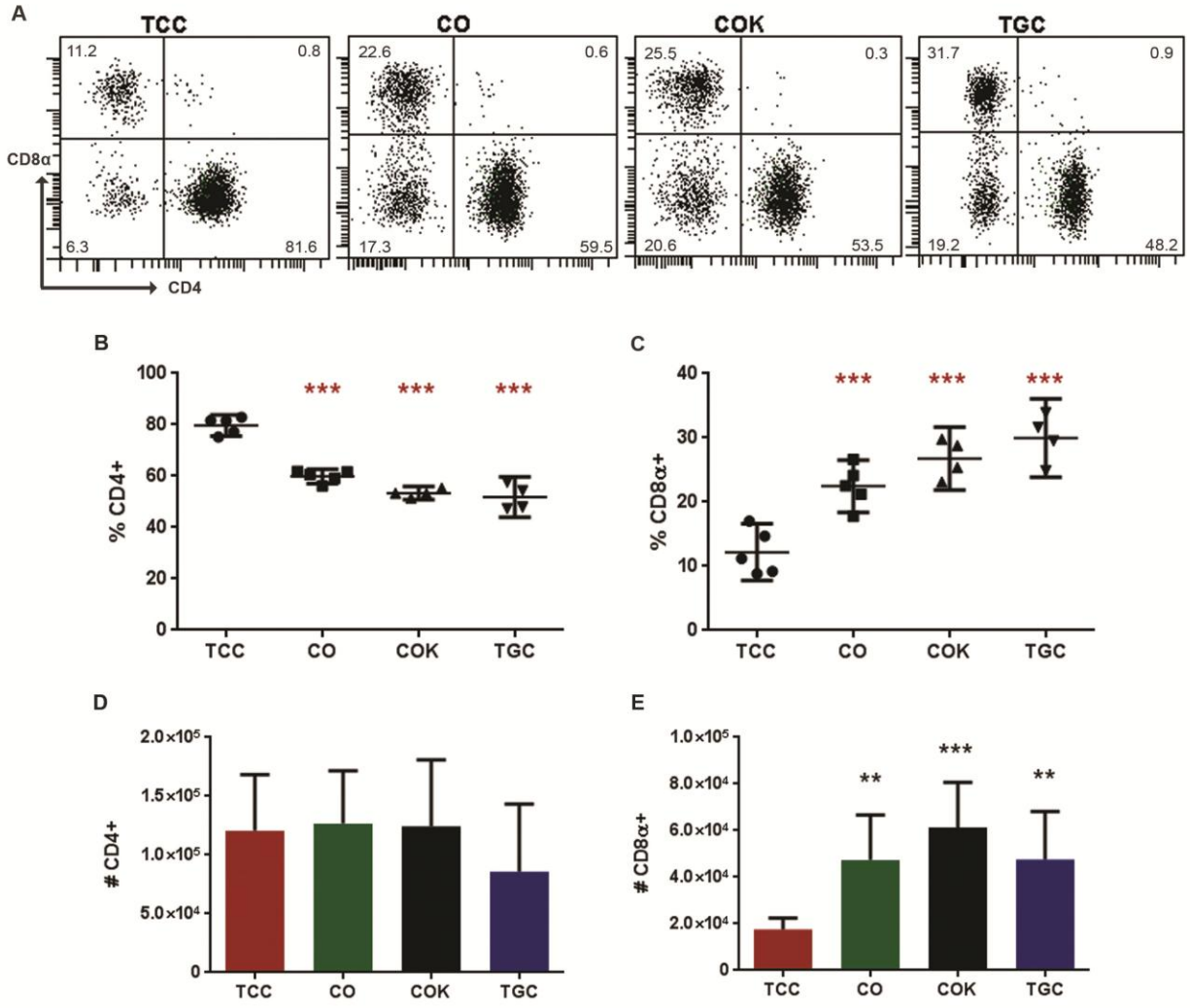


Figure 3.5. Chronically co-infected mice display a reduced ability to trap *T. canis* larvae in a *T. gondii*-dose-dependent manner. The standard dose co-infected (CO), high dose co-infected (COK) and *T. gondii* control (TGC) groups were inoculated with *T. gondii* oocysts. Eight to ten weeks later, CO, COK, and *T. canis* control (TCC) mice were given a sensitizing dose of *T. canis* eggs followed by a challenge dose four weeks afterward. Larvae were liberated from the liver by acid-pepsin digestion (A). Titer of *T. canis* excretory-secretory (TES)-specific IgG1 antibodies was determined by ELISA (B). Group means (n = 4-5) and 95% confidence intervals are shown. Each dot represents a single mouse. For Dunnett's post-test, all groups were compared to the reference group TCC. * $p < 0.05$.

Analysis of cellular infiltrates within the liver of chronically co-infected mice showed a similar picture to that of acutely co-infected mice. In short, CO, COK, and TGC groups had a lower percentage of CD4+ T cells in their livers (Figure 3.6 A-B and D) owing to the higher overall number of CD8α+ T cells in these mice (Figure 3.6 A, C, and E). Th1 cells were significantly increased in the two co-infected groups (Figure 3.7 A, D, and G); however, the possibility of additive effect from the two individual infections cannot be ruled out. CO and COK mice had as many Th2 cells as TCC controls (Figure 3.7 B, E, and H), and no differences in the Treg subset were observed (Figure 3.7 C, F, and I). In chronically co-infected mice (CO, COK), absolute numbers of IFN-γ+ CD8α+ T cells equaled that of TGC mice (Figure 3.8 A-C) rather than being intermediate between TCC and TGC control groups (Figure 3.4 A, C, and E). This change would be consistent with a long-established *T. gondii*-specific cytotoxic T cell population being more stable and less perturbable by newly introduced antigenic stimuli.

One other finding of interest is the greater abundance of IgG2a antibodies directed against *T. canis* in co-infected mice compared to TCC mice. A suggestion of this trend can be seen in the graph from an acute co-infection experiment (Figure 3.9 A); when pooled with data from a repeat experiment, the results of which were significant on their own, the tendency was confirmed (Figure 3.9 B). Results from chronic co-infection experiments also follow the same pattern (Figure 3.9 C). The IgG2a antibody isotype is associated with a Th1 response. The increased production of this isotype with specificity for *T. canis* suggests that prior infection with the Th1-promoting parasite *T. gondii* shifted the host humoral response to *T. canis* in that same direction. This would account for the correspondingly lower levels of IgG1 antibodies in co-infected mice.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B
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40
20
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TCC
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C
% CD8α+
40
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20
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TCC
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COK
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5.0×10^4
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8.0×10^4
6.0×10^4
4.0×10^4
2.0×10^4
0
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***
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TCC
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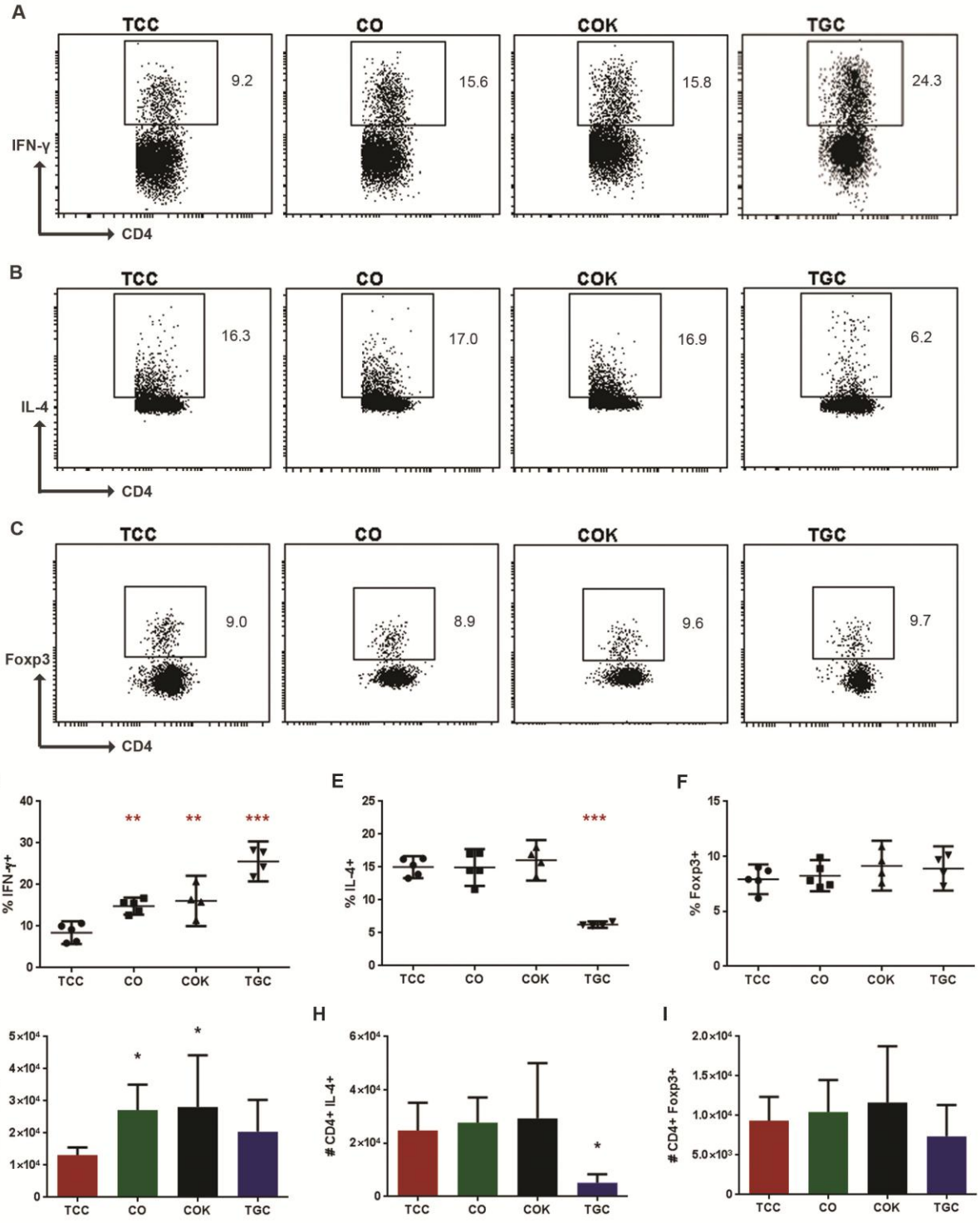
Figure 3.6. Mice chronically infected with *T. gondii* alone or in combination with *T. canis* have a significantly larger population of CD8α+ T cells in their livers compared to *T. canis* mono-infected mice. Single cell suspensions were prepared from the livers of *T. canis* control (TCC), standard dose co-infected (CO), high dose co-infected (COK), and *T. gondii* control (TGC) mice and stained for CD3ε, CD4, and CD8α expression. Plots from representative mice (A) showing percentage of CD3ε+ cells expressing CD4 and CD8α. Percentage (B-C) and total number (D-E) of CD4+ and CD8α+ T cells in the livers. Each dot represents an individual mouse (n = 4-5 per group). Graphs display means and 95% confidence intervals. For Dunnett's post-test, all groups were compared to TCC. ** $p < 0.01$, *** $p < 0.001$.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6.2
IL-4
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9.7
Foxp3
CD4
D
% IFN-γ+
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TCC
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TGC
F
% Foxp3+
TCC
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CD4+ IFN-γ+
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CD4+ IL-4+
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COK
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CD4+ Foxp3+
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CO
COK
TGC

Figure 3.7. T helper 1 cell numbers are elevated in chronically co-infected mice compared to *T. canis* controls. Mononuclear cells were isolated from the livers of *T. canis* control (TCC), standard dose co-infected (CO), high dose co-infected (COK), and *T. gondii* control (TGC) mice. (A-B, D-E, G-H) Cells were cultured for 4 hours in the presence of brefeldin A, PMA, and ionomycin and then stained for viability, CD3ε, CD4, CD8α, IFN-γ, and IL-4. (C, F, I) Uncultured cells were stained for CD3ε, CD4, CD8α, and Foxp3. Plots from representative mice (A-C) showing percentage of CD3ε+ CD4+ cells belonging to the IFN-γ+ Th1 subset, IL-4+ Th2 subset, and Foxp3+ Treg subset. Percentage (D-F) and total number (G-I) of Th1, Th2, and Treg cells in the livers of mono- and co-infected mice. Each dot represents an individual mouse (n = 4-5 per group). Graphs display means and 95% confidence intervals. For Dunnett's post-test, all groups were compared to TCC. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

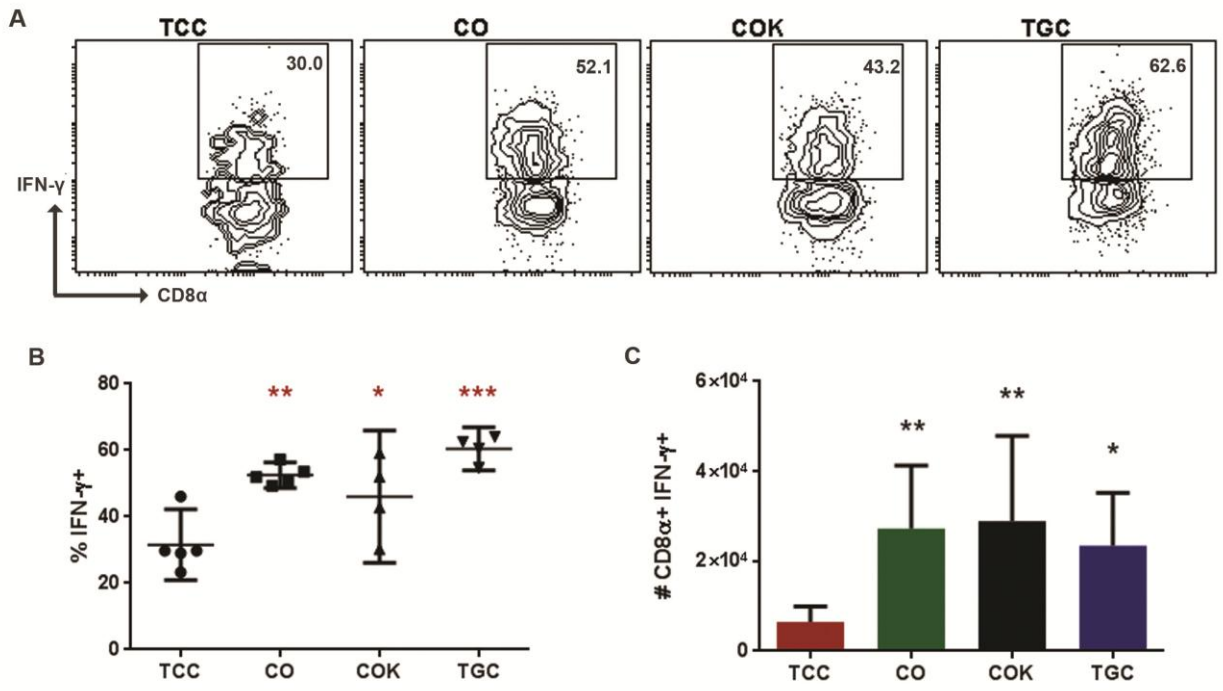

Figure 3.8. Chronically co-infected mice have an expanded population of activated cytotoxic T lymphocytes in the liver compared to *T. canis* mono-infected controls. Mononuclear cells were isolated from the livers of *T. canis* control (TCC), standard dose co-infected (CO), high dose co-infected (COK), and *T. gondii* control (TGC) mice. Cells were cultured for 4 hours in the presence of brefeldin A, PMA, and ionomycin, then stained for viability, CD3ε, CD4, CD8α, and IFN-γ. Plots from representative mice (A) displaying percentage of CD3ε+ CD8α+ cells with IFN-γ production. Percentage (B) and total number (C) of IFN-γ+ cytotoxic T lymphocytes in the livers of mono- and co-infected mice. Each dot represents an individual mouse (n = 4-5 per group). Graphs show means and 95% confidence intervals. For Dunnett's post-test, all groups were compared to TCC. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

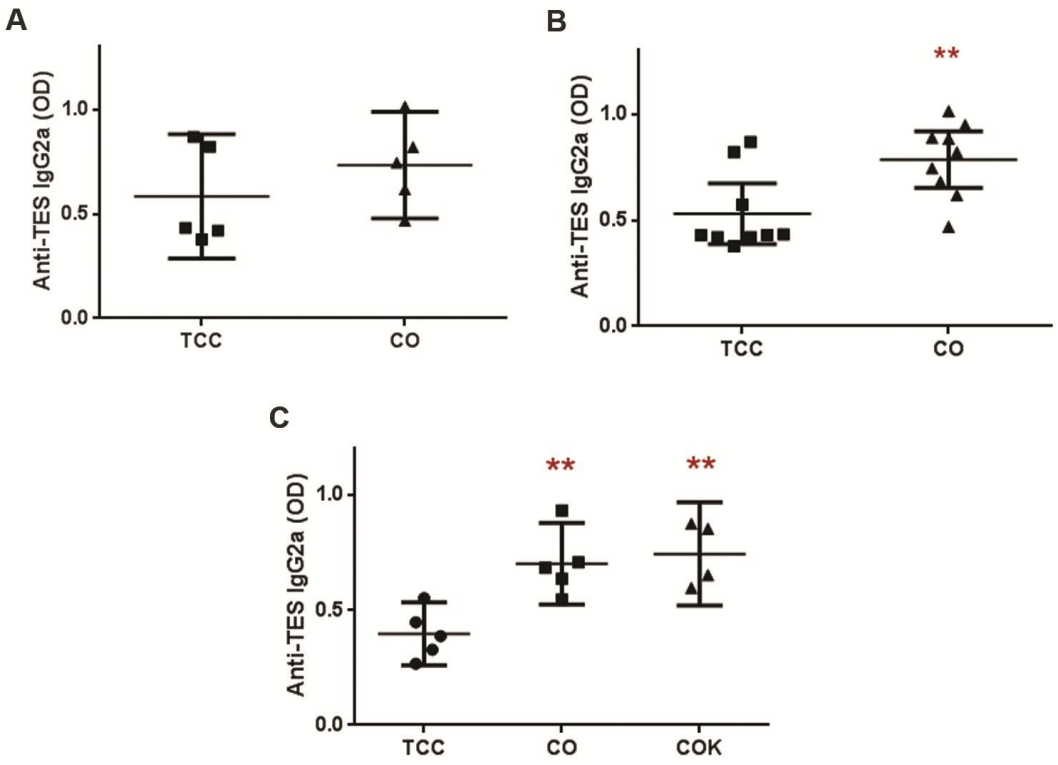

Figure 3.9. Increase in *T. canis*-specific IgG2a antibody production in co-infected mice points to a skewing of the host response against *T. canis* larvae in a Th1 direction. *T. canis* excretory-secretory (TES)-specific antibody abundance was measured by ELISA for *T. canis* control (TCC), standard dose co-infected (CO), and high dose co-infected (COK) mice. Displayed are IgG2a levels from one acute co-infection experiment (n = 5) (A), pooled results from two experiments (n = 9) (B), and results from one chronic co-infection experiment (n = 4-5) (C). Group means and 95% confidence intervals are shown. Each dot represents a single mouse. For Dunnett's post-test, all groups were compared to the reference group TCC. ** $p < 0.01$.

