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Investigation of host responses upon infection of distinct *Toxoplasma* strains

Rachel DeVonne Hill
rmcmull1@utk.edu

To the Graduate Council:

I am submitting herewith a dissertation written by Rachel DeVonne Hill entitled "Investigation of host responses upon infection of distinct *Toxoplasma* strains." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

Chunlei Su, Major Professor

We have read this dissertation and recommend its acceptance:

Todd Reynolds, Tim Sparer, Jun Lin, Gladys Alexandre

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

Investigation of host responses upon infection of distinct *Toxoplasma* strains

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Abstract

Toxoplasma gondii is the causative agent of Toxoplasmosis in human and animals. *T. gondii* isolates are highly diverse. Hundreds of genotypes have been identified, but only three clonal lineages, namely Type I, II and III are prevalent worldwide. In mouse model, *T. gondii* strains can be divided into three groups based on their virulence, including the virulent (LD_{100}=1), the intermediately virulent ($LD_{50} = 10^3$-10^4) and the non virulent ($LD_{50} > 10^5$). The clonal Type I, II and III *T. gondii* strains belong to these three groups, respectively. Epidemiologic studies suggest the difference of virulence in mice may relate to the severity of toxoplasmosis in human infection. Therefore, it is necessary to understand biological differences in genetically different *T. gondii* strains and their effect on the host responses. To date, the majority of data published on this aspect has been limited to in vitro assays. Here, we used in vivo assays to investigate host responses upon infection of distinct *Toxoplasma* strains.

Our studies examined host response to infection of the three widespread clonal lineages of *T. gondii* using a mouse model. The following results were revealed: (i) increased tissue burden in mice is the indicator of virulence of *T. gondii*. Quantification of parasite burden in the spleen of mice showed significantly more parasites for Type I strain than that of Type II and III strains, with the latter two having comparable parasite burdens. Given that the Type II strains are more virulent than the Type III strains in mice; this result suggests that difference in host response is the result of specific parasite-host interaction, which is not simply due to the difference of parasite tissue load. (ii) gene expression in the host is strongly influenced by parasite genetic background. Transcriptional profiles of mice infected with the above three types of *T. gondii* strains showed that the overall gene expression patterns are similar between Type I

and Type II infected mice and both stimulated stronger and more polarized change comparing to Type III strain. These results emphasize the importance of studying *T. gondii* pathogenesis in the host with the consideration of parasite genetic diversity. Such research could possibly aid in select appropriate regimes to treat toxoplasmosis caused by diverse *T. gondii* strains.

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List of Abbreviations

T. gondii	*Toxoplasma gondii*
Vir	Virulent
Int	Intermediate
Non	Non- Virulent

CHAPTER I. LITERATURE REVIEW

Apicomplexa phylum

The Apicomplexa phylum is a large group of protozoan microorganisms. The name characterizes key features of these organisms to include a unique organelle apical complex structure. The apical complex structure aids in penetration of the organism into host cells. Members of this phylum have great biological and veterinary significance, such as *Neospora, Hammondia*, *Plasmodium* and *Toxoplasma gondii*. For example, Plasmodium- causative agent of malaria, Neospora- pathogen in cattle and dogs and Sarcocystis- pathogen in mammals and snakes.

Toxoplasma gondii biological significance:

T. gondii is an obligate intracellular parasite that can infect any warm blooded vertebrate and birds. It is the only known species in the genus *Toxoplasma* and is considered one of the most successful eukaryotic pathogens in the world. *T. gondii* is the causative agent of toxoplasmosis. It infects up to one third of the human population (Tenter AM, 2000). In immunocompromised individuals, infection can cause severe encephalitis, potentially leading to death in some cases. Acute infections in pregnant women can result in a condition known as congenital toxoplasmosis, in which symptoms could be blindness, mental retardation or even death of the fetus (Dubey, 2009). Healthy individuals are asymptomatic and can show limited flu like symptoms.

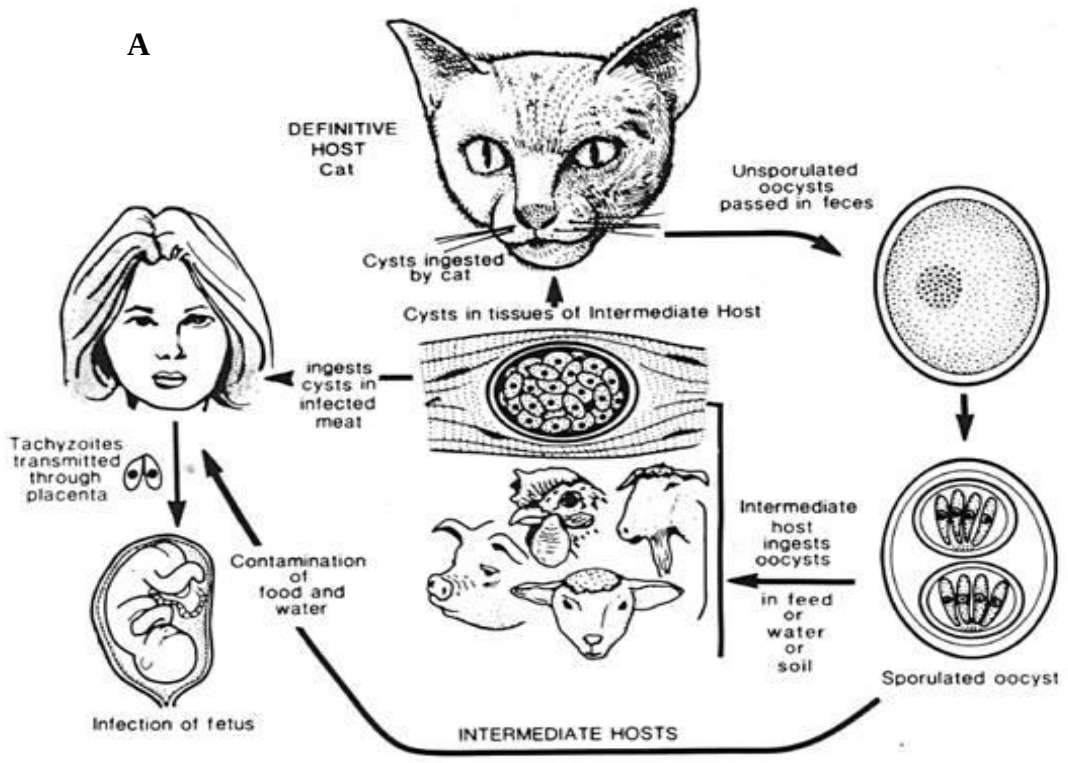
T. gondii life cycle and transmission:

T. gondii has a complex life cycle (Figure 1-1.A) that includes both sexual and asexual replication. Cats serve as the definitive host where the parasite undergoes sexual replication. An infected cat can shed millions of oocysts into its feces, which can contaminate the environment.

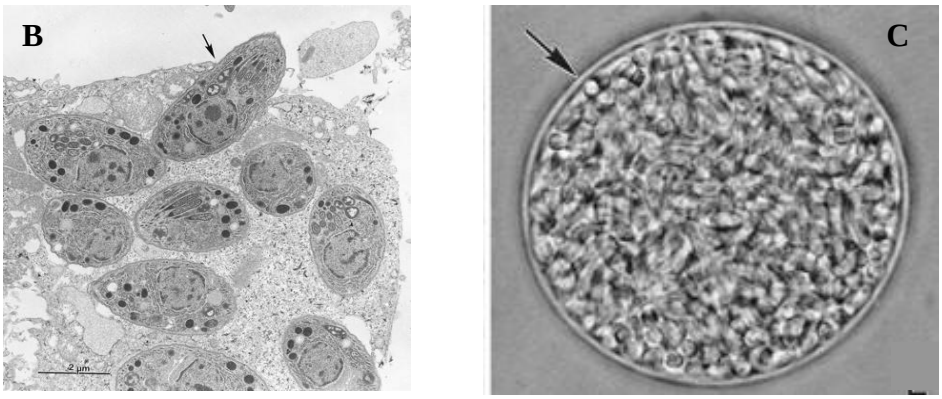
In general, oocysts have been defined as structures consisting of a thick wall which contain replicative structures and are considered the infectious form of the organism facilitating transmission. More specifically, *T. gondii* oocysts contain developing sporozoites. The thick wall structure aids in survival in harsh environments and increased viability over extended periods of time. Transmission to intermediate host can then occur via ingestion of sporulated oocysts from contaminated food or water. Within intermediate host, asexual replication occurs. There are two distinct stages of *T. gondii* during asexual replication: the tachyzoite and bradyzoite forms (Figure 1-1.A-C). In the infected animal, tachyzoites may differentiate into bradyzoites and form tissue cysts. These cysts are found predominantly in the central nervous system and muscle tissue, where they may reside for the life of the host. The development of tissue cysts throughout the body defines the chronic stage of the asexual cycle. Cysts that are ingested through eating infected tissue are ruptured as they pass through the digestive tract, causing bradyzoite release. These bradyzoites can then infect the epithelial cells of the intestine, where they can differentiate back to tachyzoite stage for dissemination throughout the body, completing the asexual cycle (Black, 2000). When this occurs in the feline, the parasite can undergo sexual replication again.

T. gondii stage conversion during asexual replication:

The tachyzoite form is characterized by rapid replication within any nucleated cell of intermediate host and non intestinal epithelial cells of the definitive host (Ajioka, 2007). Invasion of host cells by tachyzoites occurs firstly by adhering to host cells via parasite microneme proteins. Using the apical complex and its actin myosin cytoskeleton, the parasite actively penetrates the host cell without disruption or rearrangement of the host cell membrane (Sibley, 2011). Once inside the cell, the parasite resides within a protective parasitophorus vacuole (PV),



Ajioka and Soldati. 2007



Dubey 1998

Figure 1-1. A) Life Cycle of *T. gondii*. Illustration shows cat as the definitive host. Oocysts are shed in the environment and ingested by intermediate host (animals and humans). Tachyzoite form can be transmitted among intermediate host by vertical transmission. The cycle continues when cats eat cyst infected meat. B) Transmission electron micrograph of a mouse peritoneal macrophage containing several tachyzoites. C) Tissue cyst freed from mouse brain. Note the cyst wall (arrow) enclosing hundreds of bradyzoites.

it follows a lytic lifestyle and undergoes rapid replication. The host cell ruptures when it can no longer support the growth of tachyzoites. Inside the cell, tachyzoites will differentiate into bradyzoites (Lyons, 2002). Bradyzoites are the slower replicating dormant form of the parasite and is associated with the chronic infection. At this stage the parasite is protected from the host immune system. The bradyzoite is characterized by a thick tissue cyst wall that encases the replicating bradyzoites inside (Dubey, 1998; Parmley, 1994; Weiss, 2000). Tissue cysts can vary in size ranging from 5-8.5 x 1-3 μm (Mehlhorn, 1980). In vivo, the factors responsible for stage conversion have been poorly understood. Limited data have shown certain environmental factors to play a role in promoting stage conversion in vitro to include change in pH, temperature and oxidative stress (Bohne, 1994; Soete, 1994, 1993). The ability to convert to the bradyzoite stage is advantageous for parasite survival inside the host. This characteristic is strain specific and observed among intermediate and nonvirulent *T. gondii* strains and not virulent strains (Howe, 1996; Howe, 1995). Studies have shown that virulent Type I strains (ex. RH) are not able to establish chronic infection by conversion into bradyzoite latent form (Djurkovic-Djakovic, 2005). Understanding this phenomenon is of great interest, as this could elucidate key virulence factors.

Genetic diversity and virulence:

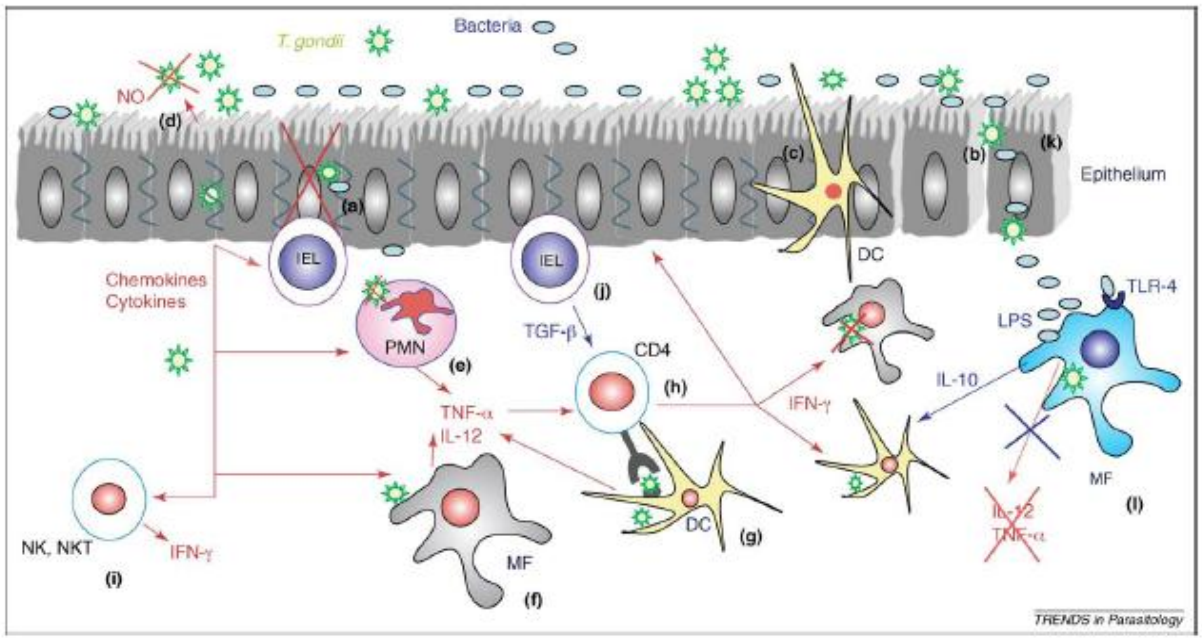
T. gondii infects up to one third of the human population (Dubey, 2009). *Toxoplasma* is a widespread pathogen with isolates found worldwide. Due to the successful nature of *Toxoplasma*, isolates have been obtained from a broad host range including mammals and birds (Corad, 2005; Dubey, 2007; Dubey, 2008). Previous dogma regarding genetic makeup of *Toxoplasma gondii* isolates have largely been classified into 3 distinct lineages as types (I, II, III) based on restriction fragment length polymorphism (RFLP) (Howe, 1995). Although this method

is useful in the identification of genotypes, there are limitations based on the number of markers used. In North America and Europe, natural isolates of animals and humans can be readily grouped into Type I, II or III lineages, with Type II strains predominant in infection (Carme B, 2002; Darde, 2004). Although the three lineages of *T. gondii* differ by only 1-2% (Howe, 1995) they display very unique virulence phenotypes (Boyle, 2006; Grigg ME, 2001). Virulence of *T. gondii* strains is usually assessed in mice. In general, Type I lineage is considered virulent in mice (LD_{100} =1 parasite), showing no survival at ~ 2 weeks. Type II lineage is considered intermediately virulent ($LD_{50}=10^3$ - 10^4) and can have similar phenotype to Type I at high dose. Lastly, Type III is nonvirulent ($LD_{50}>10^5$) showing ~ 20% mortality at day 30 (Saeij, 2006; Su, 2002). Data has revealed that there is an association with virulence in mice and disease in humans (Carme B, 2002). Carme et al, infected mice with *T. gondii* strains isolated from patients with severe toxoplasmosis and showed that these same strains were acutely virulent in mice (Carme B, 2002). Genome-wide QTL mapping revealed the loci responsible for acute virulence in 34 independent progeny generated from a genetic cross between the virulent type I strain GT-1 and the nonvirulent type III strain CTG (Su, 2002). CD1 outbred mice were infected via intraperitoneal (I.P.) injection with the parental strains and their progeny. QTL results identified Chromosome VIIa as loci controlling virulence traits such as mortality and serum responses. Further analysis revealed Rop18 as major factor controlling acute mortality (Taylor, 2006). When Type I Rop18 was transfected into Type III background, and inoculated into mice, increased mortality comparable to wild type I was observed. This Rop18 is a member of a family of parasite proteins that are secreted from apical organelles called rhoptries (Bradley, 2005). Due to this finding, interest on this family of proteins and their role in virulence has emerged.

