IN SEARCH OF TROJAN HORSES: CHARACTERIZING EARLY CELL TARGETS OF *TOXOPLASMA GONDII*

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Toxoplasma gondii is a master manipulator of the immune response, modulating proinflammatory cytokine production from host cells, blocking apoptotic pathways, and altering motility of infected cells. In these studies, I identify a Gr-1-expressing subset of dendritic cells hijacked by the parasite as a vehicle for dissemination from the inoculation site after intraperitoneal infection. These DC are preferentially infected compared to other cell types in the peritoneal cavity, and they migrate to the spleen in partial dependence on CCR2 signaling. *Toxoplasma* also suppresses TLR ligand-stimulated IL-12 production from infected cells. Subsequently, I characterize the differences in recruitment and parasitization of cell types by different strains of *Toxoplasma*, demonstrating that the avirulent Types II and III strains lead to higher levels of CD11c-expressing DC at the infection site. Finally, I elucidate the cell populations targeted in the intestine and the mesenteric lymph nodes after oral inoculation. I find that in the mesenteric lymph nodes and Peyer's patches, the cell types most strongly represented among infected cells are $CD3^+$ or $B220^+$ lymphocytes. However, the infection rate in these regions is highest in the cells expressing innate immune cell markers, such as Gr-1, CD11b, CD11c and 1A8/Ly6-G. Interestingly, infection in the lamina propria appears to be preferential for neutrophils, possibly indicating a role for neutrophils in dissemination of parasite during early oral infection.

BIOGRAPHICAL SKETCH

Allison Lee Bierly grew up in the small town of Mount Airy, Maryland. She discovered her love for biology at a young age, living on ten acres of largely-undeveloped land with plentiful flora and fauna. She received her early education at Mt. Airy Elementary School, Mt. Airy Middle School, and South Carroll High School, where she developed a dual interest in biology and language. She graduated as a valedictorian and National Merit Scholar, and was also honored with the English and Spanish awards for her senior class. She attended Ursinus College with a double major in Biology and English, learning the basics of scientific research as an undergraduate research student in the laboratory of Dr. Ellen Dawley. Her interest in biological research led her to Cornell University, where she has spent six happy years in the laboratory of Dr. Eric Denkers, furthering her training as a scientist.

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CHAPTER 1

INTRODUCTION

Toxoplasma Biology

Toxoplasma gondii is a member of the protozoan phylum Apicomplexa, so named for their unique apical complex which executes a variety of functions for cellular invasion and intracellular survival. The phylum includes many obligate intracellular pathogens, the most clinically devastating of which is the genus *Plasmodium*, which causes malaria, as well as other coccidian pathogens such as *Cryptosporidium*. *T. gondii* is the only species in its genus, and is an important organism in both human and veterinary medicine as the causative agent of disease in infants, immunocompromised populations and livestock.

T. gondii was discovered in Tunisia in 1908 by Nicolle and Manceaux, isolated from *Ctenodactylus gundi* (1), and also in Brazil by Splendore (2). It has since been found in a wide range of hosts (3-5), leading to the conclusion that the parasite can infect most birds as well as terrestrial and marine mammals. Recently, it has also been shown that oocysts taken up by Eastern oysters retain their infectivity for at least 85 days, indicating that it may also persist in invertebrates (6). The parasite is capable of infecting virtually all nucleated cell types, though sexual reproduction of the parasite only occurs within enterocytes of felids (7).

The life cycle of *Toxoplasma* begins when a member of order Felidae ingests tissue cysts from the skeletal muscle of its prey, or oocysts from the environment (8). The cyst wall is readily digested in acid and proteolytic enzymes of the stomach, and the enzyme-resistant organisms, called bradyzoites in the case of tissue cysts (or sporozoites in the case of oocysts), are released (9). Within the cat intestine, asexual

stages of development occur, called types A-E, and gametogony proceeds afterward, leading to the production of oocysts containing sporozoites, which are shed in the feces (10). The prepatent period for a cat infected with bradyzoites is 7-21 days (8), and they shed millions of oocysts during this time period. Once shed in the feces, oocysts become infective after 1-5 days (11) and can remain infectious for several months once sporulation occurs (12). Oocysts are hardy, resistant to harsh environmental conditions including cold (13) and saltwater (14). During infection of intermediate hosts, bradyzoites do not begin sexual reproduction in the epithelial cells, and instead multiply in the lamina propria as tachyzoites. Tachyzoites disseminate out of the intestine and spread throughout the body, invading and replicating inside nucleated host cells (15-17).

Toxoplasma is an obligate intracellular parasite, taking up residence in host cells for nutrient provision as well as protection from certain elements of the immune response. Entry into host cells is accomplished by a mechanism dependent on both parasite and host actin (18-19) in which specialized organelles specific to *T. gondii*'s Apicomplexan classification play distinct, sequential roles. The micronemes release proteins to adhere to the host surface, and the rhoptries release proteins from the parasitophorous vacuole (PV) (20). A ring-shaped point of contact between the parasite and host cell, called the moving junction, moves from the anterior to the posterior of the parasite, pulling the host cell plasma membrane over the organism and excluding host transmembrane proteins to form the PV (16-17). Host membrane proteins are excluded and proteins from the rhoptries and dense granules insert into the PV membrane, rendering the vacuole nonfusigenic. Therefore, the parasite is protected

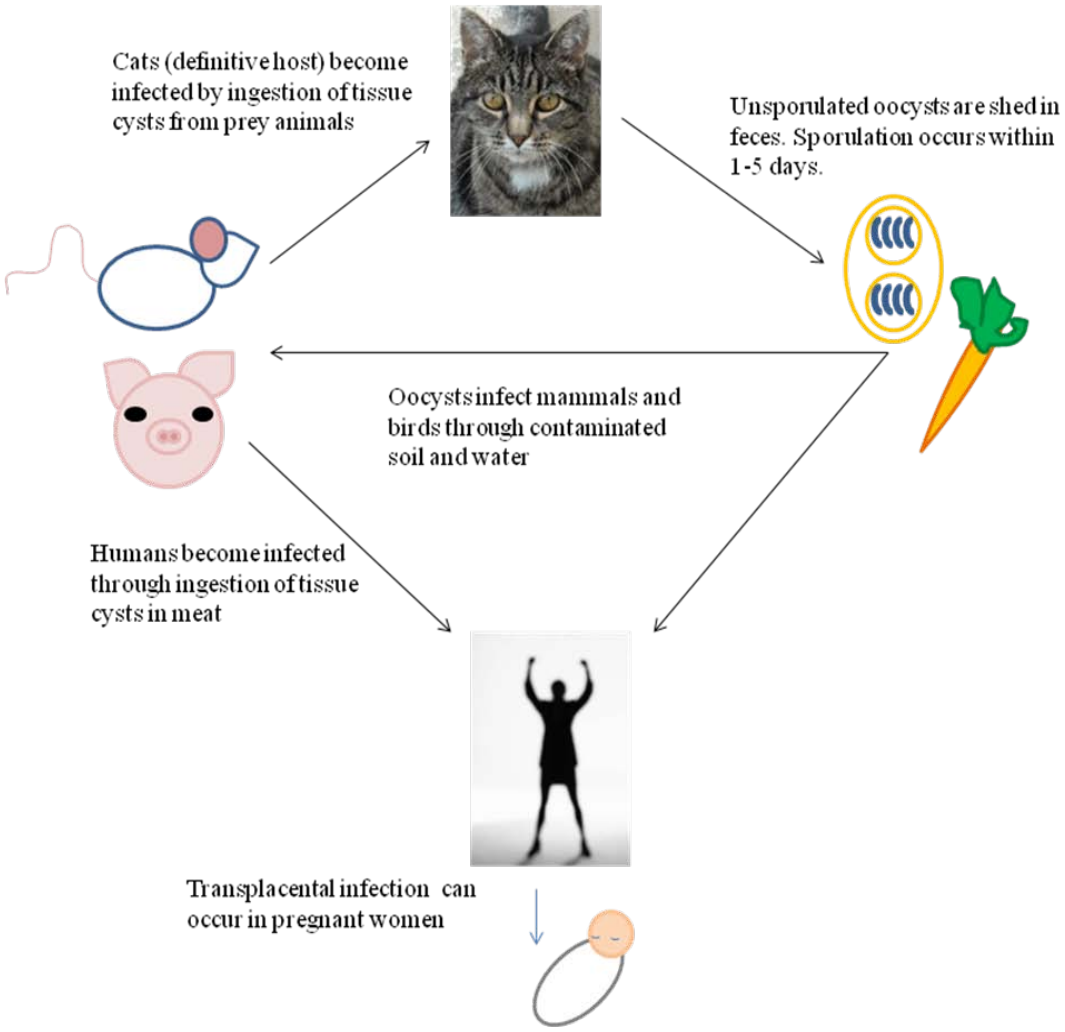

Figure 1.1. Life cycle of *Toxoplasma gondii*. A member of family Felidae ingests tissue cysts in the skeletal muscle or brain of a prey animal. Bradyzoites are released after digestion of the cyst wall, and gametogenesis takes place in the intestine. Unsporulated oocysts are shed in the feces and sporulate in the environment, where they remain infective for months. Humans and other intermediate hosts become infected by ingestion of oocyst-contaminated food such as unwashed vegetables, tissue cysts in undercooked meat, or by transplacental transmission.

from lysosomal fusion and acidification (17, 21). Studies to clarify how the parasite acquires nutrients from the host cell, however, have revealed that the parasite is not entirely sequestered from endo-lysosomal components. Within the PV, dense granule proteins are involved in the formation of a tubulovesicular network (TVN) (22). Endocytic vesicles from the host travel along microtubules to the PV, where the microtubules create invaginations in the PVM that are likely elongated by TVN lipids. Vesicles enter the vacuolar space via these invaginations, and are pinched off and sequestered within this location. In this way, the parasite can acquire many nutrients of low molecular weight (23). *Toxoplasma* also recruits host endoplasmic reticulum and mitochondria, possibly to obtain phospholipids and lipoic acid (24-25), and pores in the membrane allow passive diffusion of small molecules (26).

After invasion, tachyzoites replicate within the parasitophorous vacuole by endodyogeny, with a doubling time of approximately 7 hours (27), and eventually lyse the host cell and enter neighboring cells. Dendritic cells and monocytes have been implicated as vehicles for parasite dissemination to various organs and eventually to the central nervous system (27-28). It is hypothesized by some that upon sufficient pressure by IFN-γ-mediated immunity, tachyzoites begin to express bradyzoite antigens, undergoing stage conversion to quiescent tissue cysts which may persist for the lifetime of the host.

Three clonal lineages encompass the majority of *T.gondii* strains in Europe and North America, and they differ genetically by less than one percent. Type I is identified most commonly in immunocompetent patients who exhibit ocular disease, even though

Type II strains make up most cases of infection in humans. (29-30) Several characteristics of Type I strains may explain their enhanced virulence, including a greater ability to migrate over long distances and penetrate lamina propria and submucosa, tissues that they would encounter early in the host (31). Additionally, Type I parasites exhibit enhanced growth capability compared with Types II and III, leading to much higher parasite burden, which triggers high levels of Th1 cytokines and immunopathology (32, 33). Two virulence factors responsible for these differences have recently been identified as the kinases ROP16 and ROP18, rhoptry proteins with allelic variation between the strain types (34-35). Distinctions in parasite manipulation of host cytokine production have also been observed, including elicitation of higher levels of IL-12 from macrophages by type II parasites compared to types I and III (36-37). Type I induction of IL-12 is MyD88-independent, whereas Type II uses both MyD88-dependent and independent pathways (37). Type I parasites also elicit more neutrophils to the infection site after intraperitoneal inoculation than do Type II, which primarily induce Gr-1^{+} monocyte recruitment, and are more frequently found within neutrophils as well (38). Further characterization of the differences between infection with these strain types remains a topic of investigation.

Clinical Relevance

T. gondii infects about 9% of the United States population, but seroprevalence in other nations can be much higher (39-40). Worldwide prevalence of *Toxoplasma* infection is variable, and in some countries, such as Panama, seroprevalence by the age of 60 is as high as 90% (41). Risk factors for acquiring *T. gondii* include eating undercooked meat and unwashed vegetables, and cohabitation with felines. *T. gondii* is important in

both human and veterinary medicine, as a foodborne illness affecting humans with weakened immune systems, and a leading cause of abortion in livestock (42-43).

Transmission of *T. gondii* to intermediate hosts, including humans, can occur via three routes: fecal-oral, congenital, and carnivorism. Disease manifestation in humans can take a number of forms, depending upon the parasite strain, immune status of the patient, and route of acquisition. The most common symptom present in immunocompetent patients is lymphadenopathy (44), and infection is often subclinical. Ocular toxoplasmosis, present in approximately 2% of infected individuals in the United States (45), is characterized by recurring retinochoroiditis. The risk of developing ocular disease from *T. gondii* infection is higher in those infected congenitally, though postnatally acquired infection occasionally leads to this outcome as well (45). While most human infections are comprised of Type II strains, it has been suggested that Type I *T.gondii* is more likely to cause ocular toxoplasmosis in immunocompetent patients (29). In addition to ocular symptoms, the other three classic symptoms of congenital transmission include hydrocephalus, convulsions, and intracerebral calcifications (46). Consequences of postnatal infection are usually mild to subclinical, but immunocompromised individuals who are chronically infected can suffer reactivation of cysts in the brain, leading to inflammation and the cerebral lesions that characterize toxoplasmic encephalitis (TE) (42). This is a common opportunistic disease for AIDS patients, as one study showed the incidence of TE in this population at 15.9% over a one-year period (47). Patients with TE may experience neurological symptoms such as disorientation and convulsions, eventually leading to death (42).

Diagnosis of *T.gondii* can be achieved by several methods, including serology, PCR and histology. The original diagnostic tool for serology was the Sabin-Feldman methylene blue dye test (MBDT), which tests for the presence of antibodies in serum that can permit complement-mediated killing of tachyzoites. Because of the requirement for live parasites, however, this method is comparatively uncommon for routine testing. (48). Blood tests are available for IgM, IgA and IgG antibodies, such as enzyme-linked immunosorbent assay (ELISA), HS/AC differential agglutination test and IgM-immunosorbent agglutination assay, and indirect fluorescent antibody test (IFAT) (49-53). The IgG avidity test, an ELISA technique that utilizes urea to break the antibody-antigen bond, allows determination of whether an infection is primary or a result of reactivation (54-55), and PCR for the B1 gene of *T.gondii* is useful for detecting the parasite directly in cases where immune-based tests are unreliable, such as during advanced HIV infection (56). Serological detection of congenital toxoplasmosis is currently imprecise, as a recent study indicated that absence of IgM and IgA antibodies by enzyme-linked fluorescence assay, microparticle enzyme immunoassay and immune-fluorescent antibody technique was less than 50% accurate for predicting whether an infant was infected (57). PCR analysis of amniotic fluid, however, gives over 90% accuracy, and is currently the best method to diagnose congenital infection (58).

The most common treatment for toxoplasmosis uses pyrimethimine in combination with sulfadiazine and folinic acid, with clindamycin occasionally used as a substitute for patients who cannot tolerate the sulfonamide family of drugs. Treatment lasts from four to six weeks, or until symptoms are no longer detectable (59). However, hypersensitivity to the drugs remains a problem, and safer methods of treatment

remain a topic of study. Treatment to prevent vertical transmission and to treat ocular symptoms postnatally are less well-characterized, and further study will be needed to elucidate effective approaches for reducing both transmission and disease.

Vaccination against the parasite is currently under development, and several approaches have been tested for felines, livestock and humans. However, expense can be a limiting factor, such as with a successful vaccine for cats, T-263. A live bradyzoite mutant, T-263, prevented kittens from shedding oocysts when delivered orally, but was expensive and difficult to transport and maintain (60). A vaccine consisting of crude rhoptry proteins and the adjuvant Quil-A did diminish oocyst shedding in cats (61). Successful vaccines in livestock such as ewes, goats and pigs have been elusive, as even the licensed Toxovax vaccine, a live vaccine using strain S48 for sheep, carries the risk of reverting to a virulent strain (62). A DNA vaccine in pigs, though it did not confer strong protection, induced Th1 and humoral immunity to *T.gondii*, indicating that DNA vaccines will be an avenue worth pursuing (63). As for human vaccines, promising candidates have come from studies of rats and mice, utilizing SAG-1 as well as various microneme, rhoptry and dense granule antigens via crude protein or DNA vaccination, leading to partial protection (64-66). Recently, it has been shown that exosomes elicited from *T.gondii*-antigen-pulsed dendritic cells are capable of conferring protection against both primary (67-68) and congenital infection (69) in mice.

Host Response

The immune response to *T.gondii* is cell-mediated, depending on the critical proinflammatory cytokines IL-12, TNF-α and IFN-γ. The response can be divided into two stages corresponding with the two stages of infection in the intermediate host. The first wave of immunity aims to control replication and drive the parasite into latency, while the second phase keeps reactivation of encysted *T.gondii* in check. Anti-inflammatory responses are also produced to control immunopathology, thereby maintaining a balance between successful parasite migration to the sites of latency and survival of the host.

Immunity in the acute phase is initiated through recognition of tachyzoites by innate immune cells such as dendritic cells, macrophages and neutrophils, which are capable of sensing parasite via a number of surface receptors. TLRs 2 and 11 have been shown to recognize parasite products, leading to distinct responses. Recognition of glycophosphatidylinositols by TLRs 2 and 4 leads to NF-κB activation and TNF-α production by macrophages (70), and the TNF response from macrophages is completely abolished only when both of these receptors are absent (71). *Toxoplasma* stimulation of TLR2 is also necessary for induction of nitric oxide by macrophages (72). TLR11 recognizes the profilin-like molecule TgPRF on tachyzoites (73), eliciting IL-12 from dendritic cells *in vitro* and from splenocytes *in vivo*. G-protein coupled receptors are also capable of recognizing parasite molecules in order to mount proinflammatory responses, such as in the case of the splenic $CD8\alpha^+$ subset of DCs, which produce IL-12 in response to CCR5 ligation by the *Toxoplasma*-secreted molecule cyclophilin C-18 (74). Additionally, pertussis toxin treatment abolishes

residual STAg-induced IL-12 in $CCR5^{-/-}$ splenocytes, possibly implicating other GPCR pathways in recognition of the parasite (75).

IL-12 produced by innate immune cells activates natural killer cells to produce IFN-γ (76), which signals through STAT1 to activate microbicidal mechanisms in macrophages (77). Indeed, mice lacking STAT1 have increased susceptibility to infection, indicating a role for these mechanisms in host defense (78-79). The most important microbicidal effect during acute infection appears to be activation of p47 GTPases, which have been shown to control parasite growth *in vitro*. Recently, the mechanism of this control was elucidated, in that these molecules disrupt the membrane of the parasitophorous vacuole by vesiculation, ultimately leading to its destruction and degradation of the parasite (80-81).

While neutrophils are well-known killers of microbes via reactive oxygen intermediates, there is little evidence to show that they play this role during *T.gondii* infection. In vitro studies have shown that macrophages use nitric oxide to kill tachyzoites after activation by IFN-γ (82). However, studies using gene knockout mice have shown that iNOS is not necessary for host survival during the acute phase in mice. This leads to the conclusion that in mice, nitric oxide is likely not a critical mechanism by which parasite replication is controlled (83).

IFN-γ also acts on innate immune cells to stimulate greater production of IL-12 (84), which along with antigen presentation from dendritic cells, activates $CD4^+$ helper T cells (85). $CD4^+$ T cells differentiate into Th1 cells to produce IFN-γ and IL-2, and help activate $CD8^+$ T cells. $CD4^+$ and $CD8^+$ T cells both contribute to the IFN-γ levels

at sites of infection (86). Destruction of cells by perforin has been shown to be dispensable in controlling parasite growth (87), indicating that IFN-γ production is likely the critical component of T cell involvement during the acute phase of infection.

Chronic infection is characterized by quiescent tissue cysts in the skeletal muscle and central nervous system of the host. Depletion of $CD4^+$ and $CD8^+$ cells in mice leads to reactivation of the parasite and toxoplasmic encephalitis (86, 88), which reflects observations in AIDS patients and indicates an important role for these T cells in maintaining quiescence. This requirement for T cells is likely due in part to cytolytic activity by parasite-specific $CD8^+$ T cells, which have been demonstrated to contribute to survival during the chronic phase of Type II infection as well as reduced cyst counts in the brain (87). IFN-γ-triggered mechanisms of parasite control have also shown to be important at this stage, given that deficiency in iNOS, which is upregulated by IFN-γ signaling through STAT1, is lethal during chronic infection (83), and murine astrocytes can kill intracellular parasites *in vitro* dependent on IGTP, a p47 GTPase (89). Therefore, immunity during the chronic phase of infection combines both cell-mediated and soluble factors for parasite killing and prevention of recrudescence.