Discussion

Pre-existing infection with *T. gondii* abrogated hepatic trapping of *T. canis* larvae in co-infected mice, concurrent with a drop in *T. canis*-specific IgG1 levels. This is consistent with past work showing that larval trapping is strongly correlated with serum IgG1 (see Chapter 2, Figure 2.4 F). The possibility that larvae may have been halted in the intestine prior to entry into the liver was ruled out by the finding that the small and large intestines of CO and TCC groups contained equivalent numbers of larvae. However, it is uncertain whether larval survival might be adversely affected by concurrent *T. gondii* infection, causing the hepatic larval count to be lower due to a body-wide decline. The elevated levels of IgG2a in co-infected mice might offer a potential mechanism for such a decline in larval viability. IgG2a is a potent inducer of antibody-dependent cell-mediated cytotoxicity (ADCC).[15] This process has been implicated in the killing of other tissue-dwelling helminths such as *Strongyloides stercoralis*, *Schistosoma mansoni*, and *Trichinella spiralis*.[16–18] However, antibody- and complement-mediated binding of macrophages, neutrophils, and eosinophils to *T. canis* larvae has been demonstrated before with no perceptible injury to the larvae, so ADCC may not be effective against this parasite.[19] Nevertheless, it would be worthwhile to take full-body counts to confirm that the lower number of larvae in the liver is truly due to an escape from trapping.

The greater abundance of anti-*T. canis* IgG2a in co-infected versus TCC mice substantiates the presence of a Th1-skewing microenvironment in mice infected with *T. gondii*. B cell class switching is directed by secreted cytokines such as IL-4 and IFN-γ. A so-called bystander B cell, activated by *T. canis* antigen while receiving IFN-γ signal from the concurrent *T. gondii* infection, can be induced to switch to the IgG2a isotype rather than the typical IgG1 or

IgE associated with helminthiasis.[15] The consequent decrease in IgG1 production would account for the impaired trapping of *T. canis* larvae in the livers of co-infected mice.

Abrogation of trapping was related to the dose of *T. gondii* administered in chronic toxoplasmosis experiments. Immunological control of *T. gondii* in the chronic phase of infection is reliant on IFN-γ secretion by CD8+ T lymphocytes and Th1 cells to prevent reactivation of tissue cysts.[10] It is assumed that the larger the infecting dose, the higher the number of tissue cysts that eventually form, and the greater the need for more immune cells to keep the infection in check. The corresponding increase in IFN-γ secretion would again lead to IgG2a class switching, providing an explanation for the *T. gondii* dose dependence of hepatic trapping.

A limitation of this study was the use of PMA and ionomycin to non-specifically stimulate cells for evaluation of T cell populations within the liver. Antigen-specific stimulation would have better allowed for determination of changes in cell-mediated immunity directed against each parasite in the face of co-infection. Regardless, the flow cytometry analysis did yield some interesting results that warrant further investigation. Phagocyte numbers were high in TCC mice, reduced in acutely co-infected mice, and low in TGC mice. This may suggest a role for macrophages in trapping *T. canis* larvae, similar to what has been described for the larvae of the intestinal nematodes *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus*.[13,14] However, this pattern was not repeated in chronic co-infection experiments (data not shown), and so these results must be interpreted with caution. This concept bears further study.

In the acute co-infection experiments, the number of IFN-γ+ CD8α+ T cells in the CO group was intermediate between the TCC and TGC groups. This strongly suggests reciprocal dampening by *T. canis* of the cellular response to *T. gondii*. A similar effect was observed in mice infected with *Heligmosomoides polygyrus* followed by *T. gondii*, in that *T. gondii*-specific

CD8+ effector T cells failed to develop.[20] The cytokine IL-12 acts as the third signal for full CD8+ T cell activation, and the ability of *T. canis* to downregulate macrophage production of IL-12 may contribute to the reduction of CD8α+ effector cells in the CO group.[21–23] In chronic toxoplasmosis, where cellular memory for *T. gondii* has already been established, no such cross-regulation by *T. canis* is evident.

This study showed that co-infection with *T. gondii* shifted anti-*T. canis* antibody production from an IgG1 to an IgG2a isotype. The consequent decrease in IgG1 antibodies resulted in impaired larval trapping in the liver of co-infected mice. Also notable was the indication of reciprocal dampening of the host response against *T. gondii* as evidenced by the reduction in IFN-γ+ CD8+ T cells. The decline in CD11b+ cells in co-infected mice compared to *T. canis* control mice suggests that macrophages or another type of CD11b+ innate cell may be involved in hepatic trapping.

Future work should be aimed at delineating the antigen-specific responses of the immune infiltrates within the liver to get a better picture of the immunologic changes imposed by *T. gondii* upon the host response to *T. canis*. This would also allow for examination of any reverse effects. It could be taken a step further by altering the infection timeline such that *T. canis* inoculation occurs first, or both parasites are administered concomitantly as might happen in nature due to the common transmission routes of the two. Whole-body counts of larvae should be performed to rule out the possibility of increased larval killing in co-infected mice. Finally, the macrophage population within the liver should also be evaluated for markers of conventionally versus alternatively activated macrophages to determine if Arginase-1 and its products play a part in *T. canis* hepatic trapping.

Methods

Mice and experimental design

Eight-week-old female BALB/cJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). For acute toxoplasmosis experiments, the co-infection (CO) and *T. gondii* control (TGC) groups were inoculated orally with 100 *T. gondii* oocysts. Two weeks later, CO and *T. canis* control (TCC) groups were inoculated orally with a sensitizing dose of 200 *T. canis* eggs. After another four weeks, CO and TCC mice were inoculated with a challenge dose of 500 *T. canis* eggs. All mice were euthanized one week post-challenge. Serum was collected by cardiac puncture. Livers of a subset of each group (aside from TGC) were utilized for larval quantification. The remaining livers were processed for cell population analysis by flow cytometry. In one experiment, larvae in the small and large intestine were also counted.

The experimental design was the same for chronic toxoplasmosis experiments, with the following exceptions: a second co-infection group (COK) received 1000 *T. gondii* oocysts instead of 100, and the time interval between *T. gondii* infection and *T. canis* sensitization was lengthened to 8-10 weeks.

T. gondii oocysts and *T. canis* eggs

T. gondii type II ME49 sporulated oocysts were provided by Dr. J. P. Dubey of the USDA-ARS. Oocysts were purified by cesium chloride gradient and stored in 2% sulfuric acid at 4°C until use.[24] For preparation of inocula, oocyst concentration was determined by hemacytometer count. Serial 1:10 dilutions were made, neutralized with NaOH, and the volume adjusted to the desired concentration. Doses were prepared in a final volume of 100 uL and delivered by gavage.

T. canis eggs were collected from the uteri of female worms by dissection. Eggs were suspended in 0.6% sodium hypochlorite with frequent agitation for 10 minutes and then washed several times with water. Eggs were transferred to vented culture flasks containing 0.1N sulfuric acid and kept at 28°C for 4 weeks. Larvated eggs were stored at 4°C until use. Inocula were prepared in a volume of 100 μL and administered by gavage.

Hepatic cell population analysis

Mononuclear cells were isolated from the liver and prepared for flow cytometry as described in Chapter 2. Briefly, livers were perfused with iced-cold PBS, diced, and strained through a sterile 800 μm stainless steel mesh. Pelleted cells were resuspended in complete Dulbecco's modified Eagle's medium (cDMEM) consisting of 10% bovine growth serum (Hyclone), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 30 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin (all from Life Technologies), and 0.05 mM β-mercaptoethanol (Sigma). The suspension was digested using collagenase IV at 550 U/mL (Gibco) and passed through a 70 μm cell strainer, with two subsequent PBS washes. Mononuclear cells were separated from hepatocytes by density centrifugation with Histopaque-1077 (Sigma) and finally resuspended in cDMEM. Cells were split into two aliquots. One aliquot was surface stained with anti-CD3ε (BD Biosciences), anti-CD4 (BioLegend), anti-CD8α and anti-CD11b (both from eBioscience), then fixed and intracellularly stained with an anti-Foxp3 staining set (eBioscience). The second aliquot was incubated for 4 hours at 37°C with 3 μg/mL brefeldin A (BD Biosciences), 10 ng/mL PMA (Sigma), and 1 μg/mL ionomycin (Sigma). Cells were stained with viability dye (eBioscience) as per manufacturer instructions. CD3ε, CD4, and CD8 surface staining was followed by fixation/permeabilization using buffers from the Foxp3

staining set (eBioscience). Anti-IFN-γ and anti-IL-4 (both from BioLegend) were used for intracellular labeling. Flow cytometry was performed on a FACS Canto II (BD Biosciences), and data were analyzed using FACS Diva software (BD Biosciences).

Larval recovery from liver and intestines

The small and large intestine were removed after euthanasia and opened longitudinally to wash off luminal contents. Intestine and livers were separately homogenized in a blender and digested using 0.7% pepsin + 0.7% HCl (1-2 hour incubation with continuous agitation at 37°C). The digests were washed with PBS and neutralized with NaOH. Larvae were separated from tissue debris by density gradient centrifugation with Histopaque-1077 (Sigma) at 1000 x g for 25 minutes. Larvae were transferred onto gridded Sedgwick slides (Wildlife Supply Company, Tulee, FL) for counting on a compound microscope.

Serum antibody measurement

T. canis-specific antibodies were measured using an indirect ELISA as described in Chapter 2. Briefly, high-binding 96-well plates were coated with 10 μg/mL of *T. canis* excretory-secretory antigen. Wells were blocked with 2% bovine serum albumin in PBS. Serum was diluted 1:200 for IgG2a detection and 1:1000 for IgM. For IgG1 quantification, an antibody titer was established based on serial serum dilutions. Horseradish peroxidase-conjugated antibodies consisted of goat anti-mouse IgG1, goat anti-mouse IgG2a, or rabbit anti-mouse IgM (Jackson ImmunoResearch, West Grove, PA) diluted 1:5000 in PBS. Absorbance at 405 nm was measured with an automated spectrophotometer (model ELx800, Bio-Tek Instruments, Winooski, VT). For

antibody titer determination, the cut-off value was set at 1.5 x the average of all negative control wells (i.e. normal serum and PBS).

T. gondii cyst recovery from brains

Brains were bisected sagittally with a flat-edged razor and one half was macerated in a petri dish. The material was transferred into a 50 mL conical test tube with PBS. Using an 18 gauge blunt-tipped needle and syringe, the brain was vigorously aspirated up and down numerous times until homogeneous in appearance. The brain was pelleted by centrifugation at 1000 x g for 10 minutes then resuspended to a known volume of PBS (< 0.5 mL). Aliquots of 10 μL were examined under a microscope to enumerate cysts. If no cysts were observed in the first aliquot, additional aliquots were examined. The average number of cysts per μL was multiplied by the sample volume and then by 2 to calculate the number of cysts present in the whole brain.

Statistical analysis

Differences between two groups were analyzed using unpaired t-tests. For 3 or more groups, one-way ANOVA with Dunnett's post-test was performed, comparing all groups to the reference group TCC. Antibody titers were evaluated using the Mann-Whitney test or Kruskal-Wallis test with Dunn's comparison. ELISA values for TGC mice were negative and were excluded from statistical evaluation. Significance was set at a p-value of < 0.05. Data were analyzed using Prism software (GraphPad Software Inc., La Jolla, CA).

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CHAPTER 4

FACTORS AFFECTING *TOXOCARA CANIS* LONGEVITY AND TRAPPING, AND RECIPROCAL EFFECTS ON *TOXOPLASMA GONDII* INFECTION

Abstract

Antibody-mediated trapping of *Toxocara canis* larvae in the liver of re-infected mice has been studied in our lab using the BALB/c(J) mouse strain. Concomitant infection with *Toxoplasma gondii* has been demonstrated to abrogate liver trapping through interference with production of protective IgG1 antibodies against *T. canis*. In order to better understand the multiple host and immune factors that may contribute to *T. canis* migration and hepatic trapping, a series of experiments was carried out. Male mice exhibit enhanced trapping ability compared to females of the same strain. Eosinophils, which alternately protect the host or promote parasite growth in other instances, were shown not to influence *T. canis* longevity or migratory behavior in any discernible way when eosinophil-deficient dblGATA1-/- mice were infected. Loss of *T. canis* trapping in mice co-infected with *T. gondii* did not increase the number of larvae entering the brain of BALB/c mice, but did so in a non-statistically significant manner in mice from a C57BL/6 background. Co-infected mice possessed more *T. gondii* tissue cysts in their brains than controls, suggesting some degree of immunomodulation in host response by *T. canis*. Finally, inoculation of mice by the intravenous route during primary infection rather than by gavage appeared to result in less effective larval trapping upon challenge infection. The present work explored the effect of gender, route of sensitization, eosinophils, and mouse strain on *T. canis* longevity, distribution in host tissues, and hepatic trapping. Observed differences would inform selection of the best murine model for studying various aspects of human larval toxocariasis, moving forward.

Introduction

The immune-mediated process of *Toxocara canis* larval migratory arrest in the liver has been studied in our lab using the BALB/c(J) mouse strain. A key component of this reaction is pathogen-specific IgG1 antibodies which, when passively transferred into naïve recipient mice, confers the ability to trap *T. canis* larvae upon primary infection (see Chapter 2, Figure 2.6). *T. canis* hepatic trapping has been documented in several strains of mice, including C57BL/6J, SWR/J, C3H/HeJ, BALB/cByJ, CD1, and NIH, with some being more successful at it than others.[1,2] Gender differences in overall larval distribution within the body of the host have been noted in the mouse strains evaluated thus far (NIH, CD1, BALB/c): after a single infection, male mice had more larvae residing in their livers and less in the brain compared to females.[3,4] Castration reversed that distribution such that many more larvae were found in the brain and far fewer in the liver, suggesting a role for testosterone in controlling larval migration.[3] This male propensity for accumulating larvae in the liver extended into secondary *T. canis* infections, wherein male NIH and CD1 mice were much better able to trap larvae in the liver compared to females.[2] Whether a larger capacity for trapping exists in male BALB/c mice has not yet been evaluated.

Eosinophils were long thought to play a protective role against *T. canis* due to their massive expansion in peripheral blood after infection.[7,8] These innate immune cells provide effector function through release of cytotoxic mediators such as major basic protein, eosinophil cationic protein, and reactive oxygen species.[9,10] Yet a prior study found no difference in *T. canis* larval numbers in infected IL-5-deficient versus wild type mice.[11] Eosinophils were also suspected to contribute to *T. canis* hepatic trapping due to their frequent presence within the granulomas encasing larvae.[5,12] Anti-IL-5 treatment to deplete mice of eosinophils in vivo caused

no change in hepatic larval counts, appearing to discount this theory.[13] However, eosinophils were not completely ablated in either of these studies, so these results were not definitive. In recent years, a more symbiotic role for eosinophils in parasitic infection has been demonstrated. Eosinophils are recruited to the site of growth of the muscle-dwelling *Trichinella spiralis* larvae, which infect both animals and people.[10] They aid in growth and survival of the larvae by producing IL-10 and downregulating the harmful production of nitric oxide by neutrophils and macrophages.[10,14,15] Because *T. canis* larvae are also long-lived in host tissues, it seems plausible that eosinophils may serve a similar function of enhancing their survival. In order to conclusively answer the question of whether eosinophils benefit or harm *T. canis* larvae, studies with dblGATA1-/- knockout mice completely devoid of eosinophils were carried out.

Migratory arrest of *T. canis* larvae in the liver has been associated with reduced larval migration into the brain.[2,5,6] As shown in Chapter 3, pre-existing infection with *T. gondii* depressed IgG1 antibody production against *T. canis*, leading to impaired hepatic trapping. This should theoretically result in a higher number of larvae reaching the brains of co-infected mice. This was tested in the present study using 3 strains of mice: BALB/c, C57BL/6, and dblGATA1-/-. It was also hypothesized that the polarizing T helper 2 (Th2) response induced by *T. canis* infection would counter-regulate the T helper 1 (Th1) response generated by *T. gondii*, hampering host control of *T. gondii* replication. This would be reflected in a greater number of tissue cysts being present in the brains of co-infected mice compared to mice infected only with *T. gondii*.

Because hepatic larval trapping is reliant on IgG1 humoral immunity, which does not require a specialized site for its development, it was postulated that changing the route of primary exposure would have no effect on trapping ability. This was assessed by inoculating

mice using intravenous injection, which would bypass the liver and send larvae directly to the heart and lungs.

The present work was a preliminary exploration of the effect of gender, route of immunologic priming, eosinophils, and mouse strain on *T. canis* longevity, distribution in host tissues, and hepatic trapping. Observed differences would inform selection of the best murine model for studying various aspects of human larval toxocariasis – such as hepatic response, brain infiltration, and development of host immunity – as this research moves forward.