Host response:

Once inside the cell, the host elicits a series of responses to *T. gondii* infection. More commonly, the parasite induces a potent and long lasting protective immunity (Denkers, 1998; Jebbari, 1998; Yap, 1999). Although this protective immune response is efficient, it does not eliminate the parasite. Instead, the host and parasite achieve an equilibrium which allows for coexistence for the lifetime of the host. This equilibrium is compromised if the host has major defects in the immune system such as in AIDS and organ transplant patients. The Th1 immune response has been widely described as the mechanism responsible for maintaining this balance between host and parasite during infection. The Th1 immune response is characterized by increased levels of IL-12 and IFN-γ. The proinflammatory effects of these cytokines, including production of the microbial effects, nitric oxide, are eventually dampened by the anti-inflammatory cytokine, IL-10, TGF-β and IL-27 (Miller, 2009). Interestingly, the over reaction of this same response is responsible for mortality of mice infected with virulent *T. gondii* strain but not the intermediate ones (Mordue, 2001). Taken together, such data shows the critical relationship between host- parasite interaction and its effect on clinical outcome.

Macrophages, neutrophils and dendritic cells (DC) work together to elicit the immune responses necessary for host survival from *T. gondii* infection (Figure 1-2). Generally, dendritic cells play a role in presentation of antigen to T lymphocytes and control Th1 vs. Th2 differentiation. It has been suggested that the parasite can use the host immune system to facilitate spread during microbial infections (Denkers, 2004). During *T. gondii* infection, it was shown that the parasite has chemokine like activity which recruits immature dendritic cells (Diana, 2005). Data has suggested that when mice are infected with *T. gondii*, splenic DC undergo strong activation and produce IL-12 (Straw, 2003). DCs have also been implicated in playing a major role for IL-12



Buzoni- Gatel and Werts 2008

Figure 1-2. *T. gondii* subversion of immune system during natural infection. *Toxoplasma gondii* probably invades the mucosal intestinal epithelium of its host by infecting enterocytes (a), by penetrating through tight junctions (b), or by being sampled by DCs (c). When enterocytes are infected by the parasites, physiological and morphological disturbances occur, and the enterocytes can secrete cytotoxic molecules, such as NO (d). In addition, enterocytes respond to infection by secreting chemokines and cytokines that attract PMNs (e), MF (f) and DCs (g). When stimulated, these cells from the innate immune system can be directly microbiocidal. They also produce cytokines, such as IL-12, which trigger the adaptive CD4 immune response (h). To be elicited, a specific immune response requires the presentation of antigens, mainly through DCs. Activated T cells, in addition to NK and NKT cells (i) stimulated by cytokines produced by infected enterocytes, such as IL-15, secrete IFN-g that activates MF, DCs and enterocytes for parasite clearance. IEL (j) are cytotoxic for infected enterocytes and produce TGF-b, which might limit IFN-g production. Parasite replication in the intestine combined with inflammatory response development might lead to epithelial damage, giving the opportunity for commensal bacteria, including LPS-rich Gram-negative bacteria, to translocate beyond the epithelial layer (k). *Toxoplasma gondii* blocks numerous LPS-inducible cytokines (l) and cytokine-related genes in MF, infected in vitro as indicated by (l). Adapted from Gatel and Werts 2008.

in protection (Fischer, 2000). Like DCs, neutrophils also play a major role during *T. gondii* infection. Commonly, neutrophils are characterized by there ability to respond and migrate to invading pathogens at the site of infection. Neutrophils take up *T. gondii* by phagocytosis and release free radicals to environment. Data has shown that during *Toxoplasma* infection in neutropenic mice, both Th1 and Th2 cytokines are lower. This decrease results in lethal systemic pathology associated with elevated parasite levels (Bliss, 2001; Miller, 2009). Interestingly, when impairing the ability of neutrophils to reach the site of infection (using CXCR2-/- or CCR1-/- knockout mice), impaired resistance and enhanced growth of *T. gondii* was observed (Del Rio, 2001; Khan, 2001). In general, macrophages are important phagocytic cells and play an essential role in detection and elimination of pathogens to include *Toxoplasma*. Along with neutrophils and DCs, macrophages are an important source of IL-12 production during early infection (Aliberti, 2005) (Figure 1.2). This production of IL-12 leads to downstream production of IFN-γ (Miller, 2009) which both are hallmarks of the Th1 response, indicating an important role of macrophages in maintaining balance between host and parasite. Exposure to IFN-γ and TNF also activates macrophage mediated mechanisms that restrict intracellular replication and promote the process of autophagy. During autophagy, autophagosomes surround parts of the cytosol and organelles to fuse with lysosomes to form autolysosomes (Andrade, 2006). When parasites enter activated macrophages disruption of the PV occurs resulting in the autophagolysosomal elimination of the parasite (Yap, 2007).

Taken together, we observe that there is an extremely important role of host response and its result on clinical outcome of disease persistence during *T. gondii* infection. Understanding this

host these host responses will continue to elucidate major players responsible for pathogenesis control.

Observations That Led To Research

Toxoplasma gondii is a successful pathogen, infecting up to one third of the human population. *T. gondii* is widespread and hundreds of genotypes have been identified worldwide. PCR-RFLP analysis of hundreds of isolates collected from a broad host range revealed that Type II strain was the most common lineage worldwide, followed by Type III strain. Surprisingly, highly virulent in mice, Type I strain, though widely distributed geographically, isolation frequency is limited.

Genetic diversity among distinct *T. gondii* strains (Type I, II and III) has been shown to differ by ~ 1-2% at the DNA sequence level. Although genetically similar, they have very different phenotypes. Virulence, transmigration and growth rate are among these phenotypes that differ between strains. Results have shown that in vitro, Type I parasites have increased motility and ability to migrate when compared to Type II and III parasites (Barragan A, 2002). The correlation between *T. gondii* virulence and growth rate have been well characterized (Kaufman, 1958; Kaufman He, 1959). It has been demonstrated that Type I parasite burden is higher compared to mice infected with intermediate Type II strain (Mordue, 2001). This was supported by Jerome et al. when he showed non virulent parasites growth rate decrease after ~ 20 divisions, while Type I continued to rapidly replicate. Interestingly, in this same study, a spontaneous mutation with high virulence arose from a non virulent strain of *T. gondii* and the mutant lost the ability to switch from fast to slow growth stage (Jerome, 1998). Taken together, it is reasonable to hypothesize that maintaining fast growth phase and the ability to reach a high parasite load in mice might be a common phenomenon for virulent strains. To test this hypothesis, in Chapter II

we developed an assay to determine mouse-virulence of *Toxoplasma.* Using quantitative PCR (qPCR) approach, we determined parasite burdens of experimentally infected CD-1 outbred mice. Our results showed that parasite concentrations in spleen tissues were higher in mice infected with virulent *T. gondii* strains than mice infected with intermediately and non-virulent strains. Results confirmed that indeed increased growth rate is the indicator of virulence. In addition, we offer an alternative method for determining virulence, rather than commonly used mortality assays, which can be harsh procedures and costly.

My work presented in Chapter II laid the framework for analysis of understanding the biological differences in host response to *T. gondii* of different genetic backgrounds (Chapter III). Although virulence of *T. gondii* is different among strains, the host plays an important role in resistance to infection. Mordue et al., using limited reference strains from the clonal Type I and II lineages, showed that acute virulence was associated with an over stimulation of Th1 immune response by the Type I virulent strain which eventually kill the mice (Barragan A, 2002; Denkers, 2001; Mordue, 2001) suggesting immune pathology may play an important role in mortality.

Understanding host response to *T. gondii* has been limited to in vitro methods. Although, increased knowledge has been obtained from these studies, conclusions are limited. To overcome these limitations, in Chapter III, we use an in vivo mouse model to study host response to *T. gondii* infection. This is of relevance to human toxoplasmosis, as mouse-virulent *T. gondii* strains may potentially be associated with severe acquired toxoplasmosis in immunocompetent patients (Carme B, 2002). Using three major genotypes of *T. gondii*, we show that parasite genetic background plays an important role in host response to infection.

CHAPTER II. INCREASED PARASITE LOAD OF *TOXOPLASMA GONDII* IS THE INDICATOR OF ACUTE VIRULENCE IN MICE

This chapter has been submitted for publication as a regular article for *Veterinary Parasitology*. Rachel D. Hill and Chunlei Su*, Department of Microbiology, University of Tennessee, Knoxville, Tennessee 37996-0845. *Corresponding Author.

Introduction

Toxoplasma gondii is found worldwide and infects up to one third of human population. In general, *T. gondii* strains are highly diverse and hundreds of genotypes have been identified worldwide. In mice, *T. gondii* isolates are essentially divided into two groups: the virulent and the relatively less virulent, regardless of their genotypes. The clonal Type I lineage is an example of virulent parasite (LD_{100}=1 parasite), whereas the clonal Type II and III lineages are the relatively less virulent parasites that can be further divided into intermediately virulent (Type II, $LD_{50}=10^3 - 10^4$) and non-virulent (Type III, $LD_{50}>10^5$) strains, respectively (Saeij, 2006; Sibley, 1992b; Su, 2002)

Previous studies among limited strains have shown that virulent strains have higher parasite loads, which leads to an overproduction of Th1 cytokines (Gavrilescu, 2001; Mordue, 2001). Saeij et al. also characterized differences among virulent and less virulent strains in mice. Their results showed that the virulent strain demonstrated increased parasite burden than the less virulent strain throughout the course of infection in mice (Saeij, 2004). Also, studies have shown that the virulent strain was less capable of switching from the faster growth phase of tachyzoite, to the slow growth phase of bradyzoite (Jerome, 1998; Radke, 2006). Taken together, the data suggests that maintaining fast growth phase and the ability to reach a high parasite load in mice might be a common phenomenon for virulent strains. This trait may be explored to develop assay to determine mouse-virulence of *Toxoplasma.* In this study, we took the quantitative PCR

(qPCR) approach, and determined parasite burdens of experimentally infected CD-1 outbred mice with 19 *T. gondii* isolates, in which the virulence phenotypes have been determined previously by mortality assay in mice. Our results showed that parasite concentrations in spleen tissues were two orders of magnitude higher in mice infected with virulent *T. gondii* strains than that infected with intermediately and non-virulent strains at day 7 post infections. The results confirmed that acute virulence indeed can be inferred by tissue burden of infected mice.

Materials and Methods

Cell culture and parasite strains. Human foreskin fibroblasts (HFF) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% of heat inactivated fetal bovine serum (Hyclone #SH30070.03 HI/IR), 1% of 100X Non Essential Amino Acids (NEAA-Fisher Scientific cat. no. - SH3023801), 4 mM Hepes buffer and 10 µg/ml gentamicin (Invitrogen #15710-64) and maintained in T25 vented culture flasks at 37°C with 5% CO2.. *T. gondii* strains were expanded and maintained in confluent HFF monolayer to reach consistent two-day passages prior to use for experiment. Two groups of parasites were used in this study. Group 1 includes virulent Type I strain GT1, non-virulent Type III strain CTG and nine progeny from genetic crosses of GT1 and CTG strains, including A9SF and G2AF (non-virulent progeny), A6AF, C285-11, C285-1 and C285-4 (intermediately virulent progeny), and C285-13, C295-3 and C295-31 (virulent progeny) (Khan, 2005; Su, 2002). These progeny were selected to represent virulent, intermediately virulent and non-virulent phenotypes based on mortality assays (Khan, 2005; Su, 2002). Group 2 parasites include 10 natural isolates from a variety of hosts with five (RH, GT1, VEL, MAS and CAST) being acutely virulent, three intermediate (PTG, DEG and COUGAR) and two non-virulent (STRL and CTG) strains in mice (Khan, 2007; Sibley, 1992b; Su, 2003). Genotypic characterizations of group 2 strains are listed in Table 2.1.

In vivo parasite load determination by qPCR. Two experiments were performed to determine in vivo parasite load in the spleen of the infected female outbred CD-1 mice (Harlan Sprague). In the first experiment, virulent strain GT1, non-virulent strain CTG and the 9 recombinant clones of GT1 X CTG cross were tested in mice. *T. gondii* tachyzoites were grown in HFF cell culture, harvested and filtered through 3 μm pore size polycarbonate filters (Nucleopore Corp, Pleasanton, CA). Two hundred tachyzoites were inoculated by intraperitoneal injection (IP) into four 8-week old CD-1 mice. At days 3, 5 and 7 post infections, spleens were collected, weighed, homogenized in 5 ml PBS and diluted to 0.02g/ml in PBS. For qPCR standard controls, the spleen of a non-infected mouse was homogenized and diluted to the above concentration in PBS and *T. gondii* tachyzoites were spiked into the homogenized spleen to make a series concentrations of $1x10^7$ to $1x10^3$ parasites/ml. Aliquots of 500 μl homogenized spleens were mixed with 500 μl PBS and 50 μl of 10 mg/ml proteinase K, incubated at 55 °C for 2 hours. DNA was extracted using Qiagen DNeasy Blood and Tissue Kit (Qiagen cat no. 69504). The number of parasites in mouse spleen was determined by qPCR of extracted DNA using a TaqMan probe targeting the ITS1 sequence (GenBank Accession# AY143141). The primers for PCR amplification were ITS1-Fx: GAAGGGGCTCAATTTCTGG and ITS1-Rx: TGTTCCTCAGATTTGTTGTTTGA, which amplifies a 117 bp sequence. The ITS1 probe was 5'-/56-FAM/CGTGTCTCTGTTGGGATACTGATTTCCAGG/3BHQ-1/-3', with the 5' end labeled with FAM and the 3' end labeled with Black Hole Quencher-1 (BHQ-1) (Integrated

Table 2-1. Genotyping characterization of natural isolates of *T. gondii*

		Marker												
Strains	Hosts & Locations	SAG1	SAG2*	SAG2†	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Lineages	Virulence in mice ‡
RH	Human USA	I	I	I	I	I	I	I	I	I	I	I	Type I	Vir
GT1	Goat USA	I	I	I	I	I	I	I	I	I	I	I	Type I	Vir
VEL	Human USA	I	I	I	I	I	I	I	I	I	I	I	Type I	Vir
PTG	Sheep USA	II	II	II	II	II	II	II	II	II	II	II	Type II	INT
DEG	Human France	II	II	II	II	II	II	II	II	II	II	I	Type II variant	INT
CTG	Cat USA	II	III	III	III	III	III	III	III	III	III	III	Type III	Non
STRL	Human USA	II	III	III	III	III	III	III	III	III	III	III	Type III	Non
CAST	Human USA	I	I	I	I	I	I	II	I	III	I	III	Atypical	Vir
Cougar	Cougar Canada	I	II	II	III	II	II	II	u-1	I	u-2	I	Atypical	INT
MAS	Human France	u-1	I	II	III	III	III	u-1	I	I	III	I	Atypical	Vir

* Typing at *SAG2* locus by the method of (Howe, 1997)

† Typing at 5' end of *SAG2* locus by the method of (Su, 2006).