In addition to stimulating a Th1-biased host response, *T.gondii* also triggers anti-inflammatory cytokines to control immune pathology. Knockout studies with the anti-inflammatory cytokine IL-10 showed that mice die of massive inflammation during infection, mediated by $CD4^+$ T cells (90). Interestingly, effector $CD4^+$ T cells have also been found to be a major source of IL-10 (91), although whether they are the critical source remains to be seen, considering that other cell types also produce this

cytokine during infection (92). During chronic infection, parasite triggering of lipoxin A_4 production has been shown to dampen the immune response due to downregulation of CCR5 on dendritic cells and therefore diminished stimulation of proinflammatory cytokine production (93-94). Finally, in the absence of IL-27R, Th17 cells emerge and cause pathology during chronic infection (95), indicating that IL-27 signaling is also an important mechanism for suppressing immune pathology.

Toxoplasma Evasion of Host Response

Toxoplasma has developed a number of methods to escape host immunity, largely involving alteration of intracellular signaling pathways within the cells it has invaded in order to suppress inflammation and parasite recognition. A major consequence of this intracellular signaling manipulation is the suppression of TLR-triggered cytokine production. *In vitro* studies in macrophages have demonstrated the parasite's ability to block translocation of NF-κB to the nucleus, interfering with cellular production of IL-12 and TNF-α (96). While IL-12 production is restored after the block is lifted, TNF is still suppressed. Recent work has more fully elucidated a mechanism of TNF-α blockage by *T.gondii*, showing that the parasite blocks histone modification at the TNF promoter, preventing binding of transcription factors (97). Another mechanism of parasite-mediated cytokine suppression was found to be *Toxoplasma*'s ability to exploit the host's natural anti-inflammatory mechanisms, such as the IL-10/STAT3 signaling pathway. Upon infection of bone-marrow-derived macrophages, *T.gondii* rapidly induces phosphorylation of STAT3, typically associated with the IL-10 signaling cascade, independently of this cytokine. STAT3 phosphorylation suppresses the ability of the cells to produce IL-12 and TNF-α when stimulated with lipopolysaccharide (98). *Toxoplasma* is also capable of deactivating mitogen-activated

protein kinases (MAPK) and rendering macrophages resistant to subsequent activation by LPS, leading in turn to diminished production of IL-12 and TNF-α (99).

Toxoplasma not only dampens proinflammatory cytokine production, but also works to disable macrophages' microbidical capabilities by partially inhibiting production of nitric oxide via downregulation of iNOS (100-101). In addition to downregulating the expression of nitric oxide synthase, the parasite also diminishes antigen-presenting capability by reducing MHC Class II expression and preventing upregulation of Class I in macrophages (102-103), and prevents maturation of parasitized immature dendritic cells (104). In these ways, *Toxoplasma* helps maintain the balance between an immune response robust enough to keep the host alive, but limited enough that the parasite can survive to the chronic phase of infection.

In addition to modulation of innate immune cell signaling pathways to control inflammation, several studies have suggested that *Toxoplasma* uses various cell types to remain sequestered from host immunity and assist its dissemination throughout the host. *Toxoplasma* shuts down apoptotic pathways in parasitized cells by interfering with mitochondrial cytochrome c release, thereby diminishing the activation of the caspase cascade, as well as through activation of the G_i-dependent PI 3-kinase pathway and phosphorylation of protein kinase B (105-106). By inhibiting apoptosis, *T.gondii* may remain sequestered from extracellular immune mechanisms. Studies noted that natural killer cells are targeted for infection after killing infected dendritic cells, which become hypersensitive to NK-mediated lysis compared to uninfected DC. Newly-parasitized NK cells are resistant to killing by other NK cells and lack

intracellular microbicidal mechanisms, which may make them an ideal reservoir for parasites (107). Dendritic cells themselves have also been implicated as reservoirs and vehicles for dissemination. *In vitro* work with bone-marrow-derived DC showed that *Toxoplasma* invades immature dendritic cells more efficiently than mature DC, and blocks their ability to produce IL-12 and TNF-α in response to TLR ligands and CD40L, as well as diminishing their ability to activate naïve $CD4^+$ T cells (104). *In vivo* studies have also indicated a role for DC in dissemination of *Toxoplasma*. After intragastric inoculation with a low dose of cysts, parasitized $CD11c^+$ and $CD11b^+$ cells were able to cross the blood-brain barrier, indicating that they could deliver parasites to the brain (27), and tachyzoites induce hypermotility in dendritic cells *in vitro* both in culture and after transfer into mice (28). Recently, the ability of *T.gondii* to effectively utilize dendritic cells for transport has been suggested to be dependent on the parasite genotype (108). Combined with the ability of the parasites to downmodulate cytokine production and MHC Class II expression, the possibility exists that *T.gondii* targets dendritic cells for rapid, silent dissemination throughout the host.

Dendritic Cells

Dendritic cells are professional antigen-presenting cells that bridge the gap between innate and adaptive immunity by providing costimulation and antigenic peptide in the context of MHC Class I and II to T cells. First described in 1973 by Ralph Steinman and Zanvil Cohn, they were soon demonstrated to be the splenic accessory cells required for activation of adaptive immune responses, capable of stimulating a mixed leukocyte response up to 300 times stronger than total splenic cells (109). Several subtypes of dendritic cell are recognized, defined by location, lineage, cell surface

molecules, and function. Broadly, dendritic cells can be classified as cDCs (conventional DCs) or pDCs (plasmacytoid DCs). Immature DCs are present in the blood, peripheral tissues and lymphoid organs, and display low motility and high phagocytic capacity (110-111). Upon encounter with microbial or inflammatory stimuli such as Toll-like receptor ligands, dendritic cells become mature, upregulating costimulatory molecules such as CD80 and CD86, as well as Class II MHC. They acquire high motility and lose their phagocytic activity, migrating to secondary lymphoid organs to present antigenic peptides to T cells (111-117). They are also capable of producing cytokines important to immunity, such as IL-12, TNF-alpha, IL-10 and others (111, 118-119). Additionally, oxygen-dependent microbicidal activity has been observed in IFN-γ-activated dendritic cells during *T. gondii* infection (120).

In recent years, the role of Ly6C-expressing dendritic cells in infection has come to be appreciated through the recognition of plasmacytoid dendritic cells (pDC) and monocyte-derived DCs (mDC). Plasmacytoid dendritic cells are a unique type of DC that serve multiple functions for immunity depending on the maturation state. As interferon-producing cells (IPC) with characteristic plasma cell morphology, pDCs respond to microbial signaling through TLR7 and TLR9, rapidly producing large quantities of Type I interferon (121-123), which leads to maturation of monocytes and antigen presentation (124-125), as well as activation of natural killer cells (126). Maturation of monocytes induced by pDC-produced Type I IFNs leads to proinflammatory cytokine production and ability to cross-prime $CD8^+$ T cells (124, 127). Sequential production of interferon-α and IL-6 by pDCs also leads to plasma cell differentiation (128). Autocrine signaling by Type I interferons, as well as microbial stimuli, trigger interferon-producing pDCs to mature into an activated state

more closely resembling cDC (129-130). The mature phenotype includes expression of typical DC activation markers, such as high levels of MHC Class II, CD40, CD80 and CD86. Matured pDCs also exhibit functions in common with conventional DCs, including ability to respond to TLR4 stimulation, produce IL-12, and present antigen (129-131). In recent years, roles for pDCs in non-viral infections have been elucidated. Most notably, pDCs produce IFN-α and IL-12 after TLR11 stimulation with the profilin-like molecule of *T.gondii*, and are also capable of presenting *Toxoplasma* antigen in the context of MHC Class II (132).

Monocyte-derived DCs arise from $Ly6C^+$ inflammatory monocytes, which circulate in the blood with the phenotype $Gr\text{-}1^+CCR2^+CX_3CR1^{low}$ (133), are recruited to sites of infection by CCR2 (134-135), and differentiate into macrophages and dendritic cells (133, 136-137). Cells of this lineage play a key role in *Toxoplasma* infection, as inflammatory $Gr\text{-}1^+$ monocytes expressing the macrophage marker CD68 are important during the acute stage of infection for controlling parasite growth at the inoculation site, and also produce IL-12 and nitric oxide (38). Monocytes that have differentiated into dendritic cells have also been shown to play important roles in infection with other intracellular pathogens. During infection with *Listeria monocytogenes,* for example, monocyte-derived dendritic cells accumulate in the spleen, producing TNF-alpha and upregulating iNOS in order to clear bacteria. Due to their TNF and iNOS-dependent functions, they have been designated “TipDCs” (138). Recruitment of these cells to the spleen is dependent on CCR2 signaling. TipDCs are also critical for expansion of pathogen-specific $CD8^+$ T cells during influenza infection (139). Recruitment of inflammatory DCs to the lymph nodes has also been observed during virus infection in dependence on CCR2, producing high levels of IL-

12 and triggering strong activation of $CD4^+$ T cells, demonstrating a role for inflammatory DCs in T cell priming (140). Interestingly, it has been observed that under inflammatory conditions during infection with lymphocytic choriomeningitis virus, cells with a phenotype corresponding to plasmacytoid DCs undergo a shift in surface markers to downregulate B220, Ly6C and CD8, and upregulate CD11b and CD11c, thereby acquiring a phenotype similar to myeloid DCs. These cells shift functionally as well, becoming newly capable of responding to TLR4 stimulation by IL-12 production (141). This phenotypic and functional plasticity, in addition to evidence that pDCs and mDCs derive from a common precursor (142), suggests that the delineation between the pDC and mDC subsets is perhaps not as definitive as it once seemed.

Outline of Dissertation Research

Previous work in our laboratory on early immune responses to *Toxoplasma* led me to ask the question of which cells were parasitized during early acute infection. I utilized an intraperitoneal model of Type I infection so as to control the dose of tachyzoites more precisely, since infection with cysts can lead to irregularities based on cyst size. **Chapter 2** details the findings of these initial experiments, in which I discovered that while most cell types were parasitized by tachyzoites at the infection site, a certain population of $CD11c^+Gr\text{-}1^+$ cells were more susceptible to infection than the rest. I found that cells expressing this phenotype also comprised the majority of infected cells in the spleen at day 4 post-infection, and they expressed a number of markers related to antigen-presenting cells and plasmacytoid dendritic cells. I showed that the infected $CD11c^+Gr\text{-}1^+$ cells are capable of migrating from the peritoneal cavity to the spleen using an adoptive transfer model with congenic mice, and that this migration is

partially dependent on the presence of CCR2. Finally, I showed that cells isolated from infected mice and stimulated with TLR ligands are capable of producing IL-12, but are suppressed in this function when parasitized. I concluded that *T.gondii* targets $CD11c^{+}Gr\text{-}1^{+}$ cells at the infection site, suppresses their effector function, and uses them as Trojan horses for dissemination throughout the body.

Chapter 3 describes the continuation of this work using representative strains of the three major clonal lineages of *Toxoplasma*, Types I, II and III. Peritoneal exudate cells and splenocytes were isolated on days 3 and 5 post-infection, and cell surface phenotype and presence of parasites were examined by flow cytometry. I found that while $CD11c^{+}Gr\text{-}1^{+}$ cells were parasitized in the spleens of Type II and Type III-infected mice, they were not the majority of infected cells, indicating that the preference of the parasite for this cell type is less strong than with Type I. In the peritoneal exudates, the majority of infected cells were $Gr\text{-}1^{+}$ during Type I infection, and $CD11c^{+}$ during infection with Types II and III. Furthermore, the percentage of cells expressing $CD11c^{+}$ was significantly higher in the mice infected with less virulent strains, as was the percentage of $CD8^{+}$ cells and Siglec H^{+} cells. Given the relative speed of Type I replication and cell lysis compared to Types II and III, this variation in levels of DC and neutrophils may not indicate a difference in which cells are being targeted, but in how quickly the targeted cells are killed.

Chapter 4 examines the early targets of infection using oral inoculation with Type II cysts. While the intraperitoneal model allows us to easily recover cells from the site of inoculation and to control the parasite dose, the oral model gives a more accurate view

of how natural infection proceeds, as most infection is acquired through ingestion of tissue or oocysts. To address the question of which cells *T.gondii* targeted during oral infection, I harvested several tissues that would be encountered early on, such as the lamina propria, the Peyer's patches, and the mesenteric lymph nodes. My results showed that two populations comprised the majority of infected cells in the lamina propria: cells expressing B220, though not the B cell marker CD19, and cells expressing the neutrophil marker 1A8. In the Peyer's patches, most infected cells were $B220^+$, and in the mesenteric lymph nodes, $CD3^+$ and $B220^+$ cells were the dominant cell populations parasitized. These findings suggest that the phenomenon we observed during intraperitoneal infection, in the hypersusceptibility of $CD11c^+Gr\text{-}1^+$ cells to infection and the hijacking of these cells as Trojan horses, may not be a phenomenon that occurs during oral infection. Finally, **Chapter 5** summarizes these results and discusses conclusions and future directions.

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CHAPTER 2

DENDRITIC CELLS EXPRESSING PLASMACYTOID MARKER PDCA-1 ARE TROJAN HORSES DURING *TOXOPLASMA GONDII* INFECTION*

***This chapter reproduced, with permission, from the paper "Dendritic Cells Expressing Plasmacytoid Marker PDCA-1 are Trojan Horses During *Toxoplasma gondii* Infection", by AL Bierly, WJ Shufesky, W Sukhumavasi, AE Morelli and EY Denkers, published in 2008 in the Journal of Immunology, v181(12): 8485-91. Copyright 2008. The American Association of Immunologists, Inc.**

Abstract

Plasmacytoid dendritic cells (pDCs) play a key role in the innate immune response to viral infection, due largely to their ability to produce large quantities of type I IFNs. These cells are also notable for their ability to differentiate into conventional dendritic cells after appropriate stimulation. Here, we show that a splenic population of murine $CD11c^+$ cells expressing pDC markers Gr-1, B220, and PDCA-1 is preferentially parasitized after infection with the virulent RH strain of *Toxoplasma gondii*. Although these markers are closely associated with pDCs, the population we identified was unusual because the cells express CD11b and higher than expected levels of CD11c. By adoptive transfer of CD45.1-positive cells into CD45.2 congenic mice, we show that $CD11c^+Gr\text{-}1^+$ cells migrate from the peritoneal cavity to the spleen. During infection, these cells accumulate in the marginal zone region. Recruitment of infected $CD11c^+Gr\text{-}1^+$ cells to the spleen is partially dependent upon signaling through chemokine receptor CCR2. Intracellular cytokine staining demonstrates that infected, but not noninfected, splenic $CD11c^+Gr\text{-}1^+$ dendritic cells are suppressed in their ability to respond to ex vivo TLR stimulation. We hypothesize that *Toxoplasma* exploits pDCs as Trojan horses, targeting them for early infection, suppressing their cytokine effector function, and using them for dissemination within the host.

Introduction

Toxoplasma gondii is an intracellular protozoan known for its ability to induce strong Th1-type cytokine responses (1). IL-12 and IFN-γ are required to survive acute and chronic infection with this opportunistic pathogen, but overproduction of

these cytokines can precipitate proinflammatory pathology that results in host death (2, 3, 4, 5, 6, 7). Therefore, there is a need to tightly regulate Th1 cytokine production to allow parasite control without causing host death. Early events in initiation of immunity to *Toxoplasma* are likely to play a prominent role in determining the strength and pattern of cytokine production during infection.

Dendritic cells (DCs) are now recognized as central in immune response initiation, due to their ability to acquire Ag, migrate from peripheral tissues to lymphoid organs, activate naive T cells, and secrete immune-polarizing cytokines such as IL-12. DC biology is complex insofar as there are many distinct subpopulations with discrete phenotypic characteristics and functional activities (8, 9). Broadly, DCs can be subdivided into conventional DCs and type I IFN-producing plasmacytoid DCs (pDCs). Conventional DCs can be further subdivided based upon tissue localization and surface marker expression. Furthermore, other types of DCs arise in inflammatory or infection conditions, such as the TNF- and inducible NO synthase-expressing DCs that emerge during mouse infection with *Listeria monocytogenes* and monocyte-derived DCs that appear at the infection site during *Leishmania major* infection (10, 11, 12).

DCs are required to survive infection with *T. gondii* as shown in a recent study where diptheria toxin was used to deplete cells expressing the diptheria toxin receptor under the control of the CD11c promoter (13). Older studies showed that CD8α^+ DCs in the spleen are an important IL-12 source after i.v. injection of parasite lysate Ag (14). The identification of a *Toxoplasma* profilin molecule that induces high level IL-12

production from splenic DCs through activation of TLR11 provides a molecular explanation for this effect (15). Unexpectedly, it was recently reported that pDCs, which are usually associated with responses to viruses, acquire *T. gondii*-derived Ag and produce IL-12 during infection with this parasite (16). At the same time, other studies provide evidence that DC infection with *Toxoplasma* down-modulates their capacity to produce IL-12 (17). Discrepancies in how these cells respond to infection may result from using different DC subsets, or they may reflect differences in DC responses to extracellular parasite molecules vs their inherent ability to produce cytokines after parasite infection.

In addition to their role in initiation of immunity to *Toxoplasma*, there is evidence that DCs serve as vehicles for dissemination during infection. During early oral infection, infected $CD11c^+$ cells were identified in the lamina propria, Peyer's patches, and mesenteric lymph nodes, although $CD11c^-CD11b^+$ cells were also found to be a reservoir of infection at these locations (18). Interestingly, another study reported that infection of human monocyte-derived DCs as well as mouse bone marrow-derived DCs induce a state of hypermotility that, at least in mice, potentiates dissemination in the host (19). Therefore, it is seemingly paradoxical that *Toxoplasma* may target for infection cells that themselves play a predominant role in triggering anti-parasite immunity.

To gain insight into in vivo interactions between DCs and *Toxoplasma*, and to determine the functional consequences of intracellular infection in an in vivo situation, we examined recruitment andinvasion of DCs during the early immune response. We

chose to use an i.p. infection model so that we could precisely control delivery of parasites and examine early cell recruitment and invasion at the site of infection. Most of the infected cells in the peritoneal cavity expressed monocyte/macrophage and neutrophil markers. In striking contrast, in the spleen the major population of infected cells was largely restricted to a subset that expressed surface markers most closely associated with pDCs. These cells were present as a minor population in the peritoneal cavity, but they were markedly more susceptible to infection compared with other cell types. We examined cytokine production by these cells and found that infected DCs' ability to produce IL-12 was suppressed, whereas the corresponding noninfected population was capable of producing this cytokine. We hypothesize that *T. gondii* targets pDCs for early infection and dissemination, in the process inhibiting their ability to produce IL-12.

Materials and Methods

Mice

Female C57BL/6 mice 6–8 wk of age were purchased from Charles River Laboratories and Taconic Farms. *CCR2*$^{-/-}$, *CCR5*$^{-/-}$, and control B6.129 mice were purchased from The Jackson Laboratory. CD45.1 C57BL/6 background congenic mice (strain B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ) were maintained as a breeding colony at the Cornell College of Veterinary Medicine Animal Facility and were provided by Dr. J. Appleton (James A. Baker Institute for Animal Health, Ithaca, NY). The animals were housed in the Cornell University College of Veterinary Medicine transgenic mouse core facility, which is accredited by the American Association for Accreditation of Laboratory Animal Care.

Parasites and infections

Tachyzoites of the RH strain were originally purchased from the American Type Tissue Collection, and a transgenic RH strain expressing tandem copies of yellow fluorescence protein (RH-YFP) was a gift from D. Roos (University of Pennsylvania, Philadelphia, PA) and B. Striepen (University of Georgia, Athens, GA). Parasites were maintained by passage in human foreskin fibroblasts in DMEM (Mediatech) supplemented with 1% bovine growth serum (HyClone), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). Infections were accomplished by i.p. injection of 10^6 tachyzoites in 100 µl of PBS.

Flow cytometric analysis

Splenocytes were prepared by mechanical disruption of whole spleens, and red cells were lysed using red cell lysis buffer (8.3 g/L ammonium chloride in 0.01 M Tris-HCl buffer; Sigma-Aldrich). Splenocytes and peritoneal exudate cells were filtered through 70 µm filters, and incubated on ice for 15 min with 10% normal mouse serum in FACS buffer (1% BSA and 0.05% sodium azide in PBS). Surface staining was performed for 30 min on ice, and intracellular cytokine staining was performed using the BD Cytofix/Cytoperm fixation/permeabilization solution kit (BD Pharmingen). Cellswere stained with the following Ab obtained from BD Pharmingen: anti-CD11c allophycocyanin (clone HL3), anti-Gr-1/Ly6G PerCP (clone RB6-8C5), anti-CD4 PE (clone RM4-5), anti-B220 PE (clone RA3-6B2), and anti-IL-12p40 PE (C15.6). The following Abs were obtained from eBioscience: anti-CD40 PE (clone 1C10), anti-B7.1 PE (clone 16-10A1), anti-B7.2 PE (GL1), anti-CD3 PE (145-2C11), anti-CD11b PE (clone M1/70), anti-MHC class II PE (clone M5/114.15.2), anti-CD8 PE (clone 53-

6.7) and anti-CD45.1 PE (clone A20). Anti-mPDCA-1 PE (clone JF05-1C2.4.1) was purchased from Miltenyi Biotec, and anti-*T. gondii* p30 FITC (clone G-II9) from Argene. Cells were analyzed using CellQuest software and a FACSCalibur cytometer (BD Biosciences).