Results

Since hepatic trapping is abrogated in mice co-infected with *T. gondii* and *T. canis*, one might expect more larvae to migrate into the brain of co-infected (CO) mice compared to *T. canis* control (TCC) mice. In that case, chances of eventual larval migration into the eye would increase. To test whether trapping had an effect on larval numbers in the brain, CO mice were inoculated with *T. gondii* followed, in two weeks, by the first of two *T. canis* doses spaced 4 weeks apart. The liver and brain were digested in acid-pepsin solution to recover larvae for quantification. Due to the small group sizes, differences rarely reached statistical significance, but clear trends were discernible. BALB/c mice that were co-infected had fewer larvae in their livers but the same number in their brains as compared to TCC mice (Figure 4.1 A). For C57BL/6 mice, there was again fewer larvae in the liver of the CO group, but in this case a trend for more larvae in the brain was notable (Figure 4.1 B). In work performed by a visiting professor in our lab, Dr. Qing Lin of Northwest A & F University, China, dblGATA1-/- mice on a C57BL/6 background showed the same pattern of distribution as the C57BL/6 strain (Figure 4.1 C, unpublished data). Thus eosinophil-deficient mice remained fully capable of trapping *T. canis* larvae in the liver, and this ability was lost when co-infected with *T. gondii*, resulting in an apparent increase in the number of larvae in the brain.

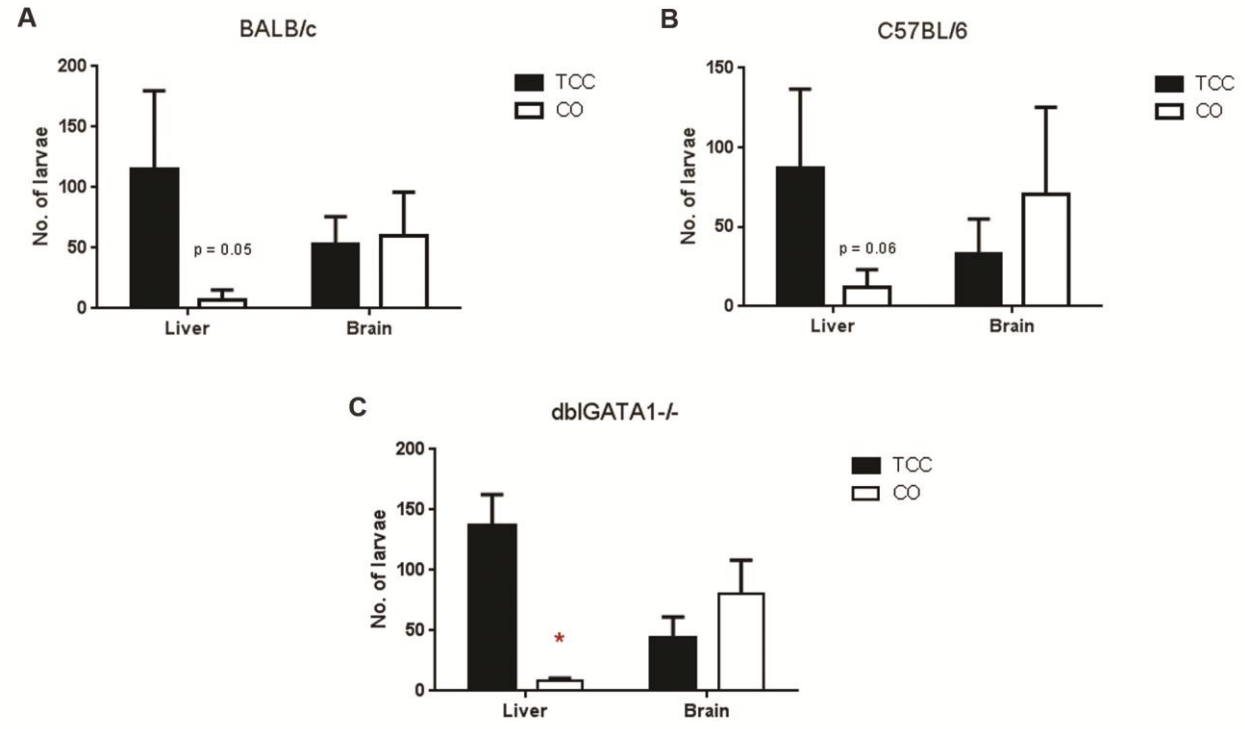

Figure 4.1. Hepatic trapping of *T. canis* larvae is lost during co-infection in 3 strains of mice. Mice were inoculated with *T. gondii* oocysts, then given two doses of *T. canis* eggs four weeks apart (n = 2-3 per group). Livers and brains were digested in acid-pepsin for larval recovery using density gradient centrifugation. Displayed are larval counts for co-infected (CO) and *T. canis* control (TCC) mice of the BALB/c (A), C57BL/6 (B), and dblGATA1-/- strain on a C57BL/6 background (C). Group means and standard deviations are shown. * $p < 0.05$.

Analysis of Dr. Lin's data on dblGATA1-/- mice also revealed a tendency toward a lower total body count of larvae than C57BL/6 control mice (Figure 4.2 A, unpublished data). To verify whether eosinophils affected *T. canis* longevity and migratory behavior in the host, the survival and distribution of larvae within dblGATA1-/- mice was assessed. Mice were euthanized 12 weeks after a single inoculation with 500 *T. canis* eggs. The brain, liver, viscera (excluding liver), upper body muscles, and lower body muscles were separately digested with acid-pepsin to recover larvae. No difference in larval numbers was observed in any of the body compartments examined (Figure 4.2 B), indicating that a lack of eosinophils had no effect on *T. canis* migration or survival.

Examination of the livers of male and female mice infected twice with *T. canis* revealed some intriguing results. Though male mice were better able than females to trap *T. canis* larvae in the liver after challenge infection (Figure 4.3 A), their *T. canis*-specific IgG1 antibody levels were equivalent to those of the females (Figure 4.3 B). Since a causal relationship has already been established between IgG1 and trapping – as supported by data presented in Chapters 2 and 3 – this finding suggests that other gender-specific factors may be influencing the outcome of larval migration through the liver.

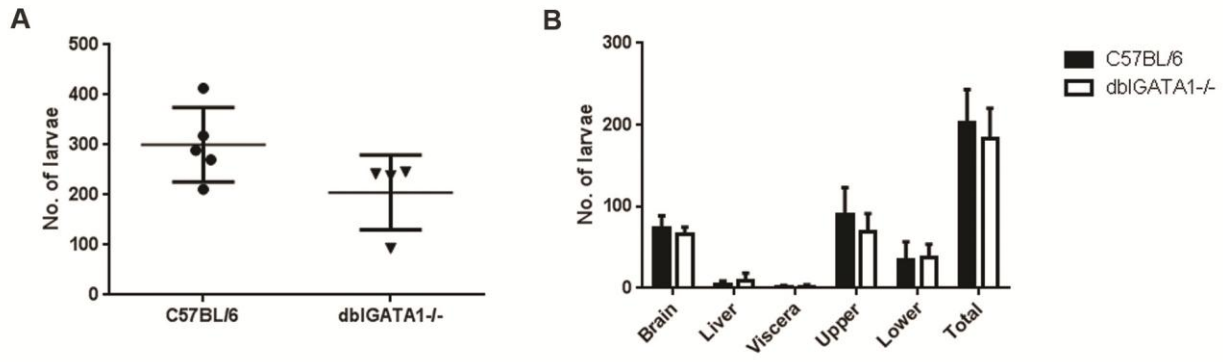

Figure 4.2. *T. canis* larval count and distribution are similar in C57BL/6 and dblGATA1-/- mice on the same background. Non-significant trend toward fewer total larvae in the body of eosinophil-deficient dblGATA1-/- mice infected with *T. canis* four and eight weeks prior (n = 4-5) (A). Comparison of larval recovery from brain, liver, thoracic + abdominal viscera (excluding liver), upper body muscles, and lower body muscles of mice (n = 5) twelve weeks after a single *T. canis* infection (B). Means and 95% confidence intervals (for scatterplot) or standard deviations (for bar graph) are displayed.

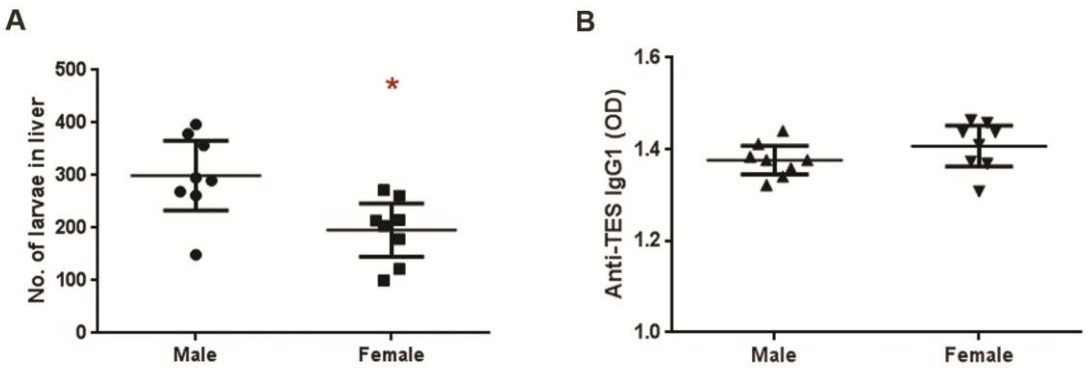

Figure 4.3. Male BALB/cJ mice are better able to trap larvae in the liver than females. Mice were inoculated with 200 *T. canis* eggs, allowed four weeks rest, then challenged with 500 *T. canis* eggs (n = 8 per group). Livers were digested with acid-pepsin. Number of larvae in the livers of male and female BALB/c mice after challenge infection (A). Means and 95% confidence intervals are shown. * $p < 0.05$

Next, to determine if the location of first contact between *T. canis* larvae and the host immune system is important for hepatic trapping to occur, mice were inoculated with *T. canis* via two different routes, by gavage (PO) or by intravenous injection into the tail vein (IV). Both groups were then challenged by gavage. As a control, a third group of mice were inoculated by gavage with the sensitizing dose only, to quantify any larvae that may remain in the liver of the PO-sensitized group that would not be present in the IV group. Unexpectedly, the IV group had fewer hepatic larvae than the PO group (Figure 4.4 A). The sensitization control group had an average of 2 larvae in the liver (range of 1-6), which would not account for the difference seen between the PO and IV groups. Even more surprising, *T. canis*-specific IgG1 levels were higher in the IV group than in the PO group (Figure 4.4 B), despite lower levels of larval trapping. This result contradicts our previous finding of a strong positive correlation between IgG1 and hepatic trapping. This preliminary experiment will need to be repeated to confirm the validity of these results.

Finally, as a first step toward evaluating the reciprocal effect of *T. canis* infection on *T. gondii* infection, the abundance of bradyzoite tissue cysts in the brains of co-infected (CO) and *T. gondii* control (TGC) mice was quantified. Inoculations were carried out using the same timeline as already described for the co-infection experiments above. Brains were homogenized and aliquots taken for microscopic examination for cysts. Though the difference was not statistically significant, ($p = 0.08$), a greater number of tissue cysts were present in the brains of CO mice compared to TGC mice (Figure 4.5), suggesting a possible role of *T. canis* in influencing host control of *T. gondii*.

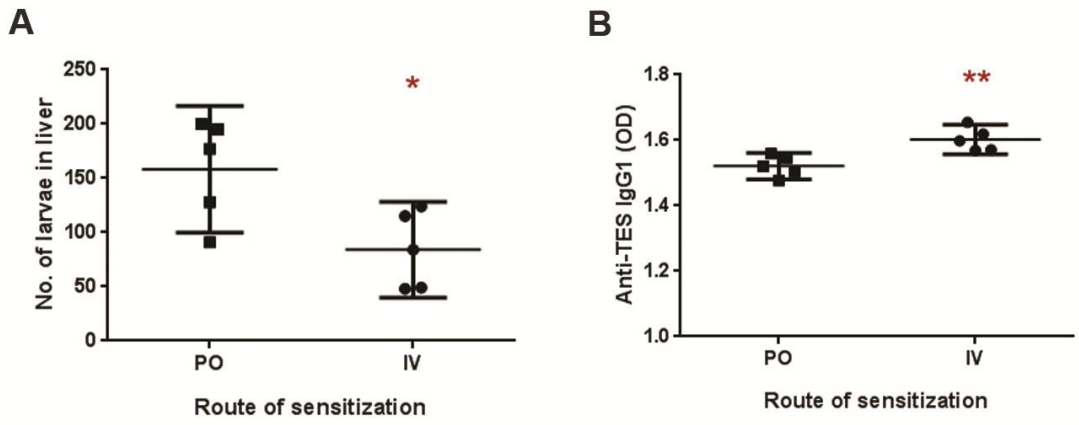

Figure 4.4. Sensitization to *T. canis* by the intravenous route results in less larval trapping upon challenge infection than peroral sensitization. Mice were inoculated with 200 eggs by gavage (PO) or 200 larvae by tail vein injection (IV), followed by a challenge of 500 eggs by gavage four weeks later (n = 5). Livers were digested by acid-pepsin. Means and 95% confidence intervals are displayed. * $p < 0.05$, ** $p < 0.01$.

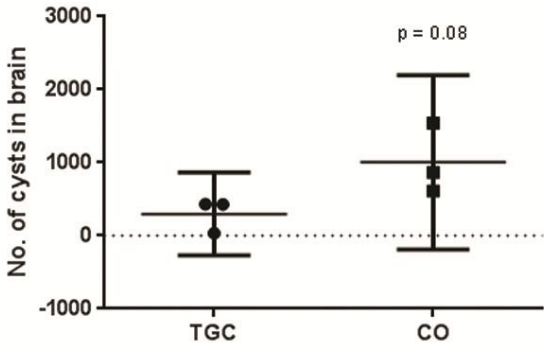

Figure 4.5. Co-infected mice have higher cyst burdens in the brain than *T. gondii* mono-infected mice. Mice were inoculated with *T. gondii* oocysts, then the CO group was given a sensitizing and challenge dose of *T. canis*. Brains were homogenized and aliquots were examined under a coverslip. Group means (n = 3) and 95% confidence intervals are shown.

Discussion

Using the eosinophil-deficient mouse strain dblGATA1-/-, it was confirmed that eosinophils make no contribution to *T. canis* larval survival, nor do they affect their migratory patterns. An absence of eosinophils likewise had no effect on the ability of dblGATA1-/- mice to trap larvae in the liver. These findings corroborate the conclusions made in previous studies with IL-5-deficient mice.[11,13]

In female BALB/c mice, no difference in brain counts was apparent between the TCC group that was able to trap larvae in the liver and the CO group that failed to do so. The BALB/c strain has been shown to have a stronger predilection for larval migration to the brain compared to other inbred (C57BL, C3H, DBA, A/J, CBA, NIH, SWR) and outbred (NMRI) mouse strains, so perhaps it is not so surprising that they have high numbers of larvae in the brain even when hepatic trapping occurs.[16,17] In contrast, mice on a C57BL/6 background displayed a trend toward reduction of larvae in the brain when hepatic trapping took place. The small group size (n = 2-3) in this experiment makes it difficult to draw firm conclusions, but this difference in brain invasion between strains is certainly worthy of further investigation.

Male BALB/c mice trapped significantly more larvae in the liver than did females. This is consistent with the report of Abo-Shehada et al. showing superior trapping ability in male NIH and CD1 mice compared to females.[2] Whether this difference between genders can be solely attributed to the male tendency to accumulate larvae in the liver is uncertain.[3] The idea that testosterone might influence larval migration dynamics is not entirely unexpected. In the canine definitive host, pregnancy hormones are believed to orchestrate an important part of the *T. canis* life cycle: vertical transmission. A substantial fraction of the larvae infecting reproductively mature dogs disseminates into body tissues, much the same as occurs in paratenic hosts, and

becomes metabolically dormant.[18,19] There the larvae remain until pregnancy triggers them to resume migration to enter the placenta and pass into the puppies.[18,19] Thus it is not difficult to believe that testosterone may have the opposite effect, hindering the migration of larvae.

An alternative explanation for this gender bias in hepatic trapping may be intrinsic differences in the immune response of male and female mice. In general, females have more T lymphocytes and circulating antibodies in comparison to males.[20] On the other hand, a recent study found that males have a greater reserve of neutrophils and macrophages in the spleen that can be quickly recruited to sites of inflammation.[21] Alternatively activated macrophages (AAM) have been demonstrated to be crucial to the trapping of other parasitic nematodes in murine skin and small intestine.[22,23] If a similar requirement for AAM activity in inducing *T. canis* larval arrest existed, it could account for the greater degree of hepatic trapping observed in male mice despite not having a correspondingly higher level of circulating IgG1.

Based on prior evidence pointing to IgG1 antibodies being the sole requirement to induce larval trapping, it was expected that the route of *T. canis* sensitization would not affect how many larvae were arrested in the liver, so long as the proper antibody isotype was generated. However, this was not the case. Furthermore, the IV group, which trapped fewer larvae than the PO control group, had a higher level of IgG1 – a finding inconsistent with other data presented thus far. Since the IV route of infection is unnatural for *T. canis*, this scenario is not relevant to real world dynamics of toxocariasis. Nonetheless, it does present an interesting conundrum. This preliminary result is not yet verified, so repeat experiments will need to be performed before conclusions are drawn.

The increased number of tissue cysts found in the brains of co-infected mice provides an indication that *T. canis* infection may also exert some immunomodulatory effects that alter how

the host responds to and controls *T. gondii* infection. This is in line with the results of Chapter 3 (Figure 3.4 E), where a reduction in the activated CD8+ T cell population was noted in acutely co-infected mice. The overall drop in IFN-γ production that would presumably accompany this decreased cell number would lead to impaired killing of infected cells, ultimately resulting in greater *T. gondii* replication and cyst formation.