‡'Virulent' (Vir) is defined to *T. gondii* isolates with LD_{100} equal to 1 viable parasite, intermediately virulent (INT) with LD_{50} equal to 10^3-10^4 parasites, and non-virulent (Non) isolates with $LD_{50}>10^4$ (Sibley, 1992; Su, 2002)

The u-1, u-2 are alleles different from that of the clonal Type I, II and III lineages.

DNA technologies, INC). qPCR had a total reaction volume of 25 µl containing 16.24 µl of ddH_20, 2 µl of 10X PCR buffer, 2 µl of 2.5mM dNTPs, 2 µl of 25mM MgCl, .5 µl of 10 mg/ ml BSA, 1.3 µl 50 µM ITS1-Fx and ITS1-Rx primers, 0.2 µl of 50 µM ITS1 probe and 0.3 µl of 5 U/µl Faststart Taq DNA polymerase (Roche Applied Science, Indianapolis, IN). One microliter of purified DNA was added as template and PCR reaction mix was transferred to 25 µl Cepheid PCR tubes. The reaction was carried out using a Smart Cycler (v2.0b, Cepheid, Sunnyvale, CA) with the following conditions: 94ºC for 60 sec, then 45 cycles of 92ºC for 15 sec, 52ºC for 30 sec and 72ºC for 40 sec. The Ct value for each sample was compared to the standard controls (10^7 to 10^3 parasites/ml and the negative) to estimate the concentration of parasite per gram of spleen.

In the second experiment, in vivo parasite load was determined for 10 natural isolates, including the five virulent *T. gondii* strains (GT1, RH88, VEL, MAS and CAST) and three intermediately (PTG, DEG and COUGAR) and two non-virulent (CTG, and STRL) strains. Parasites were grown in HFF cell culture and the tachyzoites were harvested as described above. Two hundred tachyzoites were inoculated into 4 mice for each parasite strain. At day 7 post infection, the mice were euthanized and the spleens were collected, weighed and homogenized. Genomic DNA extraction and qPCR was performed as above to determine parasite load in spleen tissue.

Competition assay. *Toxoplasma gondii* tachyzoites of Type I (GT1), Type II (PTG) and Type III (CTG) strains were prepared in HFF cell culture and harvested as the method described above. Two hundred tachyzoites of GT1 were mixed with 800 tachyzoites of PTG or CTG (1:5 ratio) and IP injected into four female outbred CD-1 mice. For positive controls, two mice were infected with 200 tachyzoites of GT1 strain, and two mice with 800 tachyzoites of CTG strain. At days 5 and 7 post infection, mice were euthanized and spleens were collected, weighed and

homogenized. Parasite numbers for all samples was determined by qPCR as described above. In a separate experiment, a 1:1 ratio of GT1: PTG, a 1:1 ratio of PTG: CTG (mixture of 200 tachyzoites of each strain), and a 1:5 ratio of GT1: PTG (200:800 tachyzoites) were also tested in mice by IP injection. For controls, two mice were infected with 200 tachyzoites each of the GT1, PTG and CTG strains. On day 7 post infection, mice were euthanized, and genomic DNA was extracted from the spleen. Parasite numbers for all samples was determined by qPCR as described above.

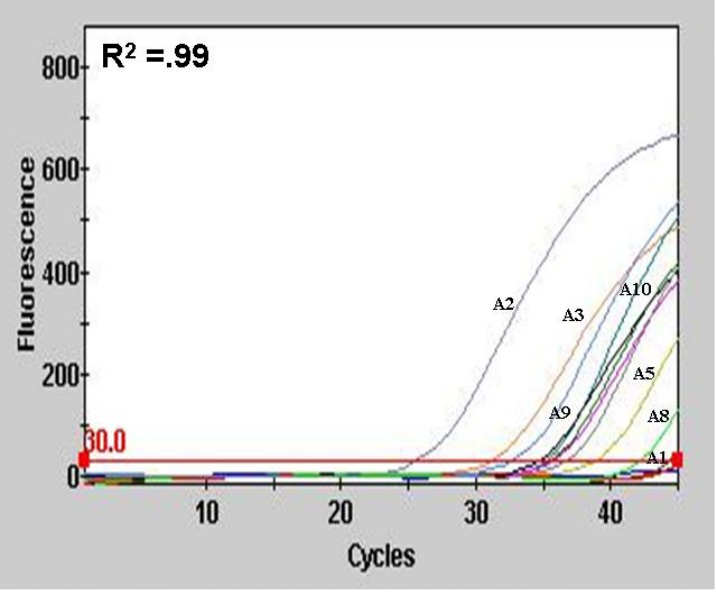
RFLP analysis. To determine the parasite types for the competition assay, a 546 bp fragment of the SAG2 gene was amplified by nested PCR and then treated with restriction enzymes HinfI and TaqI by the method described previously (Su, 2010)

Data analysis. Parasite numbers were determined by comparison to a standard curve generated by linear regression analysis of Log_{10} (number of parasites per gram of the spleen) versus FAM Ct values for each run of qPCR. Differences of parasite dissemination *in vivo* were analyzed by simple ANOVA using an F-test (Minitab v12, State College, PA).

Results

Establishment of qPCR approach as method for quantifying parasite tissue burden.

The qPCR was run for 45 cycles to quantify tissue burden of infected mice. qPCR output was calculated based on threshold cycle (C_T). C_T is defined as the PCR cycle at which the fluorescent dye becomes exponential in amplification. Therefore the lower the Ct, the greater the amount of DNA copy numbers (Schmittgen, 2008). We generated a standard curve using known concentrations of *T. gondii* to calculate absolute number of *Toxoplasma* DNA. A representative example of qPCR results were presented in Fig. 2.1. The R^2 of linear regression for all qPCR



Site	Sample ID	Ct Value
A1	NEG	ND
A2	10^7	25.6
A3	10^6	31.2
A4	10^5	35.5
A5	10^4	38.9
A6	10^3	44.4
A7	CTG M1	ND
A8	PTG M2	42.3
A9	GT1 M1	32.8
A10	GT1:PTG(1:5) M1	35.2
A11	GT1:PTG(1:5) M2	35.8
A12	GT1:PTG(1:5) M3	34.6
A13	GT1:PTG(1:5) M4	36.7

Figure 2-1. Example of qPCR output. The parasite loads were determined using DNA samples extracted from the spleen of infected mice. The qPCR was run for 45 cycles. The X axis indicates the number of PCR cycles, Y axis shows fluorescent signal of reporter dye. The threshold was set at 30. A standard curve was generated to measure parasite concentrations in experimental samples. In this example, the standard curve equation is Y = -0.219X + 12.697 and R2 = 0.99. Labeled in this figure are a few representative curves of standards and samples. ND indicates DNA not detectable.

standard curves were greater than 0.98, indicating the estimation of parasite load was acute by this approach.

Virulent strains have increased tissue burden compared to intermediate and non virulent strains.

Parasite loads in the spleen at days 3, 5 and 7 post-infection for GT1 and CTG (parental strains), A9SF and G2AF (non-virulent progeny), A6AF, C285-11, C285-1 and C285-4 (intermediately virulent progeny), C285-13, C295-3 and C295-31 (virulent progeny) are summarized in Fig. 2-2**.** Individual clones are shown in panels A and B, and the average parasite load per group in panel C. At day 3 post IP, the in vivo parasite load was low and could not be reliably detected by qPCR. At day 5 post IP, the average Log_{10} (parasites/g spleen) were 5.9, 5.1 and 5.1 for virulent, intermediately virulent and non virulent clones, respectively, the difference is not statistically significant (P>0.05). At day 7 post IP, the average Log_{10} (parasites/g spleen) were 7.4, 5.7 and 5.6 for virulent, intermediately virulent and non-virulent clones, respectively. The virulent clones reached significantly higher concentration compared to intermediately virulent and non-virulent clones ($P \leq 0.05$). On average, there are about 30 times more parasites on day 7 than that on day 5 for virulent clones. However, parasite loads only increased slightly for intermediately and non-virulent clones on day 7. At day 7, there are about 50 times more parasites in virulent clones than that of intermediately and non-virulent clones. No difference was observed between intermediately and non-virulent clones at both day 5 and day 7 (Figure. 2-2).

Parasite loads in the spleen tissues at day 7 post IP for natural isolates of five virulent, three intermediately virulent and two non-virulent strains are summarized in Figure. 2-3. The averages Log_{10} (parasites/g spleen) for GT1, RH88, VEL, MAS and CAST were 7.9, 7.4, 8.5, 8.6 and 7.1,

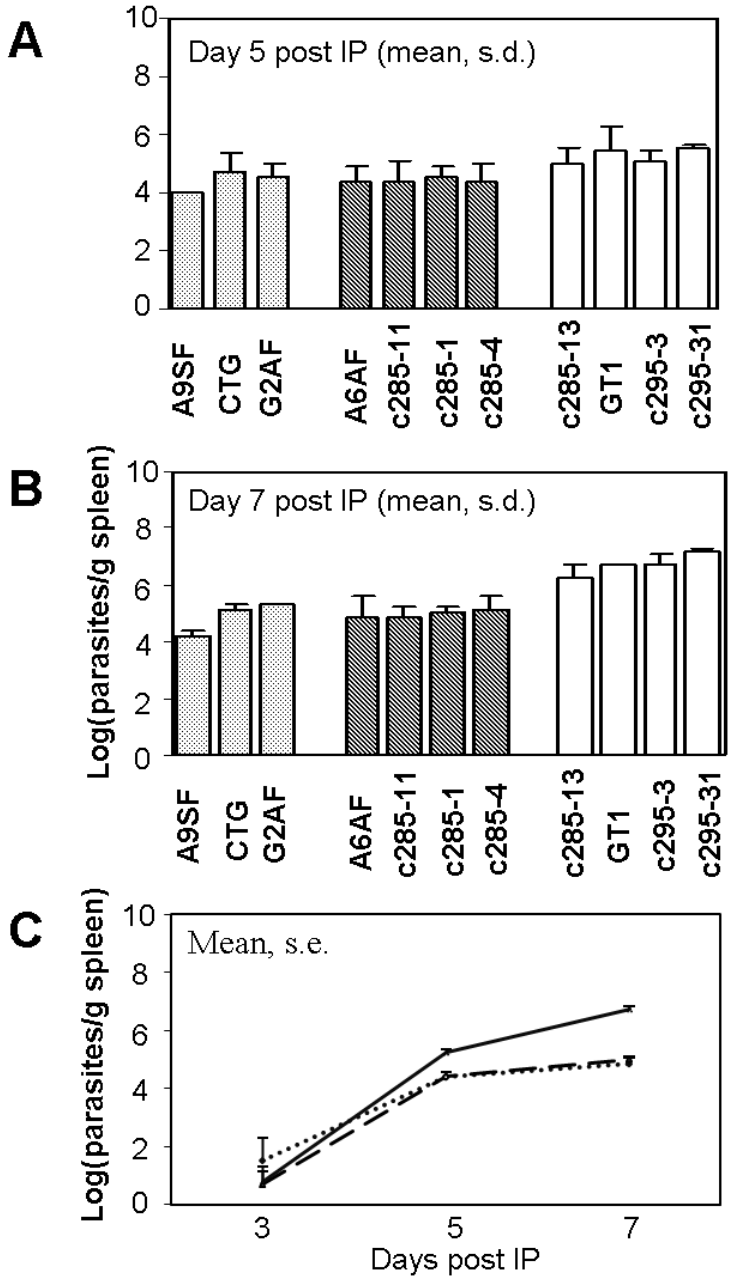


Figure 2-2. Parasite burdens in the spleens of mice. A. Day 5 post infection. Dotted bars are for non-virulent strains, hatched bars are for intermediately virulent strains, and open bars are for virulent strains. **B.** Day 7 post infection. On average, virulent strains have significantly high parasite numbers by days post infection. C. Changes in parasite load over time. Solid line is for acutely virulent strains, dotted line is for intermediately virulent and dashed line is for non virulent.

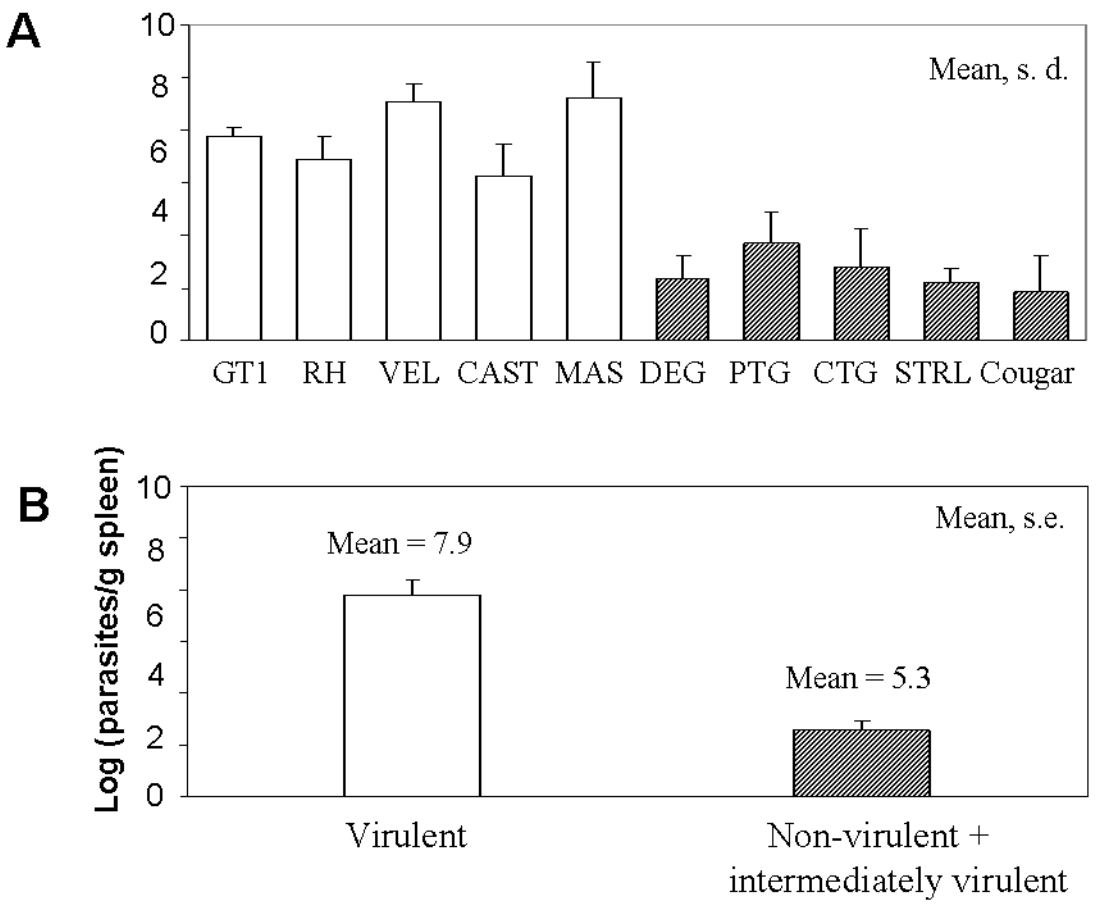


Figure 2-3. Parasite burdens Log (parasites/g spleen) in mice indicated by mean and standard deviation at day 7 post IP . **A.** Parasite load in the spleen tissues for 10 natural isolates, including GT1, RH, VEL, MAS and CAST (known virulent strains, open bars), PTG, DEG, COUGAR, CTG and STRL are intermediately virulent and non-virulent strains (hatched bars). **B.** Average tissue burden (mean, standard error) for virulent versus non-virulent and intermediately virulent strains.

respectively (Figure. 2-3.A). The average Log_{10} (parasites/g spleen) for intermediately virulent strains PTG, DEG, COUGAR, and non-virulent strains CTG and STRL were 5.9, 5.2, 4.9, 5.4 and 5.1, respectively (Figure. 2-3.A). Since parasite loads for intermediate and non- virulent strains were similar, they were combined to compare to the virulent strains. On average, the virulent strain parasite loads were more than two orders of magnitude higher (>100 times) than that of

intermediate and non-virulent strains, $P<0.0001$ (Figure. 2-3.B). Comparing the isolate CAST that had the lowest parasite load within virulent strains with the isolate PTG that had the highest parasite load within the non-virulent strains, CAST still had 15 times more parasites than PTG, and the difference is significant ($P=0.02$, two-sample t-test) (Figure. 2-3.A).