Adoptive transfer of peritoneal exudate cells

Peritoneal exudate cells were harvested from CD45.1 congenic mice on day 3 after i.p. inoculation with 10^6 RH-YFP tachyzoites, and 1.5×10^7 cells were injected i.p. into C57BL/6 mice that had been infected on the same day with the same dose of RH-YFP parasites as the donor mice. After 48 h, peritoneal cells and splenocytes were harvested, and presence and phenotype of CD45.1 cells in the spleen were analyzed by flow cytometry.

In vitro culture and analysis of splenic DCs

Splenocytes were harvested from mice 4 days after infection with RH-YFP tachyzoites. DCs were enriched using pan-DC microbeads according to the manufacturer's instructions (Miltenyi Biotec). The cells were subsequently incubated for 24 h with or without *Escherichia coli* LPS (Sigma-Aldrich) and CpG oligodinucleotide 1826 (Coley Pharmaceutical Group), including Golgi-Stop (BDPharmingen) for the last 4 h of incubation. The DC-enriched population was washed and stained for surface markers and intracellular IL-12p40, then analyzed by flow cytometry.

Immunofluorescence labeling

To visualize morphology of infected cells, splenocytes were harvested from mice infected i.p. 4 days earlier with RH-YFP tachyzoites and stained (as described above for FACS analysis) with anti-Gr-1 PE (BD Pharmingen). Cells were transferred to a glass slide via cytospin (Shandon), and nuclei were labeled with 4',6'-diamidino-2-phenylindole (DAPI) (Invitrogen, Molecular Probes). Staining of spleen sections from infected mice was performed as described previously (20). Briefly, fragments of spleen were embedded in Tissue-Tek OCT (Miles Laboratories), snap-frozen in pre-chilled methyl-butane, and stored at –80° C. Cryostat sections (8 µm) of spleen were fixed in 4% paraformaldehyde/PBS and blocked with 10% normal goat serum and the avidin-biotin blocking kit (Vector Laboratories). Tissue sections were incubated with the following reagents (Ab were obtained from BD Pharmingen, unless specified): 1) biotin-anti-MOMA-1 mAb (Bachem) followed by Cy3-streptavidin (Jackson ImmunoResearch Laboratories) or 2) anti-CD11c mAb plus Cy3-anti-hamster IgG in combination with either biotin-CD3 mAb or biotin-Gr-1 mAb followed by Cy5-streptavidin (Jackson ImmunoResearch Laboratories). Nuclei were stained with DAPI (Molecular Probes).

Statistical analyses

Statistical analysis was performed using Prism 4 software (GraphPad software) and significance was calculated using an unpaired, two-tailed Student's *t* test.

Results

A rare population of CD11c$^+$Gr-1$^+$ cells in the peritoneal cavity is preferentially targeted following i.p. infection with tachyzoites

To elucidate cell types parasitized by *Toxoplasma* during early infection, C57BL/6 mice were infected i.p. with RH-YFP, a genetically engineered parasite strain expressing tandem copies of the gene encoding yellow fluorescence protein, and the peritoneal exudate cells were analyzed by flow cytometry 3 days later. As expected for *Toxoplasma*, a parasite known for its ability to promiscuously infect a diverse range of cell types, most cells in the peritoneal cavity were parasitized at this time point, including macrophages (F4/80$^+$), neutrophils (Gr-1$^+$), DCs (CD11c$^+$), and B cells (B220$^+$) (Fig. 2.1*A*). Nevertheless, while ~50% of macrophages, neutrophils, and DCs harbored parasites, B lymphocytes and T lymphocytes were considerably more resistant to infection. In each case only a minority of cells were infected (20% and 5%

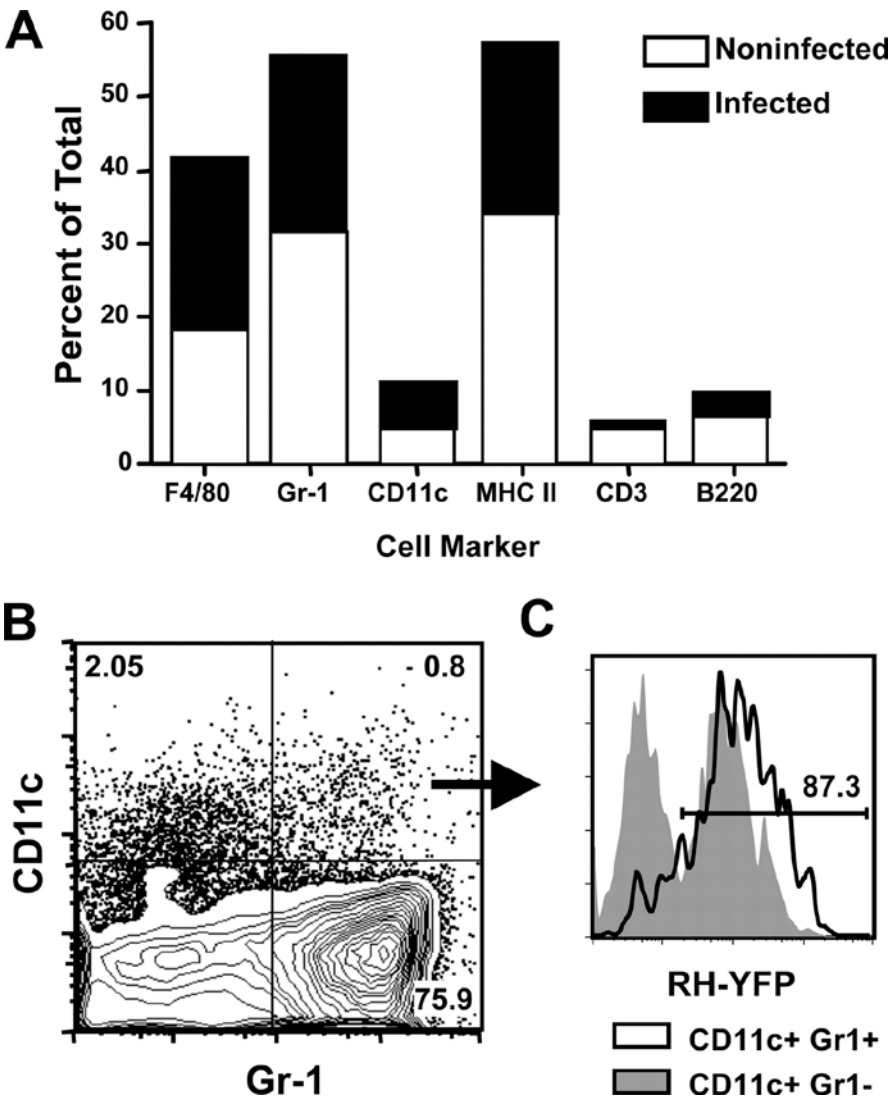

Figure 2.1. $CD11c^{+}Gr\text{-}1^{+}$ cells in the peritoneal cavity are highly susceptible to infection with *T. gondii*. *A,* Mice were infected i.p. with RH-YFP tachyzoites, and peritoneal exudate cells were harvested 3 days postinfection for phenotypic analysis and assessment of infection levels by flow cytometry. *B,* Costaining for CD11c and Gr-1 reveals a small population of $CD11c^{+}Gr\text{-}1^{+}$ cells in the peritoneal cavity 3 days postinfection. *C,* Examination of parasite levels in the $CD11c^{+}Gr\text{-}1^{+}$population reveals that these cells are highly susceptible to infection (black line histogram) compared with $CD11c^{+}Gr\text{-}1^{-}$cells (shaded histogram). The numbers indicate the percent of cells in each quadrant (*B*) or gate (*C*). This experiment was repeated three times with similar results.

for $B220^+$ and $CD3^+$ lymphocytes, respectively). There are several subsets of DCs, including some that coexpress CD11c and Gr-1. A small population of the $CD11c^+$ $Gr\text{-}1^+$ cells (<1%) was detected in the peritoneal cavity 72 h after infection (Fig. 2.1*B*). These cells were remarkable inasmuch as over 80% were positive for parasites (Fig. 2.1*C*).

The major population of infected cells in the spleen predominantly expresses CD11c and Gr-1

We next examined infection in the spleen, an organ targeted by the parasite during acute infection (2, 6). Splenocytes were obtained from mice infected 4 days before by i.p. administration of RH-YFP tachyzoites. At this time point, 5–6% of total splenocytes were positive for *T. gondii* (Fig. 2.2*A*). Gating on the infected population, we found that the majority (~70%) coexpressed CD11c and Gr-1 (Fig. 2.2*B*). We also found that ~20% of infected cells possessed a $CD11c^-Gr\text{-}1^+$ phenotype. This population is likely to be neutrophils, but it is also possible that some are $Gr\text{-}1^+$ inflammatory monocytes described by Sibley and colleagues (21, 22). We also determined the proportion of total splenic $CD11c^+Gr\text{-}1^+$ cells that were infected with *T. gondii*. As shown in Fig. 2.2*C*, ~60% of this population harbored intracellular parasites.

We wanted to investigate the phenotype of the $CD11c^+Gr\text{-}1^+$ population in more detail (Fig. 2.2*D*, *upper panels*). The cells expressed MHC class II, B220, CD11b, CD80/86, and PDCA-1. This profile, in particular expression of PDCA-1, Gr-1, and B220, was suggestive of a pDC-related population, although they are atypical in that they express

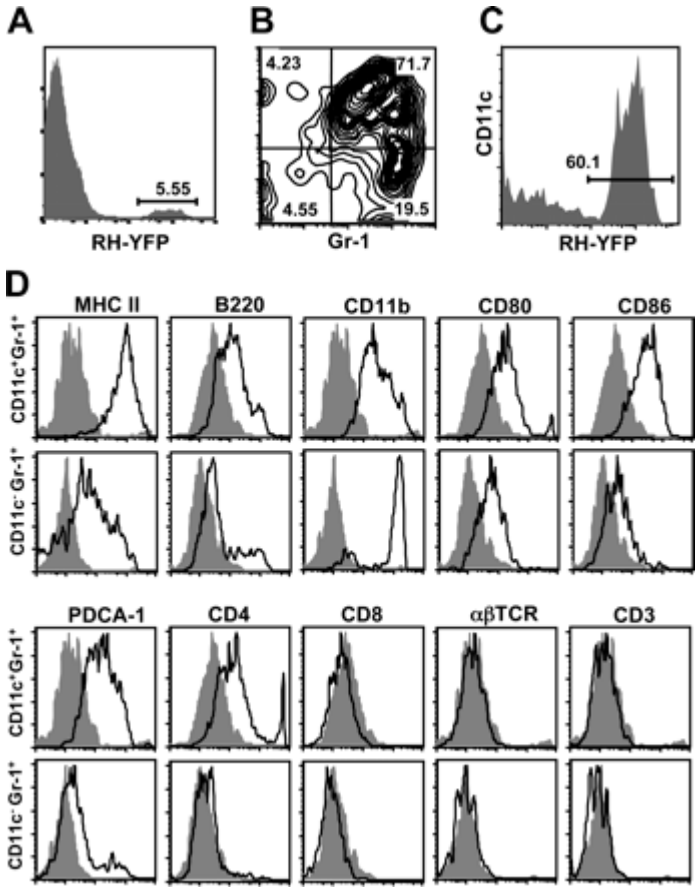

Figure 2.2. CD11c^{+}Gr-1^{+} cells are the predominant population of infected cells in the spleen, and exhibit phenotypic characteristics of both plasmacytoid and myeloid dendritic cells. *A*, Splenocytes were harvested from mice 4 days postinfection and analyzed by flow cytometry for RH-YFP tachyzoites. *B*, CD11c and Gr-1 expression on YFP-positive cells. *C*, YFP expression in the total CD11c^{+}Gr-1^{+} population in the spleen. The numbers indicate the percent of cells falling within the indicated gates (*A* and *C*) or quadrants (*B*). *D*, Phenotypic analysis of infected CD11c^{+}Gr-1^{+} (*upper panels*) and CD11c^{+}Gr-1^{-} cells (*lower panels*) shown in *B*. The solid lines represent each respective marker and the shaded histograms indicate isotype controls. One representative of two independent experiments is shown.

CD11b and higher than expected levels of CD11c (23, 24, 25, 26). We also examined the phenotype of the $CD11c^{-}Gr\text{-}1^{+}$ population (Fig. 2.2*D*, *lower panels*). These cells expressed high levels of CD11b, a molecule expressed by neutrophils. Interestingly, the cells were also positive for MHC class II and CD80.

We examined the appearance of $Gr\text{-}1^{+}$ parasite-infected cells in the spleen by fluorescence microscopy (Fig. 2.3). The vast majority of infected $Gr\text{-}1^{+}$ cells were clearly distinct from the neutrophil population, because they possessed abundant cytoplasm and non-polymorphic nuclei. However, we were also able to detect some infected neutrophils, consistent with our data showing a population of infected $CD11c^{-}$ $Gr\text{-}1^{+}$ cells (Figs. 2.2*B* and 2.3*C*).

Next, we used immunofluorescence staining of frozen tissue sections to localize infected cells in situ. In spleens of day 4-infected mice, parasites were mainly present in phagocytes of the marginal zone, red pulp, and to a lesser extent in cells of the lymphoid follicles (Fig. 2.4). Within the splenic follicles, T cell areas also contained $CD11c^{+}$ DCs infected with *T. gondii* (Fig. 2.5, *A* and *B*). In Fig. 2.5, *C* and *D*, we used 4-color fluorescence imaging to confirm that infected $CD11c^{+}$ cells (*C*) also expressed Gr-1 (*D*).

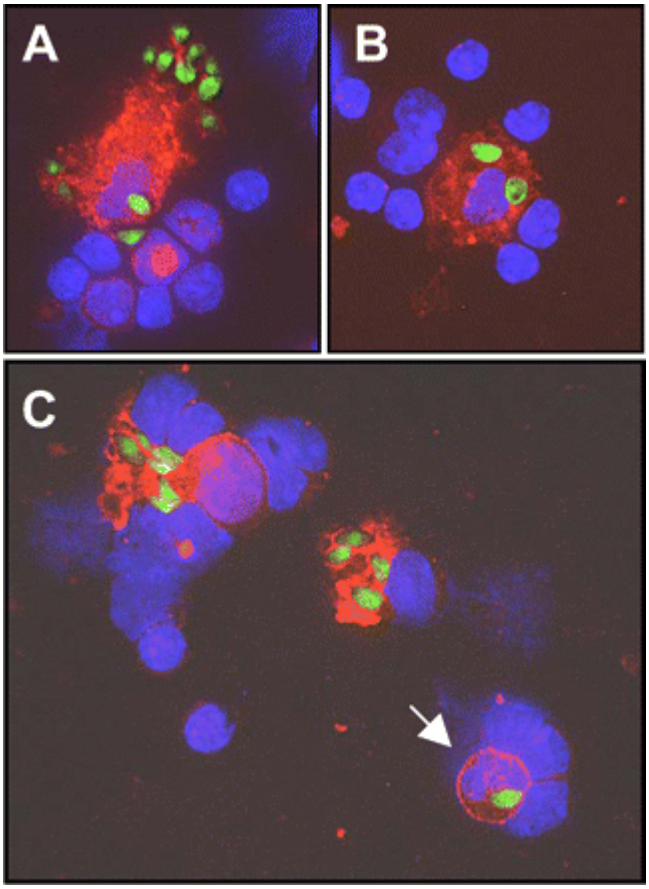

Figure 2.3. Morphology of *Toxoplasma* infected cells in the spleen. Mice were infected with RH-YFP tachyzoites (green), then 4 days later splenocytes were isolated and stained for Gr-1 (red) and DNA (DAPI, blue). Most infected cells exhibited a round or bean-shaped nucleus consistent with monocyte/macrophage/DC (*A–C*) and abundant cytoplasm, in which multiple parasites were found. Lesser numbers of infected polymorphonuclear leukocytes were also observed (*C*, arrow). The experiment was repeated twice with similar results.

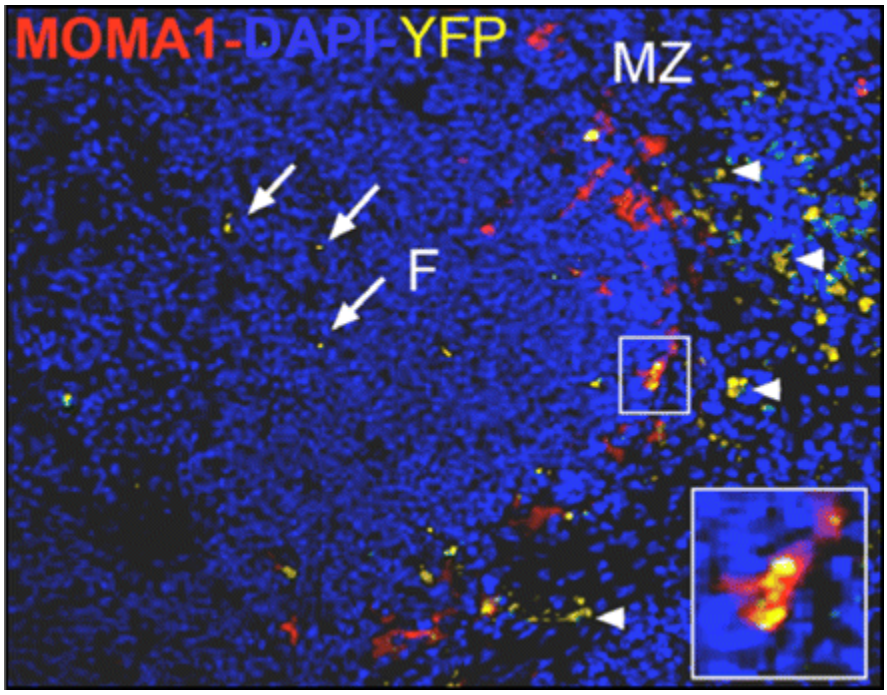

Figure 2.4. Infected cells are located primarily in the marginal zone of the spleen. Spleens were obtained from mice 4 days postinfection and immunofluorescence on frozen sections was performed. Staining for metallophilic macrophages (MOMA-1, red) delineates the edge of the marginal zone (MZ), revealing concentration of infected cells (yellow) in this area (arrowheads), with a small number of infected cells (arrows) in the lymphoid follicle (F). The *inset* shows at high magnification a MOMA-1^+ cell with intracellular parasites. Blue, DAPI stained nuclei. Original magnification x200. One representative of two independent experiments is depicted.

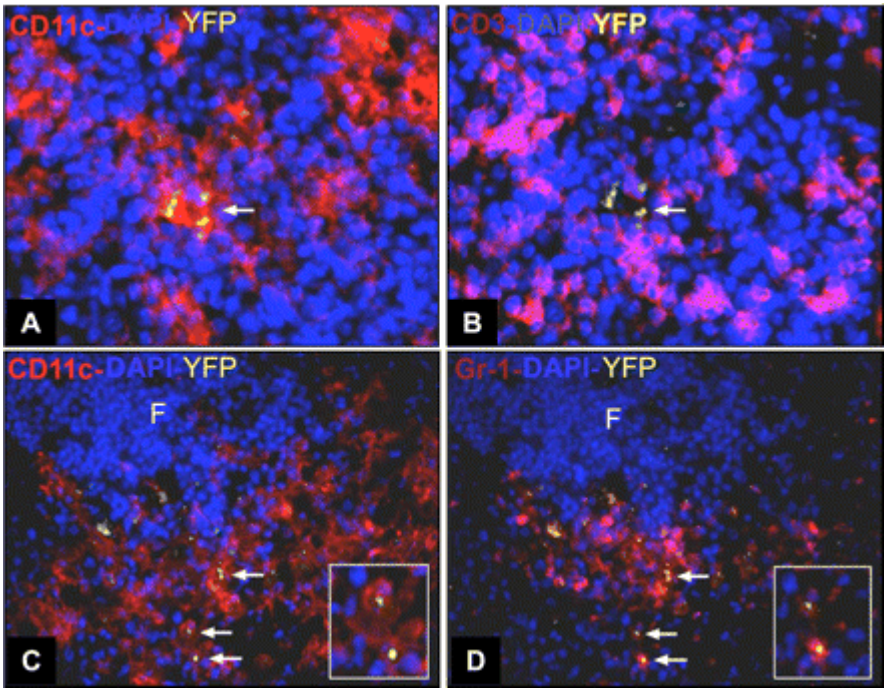

Figure 2.5. Infected cells in the T cell areas of the spleen coexpress CD11c and Gr-1. Mice were infected with RH-YFP tachyzoites and 4 days later spleens were collected for immunohistochemical staining. *A*, Four-color immunofluorescence staining of frozen sections shows $CD11c^+$ cells (red) infected with RH-YFP (yellow).*B*, In the same section, staining for CD3 (pink) reveals T cells surrounding infected $CD11c^+$ cells. Staining for CD11c (*C*) and Gr-1 (*D*) shows that most infected cells (arrows) coexpress CD11c (red) and Gr-1 (pink). *Inset*s in *C* and *D* shows an expanded view of infected $CD11c^+Gr\text{-}1^+$ cells. Original magnification x400 (*A*and *B*) and x200 (*C* and *D*). This experiment was repeated twice with similar results.