In this series of experiments, multiple factors that might influence *T. canis* larval migration or arrest were explored. It was demonstrated that eosinophils do not, in fact, alter larval longevity, distribution, or hepatic trapping. Strain differences in the propensity for larvae to migrate to the brain during co-infection highlight the importance of not making generalized conclusions when working with a single inbred mouse strain. Gender effects, too, must be kept in mind when extrapolating research findings to the general human population because males and females may react in very different ways. Finally, examination of the brain cyst burden in co-infected versus *T. gondii* mono-infected mice offered a glimpse into the possibility that *T. canis* can also alter the course of *T. gondii* infection.

Future work should address the reason for the superior trapping ability of males by assessing the speed, magnitude, and phenotype of macrophage recruitment to the liver after *T. canis* challenge. It would also be prudent to extend the model of larval toxocariasis to a mouse strain without any immunological bias to better represent a diverse human population. The IV sensitization experiment should be repeated to determine if the point of first contact truly is important for hepatic trapping, and this could encompass subcutaneous or intraperitoneal routes as well. Finally, as proposed in Chapter 3, the two-way interactions between *T. canis* and *T. gondii* during co-infection needs to be further explored to better understand the impact of chronic, subclinical infections on a host's response to all future immunologic encounters.

Methods

Mice and parasites

Six- to fourteen-week-old mice were utilized in these experiments, with groups being age-matched within individual experiments. BALB/c and C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) or The Jackson Laboratory (Bar Harbor, ME). DblGATA1$^{-/-}$ mice on a C57BL/6 background were acquired from Dr. Avery August's breeding colony at Cornell University. Unless otherwise specified, all mice were female.

T. gondii type II ME49 sporulated oocysts were provided by Dr. J. P. Dubey of the USDA-ARS. Oocysts were purified and prepared for inoculation as described in Chapter 3. *T. canis* eggs were acquired and cultured as described in Chapter 2. All inocula were administered by gavage unless otherwise stated.

T. canis hepatic trapping experiments

Male and female mice were inoculated with 200 and 500 *T. canis* eggs four weeks apart, then euthanized a week later. Livers were collected for larval recovery.

In co-infection experiments, the co-infection (CO) and *T. gondii* control (TGC) groups were inoculated orally with 50-100 *T. gondii* oocysts. Two weeks later, CO and *T. canis* control (TCC) groups were inoculated orally with 125-200 *T. canis* eggs. After another four weeks, CO and TCC mice were inoculated with a challenge dose of 500 *T. canis* eggs. Mice were euthanized 1-5 weeks post-challenge. Serum was collected by cardiac puncture. Livers from *T. canis*-infected groups (CO, TCC) were processed for larval quantification. Brains were homogenized for enumeration of *T. gondii* cysts and *T. canis* larvae.

Sensitization by IV injection

Larvae were mechanically hatched from embryonated eggs under sterile conditions in a method similar to that described by Bowman et al.[24] Briefly, embryonated eggs were cleaned using 1% sodium hypochlorite and washed several times with RPMI-1640 with L-glutamine (Lonza, Walkersville, MD), 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B (Gibco). Egg shells were disrupted using a glass tissue grinder, and larvae were filtered through cotton in a 40 μm cell strainer overnight in a 37°C incubator with 5% CO_2. Larvae were resuspended in RPMI-1640 for inoculation.

Mice were inoculated with 200 *T. canis* eggs by gavage or 200 *T. canis* larvae by injection into the tail vein. Four weeks later, both groups were administered 500 *T. canis* eggs by gavage. As a control, a third group of mice were inoculated by gavage with the sensitizing dose only. Mice were euthanized a week after and blood was collected by cardiac puncture.

***T. canis* survival in dblGATA1$^{-/-}$ mice**

DblGATA1$^{-/-}$ mice on a C57BL/6 background and C57BL/6 controls were inoculated with 500 *T. canis* eggs. Groups were of mixed gender. Twelve weeks post-infection, mice were euthanized and the following were digested for larval recovery: liver, brain, viscera (including heart, lungs, spleen, kidneys, pancreas, gastrointestinal tract, gonads), upper body (all muscles cranial to and including the diaphragm), and lower body (all muscles caudal to the diaphragm).

Larval recovery from tissues

Organs were individually homogenized in a blender and digested for 2-3 hours at 37°C in 0.7% pepsin + 0.7% HCl with continuous agitation. Samples with undigested bone fragments

were filtered through cheesecloth prior to the next steps. The digests were washed with PBS and neutralized with NaOH. Larvae were isolated from tissue sediment by Histopaque-1077 (Sigma) density gradient centrifugation. For microscopic examination and quantification of larvae, gridded Sedgwick slides (Wildlife Supply Company, Tulee, FL) were used.

T. gondii cyst recovery from brains

Brains were bisected sagittally with a flat-edged razor and one half was macerated in a petri dish. The material was transferred into a 50 mL conical test tube with PBS. Using an 18 gauge blunt-tipped needle and syringe, the brain was vigorously aspirated up and down numerous times until homogeneous in appearance. The brain was pelleted by centrifugation at 1000 x g for 10 minutes then resuspended in a known volume of PBS (< 0.5 mL). Aliquots of 10 μL were examined under a microscope to enumerate cysts. If no cysts were observed in the first aliquot, additional aliquots were examined. The average number of cysts per μL was multiplied by the sample volume and then by 2 to calculate the number of cysts present in the whole brain.

Serum antibody measurement

T. canis-specific antibodies were measured using an indirect ELISA as described in Chapter 2. Briefly, 96-well high-binding plates were coated with 10 μg/mL of *T. canis* excretory-secretory antigen. Wells were blocked with 2% bovine serum albumin in PBS. Serum, diluted 1:200 to 1:16,000 depending on the experiment and the antibody isotype being measured, was incubated for an hour. Horseradish peroxidase-conjugated antibodies against mouse IgG, IgG1, or IgG2a (Jackson ImmunoResearch, West Grove, PA) at 1:5000 dilution were incubated for an hour. ABTS substrate (KPL, Gaithersburg, MD) was incubated for 20 minutes then 1M NaF stop

solution was added. Absorbance at 405 nm was measured by an automated spectrophotometer (model ELx800, Bio-Tek Instruments, Winooski, VT).

Statistical analysis

Differences between groups were assessed using unpaired t-tests. Significance was set at a p-value of < 0.05. Data were analyzed using Prism software (GraphPad Software Inc., La Jolla, CA).

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CHAPTER 5

SUMMARY

Toxocariasis and toxoplasmosis

Toxocara canis and *Toxoplasma gondii* are very successful parasites that utilize multiple mammalian and avian species as transport hosts, including mice and people.[1–4] Their similar modes of transmission – including paratenesis and contamination of soil, food, and water – increase the odds of a host becoming infected with both parasites.[5] Within the USA alone, which has a population size of approximately 321 million people, 56 million are infected with either *T. gondii* or *T. canis*, and 11 million are infected with both.[5–7] Given that these two pathogens elicit polarizing Th1 and Th2 responses, and cross-regulation between these arms of the immune system is well documented, it was of interest to examine the immunomodulatory effects of this particular co-infection using a natural murine model. This dissertation aimed to elucidate the immunologic changes imposed by *T. gondii* infection on concurrent *T. canis* infection with respect to *T. canis* larval trapping within the liver.

Summary of findings and future directions

In Chapter 2 I showed that humoral immunity, specifically antigen-specific IgG1 antibodies, enabled host trapping of *T. canis* larvae within the liver upon secondary infection. Passive immunization using the IgG fraction from the serum of infected mice was sufficient to confer this trapping ability on naïve recipient mice. Cell depletion experiments demonstrated that CD4+ cells were necessary at the time of initial *T. canis* sensitization in order to allow B cell class switching from IgM to IgG1 production; however, their absence at the time of challenge infection did not prevent larvae from arresting in the liver, indicating that CD4+ cells do not

directly participate in the trapping process itself. Thus, this study highlighted the importance of humoral rather than cell-mediated immunity in effecting hepatic trapping of *T. canis*.

One concern with the use of BALB/c mice in this study is the fact that this strain is phenotypically Th2-biased in their responses, so the results obtained may reflect this predisposition. This issue could be resolved by validating the current findings using other mouse strains that do not have this immune bias, or administering a Th1-promoting molecule to BALB/c mice. A question left unanswered is precisely how IgG1 antibodies stop larval migration. Possibilities include sensory deprivation through antibody binding to chemoreceptor organs on the larvae, and antibody-mediated recruitment of alternatively activated macrophages that produce substances harmful to the larvae. The latter has been demonstrated for other nematode larvae such as *Heligmosomoides polygyrus.*[8] The mechanism of trapping could be investigated by examining the migratory behavior of *T. canis* larvae in vitro and in vivo after incubation with immune serum. In addition, clodronate liposomes could be administered to mice to deplete phagocytic cells, or macrophage-specific FcγR knockout mice could be used to assess macrophage involvement in larval trapping.

In Chapter 3, I presented data showing that pre-infection with *T. gondii* skewed the host response to *T. canis* away from IgG1 antibody production toward the Th1-associated IgG2a isotype instead. Co-infected mice had lower *T. canis*-specific IgG1 levels and lost the ability to trap larvae in the liver. Also associated with co-infection was a reduction in the number of CD3ε-CD11b+ phagocytes found in the liver as compared to *T. canis* mono-infected mice. Again, this suggests that innate immune cells may play a role in trapping. In chronic toxoplasmosis, the ability to trap larvae was inversely related to the dose of *T. gondii* oocysts administered. Interestingly, *T. canis* infection superimposed on acute *T. gondii* infection decreased the number

of IFN-γ-producing CD8α+ T cells compared to the *T. gondii* control group. This may be due to downregulation by *T. canis* of macrophage production of IL-12, a cytokine signal required for full CD8+ T cell activation.[9,10] This study provided evidence for reciprocal immunomodulation by *T. gondii* and *T. canis* in the context of acute toxoplasmosis, and persistent immunologic influence of *T. gondii* even in the chronic stage of infection.

A limitation of this study was the lack of antigen-specific stimulation when analyzing T cell populations in the liver, making it difficult to distinguish changes specific to each parasite. This should be addressed in future experiments using *T. gondii* tetramers and *T. canis* excretory-secretory antigen. Another question is whether lower larval counts in the liver of co-infected mice are truly due to abrogation of trapping or if they reflect enhanced larval killing. Performing whole-body counts would provide an answer. Also worthy of further investigation are the effects of *T. canis* infection on host cellular response to *T. gondii*. Given the reduction in activated CD8+ T cells in co-infected mice, and the potential for Th2 suppression of Th1 cells, one might speculate that reversing the order of infection so that *T. canis* precedes *T. gondii* could lead to poor control of tachyzoites and tissue cysts in the acute and chronic stages, respectively. This could be measured by mouse survival, parasite burden in tissues, as well as degree and character of cell recruitment.

In Chapter 4, I explored various factors that could affect host immunity and thus influence *T. canis* migration. I confirmed that eosinophil deficiency did not affect *T. canis* larval survival or distribution in host tissues. In contrast, mouse strain did appear to affect whether more larvae reached the brain of co-infected mice that failed to trap larvae in the liver: in BALB/c mice, no difference was detected in brain larval counts between co-infected and mono-infected mice, whereas in C57BL/6 mice the trend was for higher brain counts in co-infection.

Surprisingly, I found that female mice trapped fewer larvae than males despite having equivalent levels of IgG1 antibodies. If macrophages are indeed integral to the larval trapping process, then this result could be explained by male mice possessing a larger reservoir of these cells in their spleens that can be quickly dispatched to the liver when needed.[11] Another unexpected finding was that mice immunologically primed by intravenous injection of *T. canis* had decreased hepatic trapping but more IgG1 compared to mice primed by gavage. This contradicts data presented in Chapters 2 and 3 showing a strong correlation between IgG1 levels and larval trapping. Before this result is accepted it must be verified by additional experiments. Finally, preliminary data showed a non-significant rise in the number of *Toxoplasma* tissue cysts in the brains of co-infected mice, suggesting that *T. canis* infection may also modulate the host immune response to *T. gondii*. This is consistent with the decrease in CD8+ T cells producing IFN-γ in acutely co-infected mice, which could result in impaired killing of infected cells and increased cyst formation.

As previously stated, studies are needed to clarify the role of macrophages in *T. canis* larval trapping. If their involvement is confirmed, then splenectomy could be performed to test the hypothesis that males are better able to halt larval migration in the liver due to faster macrophage recruitment. With respect to intravenous inoculation, it is possible that measuring circulating IgG1 levels may be misleading in this case, and immunohistochemical quantification of local IgG1 abundance around trapped larvae would be more informative. However, if the results from such an experiment do not support a key role for IgG1 in mediating *T. canis* trapping, then the possibility that one of the thus far unmeasured IgG subclasses (i.e. IgG3 or IgG2b) is responsible will need to be considered. Based on the data presented in Chapters 3 and 4 regarding host immunity during co-infection, it would appear that there is in fact cross-talk

between the pro-inflammatory Th1 response incited by *T. gondii* and the Th2 response induced by *T. canis*. These interactions must be further studied to better understand how chronic, subclinical infections remain immunologically active.

Toxocara canis and *Toxoplasma gondii* are long-lived parasites that stay with the host for its entire life. This dissertation underscores the importance of humoral immunity in the host response to larval toxocariasis, and sheds light on cross-regulatory changes that take place during co-infection with these two pathogens. Furthermore, the data demonstrate that chronic, asymptomatic infections can still exert significant influence on a host's response to immunologic stimuli. These findings open many avenues for future inquiry.

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APPENDIX

Introduction

Included herein is a collection of research and continuing education journal articles that I have published, as first author, over the course of my doctoral program. This work constitutes part of the broader training I received as a clinical parasitology resident under the National Center for Veterinary Parasitology. As is clear from the subject of these articles, my major focus was on zoonotic parasites, including heartworms (*Dirofilaria immitis*), hookworms (*Ancylostoma caninum*), and of course *Toxocara canis*. I would like to acknowledge the financial support of Novartis Animal Health, Bayer HealthCare, and IDEXX Laboratories, which made these studies possible. These articles are presented in their published journal article format in chronological order, with corresponding citation information and DOI links listed below.

- Lee ACY, Bowman DD, Lucio-Forster A, Beall MJ, Liotta JL, Dillon R. 2011. Evaluation of a new in-clinic method for the detection of canine heartworm antigen. Vet Parasitol 177 (3-4): 387-391. http://dx.doi.org/10.1016/j.vetpar.2010.11.050

- Lee ACY, Kraus MS. 2012. Coughing cat: could it be heartworm? NAVC Clinician's Brief 10 (6): 91-95.

- Lee ACY, Epe C, Bowman DD. 2013. Comparison of *Ancylostoma caninum* worm counts acquired by endoscopy and necropsy. Vet Parasitol 196 (3-4): 538-540. http://dx.doi.org/10.1016/j.vetpar.2013.03.022

- Lee ACY, Hostetler JA, Bowman DD. 2014. Assessing the speed of kill of hookworms, *Ancylostoma caninum*, by Advantage Multi® for Dogs using endoscopic methods. Vet Parasitol 204 (3-4): 402-406. http://dx.doi.org/10.1016/j.vetpar.2014.05.028

- Lee ACY, Epe C, Bowman DD. 2015. Determination of anthelmintic efficacy against *Toxocara canis* in dogs by use of capsule endoscopy. Vet Parasitol 212 (3-4): 227-231. http://dx.doi.org/10.1016/j.vetpar.2015.08.013

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Short communication

Evaluation of a new in-clinic method for the detection of canine heartworm antigen

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ABSTRACT

Canine heartworm is endemic in many parts of the world, and veterinarians rely on rapid in-clinic antigen tests to screen for this infection. Recently, an in-clinic, instrument-based rotor employing a colloidal gold agglutination immunoassay was launched in the marketplace (VetScan VS2® Canine Heartworm (HW) Antigen Test Kit; Abaxis, Inc.). Because of the widespread use of heartworm prevention and possible false negative test results in dogs with low heartworm burdens, the performance of the VetScan VS2® HW test and a commercially available in-clinic, membrane-based ELISA test (SNAP® Heartworm RT Test; IDEXX Laboratories) was compared using samples from dogs with low heartworm burdens and/or low levels of circulating antigen.

Ninety serum samples were evaluated using the two methods. Testing was performed according to the manufacturer's product insert by personnel blinded to sample status. The samples were derived from two populations: dogs with necropsy-confirmed heartworm status (40 with 1–4 female worms, 30 with no worms), and field dogs (20) confirmed positive for antigen by microtiter plate ELISA (PetChek® Heartworm PF Antigen Test; IDEXX Laboratories). All 40 dogs with heartworms on necropsy were also confirmed to have circulating antigen by the PetChek HW ELISA.

In necropsy-negative dogs ($n = 30$), neither the VetScan VS2 HW nor SNAP HW tests detected heartworm antigen. Of the samples testing positive for antigen by PetChek HW ($n = 60$), the VetScan VS2 HW and SNAP HW tests detected antigen in 15 and 56 samples, respectively. Percent agreement (plus 95% confidence interval) for each test relative to the PetChek HW qualitative result was 50% (40–60%) for VetScan VS2 HW and 96% (89–98%) for SNAP HW. Relative to the presence or absence of female worms at necropsy, agreement was 61% (50–72%) for VetScan VS2 HW and 99% (92–99.6%) for SNAP HW tests.

It is clinically important that dogs with low heartworm burdens and/or low levels of circulating heartworm antigen be correctly identified by veterinarians in order to ensure prompt treatment, and the VetScan® VS2 HW test does not appear to be as accurate as the SNAP HW or PetChek HW tests when performed on this subset of patients.

1. Introduction

Canine heartworm (*Dirofilaria immitis*) is endemic in many parts of the world (McCall et al., 2008), and many veterinarians rely on rapid in-clinic antigen tests to screen

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for this deadly disease. Detection of circulating *D. immitis* antigen from mature female worms is highly sensitive and specific for heartworm infection in dogs (Atkins, 2003; McCall et al., 2008). However, test performance varies depending on the test format: of the commercially available in-clinic antigen assays, the microtiter plate ELISA demonstrates the highest sensitivity for canine heartworm infection, followed by the membrane-based ELISA, and finally the lateral flow immunochromatographic test (Courtney and Zeng, 2001; Atkins, 2003). Differences in assay sensitivity have also been reported when four or fewer adult female worms are present (Courtney and Zeng, 2001; Atkins, 2003). The higher frequency of false negative test results in dogs with low heartworm burdens makes diagnosis in these patients challenging.