Virulent *T. gondii* strain outgrows intermediate and non virulent strains during mixed infections.

qPCR was performed to quantify parasite concentration in the spleens of mice infected with tachyzoites mixture of GT1: CTG at 1:5 ratio (200:800 tachyzoites) (Figure. 2-4. A). On day 5 post IP, the Log (parasites/g spleen) was 6.8 (s.d. = 0.34) which was comparable to GT1 control (7.9, s.d=0.4); this was more than 10 times higher than CTG (6.5, s.d. =0.2). On day 7 post IP, the Log (parasites/g spleen) was 8.8 (s.d. =0.3) for GT1: CTG mixture, comparable to GT1 control (9.0, s. d. =0.7), this was about 80 times higher than the CTG control (6.6, s.d. = 0.4) (Figure 2-4.A). For the mixed infection and GT1 control, there were about 10 times more parasites at day 7 than that at day 5. For CTG control, the parasite loads were comparable between days 5 and 7 (Figure 2-4.A).

RFLP Analysis at SAG2 locus showed that at day 5 the parasite was not reliably detected (Figure. 2-4. B). SAG2 was not detectable for mice infected with CTG (M1, M2), and a mouse infected with 1:5 of GT1: CTG (M4). One mouse infected with 1:5 of GT1:CTG (M1) showed a

mixed infection of both Type I and III alleles with comparable intensity. Type I allele of SAG2 marker was observed for mice infected with GT1 strain (M1, M2) and a mouse with 1:5 of GT1: CTG infection (M2).

At day 7, all mice infected with 1:5 of GT1: CTG mix showed the Type I allele. The mice infected with GT1 and CTG controls showed the expected Type I and III genotypes, respectively (Figure. 2-4.B).

In a second competition assay experiment, qPCR was performed to quantify parasite concentration in the spleens infected with mixture of GT1: PTG at 1:1 ratio (200 tachyzoites each strain), mixture of GT1: PTG at 1:5 ratio (200:800 tachyzoites), and mixture of PTG: CTG at 1:1 ratio (200 tachyzoites each strain). On day 7 post IP, the Log (parasites/g spleen) for GT1: PTG (1:1 ratio) and GT1: PTG (1:5 ratio) were 7.6 (s.d. = 0.7) and 7.6 (s.d. = 0.2), respectively, which was comparable to GT1 control (7.8, s.d. = 0.8) (Fig 2-5.A). This is 20 times higher than PTG control (5.8, s.d. = 0.1). Also, on day 7 post IP, the Log (parasites/g spleen) was 5.6 (s.d. =0.6) for PTG: CTG mixture (1:1 ratio), comparable to PTG and CTG controls (5.8, s.d= 0.1 and 5.1, s.d. = 0.4). There was no statistical significant difference in parasite concentration compared to non-virulent controls (Figure 2-5.A).

RFLP analysis at the SAG2 locus revealed that mice infected with different ratios of Type I and II mixtures (1:1 or 1:5 of GT1: PTG), only revealed the Type I allele (Figure. 2-5.B). The 1:1 mixture PTG:CTG showed a Type II allele in one mouse (M4), a Type III allele in one mouse (M3), and mixed alleles of Type II and III in two mice (M1 and M2) (Figure. 2-5. B). The mice infected with GT1, PTG and CTG as controls showed the expected Type I, II and III genotypes, respectively (Figure. 2-5.B).

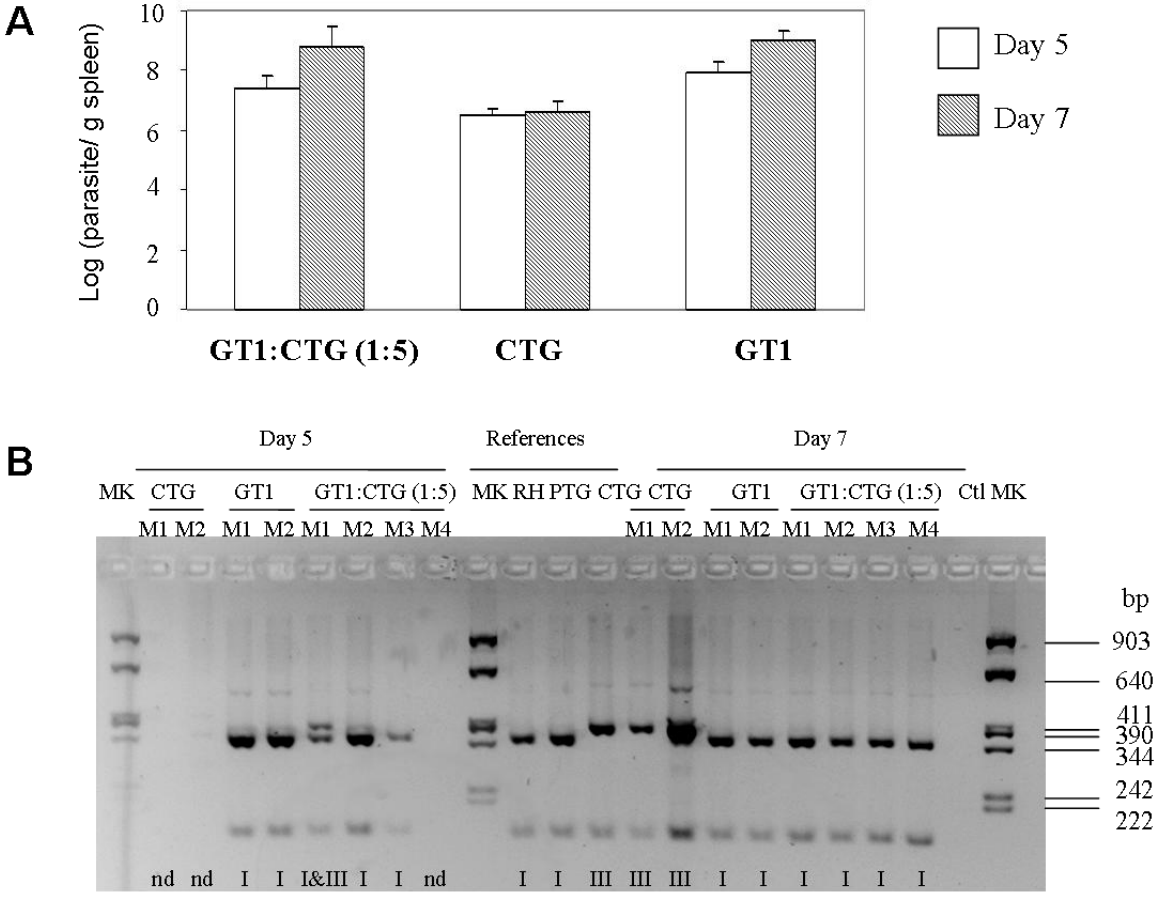


Figure 2-4. Competition assay. A. Parasite burdens Log (parasites/g spleen) in mice at days 5 and 7 post IP (mean, s.d.). GT1 and CTG are positive controls of Types I and III, respectively. GT1 (200 tachyzoites) and CTG (800 tachyzoites) were mixed to give 1:5 ratio. Parasite load was determined by qPCR. **B.** Genotyping *T. gondii* in the spleen tissues of mice infected with *T. gondii*. Marker SAG2 was amplified by nested PCR and then digested by restriction enzyme HinfI. The references RH, PTG and CTG represent Type I, II and III lineages respectively. M1, M2, M3 and M4 are the samples taken from infected mice. Competition assay was performed in four mice by infecting a 1:5 mixture of GT1 and CTG. Ctl is negative control in which no DNA template was used for PCR amplification. MK is DNA sample loading marker. nd, parasites were not detectable by PCR at the SAG2 locus.

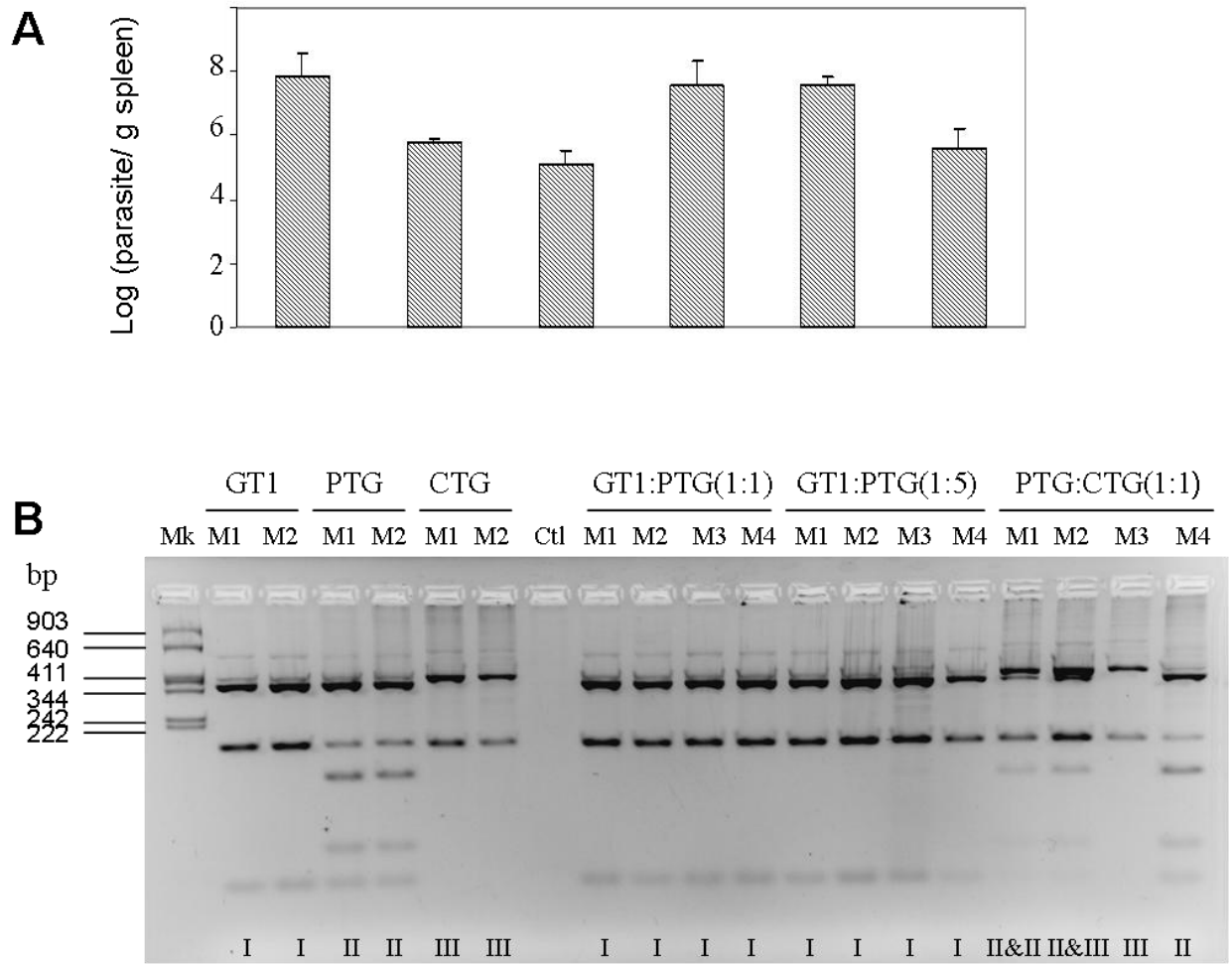


Figure 2-5. Competition assay in mice at day 7 post IP (mean, sd). **A.** GT1, PTG and CTG are positive controls of Types I, II and III, respectively. GT1 (200 tachyzoites) and PTG (200 tachyzoites) were mixed to give 1:1 ratio. GT1 (200 tachyzoites) and PTG (800 tachyzoites) were mixed to give 1:5 ratio. PTG (200 tachyzoites) and CTG (200 tachyzoites) were mixed to give 1:1 ratio. Parasite load was determined via qPCR. **B.** Genotyping *T. gondii* in the spleen tissues of mice. Marker SAG2 was amplified by nested PCR and then double-digested by restriction enzymes HinfI and TaqI. Competition assay was performed in four mice (M1 to M4) in each treatment. Ctl is negative control in which no DNA template was added for PCR amplification. MK is DNA sample loading marker.

Discussion

In this investigation we used an *in vivo* model to study the differences in parasite load in mice among distinct *T. gondii* strains. Our data suggested that virulent strains grow faster and reach a higher parasite load in mice than that of intermediately and non-virulent strains. This phenomenon is not strain specific but a general characteristic in all virulent strains tested in this study (Figure. 2-3, 2-4 and 2-5). Early studies suggested that virulent strains multiply more rapidly (Kaufman, 1958). This is consistent with in vitro growth data that showed Type I (RH and GT1) strains had increase levels of parasite growth (~70 parasites per vacuole) compared to Type II (Me49) and Type III (VEG) which had ~30 and ~25 parasites per vacuole, respectively (Radke, 2006). This finding can be explained by the ability of intermediately and non-virulent strains to convert to slow growing bradyzoite form, whereas virulent Type I strains is unable to make this conversion leading to subsequent uncontrolled growth.

High parasite load of virulent *T. gondii* strains in host tissue would suggest that virulent strains outgrow intermediately and non-virulent strains in mixed infection. This was confirmed by competition assay and RFLP analysis (Figure. 2-4, 2-5). Together our data demonstrated that regardless of the ratio in mixed infection, virulent GT1 is consistently dominant over its intermediately and non-virulent counterparts, PTG and CTG, respectively. Genotypic patterns of (GT1:CTG) infection also corresponded to GT1 controls which further suggests that the GT1 strain had taken over the culture and was now the dominant strain.