$CD11c^+Gr\text{-}1^+$ cells migrate from the peritoneal cavity to the spleen

Because we found that $CD11c^+Gr\text{-}1^+$ cells were highly susceptible to infection in the peritoneal cavity, we hypothesized that these cells may be migrating from the inoculation site to the spleen. To determine whether this was the case, we infected congenic B6.SJL mice (CD45.1) with i.p. injected RH-YFP tachyzoites. Two days later we harvested the peritoneal exudate cells from the B6.SJL mice and transferred them by i.p. injection into CD45.2 congenic C57BL/6 mice infected i.p. 2 days before with RH-YFP tachyzoites. Spleen and peritoneal exudate cells were harvested 48 h after transfer. We found a small number of transferred cells, which were primarily noninfected, remaining in the peritoneal cavity at this time (data not shown). As expected, in the spleen there was background staining for $CD45.1^+$ cells in nontransferred animals (Fig. 2.6*A*). However, we found adoptively transferred parasite-infected $CD45.1^+$ cells mobilized to the spleen in the CD45.2 congenic hosts (Fig. 2.6*B*), and as predicted, these migrating cells coexpressed Gr-1 and CD11c (Fig. 2.6*C*). We transferred cells from $CD45.1^+$ mice infected with YFP-RH into CD45.2 animals infected with nonfluorescent RH tachyzoites (Fig. 2.6*D*). As shown in the figure, we detected the appearance of YFP-RH infected, $CD45.1^+$ cells in the spleen. As expected, these cells also expressed Gr-1 and CD11c (data not shown). Also evident in Fig. 2.6*D*, most of the YFP^+ cells were derived from recipient mice, because they do not express CD45.1. The majority of these cells are positive for Gr-1 and CD11c (data not shown), and it seems likely that they originate from host pDC infected in the peritoneal cavity by RH-YFP. We cannot be sure what accounts for the low percentage of $CD45.1^+$ cells that traffic to the spleen in these situations. However, we suspect that the cells are negatively affected by the recovery and transfer protocol, and that many subsequently die after transfer. Regardless, from these data we conclude

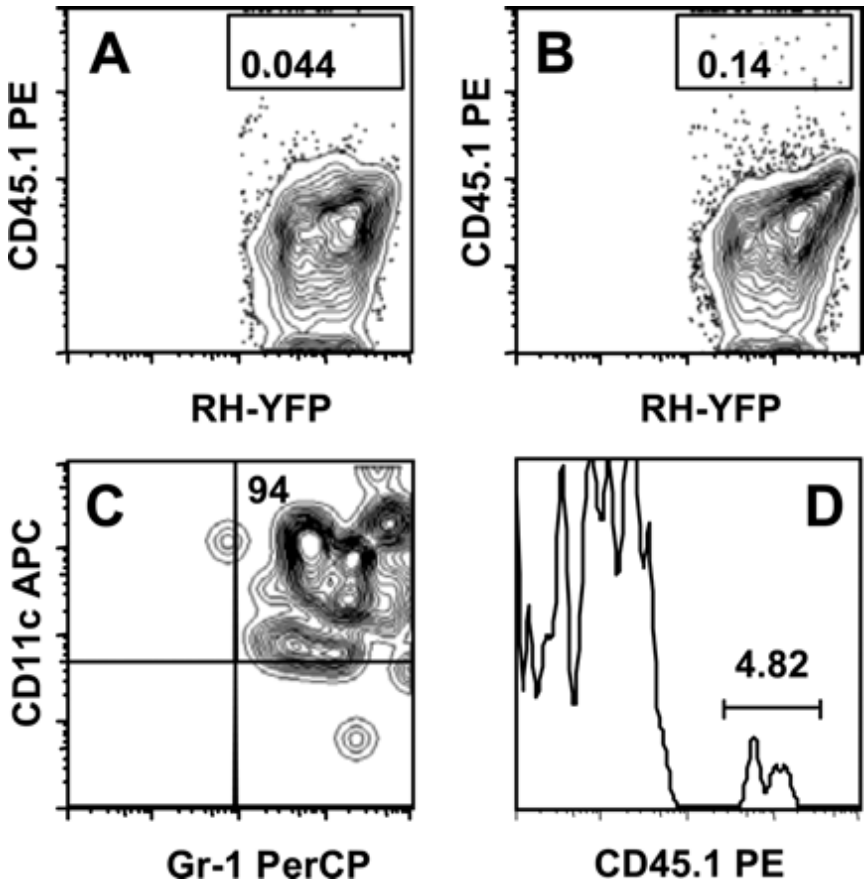

Figure 2.6. Infected CD11c$^+$Gr-1$^+$ cells migrate from the peritoneal cavity to the spleen. Peritoneal exudate cells from B6.SJL CD45.1 congenic mice were isolated 2 days after i.p. inoculation with RH-YFP tachyzoites. The exudate cells were then adoptively transferred i.p. into congenic C57BL/6 CD45.2 host mice infected 2 days earlier with RH-YFP tachyzoites. Two days later splenocytes were harvested from the host mice and three-color staining for CD45.1, CD11c, and Gr-1 was conducted. *A*, splenocytes from a control day 4-infected C57BL/6 mouse that received no congenic peritoneal exudate cells, showing background staining for CD45.2. *B*, splenocytes from a day 4-infected C57BL/6 mouse adoptively transferred with CD45.2-positive peritoneal exudate cells. *C*, CD11c and Gr-1 expression of infected CD45.2$^+$ cells in the spleen. The numbers indicate the percentage of cells falling within the indicated rectangles (*A* and *B*) and quadrants (*C*). The graphs show results from one representative mouse. In a group of four mice, the percentage of CD45.1$^+$ cells after transfer was 0.14 ± 0.03. In *D*, CD45.1$^+$peritoneal exudate cells from mice infected with RH-YFP parasites were transferred into CD45.2$^+$ mice infected with nonfluorescent parasites. Spleen cells were recovered as above and CD45.1 expression was examined on YFP$^+$ cells. The numbers in each graph indicate the percent of cells falling within the indicated quadrants or gates.

that infected $CD11c^+Gr\text{-}1^+$ cells are capable of migrating from the peritoneal cavity to the spleen over the course of infection.

CCR2 is partially involved in recruitment of $CD11c^+Gr\text{-}1^+$ to the spleen

The chemokine receptor CCR2 is involved in recruitment of inflammatory myeloid cells and DCs during several infections, including *Toxoplasma* and *Listeria monocytogenes* (10, 22). Therefore, we sought to determine whether CCR2 might play a role in recruitment of $CD11c^+Gr\text{-}1^+$ cells during infection. Accordingly, CCR2 knockout mice were infected in parallel with wild-type controls and $CD11c^+Gr\text{-}1^+$ splenocytes were analyzed 4 days postinfection. We found a major reduction in the number of $CD11c^+Gr\text{-}1^+$ cells in the spleens of infected mice in the absence of CCR2 signaling (Fig. 2.7*A*). Nevertheless, recruitment of $CD11c^+Gr\text{-}1^+$ DCs to the spleen was not completely dependent upon CCR2 because there was a low, but significant increase in the number of these cells comparing noninfected with infected $CCR2^{-/-}$ mice. We also examined the influence of CCR5, another chemokine receptor implicated in resistance to *T. gondii* (27, 28, 29), on mobilization of $CD11c^+Gr\text{-}1^+$ cells to the spleen during infection. In this case, absence of CCR5 had no impact on the migration of $CD11c^+Gr\text{-}1^+$ cells to the spleen following *T. gondii* infection (Fig. 2.7*B*).

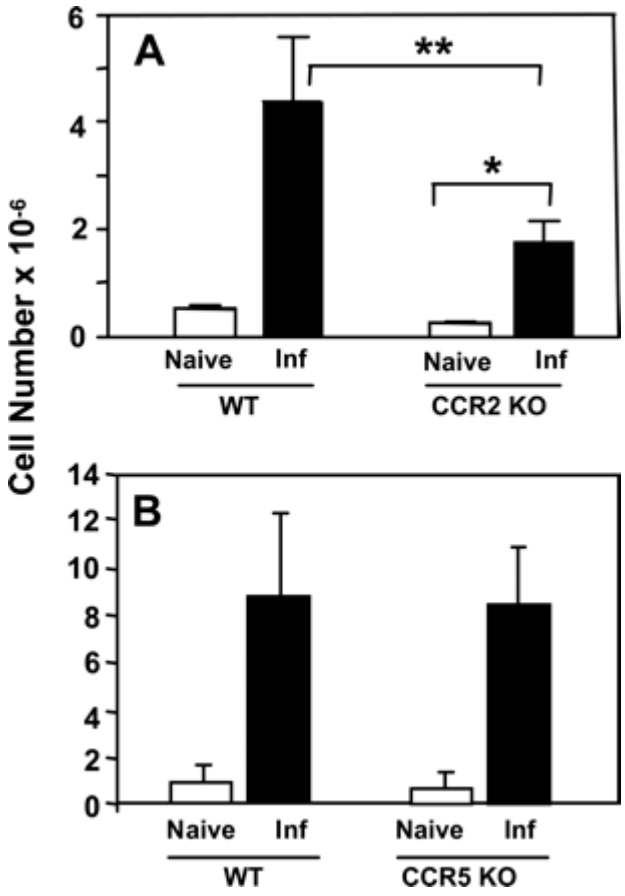

Figure 2.7. CCR2 is partially involved in recruitment of CD11c^{+}Gr-1^{+} cells to the spleen during infection. Wild-type and chemokine receptor knockout mice were infected with RH-YFP tachyzoites, and recruitment of CD11c^{+}Gr-1^{+} cells to the spleen was assessed by flow cytometry. Recruitment of CD11c^{+}Gr-1^{+} cells was defective in CCR2 knockout mice relative to wild-type controls (*A*), but unaffected in CCR5 knockout mice (*B*). Data shown are pooled from three experiments, with two mice per condition. $*$, $p < 0.05$, $**$, $p < 0.005$.

Toxoplasma-infected CD11c$^+$Gr-1$^+$ DCs are refractory to TLR-induced IL-12p40 production

Splenic CD8α^+ DCs are a well-characterized source of IL-12 after i.v. injection of *Toxoplasma* lysate Ag (14). This activity is largely due to parasite profilin-TLR11 interactions (15). However, our previous data has shown that *Toxoplasma*-infected macrophages are suppressed in their ability to produce proinflammatory cytokines such as IL-12 when subjected to TLR stimulation (30). Similar findings have been reported by others using mouse bone marrow-derived DCs (17). Therefore, we sought to determine whether the CD11c$^+$Gr-1$^+$ DCs targeted for infection by *T. gondii* were stimulated to produce IL-12, or whether the parasite actively suppressed the cytokine.

To discriminate between induction vs suppression of IL-12, splenic DCs from day 4-infected mice were enriched using pan-DC immunomagnetic beads. In this population, ~14% of the cells coexpressed CD11c and Gr-1 (Fig. 2.8*A*). The enriched DC population was cultured for 24 h in the presence of medium alone or with TLR ligands. Because the phenotype of the CD11c$^+$Gr-1$^+$ cells displayed characteristics of both pDC and conventional DC, and because these DC populations express distinct TLR ligands, we used a combination of TLR9 and TLR4 ligands (CpG and LPS, respectively) for stimulation. We then examined IL-12 p40 expression in infected and noninfected CD11c$^+$Gr-1$^+$ DCs. As shown in Fig. 8*B*, ~9% of noninfected CD11c$^+$Gr-1$^+$ cells produced IL-12 without further in vitro stimulation. After stimulation with TLR ligands, almost 40% of noninfected CD11c$^+$Gr-1$^+$ DCs expressed IL-12 (Fig. 2.8*C*). In marked contrast to these results, only 2% of infected CD11c$^+$Gr-1$^+$ stained

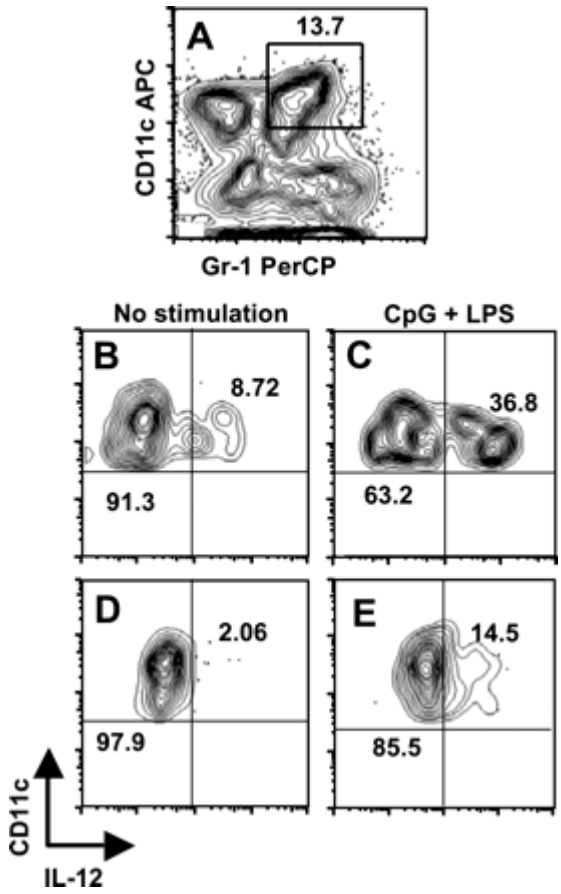

Figure 2.8. IL-12 production is defective in CD11c$^+$Gr-1$^+$ cells infected with *T. gondii*. Splenocytes were harvested 4 days postinfection with RH-YFP tachyzoites and enriched for CD11c$^+$ cells using magnetic beads. *A*, CD11c and Gr-1 expression levels in the enriched DC population. The number indicates the percent of cells in the indicated rectangle. Enriched cells were cultured 24 h in medium alone, or medium with CpG oligodinucleotide (1 μg/ml) and LPS (100 ng/ml). The cells were stained for intracellular IL-12p40 and surface Gr-1 and CD11c. *B*, IL-12 expression by noninfected CD11c$^+$Gr-1$^+$ cells cultured in medium alone. *C*, IL-12 expression by parasite-negative CD11c$^+$Gr-1$^+$ cells after in vitro stimulation with LPS + CpG. *D*, IL-12p40 expression by YFP$^+$ CD11c$^+$Gr-1$^+$ cells with no in vitro stimulation. *E*, Expression of IL-12p40 in infected CD11c$^+$Gr-1$^+$ cells after TLR ligand-stimulation. In *B–D*, the numbers indicate the percent of cells falling within the indicated quadrants. One representative of two independent experiments is shown.

positive for IL-12 during culture in medium (Fig. 2.8*D*), and with the addition of LPS and CpG, the percentage increased to only ~14% (Fig. 2.8*E*). We conclude from these data that *Toxoplasma* targets $CD11c^{+}Gr\text{-}1^{+}$ cells for infection, and the parasite suppresses IL-12 production once inside the cells.

Discussion

The results of this study show that inoculation of mice with the tachyzoite stage of *T. gondii* leads to preferential infection of Gr-1-expressing DCs. CCR2 is involved in the migration of these cells from the peritoneal cavity to the marginal zone of the spleen, where they express markers associated with pDC. Infection with *T. gondii* suppresses the capacity of these cells to produce the proinflammatory cytokine IL-12 upon ex vivo stimulation with ligands for TLR4 and TLR9, while noninfected cells retain their ability to synthesize IL-12. Recently, inflammatory macrophages have been implicated in the early anti-microbial response to *Toxoplasma*. Those cells resemble the pDC-like population identified here in that they express Gr-1 and they display CCR2-dependent recruitment (22). However, based upon expression of PDCA-1, CD11c, and B220, it is most likely that the two populations are distinct, and that the cells reported here are related to cells of the pDC lineage.

pDCs are differentiated effector cells specialized for production of anti-viral type I IFN (25). However, they possess the unusual property of being able to differentiate into conventional DCs that activate naive T cells and instruct adaptive immunity through production of mediators such as IL-12 (31). Differentiation can be induced by cytokines such as IL-3, and also microbial stimuli including bacteria and viruses (26, 32). Accompanying pDC differentiation into DCs, the cells acquire dendritic

morphology and up-regulate CD11c, MHC class II, and costimulatory molecules. However, there are likely to be functional differences between pDC-derived DCs and conventional DCs insofar as activation of naive T cells by pDC-derived DCs leads to generation of regulatory T cells in several cases (33, 34). pDCs may also differ from conventional DCs in terms of Ag uptake and processing (35, 36).

Although the cells targeted for infection express markers associated with pDCs, namely PDCA-1, Gr-1 and B220, they are nevertheless atypical in that they also express the monocyte lineage marker CD11b. A potential explanation for this unique phenotype may relate to differentiation of pDC into DC under inflammatory conditions. During infection with lymphocytic choriomeningitis virus, bone marrow-derived pDCs down-regulate B220 and Gr-1 and up-regulate CD11b, dependent upon type I IFN (37). Interestingly, this was found to be a two-step process, in which CD11b was first up-regulated, followed by down-regulation of B220. After this transformation, the cells were also newly capable of producing IL-12 in response to TLR4 activation, indicating functional as well as phenotypic conversion. In this regard, it is of interest that *Toxoplasma* was recently shown to stimulate type I IFN secretion by pDCs (16). We hypothesize that *T. gondii* infection stimulates an intermediate stage of pDC to conventional DC conversion, associated with coexpression of pDC and myeloid DC markers. Whether this is a stable phenotype associated with infection, or is a transitory state on the way to a conventional DC phenotype remains to be determined.

In addition to type I IFN secretion, pDCs also produce IL-12 upon stimulation with *T. gondii* or soluble tachyzoite Ag, and they are also capable of presenting Ag during infection (16). This observation suggests a role for pDCs in the induction of the adaptive immune response to *Toxoplasma*. However, although we did not examine Ag

presenting capability, our work argues that *T. gondii* infection suppresses pDC IL-12 production. In this regard, pDCs cultured in vitro from the bone marrow were used in the experiments showing IL-12 production in response to *T. gondii*, whereas the pDCs identified in the present study were enriched from the spleens of infected animals. Therefore, it is possible that the cells identified here are functionally distinct from pDCs generated in vitro. Additionally, we used flow cytometry to separate splenic pDCs into infected and noninfected populations. Although the infected pDC population was suppressed in IL-12 production, we found substantial IL-12 production by the noninfected pDC population. Thus, we also found that pDCs present during *T. gondii* infection are a source of IL-12. However, our study demonstrates that when pDCs are directly parasitized, their ability to produce IL-12 is suppressed.

We, and others, previously reported that *T. gondii* inhibits TLR-induced IL-12 production by bone marrow-derived macrophages and DCs (17, 38). The present study is significant because it is the first to demonstrate the suppression phenomenon in cells parasitized during in vivo infection. Inhibition of TLR signaling may be a consequence of the need to avoid activation by the parasite's own TLR ligands (15, 39). The mechanisms of suppression are not clear, although the parasite has blocking effects on both NFκB and MAPK signaling pathways (30, 40, 41, 42). More recently, IL-10-independent STAT 3 activation driven by injection of Toxoplasma rhoptry protein kinases into the host cell cytosol has been implicated in IL-12 inhibition (43, 44).

A key question is why *Toxoplasma* would target pDCs for preferential infection. As immune effectors, pDCs have two major functions, which are to produce large quantities of anti-viral type I IFN and to present Ag (25). pDCs are not known to possess strong microbicidal activity, unlike macrophages and other myeloid DC

populations. Therefore, pDCs may offer a more hospitable environment for the invading parasite relative to other cell types. Furthermore, activated DCs are highly motile, and are sensitive to chemokine-mediated recruitment to lymphoid organs draining the site of infection or inflammation. In this regard, it has long been known that *Toxoplasma* infection initially spreads through the lymphatics, and lymphoid organs become rapidly parasitized during infection (45). Therefore, pDCs may provide the parasite with a safe vehicle for dissemination during early infection.

It is an unexpected finding that pDCs play a role in the immune response to *Toxoplasma*. Although we used a virulent parasite strain that leads to 100% lethality in this study, preliminary data (not shown) suggests that low virulence strains capable of establishing long-term infection also preferentially parasitize pDCs. Other protozoa, including *Plasmodium* and *Leishmania spp*. are known to display complex interactions with DCs (46, 47). Whether these pathogens also target the pDC population remains to be determined.

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CHAPTER 3

PARASITE STRAIN DETERMINES CELL POPULATIONS TARGETED DURING ACUTE INFECTION WITH *TOXOPLASMA GONDII*

Abstract

Toxoplasma gondii is an obligate intracellular parasite that infects a wide range of warm-blooded hosts, and can lead to severe disease in immunocompromised populations. In North America and Europe, three clonal lineages comprise the majority of *T. gondii* strains, and severity of disease and host-pathogen interactions vary among these strains. Recently, I demonstrated that cells with phenotypic characteristics of plasmacytoid dendritic cells (pDC) are hypersusceptible to invasion by Type I tachyzoites, and that these parasites use pDCs as Trojan horses for dissemination to the spleen during early acute infection. In this study, I used representatives of each strain type in an intraperitoneal model to determine whether this use of dendritic cells was restricted to Type I parasites, or whether it was a general characteristic of *T. gondii* infection regardless of strain type. I found that tachyzoites of Types I and II were more likely to infect $CD11c^{+}Gr\text{-}1^{+}$ cells in the spleen than Type III tachyzoites, and that Types II and III strains induced a greater recruitment of conventional DC at the infection site compared with Type I. Therefore, I conclude that different *T. gondii* strains contribute to variations in recruitment and invasion of cells during acute infection.