Recently, an in-clinic, colloidal gold agglutination immunoassay for heartworm detection using an instrument-based rotor system was introduced in the market (VetScan VS2® Canine Heartworm Antigen Test; Abaxis, Inc.). To provide a rigorous evaluation of this new antigen detection method, the present study compared the performance of the VetScanVS2 HW test to that of an in-clinic, membrane-based ELISA test previously shown to be highly accurate (Atkins, 2003). Sera from heartworm-infected dogs with low worm burdens and/or low circulating antigen levels were utilized in order to uncover differences in assay sensitivity that may exist between the two test formats.

2. Methods

The two in-clinic heartworm antigen tests compared in this study were the VetScan VS2® Canine Heartworm Antigen Test Kit (Abaxis, Inc.; lot no. 907150) and the SNAP® Heartworm RT Antigen Test (IDEXX Laboratories, Inc.; lot no. AF452). Tests were performed according to the directions on the manufacturer's product insert, and all test kits were used prior to the printed expiration date. The VetScan VS2 instrument includes an internal quality control program, eliminating the need for routine external liquid quality controls. Instruments used in the study were updated with the most recent version of software available (2.1.23) and rotor-specific calibration information was automatically obtained from the barcode printed on the rotor.

A total of 90 canine serum samples were collected for the study from four sources: two commercial kennels, the library of serum samples at IDEXX Laboratories, and field samples from IDEXX Reference Laboratories. Of these, seventy were banked samples selected based on the number of worms identified in each dog at necropsy (no worms in 30 dogs, and 1–4 female worms plus 0–7 male worms in 40 dogs); sera were thawed, centrifuged and divided into aliquots prior to testing. Twenty additional serum samples were obtained from submissions to IDEXX Reference Laboratories where they had been determined to be antigen-positive using a microtiter plate ELISA (PetChek® Heartworm PF Antigen Test; IDEXX Laboratories, Inc.). These sera were included to permit the testing of samples not previously frozen, and were chosen to represent relatively low levels of circulating heartworm antigen (ELISA OD ≤ 2).

VetScan VS2 HW and SNAP HW testing were performed in March 2010 at Cornell University. Aliquots of all serum samples were coded and randomized so that each person performing the assays would be blinded to infection status and would test samples from both infected and uninfected dogs. VetScan VS2 HW testing of all samples was performed by a single veterinarian (ACYL), and results were recorded as positive or negative as reported by the instrument. Three licensed veterinary technicians experienced with SNAP tests were recruited from the Cornell University Hospital for Animals, and each technician tested a total of 30 serum samples using the SNAP HW assay. Technicians were instructed to consult a blinded "referee" (JLL) similar to the way they might consult the practicing veterinarian in the clinic if they were uncertain of the SNAP HW result.

The PetChek HW testing was performed by IDEXX Laboratories at the time of serum collection and was repeated, sample volume permitting, after the VetScan VS2 HW and SNAP HW tests were performed to verify the presence of detectable antigen.

Percent agreement for each test relative to the presence or absence of worms at necropsy or the qualitative result (positive/negative) of the PetChek HW ELISA was calculated, and 95% confidence intervals were determined by use of exact binomial probabilities. The Pearson correlation coefficient (r) was determined using GraphPad Prism v.5 (GraphPad Software, La Jolla, CA). McNemar's paired chi-square test was used to evaluate the degree of disagreement between the test methods. A P value <0.05 was considered statistically significant.

3. Results

In the 30 necropsy-negative samples, heartworm antigen was not detected by any of the test methods. The remaining 60 serum samples (40 from necropsy-positive dogs and 20 from the field) were all determined by PetChek HW to contain detectable levels of antigen. Of these, the VetScan VS2 HW test detected antigen in 15 (25%) samples, while the SNAP HW test detected antigen in 56 (93%). Forty-one of the 56 SNAP HW-positive samples were incorrectly identified by the VetScan VS2 test as being negative. In contrast, all 15 of the VetScan VS2 HW-positive samples were correctly identified by the SNAP HW test as being positive. Restricting the analysis to only those dogs with necropsy-confirmed heartworm infections, the VetScan VS2 HW and SNAP HW tests detected antigen in 13/40 (33%) and 39/40 (98%) dogs, respectively. Percent agreement for each test relative to the PetChek HW qualitative result was markedly lower for the VetScan VS2 HW test (50%) compared to the SNAP HW test (96%). Relative to the presence or absence of heartworms at necropsy, the percent agreement was 61% for the VetScan VS2 HW test and 99% for the SNAP HW test. Percent agreement between the VetScan VS2 HW test and the PetChek HW ELISA did not improve when the canine samples that had not previously been frozen were analyzed independently (data not shown). The overall performance of the two antigen tests is summarized in Table 1. Pairwise comparison of the VetScan VS2 HW and SNAP HW assays

Table 1
Test results and percent agreement (with 95% confidence interval) for each test relative to the PetChek HW qualitative result and to the presence or absence of heartworms at necropsy.

Reference method	Test method	True positive	True negative	False positive	False negative	Percent agreement (95% CI)
PetChek HW (n = 90)	VetScan VS2 HW	15	30	0	45	50% (40–60)
	SNAP HW	56	30	0	4	96% (89–98)
Necropsy (n = 70)	VetScan VS2 HW	13	30	0	27	61% (50–72)
	SNAP HW	39	30	0	1	99% (92–99.6)

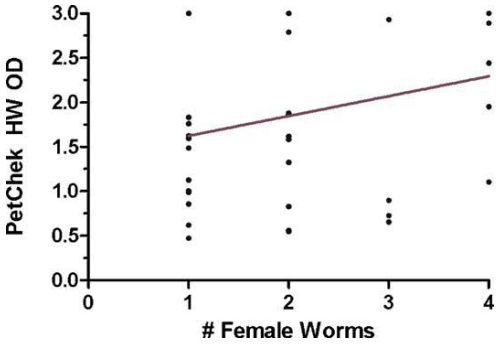

Fig. 1. PetChek HW OD values relative to the number of female heartworms found at necropsy. Regression line (r = 0.22) is represented in dark red. Dogs that were found not to have heartworm infections at necropsy were excluded from the analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

revealed significantly (P < 0.001) discordant test results as did comparison of the VetScan VS2 HW and PetChek HW tests (P < 0.001). Conversely, the SNAP HW and PetChek HW tests had only 4% discordant results and were not found to be significantly different (P = 0.13).

Based upon PetChek HW OD values, a weak positive correlation was noted between the serum antigen level and the number of female heartworms identified at necropsy (r = 0.22) (Fig. 1). In this study, the lower detection limit of heartworm antigen for the VetScan VS2 HW and SNAP HW tests correlated to PetChek HW OD values of 1.04 and 0.47, respectively. With only one exception, the SNAP HW test consistently produced positive visual results when the PetChek HW OD values were greater than 0.47, while the VetScan VS2 HW test did not consistently detect heartworm antigen even when the PetChek HW OD values were greater than 3.0 (maximum reported value) (Fig. 2). In relation to worm burden, the VetScan VS2 HW test falsely reported several dogs with 1, 2, 3, and 4 female worms as being antigen-negative. The SNAP HW test had only one false negative result, which occurred in a dog with a single female heartworm (Fig. 3).

4. Discussion

Previous studies have shown that differences in the sensitivity of in-clinic heartworm antigen tests are more apparent when testing samples from dogs with low worm burdens (Courtney and Zeng, 2001; Atkins, 2003). However, in some dogs in this study where only a single female worm was recovered at necropsy, PetChek HW OD values were at their maximum reported value, suggesting pronounced antigenemia even with this low worm burden. Therefore both the amount of measurable serum antigen and the number of female heartworms present in each dog were considered relevant to the analysis of test performance in this study. The PetChek HW OD value was used as one reference against which the VetScan VS2 HW and SNAP HW test results were assessed. This provided an indication of test performance for serum samples with known antigen levels, and demonstrated how closely each test agreed with the reference lab standard. A second comparison, with necropsy results, permitted evaluation of the accuracy of each test in determining the adult heartworm infection status of each dog, irrespective of antigenemia.

In this sample population, both the VetScan VS2 HW and SNAP HW tests correctly identified dogs without

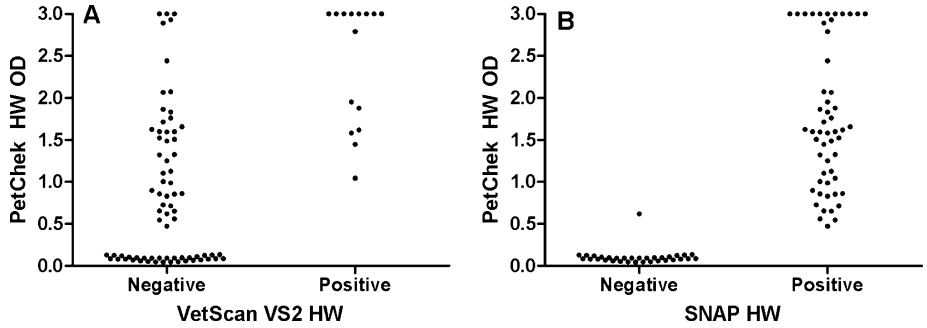

Fig. 2. Qualitative test results of (A) the VetScan VS2 HW test and (B) the SNAP HW assay plotted against the quantitative PetChek HW OD value for the same sample.

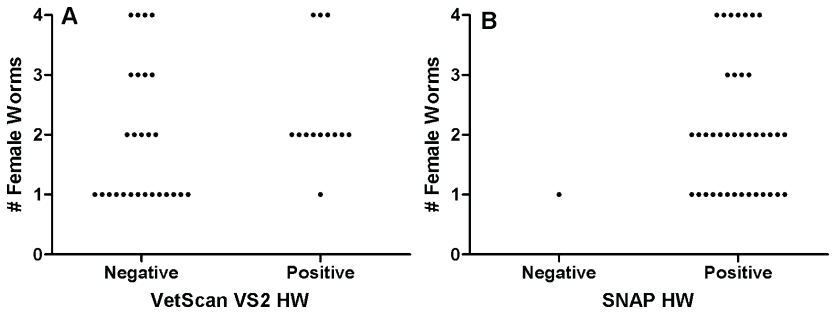

Fig. 3. Qualitative test results of (A) the VetScan VS2 HW test and (B) the SNAP HW assay plotted against the number of female heartworms found at necropsy.

heartworm infections. For the 60 dogs with detectable heartworm antigen, the VetScan VS2 HW test demonstrated a poor antigen detection rate when compared to either the PetChek HW ELISA or necropsy results. The SNAP HW test, by comparison, had a very high percentage agreement with both reference methods. In no instance did the VetScan VS2 HW test produce an accurate result that was not also accurately reported by the SNAP HW test; the reverse was not true. However, the current study was designed specifically to challenge the new instrumented test method by presenting serum samples from dogs with low worm burdens and/or low levels of circulating antigen. In a real-world clinical situation, there would be a wider range of worm burdens encountered, including some heavily infected dogs, so test performance would be expected to improve in this more general canine population. Nevertheless, dogs with low-burden infections and/or low antigenemia do present in clinical practice – and may be expected to be more common when owners are not 100% compliant in administering preventive medications – and these patients cannot be predicted based on clinical signs or geographical region. These are the cases in which test sensitivity will make a clear difference in diagnostic capability.

One limitation of the study design was the length of time the serum samples were stored in the freezer prior to testing. According to the VetScan manufacturer's product insert, serum samples should not be frozen for more than 5 weeks. Because of the difficulty in obtaining a large number of samples from dogs with necropsy-confirmed heartworm burdens, the study was carried out using banked serum samples that had been frozen, some for several years. However, an effort was made to compensate for this departure from the manufacturer recommendations by also including serum samples collected over a period of two months from the field that had never been frozen before. Furthermore, when sufficient serum volume allowed, PetChek HW testing was repeated to ensure that the heartworm antigen had not deteriorated over the course of prolonged storage.

The findings of the present study are clinically relevant because dogs with low heartworm burdens tend to be the most difficult to identify. Not only do these dogs typically have lower antigen levels, making serological diagnosis more difficult, but microfilarial tests are equivocal. Dogs with low heartworm numbers may have single-sex infections resulting in non-patency. Some dogs may also possess anti-microfilaria antibodies – either through repeat infections in areas of high heartworm prevalence (Otto, 1977), or through transplacental exposure from infected dams (Wong et al., 1973) – that promote clearance of microfilariae from the circulation. Indeed, 10–67% of dogs confirmed by necropsy to harbor adult heartworms have been reported to be amicrofilaremic (Otto, 1977). In addition, since many preventives also possess some microfilaricidal activity (McCall et al., 2008), a dog with a false negative antigen test result that is placed on chemoprophylaxis would experience a reduction in microfilaremia. For dogs in any of the aforementioned situations, a heartworm antigen test may be the only effective means of screening for infection. Therefore an accurate antigen test is pivotal to early diagnosis and treatment of infected dogs in order to prevent further progression of heartworm-induced pathology. Overall, the SNAP HW test demonstrated superior performance compared to the VetScan VS2 HW in this study. Based on the large number of false negative test results reported by the VetScan VS2 HW test in dogs with low heartworm burdens and/or low antigen levels, this new instrumented immunoassay appears to be less sensitive than ELISA-based assays for heartworm antigen detection.

Conflict of interest statement

Dr. Beall is an employee of IDEXX Laboratories, Inc. Drs. Dillon and Bowman have received research funding from IDEXX Laboratories, Inc. within the last 5 years.

Acknowledgements

The authors wish to thank the veterinary technicians who performed the SNAP HW assays: Debra Bain, Bethany Austin, and Susan Brown of the Cornell University Hospital for Animals.

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Peer Reviewed

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Coughing Cat: Could It Be Heartworm?

A 5-year-old, neutered, indoor/outdoor domestic short-haired cat presented with chronic cough.

In addition to chronic cough, the cat had periodic episodes of vomiting.

HISTORY

According to the owner, the cat, which had previously lived in Alabama, had periodic vomiting episodes but no other medical issues. He was not receiving any medications and his vaccination status was current.

EXAMINATION

The cat was bright, alert, and responsive. Examination revealed increased bronchovesicular sounds in the caudal lung fields but was otherwise unremarkable. No lungworm ova or larvae were observed by either fecal flotation or Baermann technique.

Ask Yourself…

What is the next diagnostic test of choice for this cat?

A. Echocardiography

B. Thoracic radiography

C. Transtracheal wash or bronchoalveolar lavage

D. Repeat Baermann technique

CONTINUES

CORRECT ANSWER
B. Thoracic radiography

Thoracic films are the most useful diagnostic test for identifying the primary lung disease or triggering factor that might be responsible for feline coughing, including asthma, infectious agents (eg, *Aelurostrongylus abstrusus, Dirofilaria immitis, Mycoplasma felis*), and neoplasia.

In this case, echocardiography would not be indicated because cats generally do not cough from cardiac disease. Transtracheal wash or bronchoalveolar lavage is more invasive than radiography, although it may reveal evidence of inflammation, infection, or lymphoma. If lungworm larvae are suspected, the Baermann technique may need to be repeated multiple times, as false-negative results can occur; empiric anthelmintic treatment is therefore recommended.

DIAGNOSIS

Thoracic radiographs showed lesions suggestive of heartworm or lungworm infection (**Figure 1**). An in-clinic heartworm antigen test was positive. The owners declined echocardiography.

FELINE HEARTWORM DISEASE

Heartworm infection should be considered as a differential diagnosis for respiratory signs in cats, particularly in heartworm-endemic regions. Cats are not natural hosts for heartworm and their resistance to infection translates into differences in the parasite life cycle as compared with dogs (**Table**).

1 Bronchointerstitial pattern in the caudal lung fields (**A** and **B**). Enlargement of the caudal pulmonary arteries is evident on the ventrodorsal (**B**) projection.

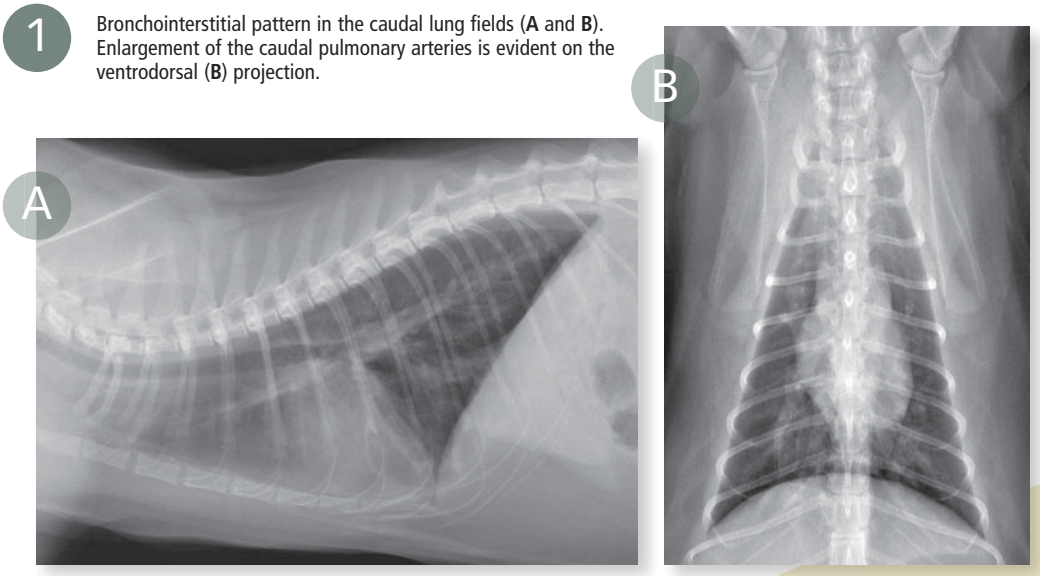


Table. Heartworm Infection in Cats Versus Dogs

	Cats	Dogs
Number of adult worms	Usually 1–4[1]	Many
Lifespan of adult worms	2–4 years[2]	5–7 years
Microfilaremia	<20%	33%–95%[3,*]
Prepatent period	7–8 months	6 months
Duration of patency	1–2 months	Years
Aberrant migration	More common in cats[4]	

*Microfilaremia persists in 10%–20% of heartworm-infected dogs receiving chemoprophylaxis.[4]

HARD = heartworm-associated respiratory disease

Clinically, cats with heartworm are difficult to distinguish from those with asthma, although asthma waxes and wanes while heartworm signs tend to persist. Cats with chronic heartworm disease (ie, adult infection) often present with vomiting in addition to respiratory signs.[2,5] Heartworm-infected cats can die suddenly without preexisting clinical signs and the presence of adult worms is not required for clinical disease.[2,5] Cats, much more so than dogs, can experience heartworm-associated respiratory disease (HARD) attributed to the death of immature adult worms in the pulmonary vasculature.[4] HARD may or may not progress to a mature heartworm infection.