RFLP analysis confirmed that intermediately and non-virulent strains have a slower growth rate and have low tissue burden than virulent strains. Mice infected with PTG: CTG (1:1) showed mixed alleles of both PTG and CTG at day 7 post infection. These results suggest that both intermediately and non-virulent strains have comparable levels of parasite present. When a

1:20 of GT1:CTG mixture was infected IP to two mice and followed by genotyping at day 9 post IP, only the allele of GT1 strain was observed (data not shown), confirming the finding that the virulent strains replicate faster than the intermediately and non-virulent ones.

Previous study reported that, the MS-J strain, a spontaneous mutant from Type III strain VEG had increased growth rate and 100% mortality in mice compared to VEG tachyzoites whose growth shifted to bradyzoite (Jerome, 1998). Data has also shown that Type I virulent (RH) strain completely lyse cells much faster than Type II and III strains in vitro (Saeij, 2005). Together, these results prove that there is a correlation between increased growth rate and virulence. There may be regulatory or genetic elements that are suppressing virulence in intermediately and non-virulent *T. gondii* strains.

In this chapter, we confirmed that increased growth of parasite in mice is a common feature of virulent strains regardless of their genetic background. This assay only needs 7 days compared to 20-30 days for mortality assay. The parasite tissue load assay is advantageous over conventional mortality assay considering the length of time needed and minimum stress on experimental mice. The limitation of this assay is that it can not distinguish parasite strains of intermediately virulent from non-virulent strains. In summary, parasite loads in spleen tissues of infected mice can be used as an alternative assay to determine acute virulence of *T. gondii* in mice.

Acknowledgements

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CHAPTER III. DIFFERENTIAL GENE EXPRESSION IN THE HOST INFECTED WITH DISTINCT *TOXOPLASMA* STRAINS

This chapter has been submitted and currently in revision for publication in *Infection and Immunity*. Rachel D. Hill[1], Julia S. Gouffon[2], Arnold Saxton[3] and Chunlei Su[1]*, [1]Department of Microbiology, [2]Affymetrix Core Facility, [3]Department of Animal Science- Experimental Statistics, University of Tennessee, Knoxville, Tennessee 37996-0845. *Corresponding Author. Work performed by coauthor Julia S. Gouffon, include microarray assay and preliminary raw data analysis. Arnold Saxton contributed to microarray statistical analysis.

Introduction

Toxoplasma gondii is the causative agent for toxoplasmosis. *T. gondii* infects almost all warm blooded vertebrates and birds. It infects up to one third of the human population (Dubey, 2009). In immunocompromised individuals, infection can cause severe encephalitis. Acute infections in pregnant women can result in a condition known as congenital toxoplasmosis in which symptoms could be blindness, mental retardation or even death of the fetus (Joynson, 2001).

In North America and Europe, natural isolates of animals and humans are divided into the Type I, II and III lineages, with Type II strains predominant in infections (Darde, 2004; Howe, 1995). In mice, the Type I lineage is highly virulent (LD_{100}=1 parasite), whereas the Type II is intermediately virulent ($LD_{50}=10^3$ - 10^4) and the Type III is non-virulent ($LD_{50}>10^5$) (Saeij, 2006; Sibley, 1992a). Using reference strains from the clonal Type I and II lineages, it was shown that acute virulence was associated with rapid parasite dissemination, high parasite load and an over stimulation of Th1 immune response by the Type I virulent strain which eventually kill the mice (Barragan A, 2002; Denkers, 2001; Mordue, 2001) suggesting immune pathology may play an important role in mortality.

Gene expression in the host infected with *T. gondii* has been characterized in vitro. In vitro gene expression analysis of macrophages infected with Type I, II or III strains revealed that

Type I infections elicited a stronger immune response compared to its non virulent counterparts (Lee, 2007). Work has shown that Type II ME49 can induce translocation of NF- κB to nucleus but not Type I RH in mice splenocytes and mice bone marrow derived macrophages (Dobbin, 2002; Robben, 2004).The nuclear factor- κB (NF- κB) is known to have a critical role in regulating inflammatory immune and anti apoptotic responses during infection. Robben et al also showed that Type II induced increased levels of IL-12p40 but not Type I and Type III in a myeloid differentiation primary response gene 88 (MyD88) dependent and TLR2/TLR4 independent manner in vitro (Robben, 2004). Altogether, this data indicates that parasite genetic background is important in inducing host gene expression.

Commonly, in vitro methods have been used to characterize host parasite interaction during *T. gondii* infection. Host response to infection has been characterized in macrophage and other immune cells (Lee, 2007; Pollard AM, 2009). Although knowledge has been gained from these studies, conclusions are limited based on the controlled conditions characteristic of in vitro systems. It is unknown how these data will translate to in vivo scenarios. To overcome these limitations, we used an in vivo mouse model to study host response to *T. gondii* infection. This is of relevance to human toxoplasmosis, as mouse-virulent *T. gondii* strains may potentially be associated with severe acquired toxoplasmosis in immunocompetent patients (Carme B, 2002). Here we revealed the differences in gene expression in mice infected with three major genotypes of *T. gondii*.

Materials and Methods

Cell culture of parasite strains and infection in mice. Human foreskin fibroblasts (HFF) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% of heat inactivated fetal bovine serum (Hyclone#SH30070.03 HI/IR), 1% of 100x Non Essential

Amino Acids (NEAA-Fisher Scientific cat. no. - SH3023801), 0.4% of 1M HEPES buffer and 0.1 % of 10 mg/ml gentamicin (Invitrogen #15710-64) and maintained in T25 vented culture flasks at 37°C with 5% CO2. *T. gondii* strains GT1 (virulent), PTG (intermediately virulent) and CTG (non-virulent) were expanded and maintained in confluent HFF monolayer to reach consistent two-day passages prior to use for experiments.

Toxoplasma gondii tachyzoites of GT1, PTG and CTG strains were harvested by filtering through 3 μm pore size polycarbonate filters (Whatman cat. no. 420400) and counted. Five hundred tachyzoites of each of the GT1, PTG and CTG strains were inoculated into four 6-8 week old female outbred CD-1 (ICR- Harlan Sprague) mice by intraperitoneal injection (IP). At day 5 post infection, mice were anesthetized to the state of unconsciousness, followed by euthanization with cervical dislocation. Five mL of ice cold PBS was injected into peritoneal cavity and then peritoneal lavage was collected. Cells of peritoneal lavage were pelleted by centrifugation. Supernatant was removed and pellet was resuspended in 1ml RNAlater, mixed and incubated in 4ºC for 1 hour, then stored in -70ºC until RNA extraction for microarray experiments. The spleens were collected from mice, weighed, homogenized and diluted in PBS to determine parasite load by real-time PCR (RT-PCR).

Microarray analysis. Total RNA was extracted from cells collected from peritoneal lavage of mice infected with GT1, PTG and CTG using Qiagen RNeasy Plus Mini Kit (Qiagen cat. no. 98 74131) following manufacturer's instruction. For host gene expression profiling, Affymetrix Mouse 1.0 ST array containing 266,220 full length mRNA was used. Microarray was carried out at the University of Tennessee Affymetrix Core Facility. RNA samples were processed according to the Affymetrix Protocol for One-Cycle DNA Synthesis using a Message Amp II102 Biotin Enhanced Kit (Ambion PN#am1791, Austin, TX). 5 μg of fragmented cRNA

was hybridized to the GeneChip, previously described. Arrays were washed and stained using the Affymetrix Hyb/Stain kit PN900720 and Affymetrix prepared wash solutions and an Affymetrix Fluidics 450 wash station. Arrays were immediately scanned using the Affymetrix 7G scanner. Array images were visually inspected for anomalies. The individual chip scans were quality checked for the presence of control genes and background signal values.

Data normalization and statistical analysis. The raw data from Affycore Analysis was extracted and normalized with Robust Multichip Algorithm (GC RMA) using Partek® Genomics SuiteTM software. A two way analysis of variance (ANOVA) was used to detect genes with statistically significant expression levels between each *T. gondii* infection compared to untreated infection samples. Gene transcripts were considered to be differentially expressed when there was at least 2 fold changes in either direction, and the ANOVA p-value is less than or equal to 0.05.

Microarray validation via RT-PCR. RNA from samples used for microarray analysis were used to quantify mRNA transcript levels of genes at the end of highly induced and the end of highly repressed in mice based on fold change signal intensity values (see TABLE 3-3). RNA was synthesized into cDNA via reverse transcription according to protocol using DyNAmo™SYBR® Green 2 Step qRT-PCR kit (New England Biolabs). In brief, RNA concentrations among infections were normalized (max 1 µg) and added to reverse transcription reaction for a total volume of 20 µl containing 10 µl of 2xRT buffer, 1 µl of 300 ng/µl random hexamer primers, 2 µl MULv reverse transcriptase and RNase free water. The reaction was carried out using the Eppendorf Mastercycler (Fisher Scientific). mRNA transcript levels were then quantitated by RT-PCR of cDNA. RT-PCR reaction had a total volume of 20 µl containing 8.6 µl of H_2O, 10 µl of 2X master mix (containing modified *Tbr*DNA polymerase), 0.2 µl of 50

Table 3-1. Primer Sequence for quantitative RT-PCR

Accession no.[a]	Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	PCR product size (bp)
NM_021400	*Prg4*	GGGAGACTCAATCAAGGC	CGTGGTGGAGATGGTGGT	170
NM_011111	*Serpinb2*	TTTCTTTCTGAGGTGTTCCATCAA	CCAGCTGCCACAGTGCC	65
NM_198028	*Serpinb10*	CCTCAGCCTGCTTCTACT	CTGGTCCACTTATCTAGTTTCT	93
NM_009696	*ApoE*	AGGCTAAGGACTTGTTTCGG	TGGTTGCTTTGCCACTCG	153
NM_021272	*Fabp7*	GCAAGATGGTCGTGACTC	CTGGCTAACTCTGGGACT	120
NM_010766	*Marco*	TGGATCACTAGCTATCGAGGACAA	GTGCTCTAGGTACCAGGTGTGTTTT	77
NM_011333	*CCL2*	CACAACCACCTCAAGCAC	AAGGGAATACCATAACATCA	151
NM_013654	*CCL7*	GCCTGAACAGAAACCAAC	TATCCCTTAGGACCGTGA	103
NM_011331	*CCL12*	ACTTCTATGCCTCCTGCTC	CACTGGCTGCTTGTGATT	159
NM_011332	*CCL17*	GCCATCGTGTTTCTGACT	GCGCTCCAAATGCCTCA	129
NM_009137	*CCL22*	CAGGCAGGTCTGGGTGAA	TAAAGGTGGCGTCGTTGG	147

[a] From the NCBI protein database

μm each of the forward and reverse primers (TABLE 3-1). The reaction was carried out using a Smart Cycler (v2.0b, Cepheid, Sunnyvale, CA) with the following conditions: 95°C for 15 min, (94°C for 10 sec, 55°C for 30 sec, 72°C for 30 sec) for 40 cycles. The addition of melting curve step was added as control for PCR product efficiency at 72-95°C. Relative quantification was used to determine the ratio between the quantity of the target normalized to GAPDH (NCBI accession no. NM_008084) reference gene using $2^{-\Delta\Delta C(t)}$ method (Schmittgen, 2008).

Data mining. Gene functional analysis was conducted for differentially expressed genes using the Onto-Express (OE) annotation database. The input into OE was a list of gene symbols corresponding to differentially expressed genes for each treatment compared to untreated samples. The output was the functional category assigned to each gene based on specific experimental evidence or by theoretical inference (Draghici, 2003). For visual representation, fold change signal intensity values were natural log transformed and values for each gene were plotted. For each functional category of genes, statistical analysis were performed to compare mean fold changes of gene expression in mice infected with the Type I, II and III strains. The formula we used is: Mean = ($\Sigma|x|/n$), where x is the natural log of fold change/negative control. The |x| is used to remove the negative sign when genes are down-regulated. Standard deviation is also calculated for each mean value.

In vivo parasite load determination by RT-PCR. For RT-PCR standard controls, the spleen of a non-infected mouse was homogenized and diluted in PBS and *T. gondii* tachyzoites were spiked into the homogenized spleen to make a series concentrations of $1x10^7$ to $1x10^3$ parasites/ml. All homogenized spleen samples were adjusted to a volume of 0.02 g/ml in PBS. Aliquots (500 ul) of homogenized spleens were stored in -20°C until use. To extract DNA from homogenized spleen samples, 500 ul PBS was added to 500 μl of the spleen lysate and samples

were digested by adding 50 µl of 10 mg/ml proteinase K and incubated at 55°C for 2 hours. DNA was extracted using Qiagen DNeasy Blood and Tissue Kit. The number of parasites in mouse spleen was estimated by RT-PCR of extracted DNA using a TaqMan probe targeting the ITS1 sequence (GenBank Accession# AY143141). The primers for PCR amplification were ITS1-Fx: GAAGGGGCTCAATTTCTGG and ITS1-Rx: TGTTCCTCAGATTTGTTGTTTGA, which amplify a 117 bp sequence. The ITS1 probe was 5'-/56 FAM/CGTGTCTCTGTTGGGATACTGATTTCCAGG/3BHQ-1/-3', with the 5' end labeled with FAM and the 3' end labeled with Black Hole Quencher-1(BHQ-1) (Integrated DNA Technologies, Inc.). RT-PCR reaction had a total volume 25 µl containing 16.24 µl of H_2O, 2 µl of 10x PCR buffer, 2.0 µl of 2.5 mM dNTP, 2.0 µl of 25 mM $MgCL_2$, 0.5 µl of 10 mg/ml BSA, 1.3 µl 50 µM ITS1-Fx and ITS1-Rx primers, 0.2 µl 50 µM ITS1 probe and 0.3 µl of 5 U/ µl FastStart Taq DNA polymerase (Roche Applied Science, Indianapolis, IN). One microliter of purified DNA was added as template and PCR reaction mix was transferred to 25 µl Cepheid PCR tubes. The reaction was carried out using a Smart Cycler (v2.0b, Cepheid, Sunnyvale, CA) with the following conditions: 94ºC for 60 sec, then 50 cycles of 92ºC for 15 sec, 52ºC for 30 sec and 72ºC for 40 sec. The Ct for each sample was compared to the standard controls (10^7 to 10^3 parasites/ml and negative) to estimate the concentration of parasite per gram of spleen.

FACS analysis. Virulent Type I (GT1), Intermediate Type II (PTG) and NON virulent Type III (CTG) strains were used for this assay. Uninfected mice served as negative control. Parasites were grown and maintained in T25 vented culture flasks at 37°C with 5% CO2 as described above. 6 -8 week old CD-1 outbred female mice were I.P. injected with 500 tachyzoites of each strain. On day 5, peritoneal lavage was collected, purified and counted as described above. 1 x 10^6 cells were transferred to a 96-well plate and washed. Cells were blocked for 1 hour with

FACS buffer (PBS + 1% BSA). Cell populations were characterized by flow cytometry using PE conjugated Ly-6G Ab (eBioscience # 12-5931-82), FITC conjugated F4/80 (eBioscience # 11-4801-82) and APC conjugated CD11c (eBioscience #17-0114-82). Samples were acquired on a FACSCalibur (BD Biosciences) cytometer collecting 50,000 events.