Introduction

Toxoplasma gondii is an intracellular protozoan parasite of the phylum Apicomplexa, which infects approximately 9% of people in the United States (1). The parasite completes its life cycle in members of the cat family (2), but can persist in a chronic state in most birds and mammals (3-4). Infection of individuals with compromised immunity, such as AIDS patients, as well as transplacental infection of a fetus, can

lead to serious clinical outcomes (5-6). Therefore, *T. gondii* is an important opportunistic pathogen and the subject of extensive research.

Three clonal lineages comprise the majority of *T. gondii* strains in North America and Europe (7). Infection with different strains can lead to different clinical outcomes, as Type II is the most common strain found in infections in the United States, but Type I is the strain most likely to be associated with severe disease such as ocular toxoplasmosis (7-9). Several features of parasite biology and host-parasite interaction vary depending on the strain type. Type I is the most virulent strain in mice, and it has been hypothesized that this high virulence is due to a number of factors, including heightened capacity for crossing endothelial barriers, enhanced migration ability and a quicker replication rate compared to Types II and III (10-11). This, in turn, may lead to greater parasite burden, increased production of Th1 cytokines, and immunopathology (12-13). The rhoptry kinase ROP18, which exhibits allelic variation between strains, has been identified as the virulence factor responsible for this enhanced growth (14).

Strain differences can also be observed in the induction and suppression of proinflammatory cytokines by *T. gondii*. Type I and Type II differ in how they induce IL-12 production, in that Type I acts solely through a MyD88-independent mechanism, while Type II uses MyD88-dependent as well as independent pathways (15). This is reflected by the higher levels of IL-12 elicited by Type II infection in vitro (16). Additionally, Types I and III, but not Type II, activate signal transducer and activator of transcription 3 (STAT3) when they invade cells, diminishing

proinflammatory cytokine production (16). The virulence factor ROP16 has been implicated in strain-specific activation of STAT3 (17). Finally, differences have been observed in cell recruitment at the site of infection after intraperitoneal inoculation with Types I and II parasites, in that the cells recruited by Type II parasites are primarily Gr-1$^+$ monocytes, whereas more neutrophils are recruited by Type I infection (18). It has been suggested that this difference is largely responsible for the difference in parasite control between Types I and II during acute infection (18).

Recently, dendritic cells have been proposed as Trojan horses during *T. gondii* infection. Parasites in the mesenteric lymph nodes and blood after intragastric inoculation are associated with CD11c$^+$ and CD11b$^+$ cells, respectively, and CD11c$^+$ and CD11b$^+$ cells in the blood of infected mice home to the brain within 7 days (19). *In vitro* studies demonstrated that *Toxoplasma* infects immature dendritic cells more readily than mature DC, and is capable of inhibiting their activation, as measured by a suppressed ability to produce IL-12 and TNF-α and activate naïve CD4$^+$ T lymphocytes (20). Furthermore, studies on dendritic cell motility have demonstrated that infection with *T. gondii* enhances migration of DCs *in vitro* by suppressing upregulation of the adhesion molecule ICAM-1. This hypermotility phenotype leads to more efficient dissemination to lymphoid organs *in vivo* compared to that of DCs activated by lipopolysaccharide, as well as more effective dissemination of tachyzoites compared to infection with parasites alone (21). Finally, one study showed that although all strain types induce hypermotility in DC, Types II and III induce this phenotype more strongly than does Type I (22), suggesting that the parasitic utilization of dendritic cells as vehicles for dissemination may be heightened based on parasite strain type.

In this study, I used an intraperitoneal model of infection to compare the three major parasite strains in terms of which cells they targeted for infection. My previous work had demonstrated that cells with a plasmacytoid DC phenotype were highly susceptible to parasitization and migrated from the infection site to the spleen, comprising the majority of infected cells at that site at four days post-infection (23). Here I find that, at day 5 post-infection in the spleen, infection has spread to other cell types in Type I-infected mice, but there are no significant strain-related differences between infection of cell types. I observe differences in the composition of the infected population in the peritoneal cavity at day 3 post-infection, as a greater percentage of these cells are $CD11c^+$ DCs in Type II and Type III infections, whereas the infected cells in the Type I peritoneal cavity are dominated by $Gr\text{-}1^+$ cells. I conclude that strain type contributes to variations in infection of certain cell types at the inoculation site.

Materials and Methods

Mice. C57BL/6 mice 6-8 weeks of age were purchased from Taconic Farms and housed in the Cornell University College of Veterinary Medicine transgenic mouse core facility, which is accredited by the American Association for Accreditation of Laboratory Animal Care.

Parasites and Infections. RH (Type I), PTG (Type II) and VEG (Type III) tachyzoites were purchased from the American Type Tissue Collection and maintained in human foreskin fibroblasts in DMEM (Mediatech) supplemented with 1% bovine growth serum (Hyclone), 100 U/mL penicillin and 100 U/mL streptomycin (Invitrogen Life Technologies). Mice were infected with 10^6 tachyzoites (TZ) intraperitoneally.

Flow Cytometry. At days 3 and 5, infected mice were sacrificed, spleens were harvested, and peritoneal exudate cells (PEC) were collected by peritoneal lavage. Splenocytes and peritoneal exudate cells were filtered through 70 µm filters, and incubated on ice for 15 min with 10% normal mouse serum in FACS buffer (1% BSA and 0.05% sodium azide in PBS). Cells were then incubated for 30 minutes on ice with fluorochrome-conjugated antibodies (BD Biosciences, BioLegend, Argene, eBioscience), washed and fixed in 3% paraformaldehyde. Permeabilization was performed with 0.075% saponin in phosphate-buffered saline, and permeabilized cells were incubated on ice for 30 minutes with antibody to parasite surface marker p30 and thoroughly washed. Cells were then read on a Becton-Dickinson FACSCalibur, and data was analyzed using FlowJo software (Treestar).

Results

Cell recruitment to the peritoneal cavity varies among strain types. Mice were sacrificed at 3 days post-infection with a high dose of Type I, II or III tachyzoites, and peritoneal exudate cells (PEC) were collected for staining of surface markers and parasites using an antibody to the *T. gondii* surface antigen p30, and analysis by flow cytometry. Slight, but reproducible differences were observed in the recruitment of

different cell populations to the peritoneal cavity (Figure 3.1, Table 3.1). Infection with Types II and III led to greater numbers of $CD11c^+$ dendritic cells in the infected population, as DCs comprised roughly 50% of the infected cells after inoculation with these strains. By contrast, DC only accounted for a third of the infected cells in Type I-infected PEC, whereas $CD11c^-Gr\text{-}1^+$ cells dominated the infected population. The $CD11c^-Gr\text{-}1^+$ group was a mixed population, with the majority of cells staining with the Ly6G-specific antibody 1A8 (neutrophils), and the rest negative for this marker (likely inflammatory monocytes). Additionally, Type I parasites induced nearly twice the percentage of 1A8-staining neutrophils compared with Types II and III, and Types II and III induced twice as many $CD8^+$ cells.

Infected cells in the peritoneal cavity are mostly myeloid DC, inflammatory DC, and neutrophils. Day 3 peritoneal exudate cells from mice infected with the three strains of parasite were gated on parasite-positive cells and analyzed for surface markers (Table 3.1, Figure 3.2). With all three strains, infected cells were composed of three major populations, $CD11c^+Gr\text{-}1^-$ cells, $CD11c^+Gr\text{-}1^+$ cells, and $CD11c^-Gr\text{-}1^+$ cells. PEC from Type I-infected mice showed a striking lack of $CD11c^+Gr\text{-}1^-$ cells in comparison to Types II and III, with $Gr\text{-}1^-$ DC comprising only 9.13% of the infected cells, compared to 21.4% and 21.3%. All three populations expressed CD11b, indicating myeloid origin, but only the $CD11c^-Gr\text{-}1^+$ cells stained with the neutrophil-specific antibody 1A8. These phenotypes are consistent with myeloid-derived dendritic cells, inflammatory-monocyte-derived dendritic cells, and neutrophils (24-26).

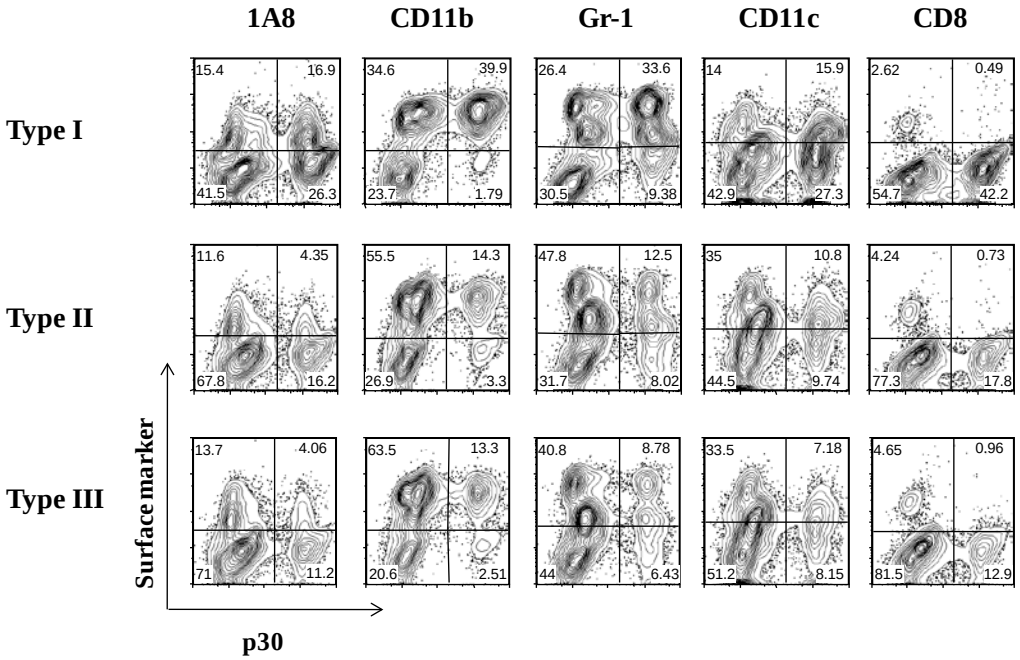

Figure 3.1. Cell recruitment and parasitization of cell populations varies on infection with different strains of *T.gondii*. Day 3 peritoneal exudate cells were harvested and stained for surface markers (y-axis) and tachyzoites (x-axis). PEC Type I-infected mice were composed of greater numbers of $1A8^+$ cells, while PEC of Types II and III had a larger $CD11c^+$ population. A higher percentage of Type I parasites were found in Gr-1^+ cells, and a higher percentage of Type II parasitized DC. These differences were statistically significant by a two-tailed Student's t-test, with p values of 0.01 and 0.043, respectively.

Table 3.1. **Strain type influences representation of different cell types among infected cells in the peritoneal cavity.** Day 3 post-infection, peritoneal exudate cells (PEC) were harvested and stained for surface markers and parasite. PEC from Type II and III-infected mice are comprised of greater numbers of dendritic cells, and DC are the major infected population as well, whereas Gr-1 cells are the greater infected population with Type I. However, the differences between percentages of DC and neutrophils recruited are not statistically significant by two-tailed Student's t-test, as the p-value for total percentage of PEC belonging to the $CD11c^+$ population is 0.33 between Types I and II, and 0.17 for the total percentage of infected PEC belonging to the $Gr\text{-}1^+$ population. The preference of Type II for infecting DC is statistically significant, however, with a p-value of 0.043 when compared to Type I, as is the preference of Type I for $Gr\text{-}1^+$ cells, with a p-value of 0.01 when compared to Type II. Data shown are the results of three independent experiments.

Day 3 PEC	**Total Cell %**		**% of Infected Cells**		
Infection	**CD11c+**	**Gr-1+**	**CD11c+ Gr-1-**	**CD11c- Gr-1+**	**CD11c+ Gr-1+**
Type I (RH)					
1	33.2	73.1	6.67	64.4	23.3
2	14.13	59.6	4.66	61.2	16.6
3	29.9	60.0	9.13	56.5	26.1
Type II (PTG)					
1	45.44	49.1	30.1	33.1	24.3
2	24.23	21.79	10.9	34.2	33.2
3	45.8	60.3	21.4	40	26.1
Type III (VEG)					
1	nd	nd	nd	nd	nd
2	40.64	35.42	21.2	33.7	27.9
3	40.68	49.58	21.3	39.6	27.0

Infection in the spleen does not exhibit significant differences between strain types. Splenocytes from infected mice were harvested 5 days after inoculation, and analyzed for tachyzoites and surface markers (Figure 3.3). Infection rate of spleens with Types II and III was much lower than with Type I, as spleen cells from the first two strains were infected at a rate of 0.38% and 0.23% respectively, while Type I infected at a rate of 3.75%. Association of parasites with $CD11c^{+}Gr\text{-}1^{+}$ cells was much weaker at day 5 than on day 4, according to my previous observations, but Type I and II tachyzoites were more strongly associated with this population than Type III; however, this result was not statistically significant. Additionally, Type III tachyzoites infected a relatively large population of $CD11c^{-}Gr\text{-}1^{-}$ cells in two out of three experiments, in contrast to Types I and II (Table 3.2). This double-negative population consists of 80% $CD11b^{-}$ cells (data not shown), and this phenotype suggests that these cells may be lymphocytes or $Gr\text{-}1^{-}$ macrophages. However, this result was not statistically significant, either. Therefore, these results suggest that variations in parasite invasion of different cell populations, or utilization of Gr-1-expressing dendritic cells as vehicles for dissemination, do not exist between strain types.

Discussion

In this study, I have shown that intraperitoneal inoculation with representative strains of the three major clonal lineages of *T. gondii* leads to variations in cell recruitment at the infection site and parasitization of cell populations in the peritoneal cavity. While Type I infection induces a greater percentage of $1A8^{+}$ neutrophils in the peritoneal cavity at three days after infection, Types II and III lead to a higher, though not

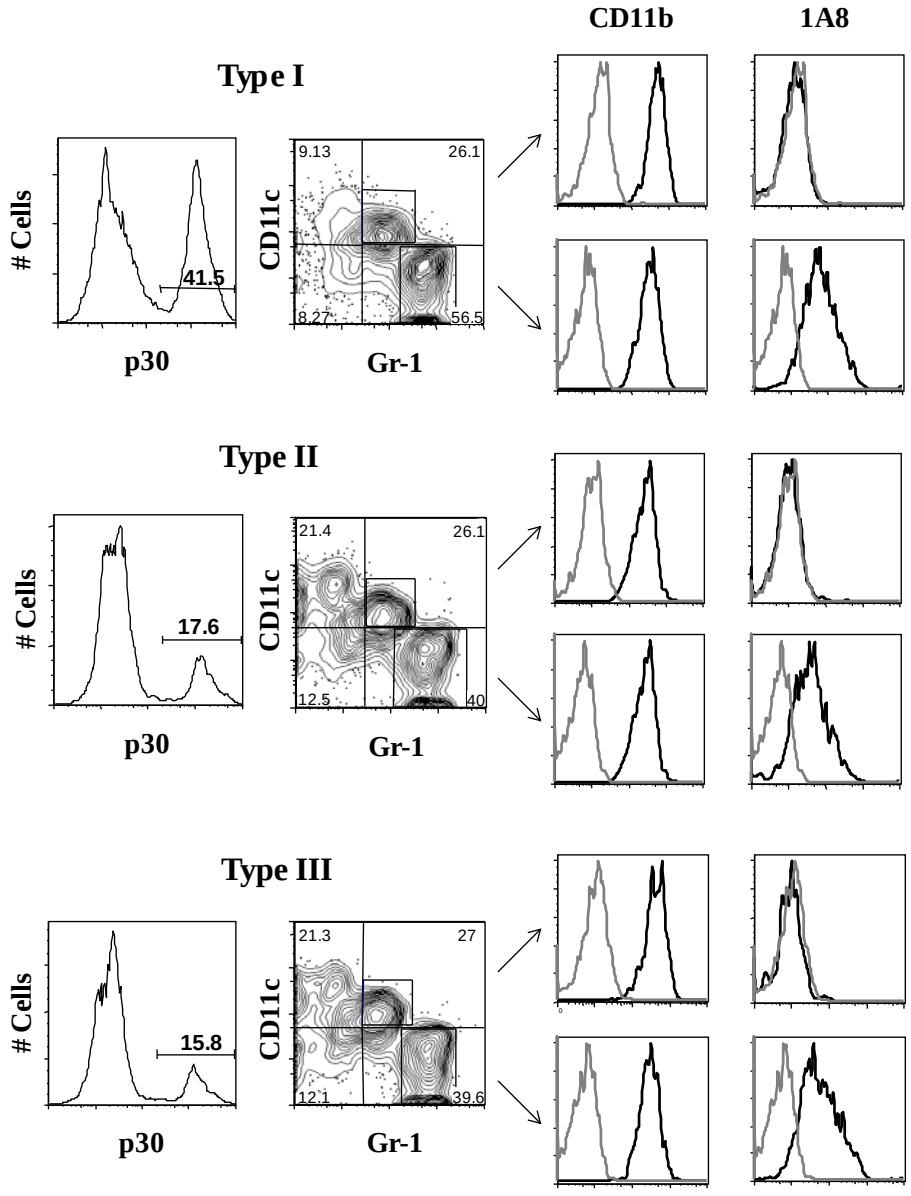

Figure 3.2. Infected cells in the peritoneal cavity on day 3 belong to three major groups: CD11c^{+} Gr-1^{-} DC, CD11c^{-}Gr-1^{+} neutrophils, and CD11c^{+}Gr-1^{+} cells. Peritoneal exudate cells were collected on day 3 post-infection and stained for surface markers and intracellular parasites (p30). Gating on parasites revealed three major groups of infected cells, CD11c^{+}Gr-1^{-} cells, CD11c^{-}Gr-1^{+} cells, and CD11c^{+}Gr-1^{+} cells. Gating on the CD11c^{+}Gr-1^{+} cells revealed that they were CD11b^{+} and 1A8^{-} (black histogram = surface molecule, grey histogram = isotype control), suggesting monocyte-derived DC, while CD11c^{-}Gr-1^{+} cells were CD11b^{+} and 1A8^{+}, indicating neutrophils.

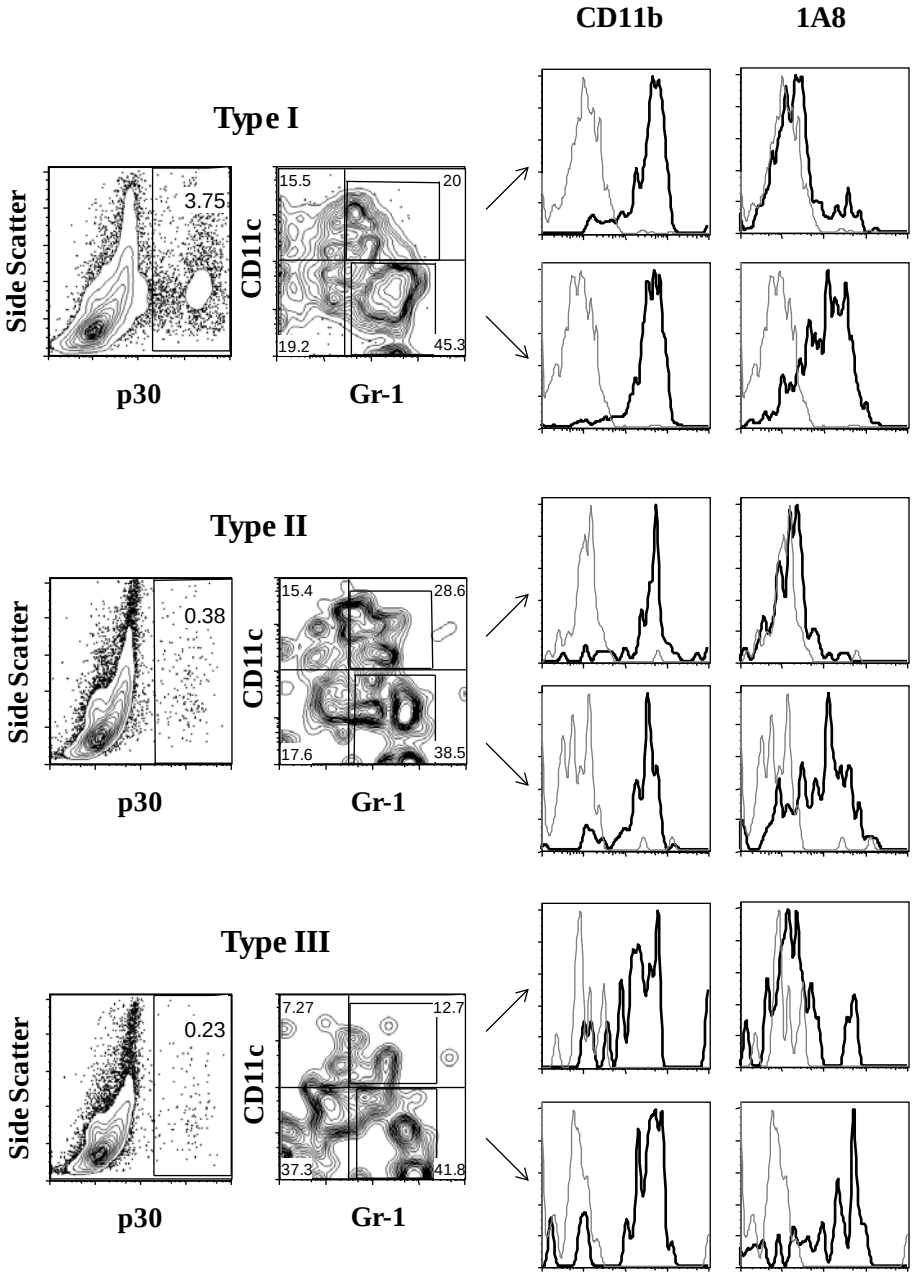

Figure 3.3. Infection of splenic cells does not vary significantly between strain types. Five days post-infection, splenocytes were harvested and stained for tachyzoites (TZ) and surface markers. Cells were gated on presence of tachyzoites, then analyzed for CD11c and Gr-1 expression. Variations in cell populations infected by different strain types were not statistically significant. (Grey histogram = isotype control, black histogram = surface molecule).