PREVALENCE

Because heartworm screening in cats is not routine practice, prevalence is estimated to be 5% to 20% of that in dogs in the same area. Shelter necropsy studies provide some indication of true prevalence (**Figure 2**). An antibody prevalence map for cats is available from the Companion Animal Parasite Council (capcvet.org).[2] Reported prevalence rates may be biased because cats are generally tested only when the index of suspicion is high; however, these values are noteworthy, as they closely resemble necropsy data.

TREATMENT

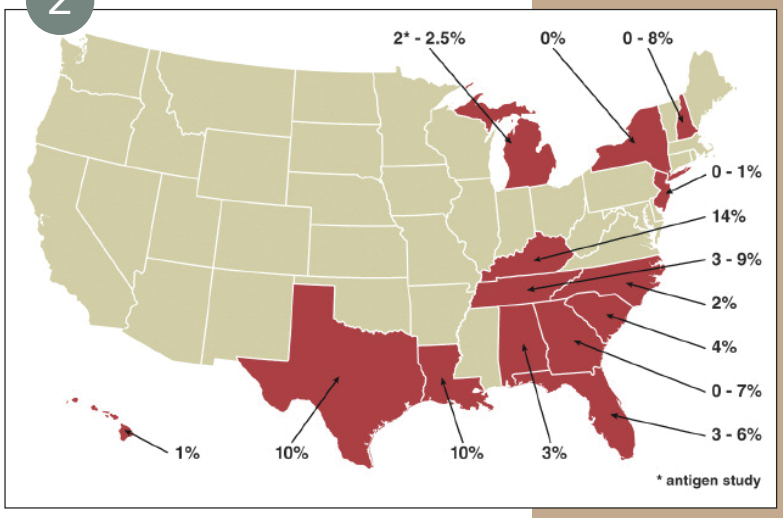
Treatment includes prednisone at 1 to 2 mg/kg q12–48h, tapered over 3 to 4 weeks. If signs recur, the lowest effective dose should be continued at q48h. Macrocyclic lactone preventive therapy should be initiated to protect against reinfection. Because cats have few to no microfilariae, there is little fear of mass microfilarial death from product administration, making macrocyclic lactone therapy acceptable.[4]

Of note, no heartworm adulticides have been approved for cats.

CONTINUES

2

Percentage of shelter cats possessing adult heartworms at necropsy, 1993–2004. Data acquired only from red-colored states.



Courtesy of Dr. Clarke Atkins & Companion Animal Parasite Council

Heartworm Diagnosis: Challenging in Cats

In symptomatic cats, a positive antigen test is considered diagnostic of a mature infection but a negative result is ambiguous. Cats have few adult worms, so the amount of circulating antigen may fall below the detection limit. Male-only infections can also lead to false-negative results. The presence of antibodies indicates exposure to heartworm but does not necessarily denote an adult infection. Conducting both antibody and antigen testing increases sensitivity of detection as compared with conducting either test alone.[1]

Radiographic lesions (eg, hyperinflation, varying patterns of parenchymal infiltrates, enlarged caudal pulmonary arteries) help support a diagnosis of heartworm but are nonspecific and can resolve within a few months of infection in cats.[5,6] Echocardiography provides a definitive diagnosis if heartworms are observed.

The American Heartworm Society has a summary table on test interpretation that can assist in diagnosing feline heartworm disease.

Surgical extraction of adult worms is possible but is associated with high risk for anaphylactic-like shock. This should be limited to cats with high worm burdens (eg, caval syndrome) and is best performed by experienced specialists.[4,7]

Prevention of feline heartworm infection is the best management strategy. Cats with outdoor access are at higher risk for infection; however, indoor-only cats represent a quarter of confirmed heartworm cases.[2,5] Cats should therefore receive chemoprophylaxis regardless of lifestyle, especially in endemic areas.

OUTCOME

The cat was treated symptomatically with a tapering dose of prednisone for 3 weeks and then maintained on q48h therapy. Year-round heartworm prevention was instituted. Three months after presentation, the cough had completely resolved.

See **Aids & Resources**, back page, for references & suggested reading.

Take-Home Messages

- The majority of coughing cats have primary lung disease, not cardiac disease.
- Unless the patient is antigen positive or heartworms are observed on echocardiography, definitive diagnosis of feline heartworm disease remains a challenge.
- Multiple test results should be used to determine whether heartworms are the cause of a patient's clinical signs.

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Short communication

Comparison of *Ancylostoma caninum* worm counts acquired by endoscopy and necropsy

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ABSTRACT

Many regulatory agencies require that the efficacy of veterinary anthelmintic medications be evaluated by enumerating parasites in treated and untreated animals after necropsy. Current ethical considerations, i.e., the 3 Rs of research, call for the replacement of this method with less invasive techniques that would not require animal sacrifice. This study tested standard gastrointestinal endoscopy as an in vivo method of quantifying the intestinal hookworm, *Ancylostoma caninum*. Worm counts were compared with those from gold standard necropsy. Thirteen dogs inoculated with third-stage *A. caninum* larvae underwent endoscopy 4–6 weeks post-infection, just prior to necropsy. Two-thirds of the adult hookworms were located in the middle section of the small intestine that could not be reached for endoscopic examination. Not surprisingly, the total worm counts obtained by endoscopy did not correlate with those from necropsy ($R^2 = 0.05$, $p = 0.464$). One method to increase small intestinal access would be to use specialized balloon or spiral endoscopes developed for this purpose in human gastroenterology. Based on the results of this study, standard endoscopy alone is unsuitable for quantification of *A. caninum* in the small intestine. Parasites in more accessible sites, such as whipworms in the cecum and colon, might be more appropriate targets for endoscopic counting.

1. Introduction

Veterinary anthelmintic drugs are commonly evaluated using controlled tests wherein treated and untreated animals are euthanized for parasite enumeration (Jacobs et al., 1994). Necropsy is required by many regulatory agencies for the purpose of drug approval because it is considered the gold standard for determining anthelmintic efficacy. As such, this method is often employed during the research and development process leading up to product registration as well. Modern times and ethical considerations – namely, the 3 Rs (reduction, replacement, and refinement) of animal use in research – call for the replacement of old-school necropsy with a less invasive technique that would achieve the same aim. For intestinal helminths, an obvious solution would be in vivo imaging of the worms. Gastrointestinal endoscopy is routinely performed in veterinary practice for the purpose of disease diagnosis and therapeutic intervention. It is a minimally invasive procedure that permits examination of the small intestine (SI) of anesthetized animals. In this study, endoscopy was tested as an in vivo method of quantifying the canine intestinal hookworm *Ancylostoma caninum*. Dogs were recruited from a separate anthelmintic efficacy trial with a terminal endpoint so that the endoscopy counts could be compared to the gold standard postmortem counts.

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2. Methods

Thirteen purpose-bred Beagle dogs (6 males, 7 females) were included in this study. Two age groups were represented: nine puppies approximately 4–6 months old, and four adults 7–9 years old. Each dog was inoculated orally with 250 third-stage larvae of a laboratory strain of *A. caninum* maintained in-house. 4–6 weeks post-infection, dogs were fasted to empty the bowels in preparation for gastrointestinal endoscopy. Water was available free choice. Endoscopy was performed under inhalant anesthesia after intramuscular premedication with a mixture of medetomidine, butorphanol, and ketamine. Antegrade (duodenal) and retrograde (ileal/cecal/colonic) endoscopy were performed and the length of small intestine accessed from either direction was estimated based on 5-cm markings on the endoscope. The number of hookworms observed in the anterior and posterior portions of the intestinal tract was separately recorded. Dogs were euthanized upon completion of endoscopy by delivery of pentobarbital via an intravenous or intracardiac route. Necropsy was performed immediately afterward. The intestinal tract was extracted and transected at the estimated limits of antegrade and retrograde endoscopic access. The mucosa was stripped from the three resulting sections and individually rinsed through a 50 μm stainless steel mesh sieve (Retsch Inc., Germany) to recover and count worms. This study protocol received approval from the Cantonal Committee on Animal Experimentation (Novartis Animal Health Centre de Recherche Sante Animale, FR401e/08 and 2010_46_FR). Student's *t*-test and linear regression were performed using Minitab 16 software (Minitab Inc., State College, PA), with significance set at $p \leq 0.05$.

3. Results

Adult *A. caninum* were present in all dogs at necropsy, however, higher worm burdens (mean ± standard deviation) were seen in puppies (115.3 ± 32.5) than in adult dogs (19.0 ± 15.2) ($p < 0.001$). On average, 66% of the worms were located within the middle portion of the SI that was not viewable by endoscopy (Fig. 1). A correspondingly strong correlation ($R^2 = 0.93$, $p < 0.001$) was observed between the necropsy counts for the middle SI and the total intestinal tract. In contrast, no relationship ($R^2 = 0.05$, $p = 0.464$) was detected between the total worm counts acquired by endoscopy and necropsy. Limiting the comparison to the anterior and posterior portions of the intestine – the parts accessible to the endoscope – improved the correlation considerably ($R^2 = 0.56$, $p = 0.003$) (Fig. 2). An endoscopic recording of two hookworms moving around in search of new mucosal attachment sites can be viewed in the Supplemental Content section of this article online (Video 1).

4. Discussion

In this study, puppies had six times the number of hookworms in their intestinal tracts as adult dogs. The adult dogs were housed in a controlled environment and had only been used for passaging non-hookworm helminths before, so they remained naïve against *A. caninum* until the time of experimental inoculation. The observed difference in worm counts probably reflects an age-related resistance to primary *A. caninum* infection in older dogs, as previously demonstrated by Miller (1965). Individual host variability may also play a part in the wide range of worm burdens. Past studies of hookworm distribution in the canine intestinal tract point to a clear site predilection. Krupp (1961) showed that the majority of adult *A. caninum* in induced infections are located in the central half of the SI, regardless of inoculum size. Similarly, Mello et al. (1977) found that the jejunum accounted for 97.5% of the total *Ancylostoma* spp. worm burden in naturally infected dogs. The results of the present experiment are in agreement with these prior studies, as evidenced by two-thirds of the worms residing in the middle portion of the SI. A lack of examination of this area greatly contributed to the low total endoscopy count compared to the total necropsy count. As expected, there was a much better correlation between the two

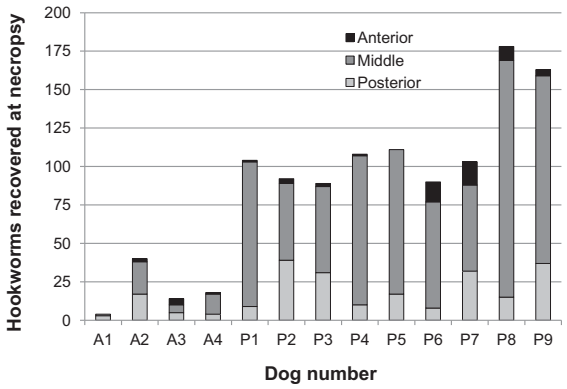

Fig. 1. Number of *A. caninum* located in the anterior, middle, and posterior sections of the intestinal tract. The majority of hookworms reside in the middle region of the small intestine, which is inaccessible by endoscopy. On the whole, adult dogs (A1–A4) were poorly infected relative to puppies (P1–P9).

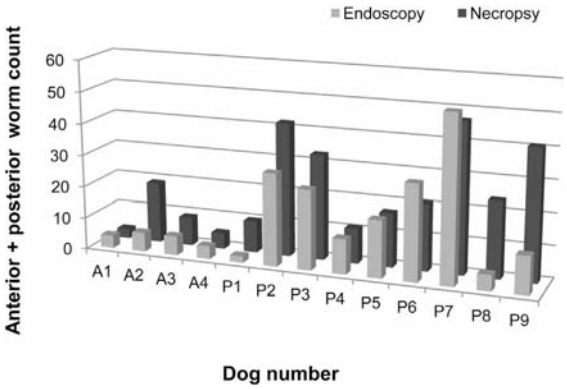

Fig. 2. Comparison of worm counts acquired by endoscopy versus necropsy for the endoscope-accessible regions of the intestinal tract. Though the two quantification methods produced similar worm numbers in some dogs, in others, the endoscopy count was much lower than the necropsy count.

quantification methods when the middle SI was excluded from analysis. However, some discordance was still observed.

Use of deep enteroscopy techniques developed for human gastroenterology may help to overcome the problem of inadequate SI inspection. These procedures rely on a specialized overtube and sequential push and/or pull motions of the enteroscope to compress the intestines like an accordion, allowing for greater SI access than standard endoscopy. Depending on the specific technique chosen (single balloon, double balloon, or spiral enteroscopy), complete SI examination can be achieved in a mean of 3–34% of human patients (Lenz and Domagk, 2012). Aside from the poor completion rate, one concern with using deep enteroscopy for SI helminth enumeration is the potential for frequent overtube maneuvering to dislodge the worms and thus impact any subsequent counts.

Based on the results of this study, standard endoscopy does not provide an accurate count of *A. caninum* worms in dogs, likely attributable to its inability to view the central portion of the SI. However, it may prove useful for enumeration of the canine whipworm, *Trichuris vulpis*, which resides in the more easily accessible cecum and colon. Therefore endoscopy bears further investigation as an in vivo quantification method for other helminth species.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2013.03.022.

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Short Communication

Assessing the speed of kill of hookworms, *Ancylostoma caninum*, by Advantage Multi® for Dogs using endoscopic methods

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ABSTRACT

Endoscopic capsules and endoscopy were used to assess the speed of kill and the clearance of hookworms in dogs experimentally infected with *Ancylostoma caninum*. A total of four adult dogs were inoculated in two separate cohorts comprised of two 4-year-old females and two 7-year-old males. Dogs were treated topically with Advantage Multi® for Dogs 13 days (Cohort 1) or 16 days (Cohort 2) after infection. Endoscopic imaging of the small intestine was carried out both pre- and post-treatment. Examination of the first cohort revealed that the worms had been cleared and the hookworm-induced lacerations were markedly diminished within 48 h of treatment. In the second cohort, endoscopic capsules were given the day of, the day after, and two days after treatment; within 24 h of product administration, the worms had been removed with a concurrent reduction in observed lesions. Topical application of Advantage Multi® for Dogs rapidly removed worms from the small intestine of the dogs in this study as early as 24 h post-treatment, with a marked reduction in the number of mucosal lesions seen.

1. Introduction

In the Freedom of Information summary of the New Animal Drug Application (NADA) made to the Food and Drug Administration (FDA) for the approval of Advantage Multi® for Dogs, the time from treatment to necropsy in the efficacy trials against intestinal helminths (*Ancylostoma caninum*, *Uncinaria stenocephala*, *Toxocara canis*, *Toxascaris leonina*, and *Trichuris vulpis*) is 10 days (NADA 141-251). FDA Guidance Document 111 for the effectiveness of anthelmintics in canines (VICH GL19) states that "with the majority of parasites seven days is a sufficient time period from the termination of treatment until the animals are necropsied," but exceptions are presented for other helminths, including the gastrointestinal dwelling species of *Physaloptera*, *Spirocerca*, *Echinococcus*, *Taenia*, *Dipylidium*, and *Mesocestoides* where the time is extended to 10 days. For approval purposes, because Advantage Multi® for Dogs is applied topically and takes 9 days to reach maximum plasma concentrations (NADA 141-251), 10 days post-treatment was a logical time point to assess efficacy based on postmortem worm counts.

The development of endoscopy, and particularly endoscopic camera capsules, has now provided a means to examine intestinal helminths in vivo at different time points after infection (Lee et al., 2011, 2013; Liotta et al., 2012). This would also potentially allow for assessment of the rate of worm clearance after treatment and, in the case of hookworms, the resultant effects on the lesions caused by feeding adult worms. Blood lost to feeding hookworms

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has been reported to occur in two peaks after infection: one during the very rapid growth of the maturing adult worms (10–15 days after infection) and one at 20 days after infection when maximal egg output begins (Miller, 1966). Advantage Multi® for Dogs is labeled for the treatment of fourth-stage larvae and both immature and mature adult worms, and thus, treatment was applied in this study earlier than the routinely used time point of >21 days post-infection to minimize blood loss by the dogs. This work documented the effects of anthelmintic treatment on populations of young *A. caninum* and subsequent resolution of hookworm-induced intestinal lesions.