Results

Type I and II modulate more host genes than Type III during infection overall.

For microarray analysis, RNA collected from peritoneal lavage of three mice infected with GT1, PTG and CTG were used for gene expression profiling during *T. gondii* infection. Differentially expressed genes were clustered and identified based on signal intensity values and fold change differences between strains compared to untreated mice. In brief, those genes with at least a two-fold change in expression values was considered. Differentially expressed genes among treatments were categorized based on biological processes via OntoExpress (vortex.cs.wayne.edu/ontoexpress). Our results showed that inoculation with Type I and II parasites affected more genes in mice compared to that of the Type III strain (TABLE 3-2). In particular, infection with Type I *T. gondii* caused the induction of 1,180 genes and the repression of 1,740genes. Type II infection induced 950 genes and repressed 1,335 genes. In contrast to this trend more genes (781 genes) were induced while less (709 genes) were repressed overall with Type III infection.

Analysis of microarray data

As described above, analysis of variance (ANOVA) statistical method was used to select significantly expressed genes in either up- or down-regulated based on signal intensity values. Using the average signal intensity value from three mice for GT1 and CTG, and two mice for PTG, cluster analysis visually revealed two levels of comparison: 1) gene expression comparison

Table 3-2. Number of host genes affected by *T. gondii* at day 5 post infection.

Functional Class	GT1		PTG		CTG	
	Induced	Repressed	Induced	Repressed	Induced	Repressed
Immune Response	82	26	73	16	75	10
Oxidation reduction	35	63	31	46	31	14
Transcription	60	168	44	132	38	51
Biological processes	63	141	49	108	34	67
Transport	76	105	57	84	51	51
Translation	10	74	5	67	4	57
Metabolic process	41	56	33	40	29	11
Signal Transduction	78	53	57	42	46	22
Apoptosis	44	40	32	32	34	17
Protein Catabolic process	1	34	0	1	1	0
Cell Cycle	51	24	46	19	40	8
Inflammatory Response	38	8	30	7	34	4
Regulation of transcription (DNA dependent)	64	170	47	133	40	50
Protein Amino Acid Phosphorylation	42	38	37	31	35	16
G coupled receptor cell signaling	52	32	41	25	26	16
Protein Transport	19	32	13	27	14	17
Unidentified	424	676	355	525	249	298
Total	**1180**	**1740**	**950**	**1335**	**781**	**709**

Induced = significantly up regulated, ≥ 2 fold (Fold change ratio of gene vs. NEG; $P \leq 0.05$)

Repressed = significantly down regulated, ≤ -2 fold (Fold change ratio of gene vs. NEG; $P \leq 0.05$)

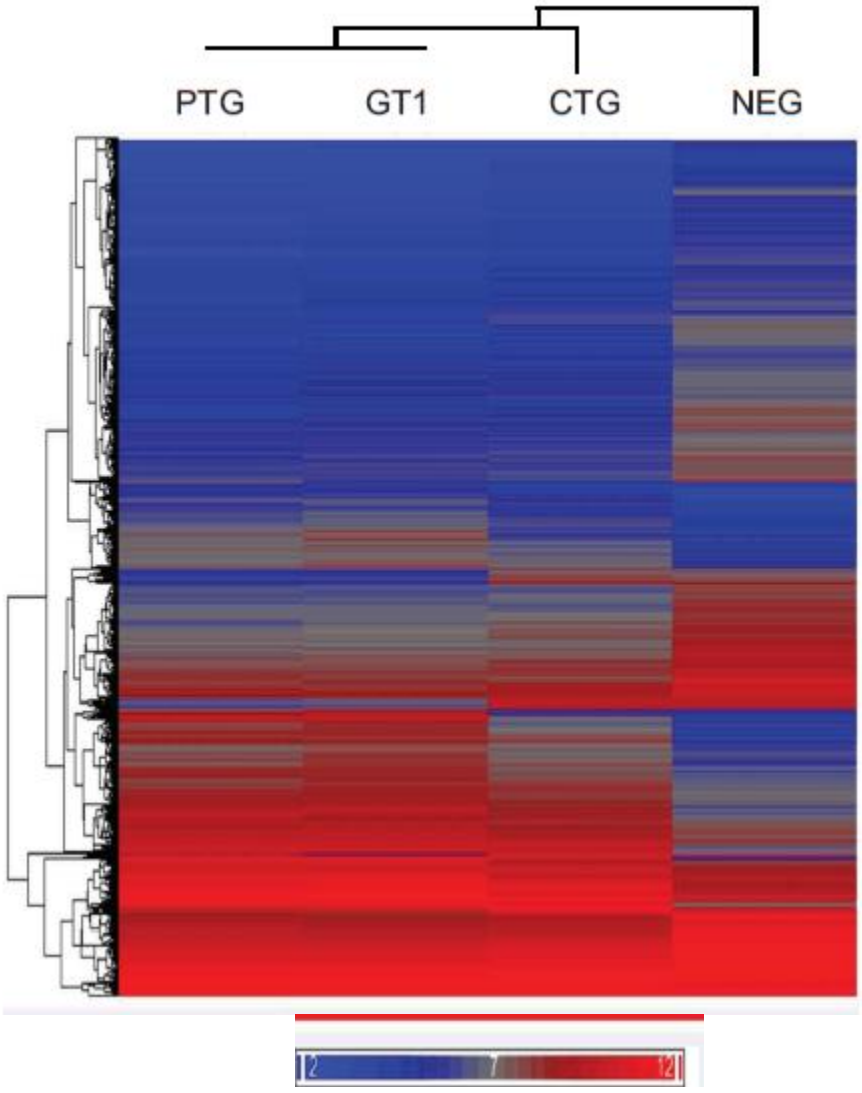
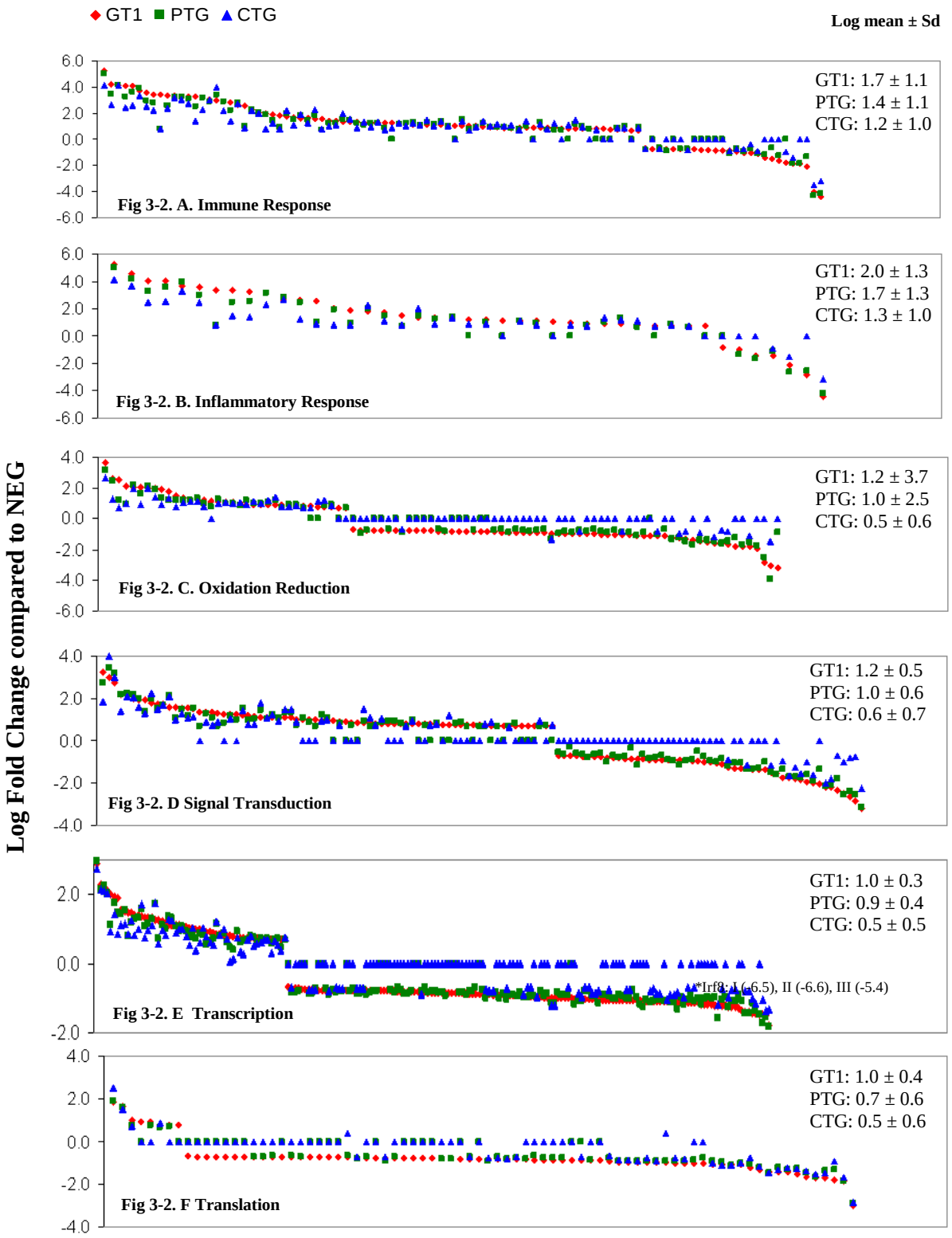


Figure 3-1. Hierarchical clustering analysis. HCA for classification of differential gene expression patterns in CD-1 outbred mice at day 5 post infection with *T. gondii* GT1, PTG and CTG strains. Gene expression signal intensity values were compared to negative control mice (NEG) that are not infected with *T. gondii*. Red indicates up-regulated signals; blue indicates down-regulated signals

of negative control mice compared to Type I, II and III infected mice and 2) gene expression comparison between *T. gondii* strain infections. Results showed that in mice infected with the three types of *T. gondii,* the overall gene expression patterns were similar when compared to the negative controls (Figure. 3-1). When comparing gene expression among mice infected with the three *T. gondii* lineages, Type III (CTG) infected mice had a distinct expression pattern from that of the Type I and II lineages (Figure. 3-1.)

Differentially expressed genes among treatments compared to untreated mice infections were further analyzed using OntoExpress (vortex.cs.wayne.edu/ontoexpress) to identify alterations to gene expression profiles for specific functional groups. We analyzed gene expression profiles for several biological processes including immune response, inflammatory response, oxidation reduction, signal transduction, transcription, translation, transport, apoptosis and cell cycle for both induced and repressed genes (Figure. 3-2. A-I and TABLE 3-5). Fold change values of differentially expressed genes were natural log transformed to generate graphs for each functional group.

The immune response category had the highest number of genes induced among all groups. Type I strain GT1 infection induced 82 genes and repressed 26 genes while PTG induced 73 genes and repressed 16 genes. Type III strain CTG induced 76 genes and repressed only 10 genes (TABLE 3-1). In this category, cytokines including *CCL22, CCL12, CCL8, CCL7, CCL3 and IFN-γ* were highly expressed compared to uninfected mice (TABLE 3-5). These genes were induced at higher levels in mice infected with Type I strain, compared to infections with Type II and III strains. Gene expression in mice infected with Type II strain remained intermediate between Type I and III infections, but more similar to Type I (FIG.3-2.A).


◆ GT1 ■ PTG ▲ CTG
Log mean ± Sd
Log Fold Change compared to NEG
GT1: 1.7 ± 1.1
PTG: 1.4 ± 1.1
CTG: 1.2 ± 1.0
Fig 3-2. A. Immune Response
GT1: 2.0 ± 1.3
PTG: 1.7 ± 1.3
CTG: 1.3 ± 1.0
Fig 3-2. B. Inflammatory Response
GT1: 1.2 ± 3.7
PTG: 1.0 ± 2.5
CTG: 0.5 ± 0.6
Fig 3-2. C. Oxidation Reduction
GT1: 1.2 ± 0.5
PTG: 1.0 ± 0.6
CTG: 0.6 ± 0.7
Fig 3-2. D Signal Transduction
GT1: 1.0 ± 0.3
PTG: 0.9 ± 0.4
CTG: 0.5 ± 0.5
*Irf8: I (-6.5), II (-6.6), III (-5.4)
Fig 3-2. E Transcription
GT1: 1.0 ± 0.4
PTG: 0.7 ± 0.6
CTG: 0.5 ± 0.6
Fig 3-2. F Translation

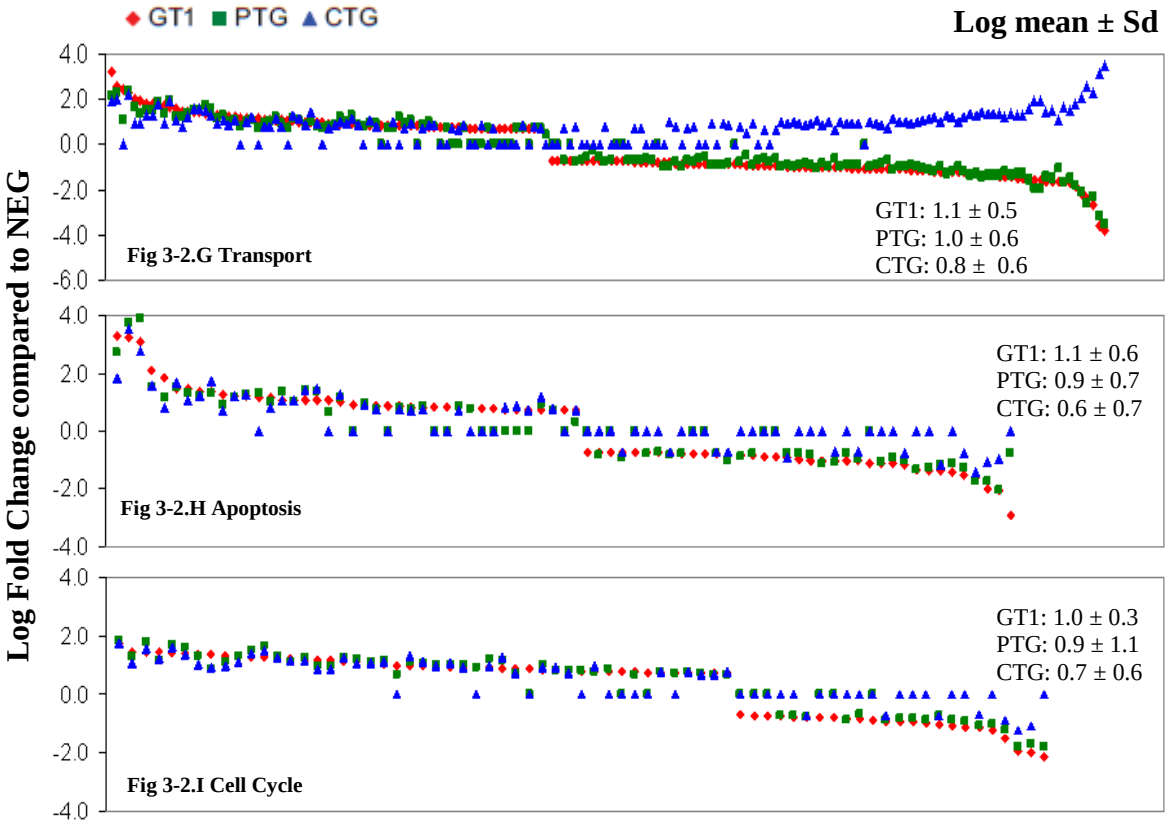


Figure. 3-2. A-I. Comparison of gene expression profiles for Type I, II and III infection based on biological processes. Shown are genes that were significantly up- or down-regulated during infection. Genes were categorized based on biological function.