Table 3.2. Strain type does not influence infection of different cell types in the spleen. Splenocytes were harvested from mice infected ip with a high dose of tachyzoites on day 5 post-infection, and stained for surface markers and cytokines. In two out of the three experiments, Types I and II tachyzoites were more frequently found in Gr-1-expressing DC compared to Type III; however, a two-tailed Student's t-test determined that the p-value of the percentage of Gr-1^{+} DC in the infected population between RH and PTG was 0.75, and was 0.37 between RH and VEG. Therefore, these variations are not statistically significant.

Spleen, day 5 **% of Infected Cells**

Infection	CD11c+Gr-1-	CD11c-Gr-1+	CD11c+Gr-1+	CD11c-Gr-1-
Type I (RH)				
Experiment 1	15.8	50.3	15.7	18.2
Experiment 2	15.5	45.3	20	19.2
Experiment 3	15.4	29.3	42	13.3
Type II (PTG)				
Experiment 1	15.4	38.5	7.69	38.5
Experiment 2	15.4	38.5	28.6	17.6
Experiment 3	8.13	42.2	30.4	19.3
Type III (VEG)				
Experiment 1	15.8	15.8	18.4	50
Experiment 2	7.27	41.8	12.7	37.3
Experiment 3	12.5	50	20.8	16.7

significantly higher, percentage of $CD11c^+$ dendritic cells. Unsurprisingly, given the higher proportion of these cells in the total population, Type I tachyzoites infect neutrophils and $Gr\text{-}1^+$ cells in general at a higher frequency than Types II and III, and Types II and III infect DC at a higher frequency than Type I. In the spleen on day 5 post-infection, Type I tachyzoites are no longer as restricted to the $CD11c^+Gr\text{-}1^+$ population as they are on day 4. Type I and especially Type II parasites, however, are more closely associated with this population of cells than are Type III in two out of three experiments. Conversely, nearly 40% of Type III tachyzoites are associated with a $CD11c^-Gr\text{-}1^-$ population, possibly lymphocytes or resident $Gr\text{-}1^-$ macrophages, that comprises less than one- fifth of the cells infected with Types I or II. However, the results from the spleen do not rise to the level of statistical significance, indicating that variations in splenic infection between strain types may simply be due to natural experimental variation.

Previous studies examining the variations between infection with different *Toxoplasma* strains have identified differences in cell recruitment, DC motility, and manipulation of cell signaling for induction of cytokines (18, 21, 14). My work confirmed a previous observation that more neutrophils are recruited in response to Type I parasites than Type II after intraperitoneal inoculation (18), and expanded on this finding by showing that Type III parasites are similar to Type II in terms of which cells are recruited to the infection site. One major difference that Type I exhibits relative to Types II and III is growth rate (10), and this is evident in the relative percentages of cells infected by each of these strains on day 3 post-infection. Growth rate may also be responsible for the variation in which cell types are infected. My work recapitulated previous studies (18) showing that Type I is more frequently

associated with neutrophils at the peritoneal cavity compared to Types II and III, but also demonstrated that a higher proportion of infected cells belong to the DC population in Types II and III infection, and that there is a higher total percentage of peritoneal cells expressing CD11c as well. This difference may be due to strain-specific variations in the induction of DC-attractant chemokines, or perhaps all strains induce recruitment of dendritic cells equivalently, but the higher growth rate of Type I leads to faster parasitization and lysis, prior to the day 3 timepoint.

Previous studies have shown that infection with all strains of *T. gondii* increases motility in dendritic cells, preventing the upregulation of the adhesion molecule ICAM-1 and causing more rapid migration to the spleen and lymph nodes compared with LPS-activated DC (21). Out of all the strains, however, Type II induced this hypermotility most strongly (22). My work with Type I intraperitoneal infections identified a population of $Gr\text{-}1^{+}$ dendritic cells that migrated to the spleen from the peritoneal cavity after infection with tachyzoites. At day 5 post-inoculation in the spleen, I found that Type I and II parasites were associated more strongly with $CD11c^{+}Gr\text{-}1^{+}$ cells than Type III parasites; however, this difference was reproduced only twice out of three experiments, and was not statistically significant. The lack of variation between strains in terms of dendritic cell migration to the spleen is surprising, given the strain-specific differences in dendritic cell motility described in other studies (22); nevertheless, the question of whether Types II and III use Gr-1-expressing DC for dissemination in the same manner as Type I remains open.

Further work will be needed to determine whether the presence of a highly-infected splenic population of $CD11c^{+}Gr\text{-}1^{+}$ cells during Type II infection means that these cells are utilized by the parasite in the same fashion as they are by Type I. Nevertheless, the strain type of *T. gondii* clearly influences the cell populations infected in the peritoneal cavity, and these differences may point to strain-specific variations in dissemination of *Toxoplasma*.

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CHAPTER 4

CHARACTERIZATION OF EARLY CELL TARGETS OF *T. GONDII* DURING ORAL INFECTION

Abstract

Toxoplasma gondii invades host cells in a promiscuous manner, and is capable of replication inside all nucleated cell types. In recent years, however, evidence has suggested that *T. gondii* uses specific cell populations such as dendritic cells and natural killer cells for enhanced dissemination throughout the host. In this study, I aim to elucidate the earliest cell populations targeted for invasion in the intestine and the intestinal lymphoid regions, to determine whether the preference for DC manifests at early stages after the natural route of infection. Surprisingly, I found that *T. gondii* infection is mostly in $CD3^+$ cells in the mesenteric lymph nodes, and the largest population of infected cells in the Peyer's patches are $B220^+$, while infection in the lamina propria occurs in $B220^+CD11c^-$ and $Gr\text{-}1^+CD11c^-$ cells. Infection of $MyD88^{-/-}$ mice revealed a diminished recruitment of dendritic cells to both the mesenteric lymph nodes and Peyer's patches, as well as increased infection rates among total cells, but the absence of this adaptor molecule did not appear to affect infection patterns. I conclude that dendritic cells are not the major targets of infection at these time points after oral inoculation in the intestine, but that further work will be needed to determine whether they are targeted at other times during infection.

Introduction

Toxoplasma gondii is a protozoan parasite that infects mammals and birds (1-2) through ingestion of environmentally-hardy oocysts, or tissue cysts in the muscle of prey animals (3). The cyst wall is dissolved in the stomach, releasing the slow-growing form of the parasite, the bradyzoite, into the small intestine (4). Within 18 hours, bradyzoites convert to the rapidly-dividing form, the tachyzoite, which

disseminates throughout the body and leads to chronic infection in the central nervous system and skeletal muscle (5). *T. gondii* is an obligate intracellular parasite, relying on the cells of its host for nutrients as well as for protection from extracellular immunity, and is capable of surviving and replicating inside any nucleated cell type (6-8). Some studies *in vitro* have shown preferential invasion of certain cell types, however, such as a marked preference for immature compared to mature DCs (9), and during *in vitro* infection of macrophages, some cells are invaded by large numbers of parasites while some remain entirely parasite-free (unpublished observations). This indicates that the parasite is perhaps capable of selective invasion, despite not being necessarily restricted to certain cells.

Recent studies have addressed the question of whether *T. gondii*, like some other intracellular pathogens (10), utilizes specific cell types as Trojan horses for spreading to distant tissues. Natural killer cells have been implicated in *in vitro* studies as potential safe havens for *T. gondii* infection, as they are highly susceptible to infection after they lyse infected dendritic cells, and are resistant to killing by non-infected NK cells (11). Studies in macrophages have demonstrated *Toxoplasma*'s capability to suppress various immune-activating functions by innate immune cells, creating a more hospitable environment for the organism. *In vitro* studies have shown that *T. gondii* can downregulate surface molecules necessary for antigen-presentation, such as MHC Class II (12-13), as well as molecules involved in parasite killing, such as iNOS (14-15). The parasite also interferes with apoptotic pathways via prevention of cytochrome c release and therefore disruption of the caspase cascade, as well as activation of the G_i-dependent phosphoinositide 3-kinase pathway and phosphorylation of protein kinase B (16-17). Additionally, intracellular *T. gondii* is capable of dampening the

proinflammatory cytokine response in macrophages by inhibiting chromatin remodeling and blocking translocation of NF-κB to the nucleus, as well as through activation of the signal transducer and activator of transcription 3 (STAT3) (18-20).

Dendritic cells are also likely Trojan horse candidates during oral infection, as *T. gondii* has been shown to modify their behavior in several important ways. *In vitro* studies have shown that dendritic cells acquire a hypermotility phenotype when infected with live parasites, and when transferred to mice, arrive more swiftly to the spleen and the lymph nodes than do LPS-activated DC. Additionally, transfer of tachyzoite-infected DC leads to more rapid parasite dissemination to distant sites in the body than infection with parasites alone (21). Studies of *in vivo* infection by intragastric inoculation of cysts showed that blood $CD11b^+$ and $CD11c^+$ cells bearing parasites cross into the brain (22). Finally, *in vivo* work with the intraperitoneal model of infection has shown a distinct population of Gr-1-expressing DC as hypersusceptible to infection at the inoculation site. The infected $Gr\text{-}1^+$ DC migrate to the spleen, where they are the majority of the infected population. These cells produce IL-12 upon TLR ligand stimulation, but are suppressed in this capability when parasitized (23).

Using an oral model of inoculation with Type II cysts, I find that the infected cell population in the mesenteric lymph nodes appears to be dominated by $CD3^+$ T lymphocytes, which would be expected if infection was nonpreferential as these are the dominant cell population in the lymph nodes. Similarly, the largest cell population in the Peyer's patches, $B220^+$ cells, are also the majority infected population, again

suggesting nonpreferential infection. In the lamina propria, however, the largest infected cell populations are $B220^+$ and $1A8^+$ cells, the latter of which is striking because $1A8^+$ neutrophils only comprise about 1% of the total cell population. These results suggest that cells other than dendritic cells are the targets of infection at early timepoints in the intestinal lymphoid tissues and lamina propria. However, given the findings of Courret *et al*. regarding dendritic cell involvement in transport of tachyzoites to the brain (22), this should not rule out the possibility that dendritic cells are utilized as vehicles for dissemination at other points during oral infection.

Materials and Methods

Mice

Female C57BL/6 mice 6-8 weeks in age were purchased from Taconic Farms (Hudson, NY). $MyD88^{-/-}$ mice on a C57BL/6 background were provided by Dr. E Perlman (Case Western Reserve University, Cleveland, OH) and originally generated by S. Akira (Osaka University, Osaka, Japan). We produced homozygous mice by crosses of heterozygotes, and genotype of the progeny was determined by RT-PCR using the following primers: MyD88(forward) 5'-TGG-CAT-GCC-TCC-ATC-ATA-GTT-AAC-C-3', (reverse) 5'-GTC-AGA-AAC-AAC-CAC-CAC-CAT-GC-3', and (neo) 5'-ATC-GCC-TTC-TAT-CGC-CTT-CTT-GAC-G-3'.

Parasites and Infections

ME49 cysts were maintained by chronic infection in Swiss Webster mice (Harlan). Isolation of cysts was performed by removing the brain and homogenizing with the

plunger of a syringe and repeated passage through an 18-gauge needle. Brain homogenate was then mixed with 40% Percoll and underlaid with an equivalent amount of 80% Percoll, and centrifuged at 1600 RPM for 20 minutes to separate cysts, tissue and red blood cells. Cysts were counted and administered to mice by oral gavage.

Isolation of Lamina Propria Cells

Intestines were flushed with ice-cold PBS and sliced longitudinally, then minced with dissection scissors. Tissue pieces were incubated with PBS + dithiothreitol (Sigma) under rotation for 20 minutes at 37^{o} C, vortexed, and supernatants containing epithelial layers were removed. This step was repeated twice. Remaining tissue was incubated for 20 minutes at 37^{o} C with Liberase CI (0.2 mg/mL) and recombinant DNAse I (0.5 mg/mL) two times, or until all tissue had been digested. Supernatant was immediately centrifuged after each collection and the cell pellet was resuspended in serum-containing PBS. Cell viability was tested using Trypan blue.

Flow Cytometry

Mesenteric lymph nodes and Peyer's patches were obtained from infected mice and digested with Liberase CI for 20 minutes (Roche). Cells were then blocked in a 96-well plate with buffer (1% fetal calf serum in phosphate-buffered saline) and 10% normal mouse serum. Cells were then incubated with antibodies to surface markers (BD Biosciences, BioLegend, Argene, eBioscience) on ice for 30 minutes, washed and fixed in 3% paraformaldehyde. Permeabilization was performed with 0.075% saponin

in phosphate-buffered saline, and cells were then incubated with antibody to *T. gondii* surface antigen, p30, and washed before being analyzed on a FACSCalibur (Becton-Dickinson), and the data was analyzed with FlowJo software (TreeStar).

Immunofluorescence

Intestines were removed from mice and flushed with PBS, then cut into small sections and embedded in OCT freezing medium (Miles Laboratories), and frozen on dry ice. Cryostat sections of 8μM thickness were fixed in acetone and stored at -80^{o} C. Tissue sections were blocked using 2x casein and goat serum, then stained with mouse anti-*Toxoplasma gondii* p30, rat anti-mouse CD3 and rat anti-mouse 1A8. Sections were washed twice in PBS, then stained with AlexaFluor 488 goat anti-mouse, and AlexaFluor 647 goat anti-rat. Slides were washed again and fixed in 4% paraformaldehyde, rinsed in water and mounted with ProLong Anti-fade with DAPI (Invitrogen). Stained sections were visualized on a Leica SP5 confocal microscope.

Histology and Immunohistochemistry

Intestines were removed from mice and flushed with PBS. They were then placed in 10% formaldehyde overnight, and then cut and sent to the Histology Unit, Cornell University College of Veterinary Medicine (Ithaca, NY) for embedding in paraffin and staining for hematoxylin and eosin, as well as staining with rabbit anti-*Toxoplasma gondii* antiserum. Sections were visualized on an Olympus BX51 microscope.

Results

Infection is promiscuous at day 3 post-infection in the mesenteric lymph nodes and Peyer's patches. Three days after oral infection with 100 ME49 cysts, mesenteric lymph nodes and Peyer's patches were isolated and stained for surface markers and parasites (Figure 4.1). In the mesenteric lymph nodes, the cell type most highly represented in the infected population was $CD3^+$ T lymphocytes, accounting for 69% of infected cells, and T cells were also the largest population in the lymph nodes overall, at 57.3%. However, the proportion of infection in innate immune cells was higher due to their lower total frequency, particularly in $CD11c^+$ cells. While $CD3^+$ cells were infected at a rate of only 0.02%, the infection rate in $CD11c^+$ cells was 0.2%. A similar situation was observed in the Peyer's patches, in that $B220^+$ cells were the largest total population, and also the largest population of infected cells, but other cell types were, proportionally, more heavily infected. Overall, though a preference may exist for innate immune cells, all cell types examined are infected at this time point.

Infection is dominated by lymphocytes at days 5 and 7 post-infection. Five and seven days after oral inoculation, cells from mesenteric lymph nodes and Peyer's patches were harvested for flow cytometric analysis of surface markers and parasites (Figure 4.2). On day 5 in the mesenteric lymph nodes, $CD3^+$ T lymphocytes were the primary infected population, comprising 61% of infected cells, followed by $B220^+$ cells at 33%. Cells expressing CD11b, Gr-1, CD11c and 1A8 were infected at a higher rate proportionally to their overall representation in the lymph nodes, however. While $CD3^+$ T cells were only infected at a rate of 0.8%, and $B220^+$ cells at a rate of 0.4%,

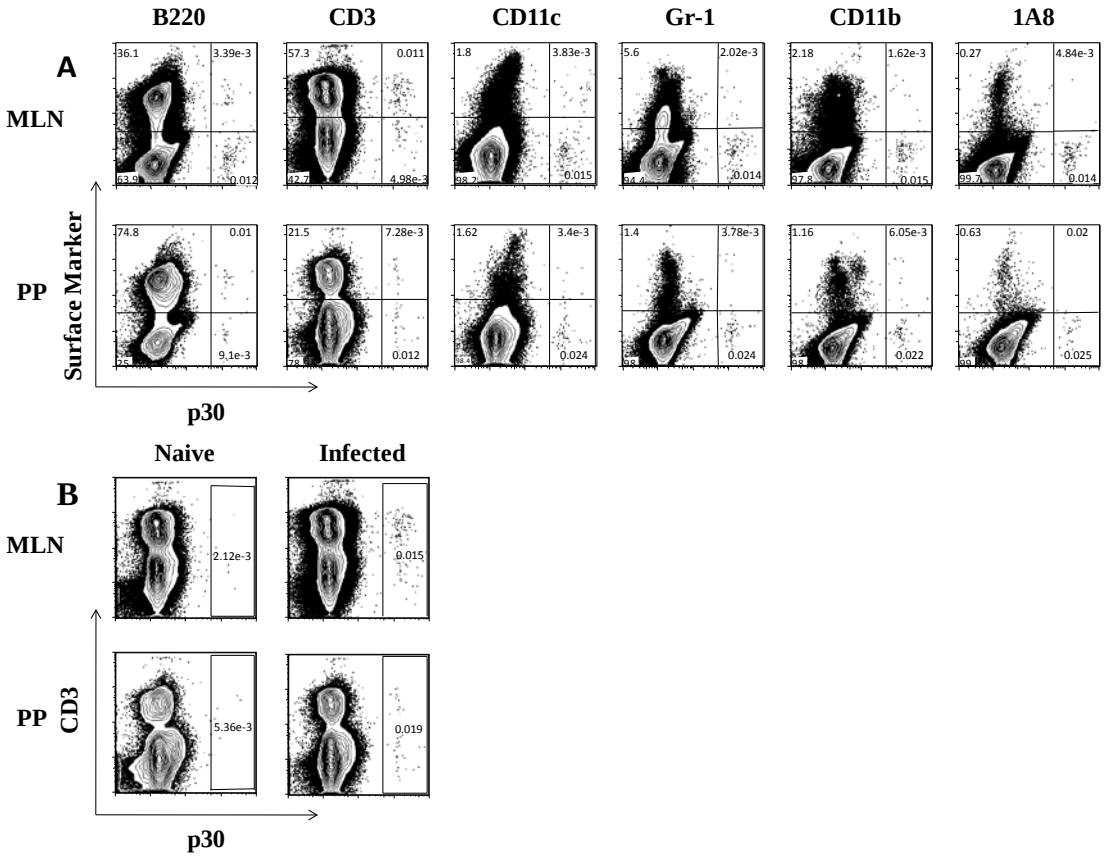

Figure 4.1. Infected cells are primarily T lymphocytes in the mesenteric lymph nodes on day 3 post-infection. Mice were infected with 100 cysts by oral gavage, and sacrificed three days post-infection. Mesenteric lymph nodes (MLN) and Peyer's patches (PP) were removed and digested to a single-cell suspension, then stained for surface markers and intracellular parasites. One million cells were collected per sample. Infection in the mesenteric lymph nodes was mostly in the $CD3^+$ lymphocytes, whereas the infected population was evenly divided among the cell types in the Peyer's patches (A). Infection rates per cell type (as defined by surface marker) are as follows: **MLN** B220: 0.009% CD3: 0.02% CD11c: 0.2% Gr-1: 0.04% CD11b: 0.09% 1A8: 1.8% **PP** B220: 0.02% CD3: 0.05% CD11c: 0.4% Gr-1: 0.7% CD11b: 0.9% 1A8: 3.7% In panel B, noninfected MLN and spleen show very low levels of background staining for parasite p30 (0.00212% in MLN and 0.00526% in PP). This experiment was repeated 4 times.