2. Materials and methods

2.1. Animals

All work was performed under a protocol approved by Cornell University's Institutional Animal Care and Use Committee. The four Beagle dogs used in this study had been acquired from an approved Class A dealer (Marshall BioResources, North Rose, NY) and had resided in Cornell's research facility for a number of years as part of an unrelated investigation into canine herpesvirus-associated ocular disease. The work was performed in two cohorts with two 4-year-old females in Cohort 1 and two 7-year-old males in Cohort 2. At the beginning of the trial utilizing Cohort 1, all dogs were transferred from the room in which they had been housed to a different room within the same facility. The female dogs were housed together, but due to between-dog aggressive tendencies, the male dogs were housed individually. Although transferred at the same time, dogs were monitored for the study such that there was a 4 day (Cohort 1) or 7 day (Cohort 2) acclimation period to the new room before the study began, i.e., the day on which the dogs received their hookworm infections (Day 0). Dogs received initial physical examinations between Days −5 and −2; all were clinically healthy. The study ended on Day 22 (Cohort 1) or 25 (Cohort 2) and, this being a non-terminal study, at study completion the dogs were transferred back to the previous investigator.

2.2. Infection and treatments administered

On Day 0 for each cohort, the dogs were orally inoculated with 500 third-stage larvae of *A. caninum* that had been grown in charcoal cultures (Bowman et al., 1991). The source of the larvae was a fecal sample from a random source dog supplied by Cheri-Hill Kennel and Supply, Inc., Stanwood, MI. The larvae were grown in culture for 6 days prior to harvest and were stored in water at room temperature in T-25 vented tissue culture flasks until used. Aliquot counts were made for preparing the doses to infect the dogs in Cohorts 1 and 2, respectively. The dogs were treated with Advantage Multi® for Dogs at the dose band recommended by the manufacturer for their weight. For Cohort 1, treatment (equivalent to 6.8 mg/kg moxidectin) was administered on Day 13 of the trial, and for Cohort 2, treatment (4.6–5.5 mg/kg moxidectin) was applied on Day 16. Fecal samples from these dogs were examined by sucrose centrifugal flotation on Days 19 and 24 for Cohorts 1 and 2, respectively.

2.3. Endoscopic examination

Dogs were fasted overnight prior to each endoscopic examination, then fed after completion of the procedure. Capsule endoscopy was performed according to the method previously described (Lee et al., 2011). Briefly, dogs were instrumented with external antennae along the ventral body wall, fitted with a jacket and pouch holding the image recording device, and the endoscopic capsule was then given by mouth. After image acquisition, instruments were removed and the dog's feces were checked for capsule expulsion. Stored images were downloaded onto a computer for review. For conventional endoscopy, dogs were premedicated with a butorphanol–midazolam mixture, induced with propofol, and maintained under general anesthesia with isoflurane inhalant gas. Examination of the duodenum was carried out as per standard endoscopic protocols. Dogs were monitored until fully recovered from anesthesia. In Cohort 1, the dogs were examined with endoscopic capsules on Days 7, 9, 12, and 15, and with a conventional endoscope on Days 11 and 16. The dogs in Cohort 2 were examined by capsule endoscopy on Days 9, 12, and 15–18, and by conventional endoscopy on days 14 and 19.

3. Results

In the examination of the images collected from Cohort 1, hookworm-induced lacerations were observable as early as Day 7 (Fig. 1A), the first day that endoscopic capsules were given to the dogs. Worms were seen on Day 9 (Fig. 1B), and this was verified with endoscopy on Day 11 (Fig. 1C and D; see also Video 1 online). Worms were larger and more numerous on Day 12, with mucosal hemorrhages still being present as would be expected (Fig. 1E and F). Cohort 1 dogs were treated on Day 13, and the endoscopic capsule images captured on Day 15 (2 days post-treatment, DPT) showed the presence of healing scars only (Fig. 1G); this was supported by the endoscopy views collected on Day 16 (3 DPT) (Fig. 1H and I). Centrifugal flotation of feces collected on Day 19 was negative for hookworm eggs.

In the case of Cohort 2, worms and lesions were obvious from the images collected from Day 9 (Fig. 2A) and Day 12 (Fig. 2B) through Day 14 (Fig. 2C and D; see Video 2). These dogs were treated on Day 16, and images collected by capsule endoscopy on this day showed that worms and lesions were abundant (Fig. 2E and F; see Video 3). However, by Day 17 (1 DPT), worms were no longer present in the small intestine and lesions were few in number (Fig. 2G). Again, this was verified by endoscopy performed on Day 19 (3 DPT), which showed only a few small lesions remaining as compared to the extensive lesions present before treatment (Fig. 2H and I; see Video 4). Fecal examination on Day 24 revealed no hookworm eggs.

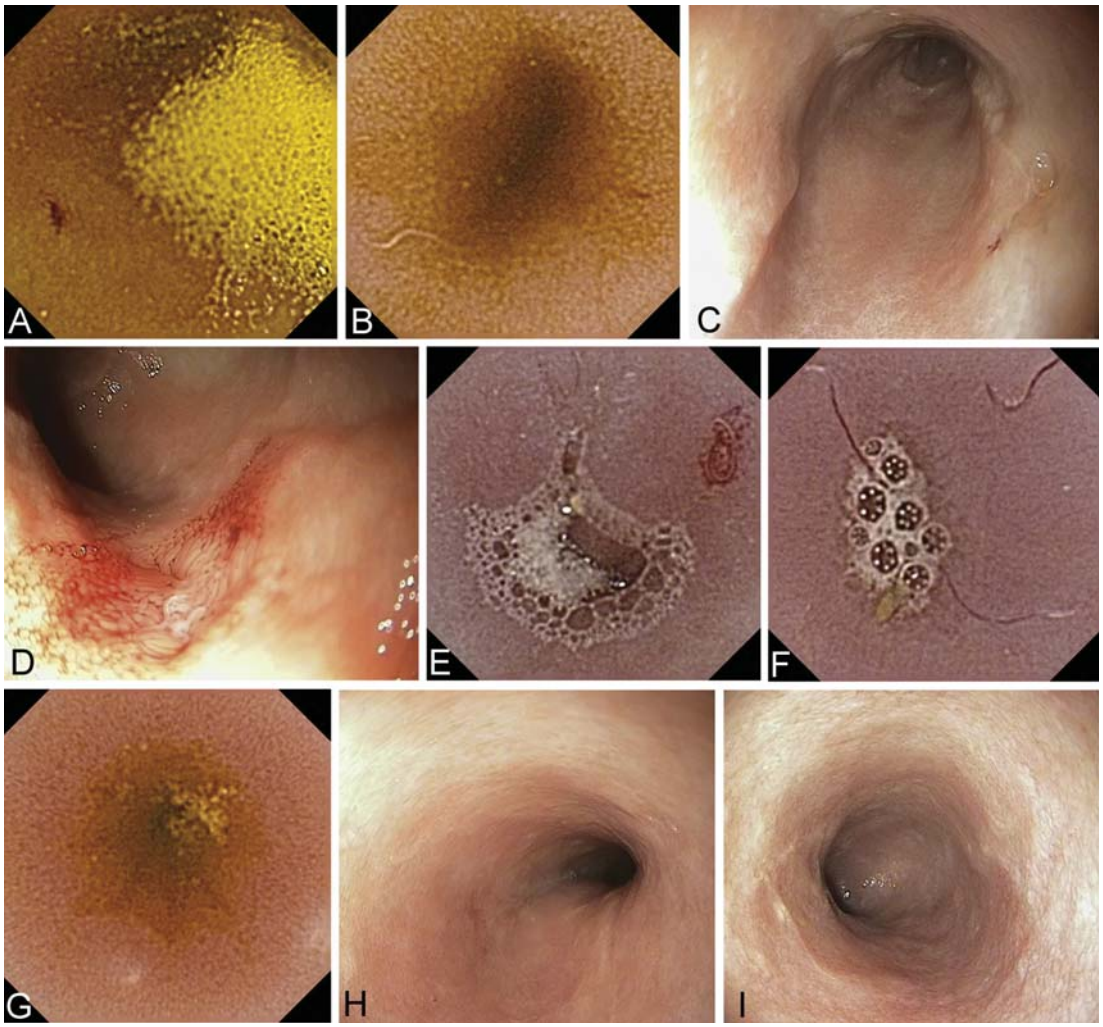

Fig. 1. Immature adult *Ancylostoma caninum* and associated intestinal bleeding in dogs of Cohort 1, showing resolution after treatment with Advantage Multi® for Dogs. (A) Small bowel mucosal hemorrhage, Day 7 post-infection. (B) Young *A. caninum*, Day 9. (C) Abandoned feeding site, Day 11. (D) Immature adult hookworm (bottom center), Day 11, camouflaged by the surrounding intestinal villi and a large amount of frank blood (see corresponding Video 1). (E) Solitary hookworm nestled in blood, Day 12. (F) Three *A. caninum* attached to mucosa, Day 12. (G) Focal blanched areas on the intestinal wall, Day 15 (2 days post-treatment), presumed to be healing lacerations. (H and I) Normal-looking bowel, Day 16 (3 days post-treatment). Panels A, B, and E–G are capsule endoscopy images and panels C, D, H, and I are conventional endoscopy images.

4. Discussion

The discovery that the worms and associated hemorrhage in dogs of Cohort 1 had been cleared by the second day post-treatment prompted a modification of the schedule of events for the next set of dogs. Thus, in Cohort 2, two additional endoscopic capsules were given: one on the day of treatment and another on the following day. Images from Cohort 2 clearly demonstrated that as early as 24 h after topical treatment with Advantage Multi®, *A. caninum* worms were removed and lesions were reduced in number. Another difference between the two cohorts was the delaying of treatment for Cohort 2 to Day 16; this three-day postponement permitted the development of extensive and severe lesions by Day 14 (Fig. 2C) that were not present in Cohort 1 (Fig. 1G–I). The speed with which the worms were removed from the intestine after treatment was much more rapid than had been anticipated when the study was designed. It is possible, however, that a few worms may have still been present in the colon 24 h after treatment, because this segment of the intestinal tract is not examined by the small bowel endoscopic capsule, and colonoscopies were not performed.

Fecal examinations were not performed on these dogs except for the purpose of verifying that they were egg-negative before being returned to the care of their original investigator. Because the worms were young, eggs may not have been present in the feces of the dogs in Cohort 1 that were treated on Day 13 post-infection, and the females would not have yet reached their maximal egg production

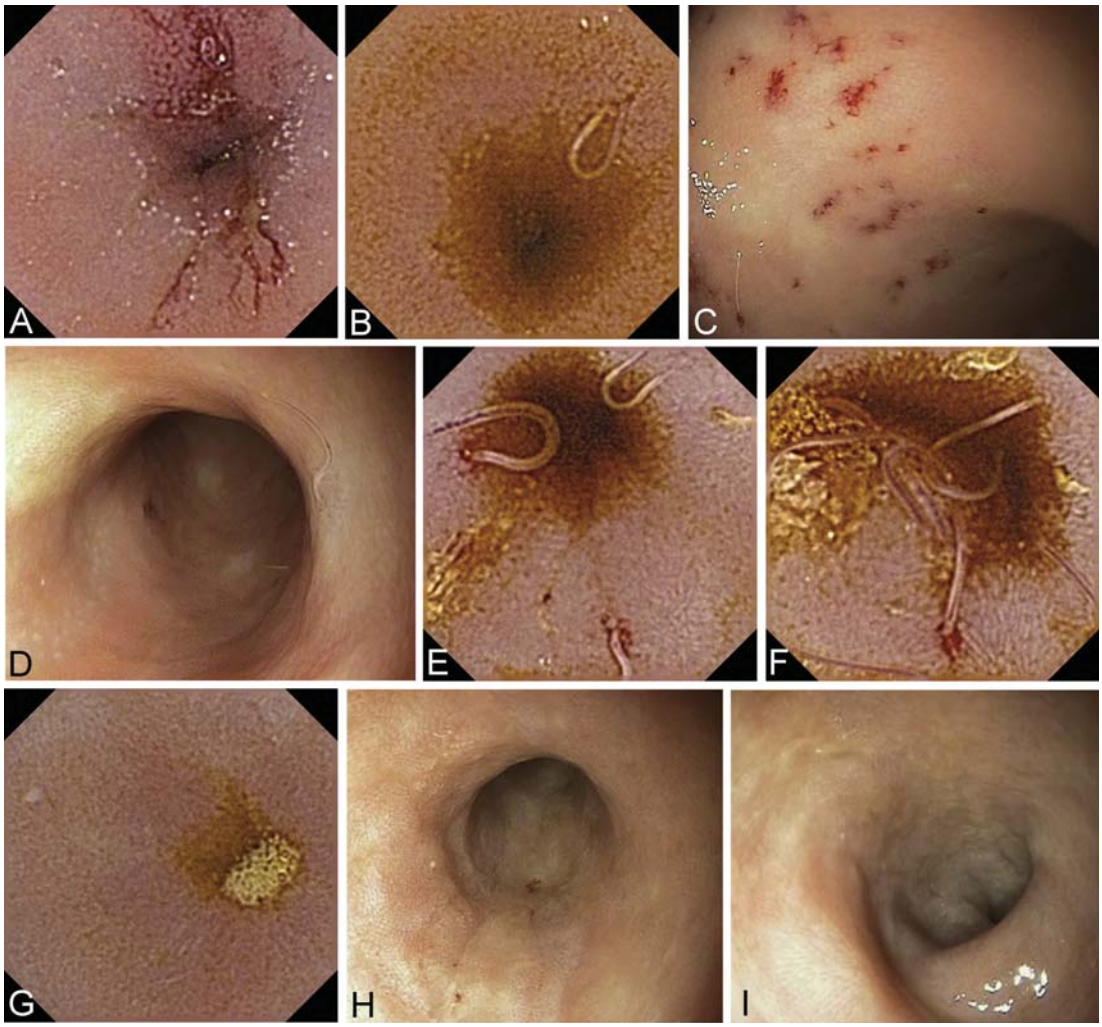

Fig. 2. Intestinal lacerations in *Ancylostoma caninum*-infected dogs of Cohort 2, with rapid healing after treatment with Advantage Multi® for Dogs. (A) Young hookworm on Day 9 post-infection, curled up in pooling blood. (B) Immature adult *A. caninum*, Day 12. (C) Numerous hookworm-induced lacerations, Day 14 (see Video 2). (D) Hookworm (top right) attached to intestinal mucosa, Day 14; a previous attachment site can be seen in the background. (E and F) Multiple adult hookworms causing hemorrhage as they feed, Day 16 (see Video 3). (G) Healing laceration, Day 17 (1 day post-treatment). (H) Minor residual lesions, Day 19 (3 days post-treatment). (I) Normal-looking bowel, Day 19 (3 days post-treatment) (see Video 4). Panels A, B, and E–G are capsule endoscopy images and panels C, D, H, and I are conventional endoscopy images.

until four weeks after infection (Bowman et al., 1991). After the treatment with Advantage Multi® of dogs with experimentally induced *Ancylostoma ceylanicum* infections, daily eggs counts were negative for all treated dogs four days after treatment (Taweethavonsawat et al., 2010). The rapid clearing of the eggs in these dogs would suggest that, as was seen in the present study, there was likely removal of the worms as a source of eggs within a day of treatment.

The endoscopic camera capsules have the capacity to image the entire small intestinal tract, and it would be of interest to know when other helminths – and perhaps most interestingly, tapeworms – are actually killed by treatment. It would also be of interest to see the response of larger helminths to treatment; this was not examined in this case. *A. caninum* grows rapidly, but the worms were still quite small at the time this study was performed. According to the careful work by Matsusaki et al. (1965), the respective lengths in millimeters of male and female *A. caninum* at different days post-infection are 3.0–4.1, 3.3–3.8 (6 days); 5.0–6.5, 5.0–7.6 (10 days); 7.5–10.0, 8.0–11.5 (14 days); 8.1–10.1, 11.0–14.0 (18 days); 10.9–11.6, 13.5–14.8 (25 days); 10.2–12.2, 14.1–19.1 (35 days); and 11.0–12.9, 18.4–19.4 (45 days). Thus, most of the worms seen in this study were less than half (3.3–14.0 mm) of what their lengths would have been (10.9–14.8 mm) at the typical time of necropsy 28–30 days after infection, and still much smaller than they would have been (11.0–19.4 mm) when fully mature at one-and-a half months of age.

Role of funding source

The sponsor had no role in study design, implementation, or study report preparation.

Conflict of interest

Dr. Bowman has been paid by Bayer HealthCare LLC for work relative to consultancies and honoraria for speaking; Cornell has received funds for research and contractual work from Bayer with Dr. Bowman as the principal investigator.

Dr. Lee has received compensation for educational articles written for Bayer.

Dr. Hostetler is employed by Bayer HealthCare LLC.

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Appendix A. Supplementary data

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Research paper

Determination of anthelmintic efficacy against *Toxocara canis* in dogs by use of capsule endoscopy

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ABSTRACT

Industry guidelines for anthelmintic testing call for postmortem inspection of animals to verify treatment efficacy. A previous study showed that capsule endoscopy (CE) can be performed on dogs in vivo to quantify hookworms in the small intestine. Adoption of a minimally invasive procedure such as this could reduce the need for necropsy in efficacy trials. The present study employed CE to enumerate *Toxocara canis* in dogs, with two main goals: to determine if multiple capsule examinations improves the accuracy of worm counts compared to a single examination, and to establish if the efficacy of an anthelmintic compound is the same whether calculated using CE or necropsy data. To avoid needless animal sacrifice, the study was carried out on beagle dogs already in a product development trial with a planned terminal endpoint. Dogs were infected by oral inoculation with *T. canis* eggs. Untreated control dogs ($n = 8$) were evaluated by CE three times while dogs treated with test compounds (3 groups of 4) were examined only once. Utilizing either the average count or just the last complete capsule examination, a robust correlation was found between CE and postmortem numbers ($r = 0.94$, $p < 0.001$). Calculated anthelmintic efficacy was essentially identical for the two enumeration methods, ranging from 94% to 100% for the three research compounds. CE may therefore be a viable alternative to necropsy for *T. canis* parasiticide trials.