Inflammatory response was a specific immune response significantly altered as a result of infection. Virulent strain GT1 and intermediately virulent strain PTG overall expression patterns were very similar. CTG again elicited a milder response compared to virulent and intermediate infections for genes both induced and repressed. Specifically, Type I strain GT1 infection induced 38 genes and repressed 8 genes while PTG induced 30 genes and repressed 7 genes. Type III CTG induced 4 genes and repressed only 10 genes. The same 3 genes, Tryptase beta-2 (Tpsb2), Thrombospondin 1 (Thbs1) and C-X-C motif chemokine 13 (Cxcl13) were dowregulated by 2 fold among all three infections. The number of differentially expressed genes down-regulated is significantly lower than those up-regulated (TABLE 3-1). Among differential expressed genes induced, Type I and II infections share similar expression profiles. Type III expression does not follow a clear trend (Figure. 3-2.B).

Oxidation reduction was another process determined to be altered during *T. gondii* infection. A total of 35, 31 and 31 genes belonging to this group were induced during Type I, II and III infection, respectively. Many genes overlapped between infections. The number of genes repressed during Type I (63 genes) and Type II (46 genes) infections surpassed the number of genes induced. Type III CTG only repressed 14 genes (TABLE 3-1). Repressed genes involved in oxidation reduction biological process followed a similar expression profile among Type I and II infection. Gene profiling of Type III repressed genes show the similar suppression trend of Type I and II but to a lesser extent (Figure.3-2. C).

In the remaining categories (signal transduction, transcription, translation, transport, apoptosis and cell cycle), we observed a similar phenomenon described above (Figure. 3-2. D-I). Type I and II had similar expression patterns compared to Type III. Collectively, an overall trend of polarized expression among the type I, II and III strains was detected. This polarization

characteristic was highly observed among repressed genes. Our results showed that expression is driven in two different directions, dependent on parasite genetic background.

Microarray validation via RT-PCR

In order to validate our microarray data, 5 most highly up-regulated and 6 most down-regulated genes were selected. The 5 most up regulated genes selected were *CCL22, CCL17, CCL12, CCL7* and *CCL2*, and the 6 highly repressed genes selected were *Prg4, Serpinb2, Serpinb10, ApoE, Fabp7* and *Marco* (TABLE 3-3). The RT-PCR expression data of these 11 genes showed similar trends during Type I and II infections. Although, the highly repressed genes share similar trends during Type III infection, the highly upregulated genes were downregulated based on RT-PCR data (Table. 3-4).

Determine parasite load in the spleen tissues of mice by RT-PCR

Parasite load in the spleen of mice infected with 500 tachyzoites of GT1, CTG and PTG was quantified. On day 5 post IP, the Log (parasites/g spleen) was 6.7 (s.d. = 1.0) for virulent strain GT1. This was more than 300 times higher than intermediate PTG which was 3.6 (s.d= 0.8) and non virulent CTG which was 2.4 (s.d. = 0.2) (FIG. 3-3).

Characterization of host cell population

FACS analysis revealed that Type I GT1 (Figure 3-4) infection dendritic cells comprised ~ 46 % of live cells gated, while macrophages were only 5% and neutrophils were ~ 45 %. Type II PTG (Figure 3-4) peritoneal lavage cells followed a very similar trend to Type I infection being composed of composed of ~ 50% , ~8% and 22% of dendritic cell, macrophages and neutrophils, respectively. Lastly, during Type III CTG, dendritic cells were composed of 57% of cell population, followed by neutrophils with 29% and macrophages being ~ 3% (Figure 3-4.A-I).

Table 3-3. Most highly up or down regulated genes

Gene	Accession no.	Fold Change (signal intensity value) GT1	PTG	CTG
Prg4	NM_021400	-139.5	-86.7	-1.9
Serpinb2	NM_011111	-61.4	-31.7	-1.4
Serpinb10	NM_198028	-83.5	-94.2	-6.7
ApoE	NM_009696	-43.7	-32.8	-6.5
Fabp7	NM_021272	-35.6	-23.6	-1.7
MARCO	NM_010766	-1.3	1.8	-14.1
CCL2	NM_011333	29.4	11.8	4.3
CCL7	NM_013654	26.0	12.3	4.2
CCL12	NM_011331	57.9	23.8	12.0
CCL17	NM_011332	3.9	2.0	1.6
CCL22	NM_009137	12.9	2.8	2.4

GT1– Type I, virulent **PTG** – Type II, intermediate ly virulent **CTG**– Type III, non-Virulent

Table 3-4. Microarray validation

Gene	RT-PCR ($2^{-\Delta\Delta CT}$) GT1	PTG	CTG
Prg4	-9.8	-8.6	-3.0
Serpinb2	-9.2	-7.3	-2.9
Serpinb10	-8.7	-10.1	-1.9
ApoE	-7.6	-9.5	-3.1
Fabp7	-9.7	-5.1	-1.9
Marco	-8.6	-7.2	0.2
CCL2	3.7	4.1	-0.9
CCL7	3.8	4.9	0.4
CCL12	4.1	5.0	-3.4
CCL17	1.9	-5.1	-5.8
CCL22	2.6	1.4	-3.9

Table 3-4. Validation of microarray using reverse transcriptase PCR (RT) data. The table shows 2^-ΔΔCt values obtained from RT-PCR for 11 genes up or down regulated during GT1, PTG and CTG infection. Gapdh was used as an internal control for expression comparison.

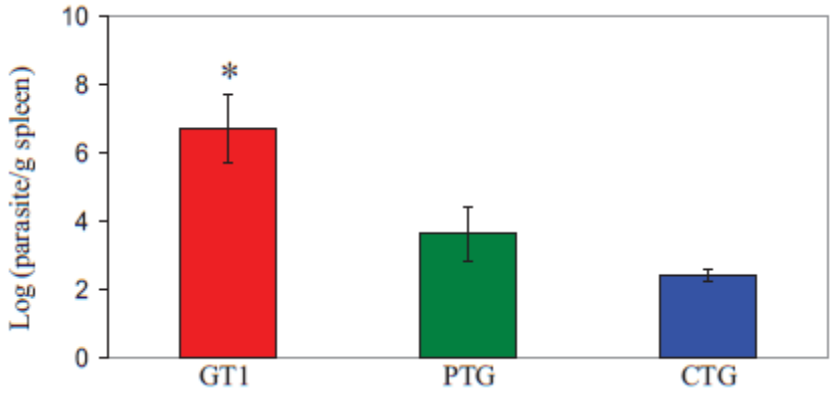


Figure 3-3. Parasite burdens (Log parasites/g spleen) in mice at day 5 post IP. Parasite load was determined via RT-PCR. GT1 has a significantly high parasite burden than that of PTG and CTG (*P = 0.001, ANOVA).

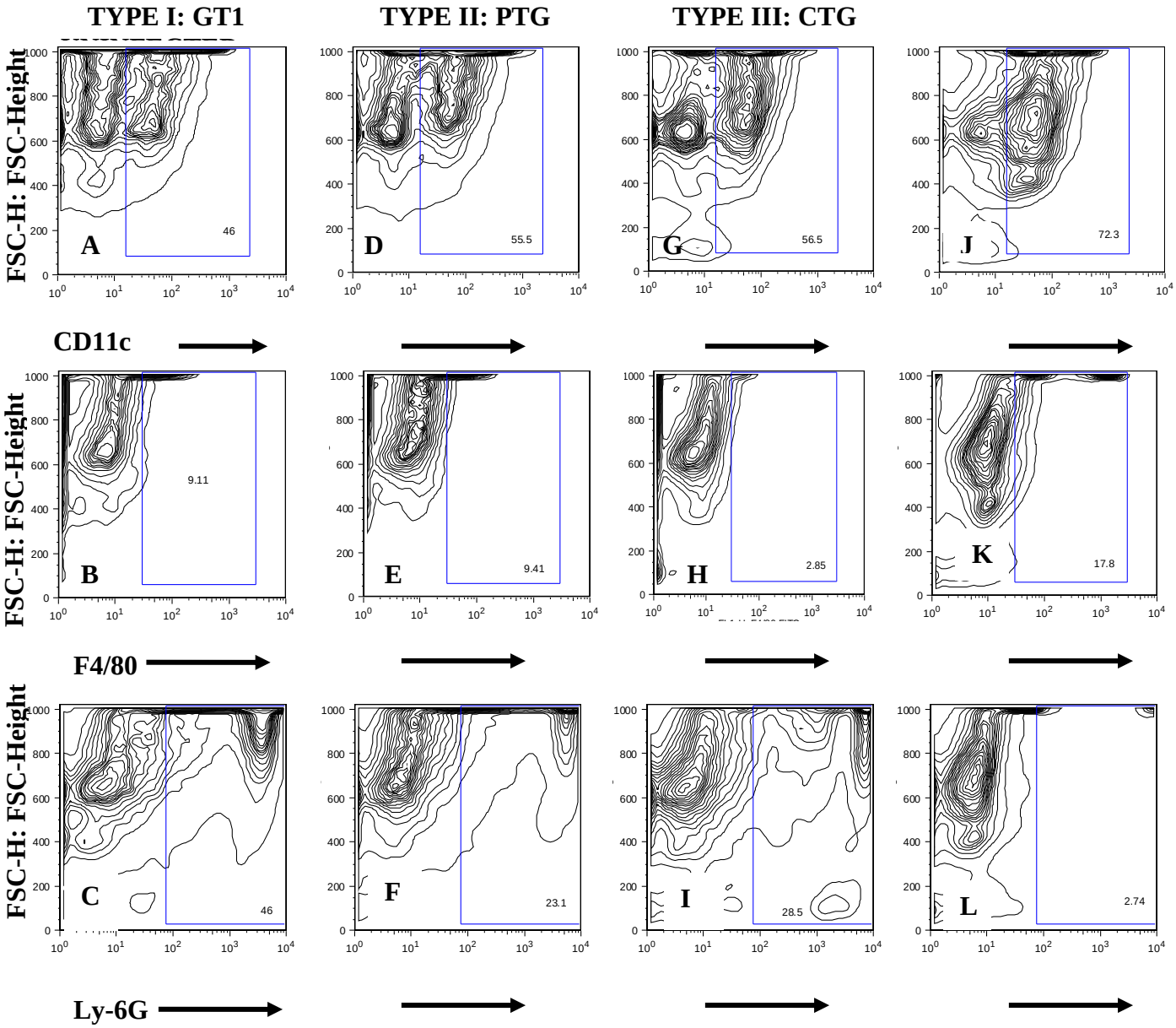


Figure. 3-4.A-L. Host cell population from peritoneal lavage during in vivo infection. Flow cytometric contour plots show expression of CD11c , F4/80 and Ly-6G in Type I (A-C), Type II (E-F), Type III (G-I) compared to uninfected (J-L). Data from a representative experiment performed in duplicate are shown.

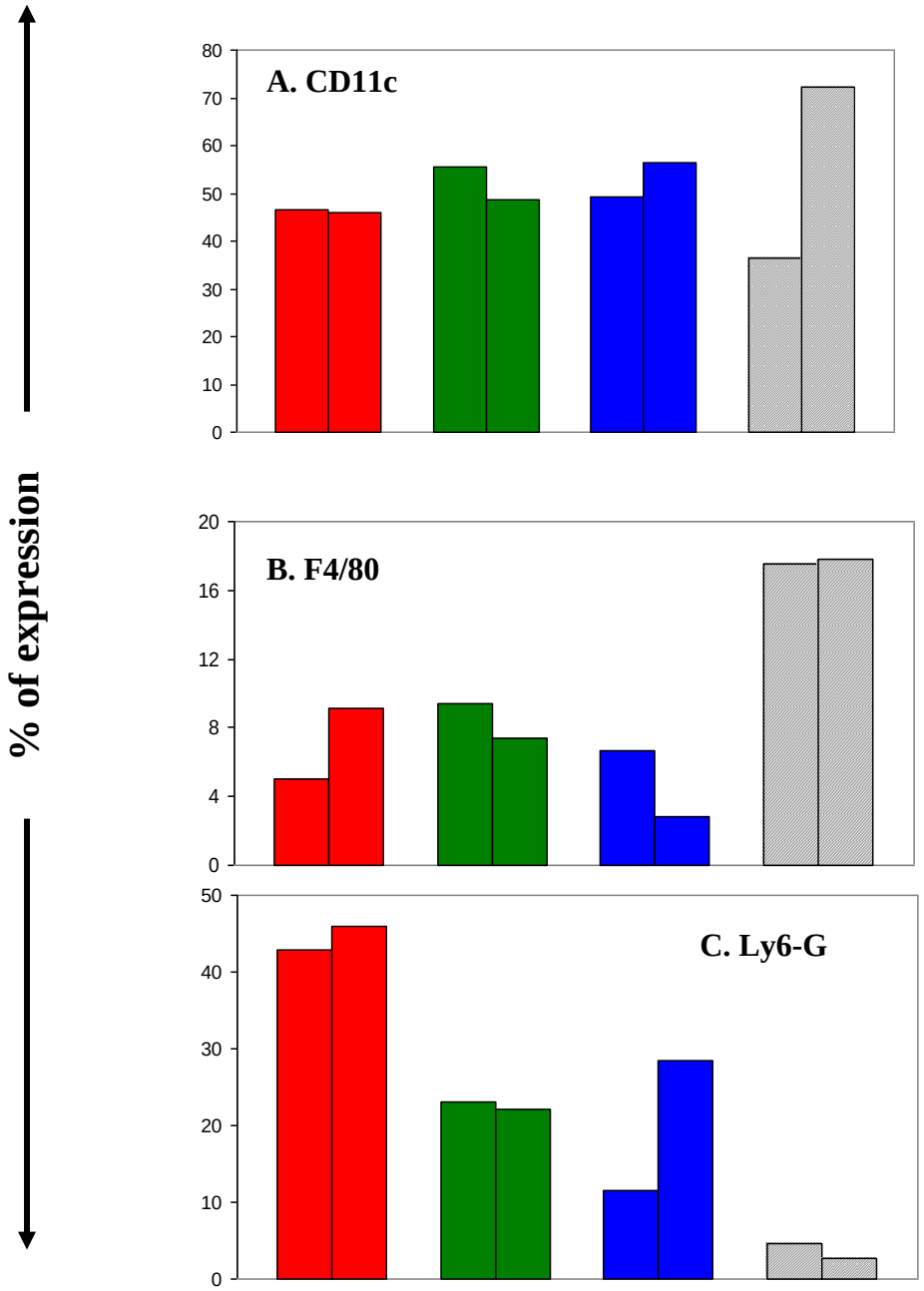


Figure 3-5 A-C. Bar diagrams of FACS analysis after *T. gondii* infection in vivo. Bar diagrams show the percent host cell expression of individual mice samples. Each bar represents one individual mouse sample. Cells were stained with CD11c (A), F4/80 (B) or Ly-6 (C) for each treatment.