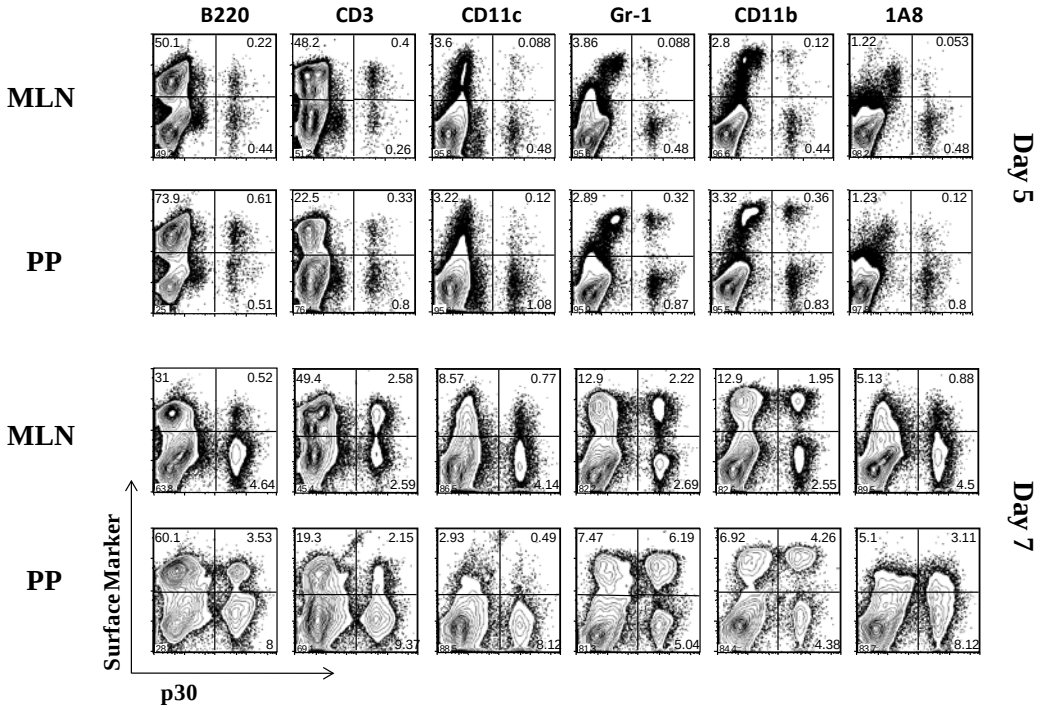

Figure 4.2. Infection in the mesenteric lymph nodes and Peyer's patches is not restricted to a specific population of cells. On day 5 and day 7 after oral infection, mesenteric lymph nodes and Peyer's patches were harvested, digested into single-cell suspensions, and stained for surface markers and parasites. The infected population was dominated by T and B lymphocytes at day 5 in both organs, but the infection in both organs on day 7 was evenly divided between Gr-1^{+} cells and lymphocytes. Infection rates per cell type (as defined by surface marker) are as follows: **Day 5 MLN** B220: 0.4% CD3: 0.8% CD11c: 2.4% Gr-1: 2.2% CD11b: 4.1% 1A8: 10% **PP** B220: 0.8% CD3: 1.4% CD11c: 3.7% Gr-1: 10% CD11b: 9.7% 1A8: 8.9% **Day 7 MLN** B220: 1.6% CD3: 5.0% CD11c: 8.2% Gr-1: 14. 7% CD11b: 13.1% 1A8: 14.6% **PP** B220: 5.6% CD3: 10.0% CD11c: 14.3% Gr-1: 45.3% CD11b: 38.1% 1A8: 37.9% This experiment was repeated 4 times with similar results.

the rates of infection in CD11b, Gr-1, CD11c and 1A8-expressing cells were 4.1%, 2.2%, 2.4% and 4.2%, respectively. This may indicate that although infection is promiscuous, innate immune cells, as defined by expression of CD11c, CD11b, Gr-1 and/or 1A8, are preferred by the parasite. Peyer's patches exhibited a similar pattern on day 5, with $B220^+$ cells making up most of the infected population while innate immune cells showed a higher rate of infection, especially $CD11b^+$ and $Gr\text{-}1^+$ cells, which were infected at rates of 10% and 9.8% respectively, compared to the infection rate of 0.82% in $B220^+$ cells. By day 7, innate immune cells accounted for more of the total cell population in both lymphoid sites, and represented about half of the total infected cells in lymph nodes, and nearly two-thirds in the Peyer's patches. Overall, infection in the lymph nodes and Peyer's patches appeared to favor cells expressing innate immune cell markers over lymphocytes; however, all cell types were invaded at these timepoints.

MyD88 expression affects recruitment of dendritic cells and total infection rate, but not composition of the infected cell population. $MyD88^{+/+}$ and $MyD88^{-/-}$ mice were infected with 100 cysts and mesenteric lymph nodes and Peyer's patches were stained for flow cytometry on days 5 and 7. Infection rate in $MyD88^{-/-}$ animals was heavier at both timepoints, as about 0.3% of cells were infected in wild-type MLN compared to greater than 5% in the knockout, and 0.32% of cells were infected in wild-type Peyer's patches compared to 1.27% in the knockouts (Figure 4.3 for day 7, data not shown for day 5). This confirmed previous results showing that parasite burden is higher in MyD88-deficient animals (24-25). Nevertheless, the absence of this signaling molecule did not appear to affect which cells were parasitized, suggesting that MyD88

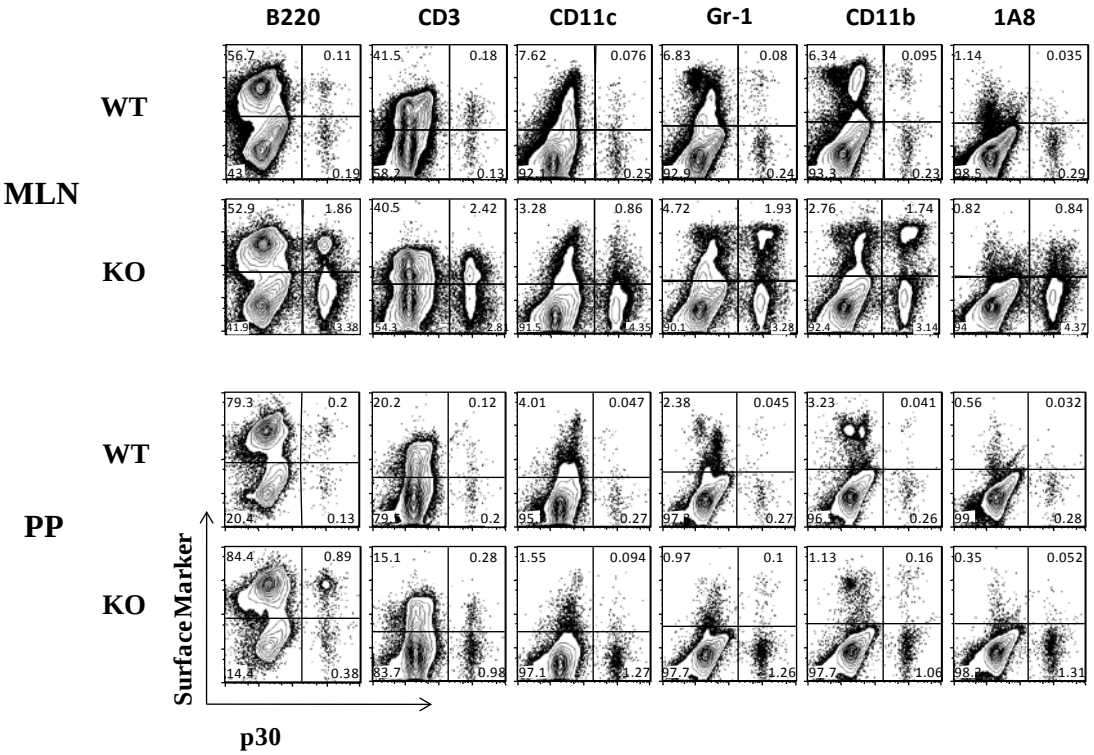

Figure 4.3. MyD88 expression does not affect infection of cells by *T. gondii*. MyD88$^{+/+}$ and MyD88$^{-/-}$ mice were infected with 100 cysts, and mesenteric lymph nodes (MLN) and Peyer's patches (PP) were isolated for flow cytometry 7 days post-infection. While MyD88$^{-/-}$ mice exhibited slight deficiency in recruitment of DC to these sites, the cell types comprising the infected population were not noticeably different in the absence of MyD88. Experiment was performed three times, with similar results.

signaling pathways are not a factor in parasite preference for particular cell types. However, at day 7 post-infection, there was a small but reproducible decrease in the dendritic cell population in the MLN of MyD88$^{-/-}$ mice, from 7.62% in wild-type lymph nodes to 3.28% in the MyD88-deficient animals. Over three experiments, the average decrease between wild-type and knockout mesenteric lymph nodes was 5.8 to 3.01.

1A8 cells are disproportionately parasitized in the lamina propria, and comprise half of the infected population. To examine infection in the lamina propria, I used two high doses of cysts, 500 and 1500 per mouse, as the 100 cyst dose yielded an extremely low percentage of infection in this tissue (Figure 4.4 and data not shown). Infection rate was too low to draw definitive conclusions from the 500 cyst dose. However, these results show a similar pattern to what is observed in the 1500 cyst dose. In both cases, the infected population was mostly divided between B220^{+} cells and 1A8^{+} cells. B220^{+} cells comprised 39%, and 1A8^{+} cells comprised 44% of the infected population after the 500 cyst infection, while B220^{+} cells accounted for 41% of infected cells after the 1500 cyst dose, and 1A8^{+} cells for 30%. Although 60.8% of total LP cells express B220, however, less than 7% express the B cell marker CD19 (data not shown), and none of the CD19-expressing cells harbor parasites, leaving the identity of the larger B220-expressing population, including the infected cells, ambiguous. Nevertheless, as B220^{+} cells are the dominant total population, their strong representation in the infected population was unsurprising. However, the strong presence of infected 1A8^{+} neutrophils, which comprised only 1% of the lamina propria

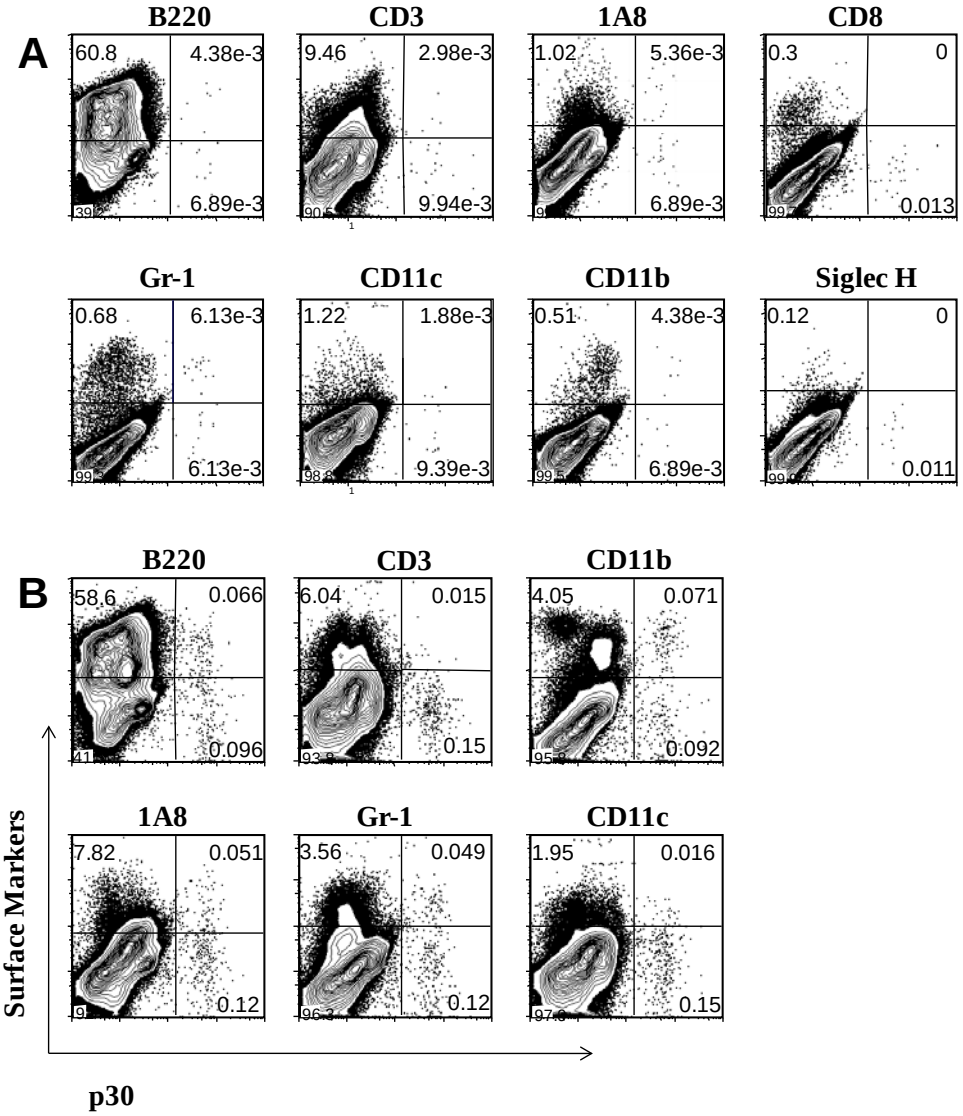

Figure 4.4. Infected cells in the lamina propria are mostly B220^{+} cells and neutrophils. Lamina propria from mice infected with 500 (A) or 1500 (B) cysts were harvested on day 4 post-infection, digested into a single-cell suspension and stained for surface markers and tachyzoites. 200,000 cells were collected for analysis. Dendritic cells and T cells were less strongly represented in the infected population, whereas neutrophils and B220^{+} cells were the major cell types parasitized. Infection rates per cell type as determined by surface marker are: **500 cyst dose**: B220: 0.007% CD3: 0.03% 1A8: 0.5% Gr-1: 0.9% CD11c: 0.15% CD11b: 0.9% **1500 cyst dose:** B220: 0.1% CD3:.2% CD11b: 1.7% 1A8: 0.6% ‘ Gr-1: 1.4% CD11c: 0.8% The 1500-cyst dose was repeated 3 times.

cells after a 500-cyst dose, and only 8% with 1500 cysts, was striking in comparison to the relatively non-preferential infection in the mesenteric lymph nodes and Peyer's patches.

Infected cells are located primarily in the lamina propria region.

The method that I used to isolate lamina propria cells for FACS analysis requires removal of the epithelial cells, and therefore does not give a complete picture of the non-Peyer's patch cells in the intestine. To determine whether epithelial cells were targets of infection, or whether infection was mostly located in the lamina propria, I infected mice for four days with 1500 cysts, and had sections of intestine stained for *Toxoplasma* by immunohistochemistry. My results showed parasite staining in the LP regions of the villi (Figure 4.5), and only infrequent staining in epithelial cells, confirming that the lamina propria was the primary location of infected cells on day 4 post-inoculation.

Evaluation of flow cytometry staining by immunofluorescence for CD3

Digestion of the lamina propria for flow cytometry, while it can be a useful technique, also carries drawbacks. One of the primary difficulties with using this method for assessing populations of infected cells is the fact that the enzymes can digest surface molecules, leading to inaccurate results (26). The cells can recover their surface marker expression in culture, but culturing the cells carries the risk of parasites escaping the cells infected in the mouse, infecting other cells in culture, and rendering false results. My lamina propria preparations showed an unusually small population of

$CD3^+$ cells, and I hypothesized that this may be due to the loss of surface molecules. To address this problem, I stained frozen intestinal tissue sections for CD3, to confirm whether the percentage of these cells that I observed by flow cytometry seemed comparable to the proportion in whole tissue. Previous characterizations of cell populations in the lamina propria have indicated that about 40% of the cells should be T lymphocytes (27). The immunofluorescence, like the flow cytometry data, did not show a majority of cells expressing $CD3^+$ in either the proximal (Figure 4.6 A) or the distal (Figure 4.6 B) regions of the intestine. However, the proportion of $CD3^+$ cells to total LP cells did appear to exceed the 6 to 10% that I had seen in my flow cytometry experiments, and therefore testing of shorter incubation times or different digestion enzymes may be desirable for optimizing the accuracy of the lamina propria flow data.

Discussion

Hijacking and manipulation of host cells by *Toxoplasma gondii* has been a topic of much investigation over the last several years. *In vitro* work in macrophages demonstrated that *Toxoplasma* infection can downregulate MHC Class II and block apoptosis (12-13, 16-17), and a recent study showed that natural killer cells are readily infected and resistant to lysis by uninfected NK cells afterward (11). These studies suggest that macrophages and NK cells could be reservoirs for the parasite. However, several studies point to modulation of motility and effector function in dendritic cells as indicators that *T. gondii* may use these cells as vehicles for silent dissemination throughout the body (21-23). My work with an intraperitoneal model of infection showed that Type I tachyzoites preferentially infect Gr-1-expressing DC compared to other cell types in the peritoneal cavity, and suppress their ability to produce IL-12.

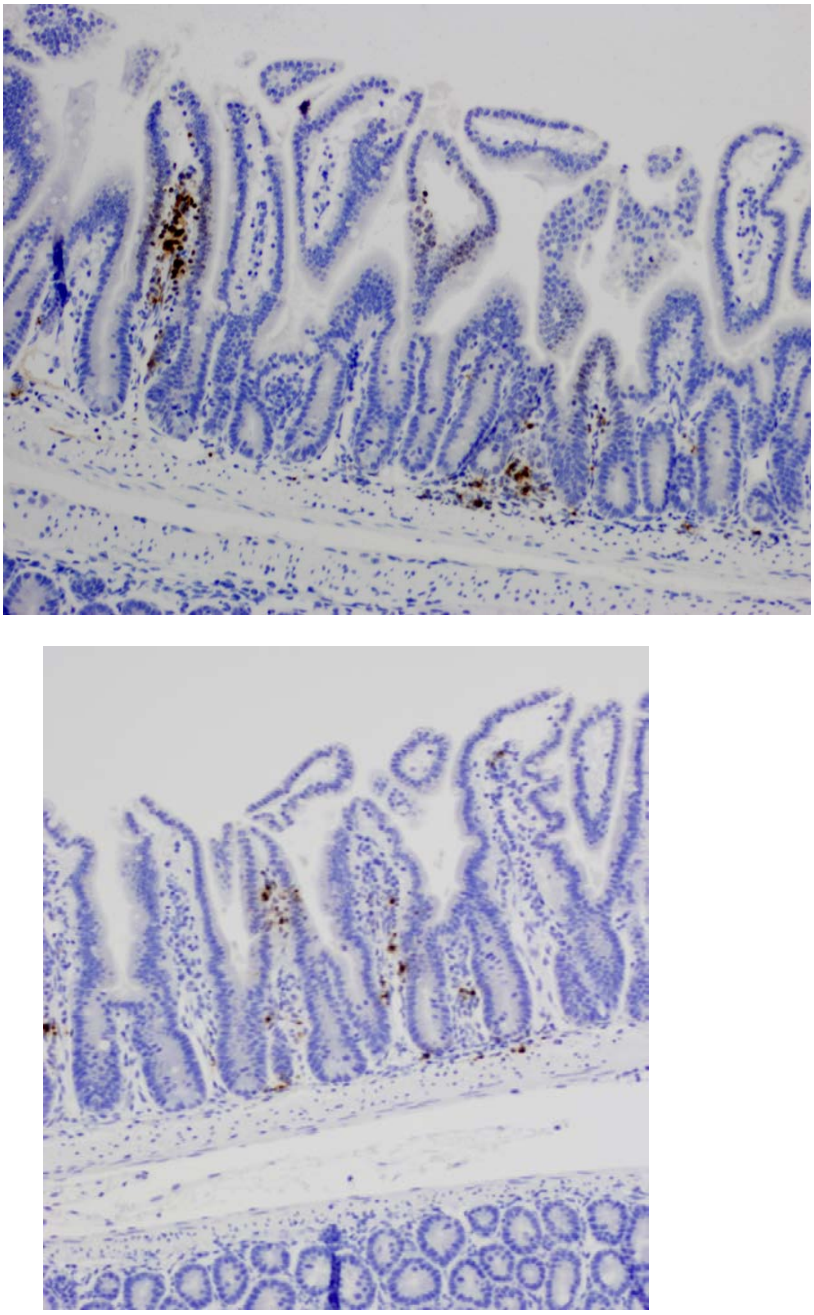

Figure 4.5. Parasites are mostly located in the lamina propria of the intestine. Intestines were harvested on day 4 post-infection, fixed in formalin, and embedded in paraffin for immunohistochemical staining. Staining for *Toxoplasma* was performed using rabbit anti-*T. gondii* serum. Patches of *T. gondii* staining (brown) are visible in the lamina propria regions of the villi. Parasite staining was infrequently observed in epithelial cells.