1. Introduction

Current anthelmintic testing guidelines call for postmortem examination of test animals to prove that target helminths are successfully eliminated by treatment. This translates into hundreds of euthanasias for each product that reaches market. Recently, gastrointestinal endoscopy has been investigated for its potential to assess anthelmintic drug efficacy in living animals, including dogs in a research setting and European shags (*Phalacrocorax aristotelis*) in the field (Burthe et al., 2013; Lee et al., 2013). Although conventional endoscopy allows for observation of helminths in the upper and lower gastrointestinal tract, it does not provide a good representation of worm burdens in the small intestine (SI) due to a lack of jejunal access (Lee et al., 2013). Capsule endoscopy (CE)—the use of wireless ingestible cameras—has been tested in dogs as a means of overcoming this limitation. Pilot work demonstrated the utility of CE for detection and enumeration of *Ancylostoma caninum* in the SI, showing a clear but imperfect correlation to worm counts acquired by necropsy (Lee et al., 2011).

The present study applied CE to another common SI nematode of dogs, *Toxocara canis*. Objectives were twofold: (1) Assess whether averaging the counts from three capsule passages in the same dog would better reflect true worm burden compared to a single count; and (2) Determine if the anthelmintic efficacy calculated using CE versus necropsy data is comparable. The latter is important because countries that adhere to the VICH guidelines for anthelmintic testing (VICH, 2001), including the United States and members of the European Union, require a minimum efficacy of 90% before a drug label claim can be made. Any method intended to replace gold-standard necropsy in the testing process must therefore produce similar results in terms of efficacy evaluation. In order to avoid sacrificing dogs expressly for this investigation, CE was incorporated into an existing product development trial that had a terminal endpoint as per the industry norm.

2. Materials and methods

The study protocol was approved by the institutional animal care and use committees of Cornell University and Novartis Ani-

Abbreviations: CE, capsule endoscopy; SI, small intestine; VICH, International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products.

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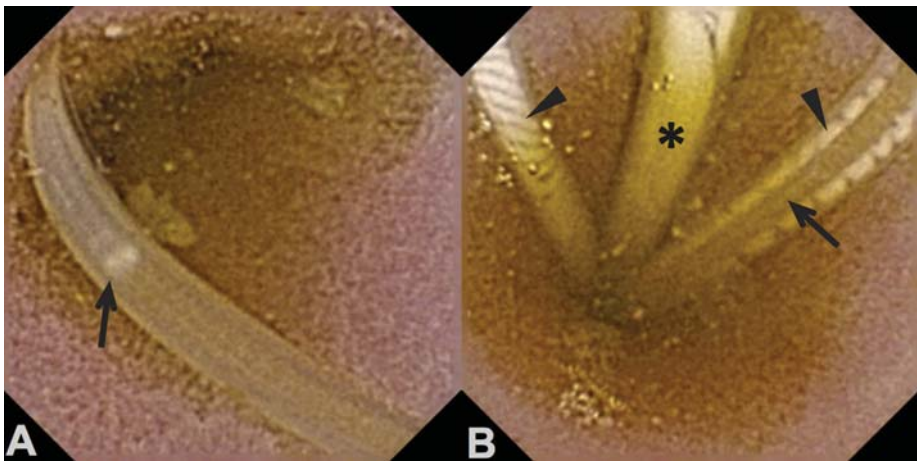

Fig. 1. Morphological features of *T. canis* visible on capsule endoscopy. (A) Anterior end of a worm with a conspicuous, white-colored ventriculus (arrow). (B) Clearly discernible within the bodies of the centrally located female and two flanking males are the uterus (asterisk), testis (arrowheads), and intestine (arrow).

mal Health in St-Aubin, Switzerland. Four-month-old beagle dogs were orally inoculated with eggs cultured from the feces of a dog naturally infected with *Trichuris vulpis* and *T. canis*. The inoculum volume was chosen based on the main target species of the anthelmintic trial, *T. vulpis*; *T. canis* eggs were present but not quantified, so the exact dose administered to the dogs was unknown. Three months after inoculation, dogs were allocated based on *T. vulpis* fecal egg counts into a control group ($n = 8$) or one of three treatment groups ($n = 4$ each), all comprised of an equal number of males and females. All dogs were verified to be shedding *T. canis* eggs.

Over a period of 15 days, control dogs were examined by CE (Endo Capsule System, Olympus America Inc., Center Valley, PA) on three separate occasions and treatment dogs were examined once. The CE protocol has been described elsewhere (Lee et al., 2011). Briefly, dogs were fasted the day before CE, instrumented with the external antennae and recorder, and given the capsule by mouth. Instruments were removed once the real-time monitoring device showed the capsule was in the colon or when the capsule battery was exhausted. Recorded images were downloaded and analyzed. Capsules were expelled in fecal matter within 1–2 days and were discarded. Postmortem examinations were carried out 5–7 days after treatment groups were administered research compound A, B, or C. Dogs were sedated with an intramuscular injection of medetomidine, butorphanol, and ketamine, followed by intravenous delivery of 400 mg/kg pentobarbital. The intestinal contents and mucosa were rinsed through a 50 μm steel mesh sieve. *T. canis* worms recovered were counted. There was a 5-day interval between the last CE performed on each control dog and the day on which it was euthanized. For treatment dogs, this period was 1–3 days. All CE images were reviewed by a single endoscopist blinded to the necropsy results.

Anthelmintic efficacy for the three compounds was calculated as follows: 100% × (geometric mean of control–geometric mean of treated)/geometric mean of control. Pearson correlation coefficient was determined using GraphPad Prism v.6 (GraphPad Software, La Jolla, CA) with significance set at $p < 0.05$. Incomplete CE examinations (i.e., lack of SI images as a consequence of protracted gastric transit of the capsule) were excluded from analysis. For evaluation of single CE counts in control dogs, incomplete examinations were replaced with data from the next available complete CE for that dog. No substitutions were made when taking the average across three capsule passages.

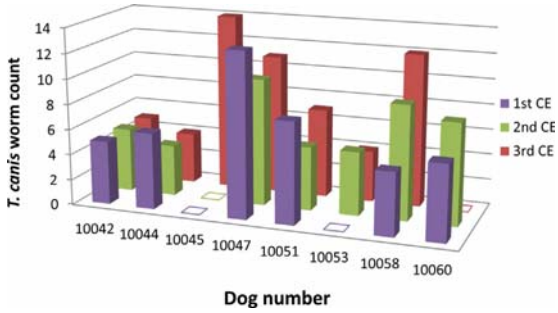

Fig. 2. Intra-dog variability in capsule endoscopy counts for *T. canis*. The number of worms observed during each capsule passage in the 8 control dogs is presented. Open squares correspond to incomplete examinations wherein the capsule remained in the stomach until the battery was depleted and so no images of the small intestine were available for review. Within-dog counts tended to be similar, though a marked difference was observed between the first and third CE for dog no. 10058.

3. Results

Due to a scheduling error relative to the timing of CE, two dogs each from treatment groups A and C were excluded from analysis. Fifteen dogs in total—8 control and 7 treated—had at least one complete CE. A combined 66 worms were observed in the last complete CE performed prior to necropsy, i.e., the third CE for most control dogs. Of these, 62 (93.9%) were located in the first half of the SI images. *T. canis* adults were easily detected by CE due to their large size. Additionally, various morphological features such as the digestive and reproductive tracts could be identified (Fig. 1, Supplementary Video 1 online). In the control group, repeated CEs revealed only slight variability in worm numbers measured within the same animal aside from dog no. 10058, which showed a distinct increase in the count between the first and third capsule passage (Fig. 2). The median and range for the first, second, and third CE counts were 6 (5–13), 5 (4–10), and 7 (4–14), respectively; for necropsy it was 8.5 (4–18). In the treated groups, one dog had a single worm that was detected by both CE and necropsy (Fig. 3). The median and range were thus the same for the two methods: 0.0 (0–1).

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2015.08.013.

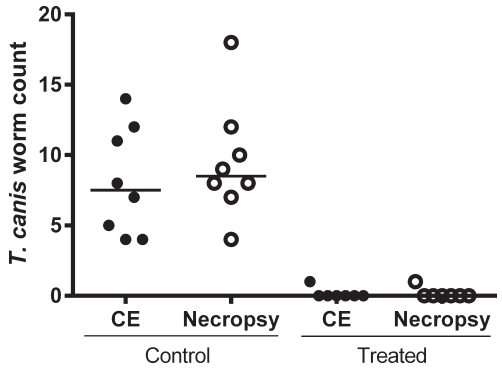

Fig. 3. *T. canis* worm counts for all dogs plotted by treatment and quantification method. Horizontal bar indicates the median. For control dogs, CE counts represent the last complete examination that took place prior to necropsy (i.e., the third CE in most cases). Among the treated dogs, CE and postmortem values were completely in accord, with a single worm found in the same dog.

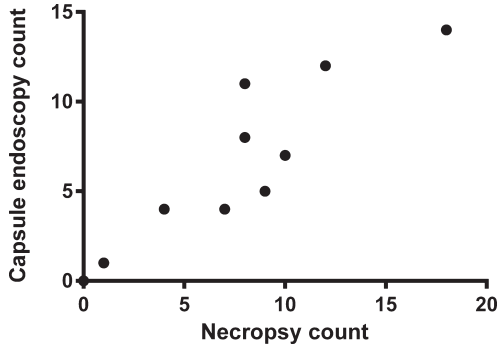

Fig. 4. *T. canis* enumeration by capsule endoscopy versus necropsy. CE values for the control dogs represent the last complete examination that took place prior to necropsy (i.e., the third CE in most cases). A strong correlation ($r = 0.94$, $p < 0.001$) was evident between the two quantification methods.

Table 1
Anthelmintic efficacy of the three tested compounds as determined by capsule endoscopy versus necropsy.

Research compound	Percent efficacy (%)	
	Capsule endoscopy	Necropsy
A	100%	100%
B	100%	100%
C	94%	95%

Pairwise comparison of the first complete CE examination for each dog with the corresponding postmortem result revealed a strong correlation ($r = 0.87$, $p < 0.001$). The relationship was even more robust using the last complete CE ($r = 0.94$, $p < 0.001$) (Fig. 4) or the average of all three CEs ($r = 0.94$, $p < 0.001$). More importantly, the calculated anthelmintic efficacy for the three research compounds was nearly identical for the two enumeration methods (Table 1). The results were the same whether a single or an average count was applied for the control group.

Remarkably, over the course of three capsule passages in one of the control dogs (no. 10051), the gradual maturation of a young adult *T. canis* was observed. During the initial CE, the worm was unquestionably immature based on its small size and lack of perceptible reproductive organs (Fig. 5a). The second CE, performed 5 days after the first, showed obvious growth as well as uterine branch development, marking this worm as a female (Fig. 5b). The final CE occurred two days afterward and revealed further worm maturation; however, this young female was still dwarfed by an adjacent full-grown female (Fig. 5c).

4. Discussion

The authors' prior experience examining canine hookworms by CE showed that visibility within the SI was reduced in the presence of foam, bile, and debris, and also when the capsule's camera was oriented toward the intestinal wall instead of the lumen (Lee et al., 2011). The same was true in this study, where foam and luminal debris in particular tended to obscure the *T. canis*. This was considered the probable cause for the low initial CE count for dog 10058, which had a much higher count during subsequent CEs as well as during postmortem examination. Likewise, other instances of CE values falling below those of necropsy may have resulted from poor visibility.

An added challenge during image review was the relative immensity of *T. canis* compared to the capsule. It was not possible to capture an entire worm in a single frame, so when several parasites were intertwined it became more difficult to distinguish individuals. This might have predisposed to overcounting in these situations. Spontaneous fecal elimination of adult ascarid worms is also known to occur (Stephenson et al., 1977; Lloyd, 1987), and this could be another reason for the occasional observation of more worms in CE than at necropsy. For natural *T. canis* infections, this is reported to occur most commonly in 4–6 week old puppies with heavy burdens acquired prenatally (Lloyd, 1987); a second expulsion period typically follows at 2–6 months of age (Fernando, 1968; Lloyd, 1987). Elimination rates range from 21% to over 50% of the total worm population (Burke and Roberson, 1978; Roberson and Burke, 1982; Lloyd, 1987). For induced *Ascaris suum* infection of pigs, expulsion occurred both in pigs with light burdens of 9–17 worms and in those with heavier burdens (Stephenson et al., 1980). Worms were also expelled at a greater frequency after a period of diet restriction (Stephenson et al., 1980). In the present study, CE procedures had to be spread out over time due to limited availability of recording devices, so there was an unavoidable lag period preceding necropsy when natural worm attrition may have occurred. Moreover, the enforced fasting period prior to CE may have acted as a trigger for worm elimination. It is therefore possible that a small number of worms could have been lost in this manner.

CE counts from control dogs were evaluated against necropsy either as a set of single capsule passages or as an average of all three. Unfortunately, in each set of CEs there was at least one incomplete SI examination due to prolonged gastric transit of the capsule. During an actual anthelmintic trial, when it is necessary to acquire data from each dog, CE would naturally be repeated as needed until the data had been collected. Hence for comparing single passages to necropsy, missing values associated with incomplete examinations were substituted with counts from the next closest complete CE in the same dog. Use of the average rather than the first complete CE did improve concordance between the two counting methods, but it was not superior to using the last complete CE. Logically, it makes sense that the last complete CE would most closely match postmortem findings because it was temporally nearest to necropsy. Any variability resulting from potential worm loss over time would have been minimized.

CE correctly determined the infection status of all 15 dogs in this study; it did not produce any false positive or false negative results. It was also able to detect the lone *T. canis* worm that remained in one of the treated dogs. Given this success, CE could be effectively applied to anthelmintic evaluation in many ways.

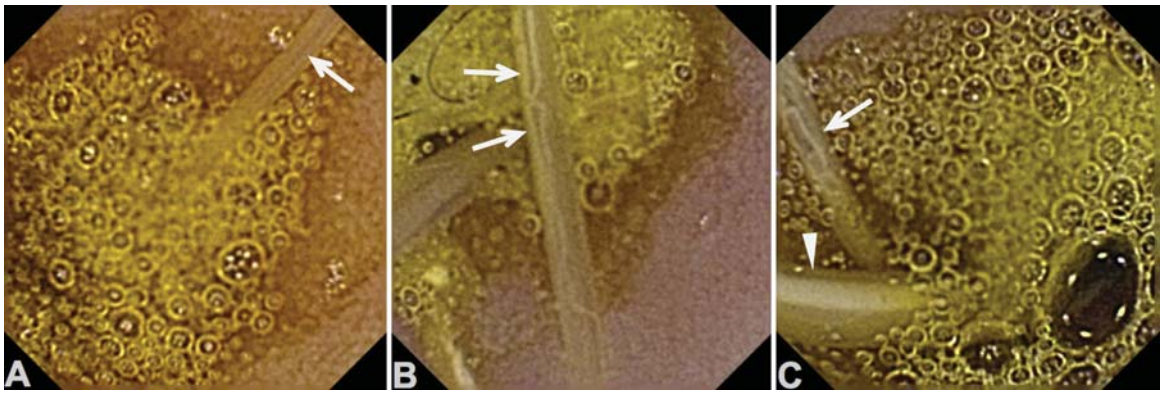

Fig. 5. Evidence of worm maturation over time in control dog 10051. (A) Immature adult *T. canis* (arrow) exhibiting little to no internal structures during first capsule passage. (B) Five days later, the worm is identifiable as a female due to the emergence of uterine branches (arrows). (C) After another two days, the female worm (arrow) has developed further, though it is still noticeably smaller in diameter than a fully mature female (arrowhead).

For example, dogs could be allocated to treatment groups based on direct worm quantification rather than relying on fecal egg counts. Alternatively, by eliminating separate control groups altogether, animal use in these trials could be greatly reduced. VICH guidelines describe critical tests wherein animals act as their own control, with the number of helminths expelled post-treatment being added to the number found at necropsy to establish the pre-treatment worm count (VICH, 2001). CE could easily be employed in this scenario to obtain counts before and after treatment instead of having to sieve feces for worms and euthanize animals. CE would also allow for use of the critical test on tapeworms, something that cannot be done currently because dead tapeworms are digested and thus not expelled intact (Burge and Burge, 1915). Another advantage of in vivo imaging is that worm counts can be acquired in field studies with client-owned animals. Availability of such data could lower the need to carry out multiple efficacy trials with research animals. Moreover, natural infection studies better represent the genetic diversity of parasites found in the real world and would more accurately predict how the anthelmintic drug would perform in practice. Additional roles for CE in parasiticide trials may very well be discovered with further exploration of this imaging modality.

A limitation of this study was the lack of a defined inoculation dose of *T. canis*, leading to a relatively low worm burden in control dogs. As noted earlier in the discussion, congregation of worms in large masses makes enumeration more difficult, and the probability of such masses forming would increase with higher worm numbers. Attempting to quantify worms in heavily infected dogs would presumably present a greater challenge. Despite the overall low worm burden, 7 of 8 control dogs did meet the threshold of adequate infection (i.e., a minimum of 5 worms) defined by industry guidelines (VICH, 2001), thus this limitation does not represent a major impediment to the application of CE in anthelmintic testing.

One of the goals of this study was to assess whether obtaining an average worm number by sequential CEs would better establish worm burden relative to gold-standard necropsy. There was no improvement in correlation when using the average count as opposed to the last complete CE. From a practical standpoint, therefore, performing a single CE at the time when necropsy normally takes place is the most cost-effective option. A second objective was to compare the CE- and postmortem-derived efficacies of three research compounds against *T. canis*. Final interpretation of anthelmintic activity was the same regardless of the manner of data collection. The two helminth enumeration methods were thus considered equivalent.

5. Conclusion

A single CE count at the end of the anthelmintic trial was sufficient to accurately determine drug efficacy against *T. canis* in the present study. CE produced virtually the same percent efficacy as necropsy for the three research compounds tested. It also allowed for observation of worm development over time within the same dog, which is not possible with necropsy. In most cases, the gender of each worm could be identified from the capsule images, though the true proportion of male and female worms was not confirmed postmortem. Future studies should aim to address this gap. If CE is capable of differentiating *T. canis* worm gender in addition to providing accurate anthelmintic efficacy values, then it may indeed prove to be a viable alternative to necropsy for parasiticide trials for this worm species.

Conflict of interest

The authors have no conflicts of interest to declare.

Role of the funding source

The sponsor was involved in designing the study and collecting data.

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