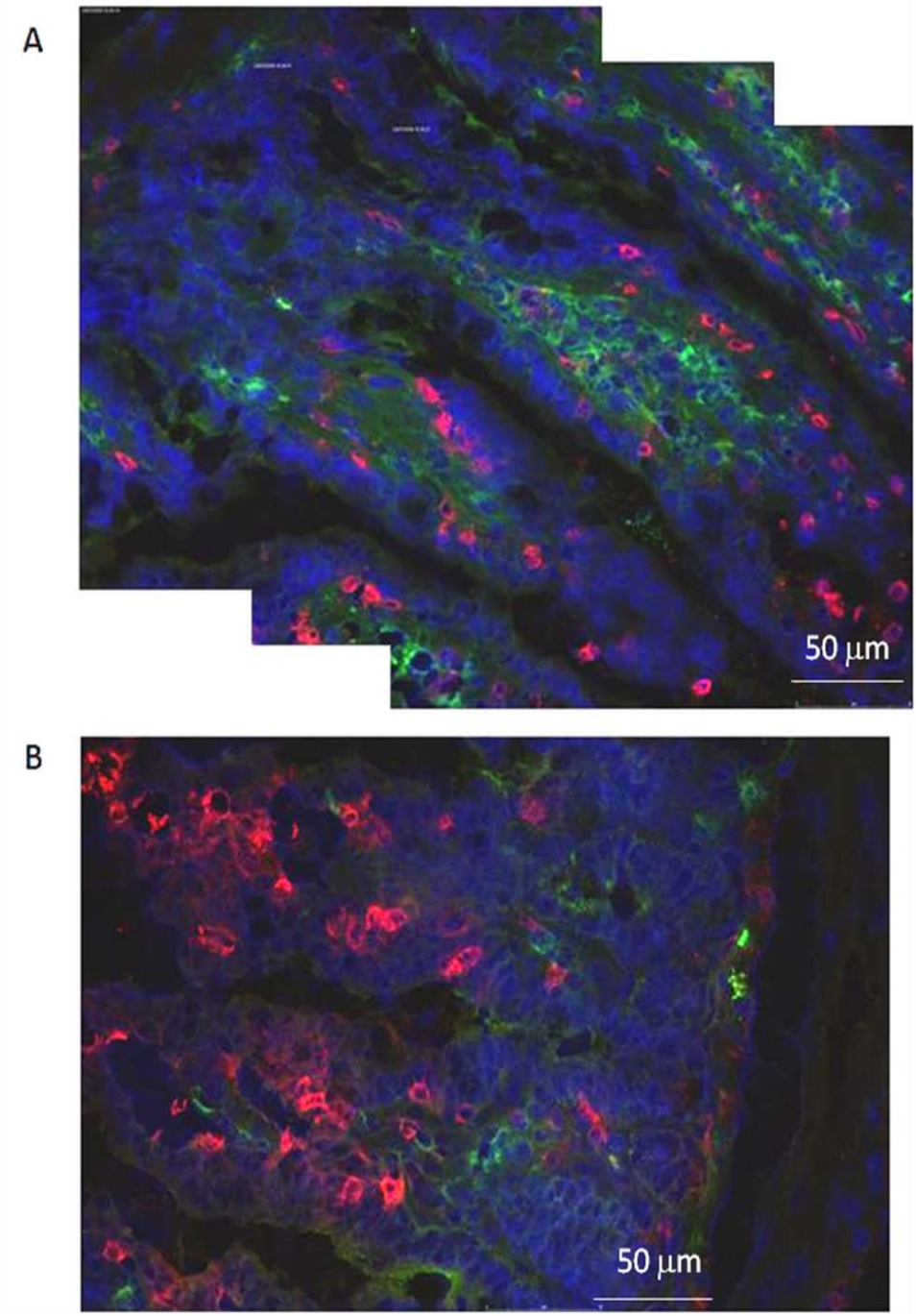

Figure 4.6. CD3$^+$ cells are a substantial population in the proximal and distal sections of the intestine. Frozen intestinal tissue was cut into 8μM-thick tissue sections and stained with antibodies to CD3 (red) and DAPI (blue). Background staining with AlexaFluor 488 anti-mouse antibody is in green, and images are composites of sequential sections. CD3$^+$ cells were visible in the proximal (A) and distal (B) regions of the intestine, but did not appear to comprise the majority of cells.

These DC migrate from the inoculation site to the spleen, where they are the primary population of infected cells. Later work revealed that Type II tachyzoites are also strongly associated with Gr-1-expressing DC in the spleen during acute infection, but that Type III parasites are not, possibly indicating that strain type determines whether tachyzoites use DCs for this purpose (Chapter 3).

In this study, I used the oral model of infection with cysts of a Type II strain, ME49. The oral model, while not allowing as precise and consistent a dosage as the intraperitoneal route of infection, is an important one in *T. gondii* research, as it mimics the natural route of inoculation. The question of whether DC were Trojan horses not only in the intraperitoneal model, but also in the oral model, was therefore critical to discerning whether this phenomenon is relevant in natural infections. I examined several tissues from the gut, where bradyzoites transform into tachyzoites and begin their migration to other sites of the body. In lymphoid regions such as the mesenteric lymph nodes and Peyer's patches, I found that innate immune cells had a higher frequency of infection than lymphocytes, but that lymphocytes comprised a greater percentage of the overall infected population, likely due to the fact that they were the major cell population in these tissues overall. This indicates that although *T. gondii* may exhibit a preference for innate immune cells, infection is promiscuous at early sites. Promiscuity at early sites of infection is consistent with my findings in the intraperitoneal model, where most cell types were infected in the peritoneal cavity.

Interestingly, however, I found that infection was mostly restricted to two populations in the lamina propria: B220-expressing cells, and $1A8^{+}$ cells. I increased the infection

dose in order to produce a larger population of infected cells for visualization by immunofluorescence. I also noted that the $CD3^+$ T lymphocyte population was unexpectedly small in my lamina propria preparations, as they typically represent almost half of the immune cells in this tissue (27). This result suggested to me that perhaps the long incubation of the lamina propria tissue in digestive enzymes was altering the expression of cell surface molecules, potentially leading to inaccurate results. However, this digestion was necessary to obtain a single-cell suspension for flow cytometry. Therefore, to confirm the validity of my flow data, I stained frozen sections of intestine for CD3. In contrast to the large numbers of $CD3^+$ cells I expected to see, I found a minor population consistently throughout the intestine, mostly in the villi, and at similar levels in both the proximal and distal regions. Still, the proportion of CD3-expressing cells appeared to be higher than the proportion in my cell preparations for flow cytometry, indicating that further optimization of the digestion steps, including adjustment of time and perhaps varying the enzymes or enzyme concentrations, would be helpful. Additionally, further immunofluorescence would be desirable in order to determine the location of the infected neutrophils and $B220^+$ cells.

The preferential infection of neutrophils in the lamina propria raises the question of what role these cells are playing. In relation to the Trojan horse question, neutrophils have been observed to be important reservoirs for establishing infection with other intracellular pathogens, such as during infection with species of *Leishmania (*28-30*)*. Parasites infect neutrophils, which produce macrophage inflammatory protein (MIP-1β) and undergo apoptosis, and are then phagocytosed by the recruited macrophages where the parasite propagates (28). An alternative model has also been proposed in

which viable parasites are released from apoptotic neutrophils and then taken up by macrophages, but depletion of neutrophils impairs establishment of infection, implying that neutrophil infection or apoptosis is a critical step regardless (29). Nevertheless, depletion of neutrophils during *Toxoplasma* infection leads to higher parasite burden, which seems to rule out the possibility that a similar phenomenon is at work (31-32). Additionally, as-yet-unpublished results in our laboratory have confirmed the susceptibility of neutrophil-depleted mice using the neutrophil-specific antibody 1A8. Therefore, further work will be necessary to determine the significance of the apparent preferential infection of neutrophils in the lamina propria, including examination of chemokines being produced to determine whether infected neutrophils attract other cells to assist in parasite dissemination, observation of whether neutrophils infected in this tissue kill parasites or whether parasites persist, and tracking of neutrophils to determine whether they migrate from the lamina propria once infected.

Myeloid differentiation factor 88 (MyD88) plays a number of roles in *T. gondii* infection. As an adaptor molecule for Toll-like receptor signaling, it is involved in the induction of proinflammatory cytokines by recognition of *Toxoplasma*, and is therefore important for survival of infection. Knockout studies in mice show that in the absence of this molecule, animals show increased susceptibility to intraperitoneal and oral infection in that parasite replication is uncontrolled, and IL-12 production is defective in innate immune cells, with reduced levels of IL-12 overall in the blood (33). However, work with oral infections has shown that induction of adaptive immunity is independent of MyD88 (34). Additionally, infected MyD88 knockout mice display defective recruitment of neutrophils to the lamina propria after oral

inoculation and to the peritoneal cavity after intraperitoneal infection (34), implying that signaling through this molecule plays a part in determining what cells are present at the infection site. Therefore, MyD88 signaling may indirectly influence which cell types are targeted by tachyzoites. However, my work showed that there was no defect in cell recruitment to the mesenteric lymph nodes or Peyer's patches on day 5 post-infection, and while there was a reproducible defect in recruitment of $CD11c^+$ DC to these regions on day 7, there did not appear to be major differences in the cell types targeted by the parasite. Therefore, MyD88 signaling does not appear to be involved in parasite preference for certain cell types.

Previous studies with oral infections have suggested that $CD11c^+$ cells are infected by tachyzoites shortly after infection in the lymphoid regions of the intestine. However, these studies were based on isolation of dendritic cells and subsequent PCR to test for the presence of parasites, a technique that could easily render misleading results if the dendritic cell isolation was not completely pure (22). My work using flow cytometry identifies infected cells more directly, and is therefore a more accurate picture of which cell types are targets of the parasite in these tissues. My work does not show preferential targeting of $CD11c^+$ cells in the mesenteric lymph nodes, Peyer's patches or lamina propria in the first week after infection, compared with other innate immune cells. However, studies have shown that *Toxoplasma* infection induces a state of hypermotility in dendritic cells, and that Type II parasites are the most efficient inducers of this increase in migration ability (21, 35). Therefore, although *T. gondii* may infect cells promiscuously in early tissues, this does not indicate that dendritic cells are not being used as Trojan horses; rather, it is possible that while all cell types get infected, dendritic cells are the only cell type in which increased migration is

promoted. Additionally, four days post-infection is likely too late to identify the very earliest targets of infection, as parasites have been detected in the intestine at 2 days post-infection (22). However, detecting very small numbers of infected cells at these early timepoints has proved technically challenging. Therefore, the possibility remains that the earliest cell targets may be dendritic cells, and this will require further study.

This study has elucidated the cell types parasitized at the early sites of infection after oral inoculation, showing that *T. gondii* infects all cell types in the mesenteric lymph nodes and Peyer's patches, and shows a preference for neutrophils in the lamina propria. Although these findings do not definitively point to a role for any cell type in enhancing the spread of *Toxoplasma*, they are useful in that this is the first study to directly characterize infected cells in the intestine by flow cytometry.

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Chapter 5

DISCUSSION

Summary of Findings

Toxoplasma gondii infects mammals and birds by ingestion and is released from cysts into the intestinal tract as bradyzoites. Bradyzoites convert to tachyzoites and migrate to distant sites throughout the body before transforming into quiescent tissue cysts in the skeletal muscle and brain (1-5). An obligate intracellular parasite, *T. gondii* infects host cells and suppresses their proinflammatory cytokine production, ability to stimulate naïve T cells, and apoptosis pathways in order to evade killing by the immune system (6-11). Several *in vitro* and *in vivo* studies have attempted to elucidate whether *T. gondii* uses certain cell types as Trojan horses (12-13). In Chapter 2, I describe a population of Gr-1-expressing dendritic cells that are preferentially parasitized by *T. gondii* after intraperitoneal infection at the inoculation site, and comprise the majority of the infected cells in the spleen at day 4 post-infection. Using adoptive transfer with congenic mice, I demonstrate the ability of these infected DC to migrate from the peritoneal cavity to the spleen, and show with CCR2 knockout mice that the splenic population of $CD11c^{+}Gr\text{-}1^{+}$ cells is diminished, suggesting a role for this chemokine receptor in either the recruitment of these cells to the site of infection, or in migration from the infection site to the spleen. Finally, I cultured splenocytes with LPS and CpG and examined IL-12 production by flow cytometry, finding that *T. gondii* infection suppressed IL-12 induction in $CD11c^{+}Gr\text{-}1^{+}$ cells.

The three major *Toxoplasma* strain types found in human infection in Europe and North America, Types I, II and III, show differences in growth rate and ability to modulate cytokine production, as well as in the ability to stimulate dendritic cell motility (14-17). In Chapter 3, I examined whether the preferential infection of $Gr\text{-}1^{+}$ DC by Type I tachzyoites that I had noted in Chapter 2 was a strain-specific

phenomenon. I found that there were no significant differences between the strain types on day 5 post-infection in the spleen, with regard to which cells comprised the infected population. More work would be needed to determine whether Types II and III use this cell type for dissemination in the same manner as Type I, but these experiments did not demonstrate differences between strain types in terms of which cell populations are parasitized during acute infection in the spleen. I did, however, observe differences between strain types in the percentages of dendritic cells and neutrophils comprising the infected population at the inoculation site three days post-infection, in that Type I-infected mice showed greater percentages of neutrophils in the infected population, and Type II- and Type III-infected mice showed greater percentages of DCs. This may indicate a difference in recruitment of cells to the infection site depending on strain type, or perhaps that all strain types preferentially infect DC, but the higher growth rate of Type I tachyzoites leads to lysis of infected DC prior to this timepoint.

The intraperitoneal model of infection is a useful tool for keeping dosages consistent for *T. gondii* infection, as tachyzoites can be counted and delivered in precise quantities, whereas tissue cysts can contain varying numbers of bradyzoites, and therefore do not always give a consistent infection rate. Additionally, the intraperitoneal model allowed me to easily recover cells from the inoculation site. For the work described in Chapter 4, however, I wanted to assess the relevance of my work in the intraperitoneal model to the natural route of infection, which is simulated in the laboratory by delivery of tissue cysts to the stomach via oral gavage. I examined early sites of infection by flow cytometry, such as the lamina propria, the mesenteric lymph nodes and the Peyer's patches, and determined that in the latter two regions,

infection appeared nonpreferential at days 3, 5 and 7 post-infection. All the immune cell populations that I examined were infected, and the largest total cell populations were also the most strongly represented cell types in the infected population; nevertheless, preferential infection may take place earlier in these regions. However, this was not the case in the lamina propria, where infected cells were mostly $B220^+$ and $1A8^+$. I was concerned about the validity of my flow cytometry data, however, because the $CD3^+$ population in my lamina propria preparations was unexpectedly low. Long enzymatic digestion of the tissue can destroy surface molecules (18), and so I sought to confirm the low numbers of $CD3^+$ cells by immunofluorescence. I also used immunohistochemistry to confirm that only the lamina propria was infected, as I washed off the epithelial cells during preparation, and indeed found that epithelial cells were not targeted by *Toxoplasma*.

Trojan horses during *T. gondii* infection

The possibility that *Toxoplasma gondii* utilizes innate immune cells as safe havens or vehicles for dissemination is supported by a great deal of *in vitro* and *in vivo* evidence. The parasite not only prevents apoptosis in macrophages (10-11), but also diminishes the expression of the major histocompatibility complexes on macrophages and DC and prevents immature dendritic cells from upregulating CD40 (8-9), thereby avoiding antigen presentation. *In vitro* studies also showed the capacity of *T. gondii* to prevent TLR-triggered cytokine production by bone-marrow-derived macrophages (19-22). My work confirmed this latter finding, showing that IL-12 from splenic Gr-1-expressing dendritic cells is diminished when these cells are parasitized during infection, suggesting that the parasite's ability to block cytokine production induced by microbial stimuli is a relevant phenomenon during infection.

The downmodulation of immune functions in parasitized cells suggested the possibility that the Trojan horse phenomenon, in which intracellular pathogens hijack host cells for use in silent dissemination, is present in *T. gondii* infection. Previous studies showed evidence for dendritic cells as the target, showing that *Toxoplasma* infection increased the ability of bone-marrow-derived dendritic cells to cross endothelial cell monolayers *in vitro*, and transfer of infected DC to mice led to more rapid parasite dissemination *in vivo* (17). My work demonstrates that in an intraperitoneal infection model with Type I parasites, infected DC transferred via the peritoneal cavity do traffic to the spleen. This suggests that the large infected $CD11c^{+}Gr\text{-}1^{+}$ population that I observe in this organ on day 4 after infection is due to migration of parasitized cells from the inoculation site. When considered in conjunction with the studies showing increased DC motility after infection, this finding suggests that *Toxoplasma* hijacks dendritic cells specifically at the inoculation site to enhance its dissemination to other sites in the host.

The differences in pathogenesis between strain types has been a topic of much research recently, as infection with different strains leads to distinct disease outcomes (23-25). Disparities in growth rate and cytokine suppression, two of the major strain differences, have been linked to recently-identified virulence factors (26-27), and a number of other strain variations have yet to be explained. For example, at low doses of infection, Type I tachyzoites induce greater influx of neutrophils to the inoculation site in intraperitoneal infection, whereas Type II parasites induce more $Gr\text{-}1^{+}$ monocytes (28). My work showed a greater percentage of neutrophils on day 3 post-inoculation with Type I parasites in the peritoneal cavity, but I also noted higher levels

of dendritic cells among the peritoneal exudate cells with Types II and III. While this may indicate that strain types induce recruitment of distinct cell populations, it may also simply be a result of the faster growth rate of Type I parasites, in that all strains may infect dendritic cells preferentially, but Type I lyses them prior to day 3.

Previous work examining the cellular targets of *T. gondii* infection after oral inoculation with cysts has identified $CD11c^+$ cells as targets in the mesenteric lymph nodes, and $CD11c^+$ and $CD11b^+$ cells as parasite-bearing cells in the blood and brain. (29). However, this study examined infection by isolating dendritic cells and performing PCR to determine infection, a method which is only as reliable as the purity of the cell preparations. My work using flow cytometry shows that after a dose of 100 cysts, most cell types are infected in the lymph nodes and Peyer's patches on days 3, 5 and 7 post-infection, with lymphocytes, which are the largest populations in those sites, accounting for the largest populations of infected cells at days 3 and 5. This seems to suggest that at these timepoints, and at these sites, infection is not preferential. However, this does not rule out the possibility that preferential infection of DC takes place at other timepoints, or with different dosages of cysts.

Infection of the lamina propria seems to be preferential toward $1A8^+$ cells, as these and $B220^+$ cells comprise most of the infected population. Work in other models of infection with intracellular pathogens, including *Leishmania major* and *Chlamydia pneumoniae,* have proposed a role for neutrophils in establishing productive infections, allowing pathogens to silently enter and propagate in macrophages (30-31).

It is unlikely that this phenomenon occurs in *Toxoplasma* infection, as depletion of neutrophils with an anti-Gr-1 antibody leads to overwhelming parasite proliferation and therefore they do not seem to be necessary for establishment of infection (32). Caution must be exercised when interpreting this result, however, as Gr-1 is expressed on many other cell types, and therefore the lack of neutrophils is likely not the only factor in this enhanced susceptibility. Additionally, studies in our lab have shown that neutrophils stimulated with *T. gondii* release chemokines that are attractants for immature dendritic cells (33). Therefore, the possibility exists that neutrophils in the lamina propria, acting as parasite reservoirs, attract dendritic cells in order to infect them with tachyzoites, which then potentiate the dendritic cell migration to other areas.

Future questions

Several points still require study and clarification. Although I found fairly strong association of Type II parasites with $CD11c^{+}Gr\text{-}1^{+}$ cells in the spleen after intraperitoneal infection, this does not necessarily mean that the phenomenon I observed with Type I tachyzoites has been recapitulated. In order to gather more evidence that Type II parasites are also using this cell population as Trojan horses, I would need to perform at minimum an adoptive transfer experiment of Type II infected peritoneal cells into infected mice to determine whether they migrate to the spleen. Additionally, studying whether Type II parasites inhibit IL-12 production from these cells, as do Type I tachyzoites, would give further evidence for or against a Trojan horse hypothesis. The other major question raised by the strain comparison experiments is the reason behind the higher total percentages of DC at the infection site, and the higher percentage of DCs in the infected population, after inoculation

with Types II and III. There are two major possibilities, one of which is that Types II and III induce different chemokines than Type I, leading to greater recruitment of DCs, and that these parasites infect DC preferentially. The other possibility is that all strain types recruit and infect dendritic cells equally, and the disparity is simply the result of higher growth rate and faster lysis by Type I tachyzoites. The simplest approach to answering this question would be to harvest peritoneal cells on days 1 through 3 post-infection, to determine whether dendritic cell levels with Type I are comparable to those during infection with Types II and III at earlier timepoints. Additionally, analysis of variations between strain types in terms of induction of chemokine production by peritoneal cells could point to differences in recruitment as well.

A major question arising from the oral infection experiments is the rationale behind the preferential infection of neutrophils in the lamina propria. A small population of cells, they are nonetheless as strongly represented among infected cells as the largest total cell population, B220-expressing cells. Determining the reasons behind the apparent preference of *Toxoplasma* for infecting neutrophils will be necessary in order to decipher whether they play a role in dissemination. If neutrophils are acting as reservoirs for parasite and simultaneously recruiting immature DC, perhaps to act as vehicles for carrying the parasite elsewhere, depletion of neutrophils may diminish the levels of DC found in the lamina propria after infection, which could be examined by flow cytometry or immunofluorescence. Alternatively, comparing cell motility of isolated infected vs noninfected neutrophils would test the possibility that they are themselves disseminators of parasites.

In summary, I have elucidated a mechanism for dissemination by *T. gondii* in the intraperitoneal model of infection, observed differences in cell recruitment and targeting by varying strains of *Toxoplasma*, and have identified the early cellular targets of the parasite after oral inoculation. Several questions remain, and will need to be answered in order to determine whether DC are used as Trojan horses in different *Toxoplasma* strains as well as during oral infection, and what role neutrophils play as the early targets of infection in the intestine.

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