Table 3-5. Gene expression ratio of category specific genes modulated during *T. gondii* infection compared to NEG

Functional Class	**Accession no.**	**Gene Name *(Gene symbol)***	**Fold Change in Transcription vs. NEG mice**		
			GT1	PTG	CTG
Immune Response	NM_021274	Chemokine (C-X-C motif) ligand *(Cxcl10)*	*191.8*	*156*	*62.6*
	NM_15351	Interleukin 1 family, member 9 *(Il1f9)*	*67*	*32.2*	*13.9*
	NM_011579	T-cell specific GTPase *(Tgtp)*	*65.6*	*63.5*	*63.4*
	NM_011331	Chemokine (C-C motif) ligand 12 *(CCL12)*	*57.9*	*26.4*	*11.9*
	NM_008361	Interleukin 1 beta *(Il1b)*	*57.2*	*37.5*	*12.9*
	NM_008599	Chemokine (C-X-C motif) ligand 9 *(Cxcl9)*	*40*	*52.4*	*28.1*
	NM_011337	Chemokine (C-C motif) ligand 3 *(CCL3)*	*35.6*	*19.4*	*12.2*
	NM_001038604	C-type lectin domain family 5, member a *(Clec5a)*	*31*	*16.7*	*9.1*
	NM_138648	Oxidized low density lipoprotein (lectin-like) receptor 1 *(Olr1)*	*29.7*	*2.2*	*2.2*
	NM_020001	C-type lectin domain family 4, member n *(Clec4n)*	*28.2*	*13.6*	*10.7*
	NM_153564	Guanylate binding protein 5 *(Gbp5)*	*28.1*	*26.9*	*24.3*
	NM_010260	Guanylate binding protein 2 *(Gbp2)*	*27*	*26*	*21.6*
	NM_011854	2'-5' oligoadenylate synthetase-like 2 *(Oasl2)*	*27*	*22.5*	*15.8*
	NM_013654	Chemokine (C-C motif) ligand 7 *(CCl7)*	*26*	*12.3*	*4.2*
	NM_021443	Chemokine (C-C motif) ligand 8 *(CCl8)*	*23.8*	*23.8*	*10*
	NM_008326	Immunity-related GTPase family M member *(1Irgm1)*	*20.1*	*18.6*	*20.7*
	NM_010259	Guanylate binding protein 1 *(Gpb1)*	*19.9*	*30.9*	*55*
	NM_008337	Interferon gamma *(Ifng)*	*17.8*	*18*	*9.5*
	NM_031167	Interleukin 1 receptor antagonist *(Il1rn)*	*16.8*	*8.9*	*4.1*
	NM_009778	Complement component 3 *(C3)*	*14.2*	*16.8*	*15.1*
	NM_009137	chemokine (C-C motif) ligand 22 *(CCL22)*	*12.9*	*2.8*	*2.4*
	NM_013683	Transporter 1, ATP-binding cassette, sub-family B *(Tap1)*	*9.6*	*10.3*	*9*
	NM_030150	DEXH (Asp-Glu-X-His) box polypeptide 58 *(Dhx58)*	*8.2*	*7.8*	*7.3*
	NM_019494	Chemokine (C-X-C motif) ligand 11*(Cxcl11)*	*7.4*	*6.9*	*2.2*
	NM_145634	CD300 antigen like family member F *(CD300lf)*	*7.1*	*4.4*	*3.4*
	NM_018866	Chemokine (C-X-C motif) ligand 13 *(Cxcl13)*	-81.4	-65.6	-23.4
	NM_019932	Platelet factor 4 *(Pf4)*	-55.2	-73.7	-31.7
	NM_007572	Complement component 1, q subcomponent, alpha polypeptide *(C1qa)*	-7.9	-3.7	NS
	NM_009888	Complement component factor h *(Cfh)*	-6.4	-6.6	NS
	NM_130449	Collectin sub-family member 12 *(Colec12)*	-6.2	-6.4	-4.0
	NM_007574	Complement component 1, q subcomponent, C chain *(C1qc)*	-5.8	NS	2.7
	NM_010016	CD55 antigen *(Cd55)*	-5.3	-3.4	NS
	NM_009777	complement component 1, q subcomponent, beta polypeptide (*C1qb)*	-4.2	-2.0	NS
	NM_007758	Complement receptor 2 *(Cr2)*	-4.2	-3.2	NS
	NM_009884	CCAAT/enhancer binding protein (C/EBP), gamma *(Cebpg)*	-3.1	-3.0	-2.5

Table 3-5. Continued

Functional Class	Accession no.	Gene Name *(Gene symbol)*	Fold Change in Transcription vs. NEG mice		
			GT1	PTG	CTG
	NM_033622	Tumor necrosis factor (ligand) superfamily, member 13b *(Tnfsf13b)*	-2.4	-2.5	-3.1
	NM_001037719	B and T lymphocyte associated *(Btla)*	-2.4	NS	NS
	NM_013499	Complement component (3b/4b) receptor 1-like *(Cr1l)*	-2.2	NS	NS
	NM_133829	Major facilitator superfamily domain containing 6 *(Mfsd6)*	-2.2	NS	NS
	NM_008339	CD79B antigen *(Cd79b)*	-2.2	NS	NS
	NM_001012477	Chemokine (C-X-C motif) ligand 12 *(Cxcl12)*	-2.2	-2.1	-2.3
	NM_027987	CD300 antigen like family member G *(Cd300lg)*	-2.2	-2.0	NS
	NM_009403	Tumor necrosis factor (ligand) superfamily, member 8 *(Tnfsf8)*	-2.1	NS	NS
	NM_199221	CD300 antigen like family member B *(Cd300lb)*	-2.1	-2.4	NS
	ENSMUST00000060105	Retinoic acid early transcript 1E *(Raet1e)*	-2.1	-2.0	-2.1
	NM_010389	Histocompatibility 2, O region beta locus *(H2-ob)*	-2.0	NS	NS
	NM_008176	Chemokine (C-X-C motif) ligand 1 *(Cxcl1)*	-2.0	-2.3	-2.0
Inflammatory Response	NM_021274	Chemokine (C-X-C motif) ligand *(Cxcl10)*	*191.8*	*156.0*	*62.6*
	NM_009892	Chitinase 3-like 3 *(Chi3l3)*	*96.4*	*68.3*	*38.3*
	NM_011331	Chemokine (C-C motif) ligand 12 *(CCL12)*	*57.9*	*26.4*	*11.9*
	NM_008361	Interleukin 1 beta *(Il1b)*	*57.2*	*37.5*	*12.9*
	NM_008599	Chemokine (C-X-C motif) ligand 9 *(CxCl9)*	*39.9*	*52.4*	*28.1*
	NM_011337	Chemokine (C-C motif) ligand 3 *(CCL3)*	*35.6*	*19.4*	*12.2*
	NM_138648	Oxidized low density lipoprotein (lectin-like) receptor 1 *(Olr1)*	*29.7*	*2.2*	*2.2*
	NM_011333	Chemokine (C-C motif) ligand 2 *(Ccl2)*	*29.4*	*11.8*	*4.3*
	NM_013654	Chemokine (C-C motif) ligand 7 *(CCL7)*	*26.0*	*12.3*	*4.2*
	NM_021443	Chemokine (C-C motif) ligand 8 *(CCL8)*	*23.8*	*23.8*	*10.0*
	NM_009778	complement component 3 *(C3)*	*14.2*	*16.8*	*15.1*
	NM_010927	nitric oxide synthase 2, inducible *(Nos2)*	*13.4*	*12.1*	*3.5*
	NM_009137	Chemokine (C-C motif) ligand 22 *(CCL22)*	*12.9*	*2.8*	*2.4*
	NM_019494	Chemokine (C-X-C motif) ligand 11 *(Cxcl11)*	*7.4*	*6.9*	*2.2*
	NM_013652	Chemokine (C-C motif) ligand 4 *(CCL4)*	*6.5*	*2.6*	*2.3*
	NM_009915	Chemokine (C-C motif) receptor 2 *(CCr2)*	*6.0*	*7.4*	*9.5*
	NM_013693	Tumor necrosis factor (Tnf)	*5.4*	*4.6*	*3.0*
	NM_020008	C-type lectin domain family 7, member a (Clec7a)	*4.6*	*2.1*	*2.3*
	NM_013653	Chemokine (C-C motif) ligand 5 *(CCl5)*	*3.9*	*4.6*	*7.5*
	NM_010851	Myeloid differentiation primary response gene 88 *(Myd88)*	*3.8*	*3.6*	*2.4*
	NM_011610	Tumor necrosis factor receptor superfamily, member 1b *(Tnfrsf1b)*	*3.8*	*4.3*	*3.7*
	NM_010554	Interleukin 1 alpha *(Il1a)*	*3.4*	*NS*	*2.5*
	NM_145126	Chitinase 3-like 4 *(Chi3l4)*	*3.2*	*2.9*	*2.4*
	NM_009140	Chemokine (C-X-C motif) ligand 2C *(Cxcl2)*	*3.1*	*NS*	*NS*

Table 3-5. Continued

Functional Class	Accession no.	Gene Name *(Gene symbol)*	Fold Change in Transcription vs. NEG mice		
			GT1	PTG	CTG
	NM_145636	Interleukin 27 *(Il27)*	*3.1*	*3.0*	*3.1*
	NM_018866	Chemokine (C-X-C motif) ligand 13*(Cxcl13)*	-81.4	-65.6	-23.4
	NM_011347	Selectin, platelet *(Selp)*	-17.7	-13.0	NS
	NM_010781	Tryptase beta 2 *(Tpsb2)*	-4.2	-3.1	-2.4
	NM_021439	Carbohydrate sulfotransferase 11 *(Chst1)*	2.3	NS	NS
	NM_011580	Thrombospondin 1*(Thbs1)*	-8.4	-13.4	-4.5
	NM_007553	Bone morphogenetic protein 2 *(Bmp2)*	-2.7	-3.8	NS
Oxidation Reduction	NM_009890	Cholesterol 25-hydroxylase (*Ch25h)*	*39.4*	*23.1*	*14.8*
	NM_010927	Nitric oxide synthase 2, inducible *(Nos2)*	*13.4*	*12.1*	*3.5*
	NM_011198	Prostaglandin-endoperoxide synthase 2 *(Ptgs2)*	*12.7*	*3.3*	*2.0*
	NM_019698	Aldehyde dehydrogenase 18 family, member A1 *(Aldh18a1)*	*7.7*	*8.6*	*7.3*
	NM_054098	STEAP family member 4 *(Steap4)*	*7.6*	*5.1*	*2.5*
	NM_009104	Ribonucleotide reductase M2 *(Rrm2)*	*7.0*	*8.2*	*7.1*
	NM_013671	Superoxide dismutase 2, mitochondrial *(Sod2)*	*7.0*	*6.9*	*4.1*
	NM_145533	Spermine oxidase *(Smox)*	*6.9*	*3.9*	*2.6*
	NM_175521	Dehydrogenase/reductase (SDR family) member 9 *(Dhrs9)*	*5.7*	*3.4*	*3.7*
	NM_010442	Heme oxygenase (decycling) 1 *(Hmox1)*	*3.8*	*3.3*	*2.9*
	NM_001012306	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and ste *(Hsd3b3)*	*3.3*	*3.4*	*3.1*
	NM_010344	Glutathione reductase *(Gsr)*	*3.3*	*3.7*	*3.2*
	NM_008638	Methylenetetrahydrofolate dehydrogenase (NAD+ dependent) *(Mthfd2)*	*3.3*	*2.6*	*2.1*
	NM_008898	Perforin 1 (pore forming protein) *(Por)*	*3.2*	*2.2*	*NS*
	NM_010274	Glycerol phosphate dehydrogenase 2, mitochondrial *(Gpd2)*	*3.1*	*3.5*	*2.8*
	ENSMUST00000082392	NADH dehydrogenase subunit 1 *(ND1)*	3.2	2.5	*NS*
	NM_013759	Selenoprotein X 1 *(Sepx1)*	*3.0*	*2.8*	*3.0*
	NM_008084	Glyceraldehyde-3-phosphate dehydrogenase *(Gapdh)*	*2.9*	*2.7*	*2.8*
	NM_001081274	Phosphogluconate dehydrogenase *(Pgd)*	*2.7*	*2.3*	*3.0*
	NM_008324	Indoleamine 2,3-dioxygenase 1 *(Ido1)*	*2.6*	*3.4*	*2.6*
	NM_019468	Glucose-6-phosphate dehydrogenase 2 *(G6pd2)*	*2.6*	*2.7*	*3.1*
	NM_009103	Ribonucleotide reductase M1 *(Rrm1)*	*2.6*	*2.8*	*2.7*
	NM_008062	Glucose-6-phosphate dehydrogenase X-linked *(G6pdx)*	*2.5*	*2.9*	*3.3*
	ENSMUST00000054963	Farnesyl diphosphate farnesyl transferase 1 *(Fdft1)*	*2.5*	*3.3*	*4.1*
	NM_008617	Malate dehydrogenase 2, NAD (mitochondrial) *(Mdh2)*	*2.5*	*2.0*	*2.1*
	NM_009660	Arachidonate 15-lipoxygenase *(Alox15)*	-21.2	-51.2	-4.2
	NM_178767	Transmembrane protein 195 *(Tmem195)*	-17.0	-12.1	NS
	NM_008969	Prostaglandin-endoperoxide synthase 1 *(Ptgs1)*	-6.8	-5.6	NS

Table 3-5. Continued

Functional Class	Accession no.	Gene Name *(Gene symbol)*	Fold Change in Transcription vs. NEG mice		
			GT1	PTG	CTG
	NM_178600	Vitamin K epoxide reductase complex, subunit 1 *(Vkorc1)*	-6.3	-4.8	-2.9
	NM_183158	Cytochrome P450, family 2, subfamily ab, polypeptide 1 (*Cyp2ab1)*	-6.0	-5.3	NS
	NM_010497	Isocitrate dehydrogenase 1 (NADP+), soluble *(Idh1)*	-5.9	-3.6	NS
	NM_011830	Inosine 5'-phosphate dehydrogenase 2 (*Impdh2)*	-5.2	-4.2	-2.4
	NM_030004	Crystallin, lambda 1 *(Cryl1)*	-4.9	-4.9	-2.1
	NM_019779	Cytochrome P450, family 11, subfamily a, polypeptide 1 *(Cyp11a1)*	-4.8	-4.2	-3.3
	NM_025522	Dehydrogenase/reductase (SDR family) member 7 *(Dhrs7)*	-4.7	-3.9	NS
	NM_001136084	Tryptophan hydroxylase 1 *(Tph1)*	-4.3	-4.1	-2.4
	NM_009662	Arachidonate 5-lipoxygenase (*Alox5)*	-4.1	-5.2	NS
	NM_013778	Aldo-keto reductase family 1, member C13 (Akr1c13)	-3.7	-4.4	-2.3
	NM_172778	Monoamine oxidase B *(Maob)*	-3.5	-3.5	-2.7
	NM_010008	Cytochrome P450, family 2, subfamily j, polypeptide 6 *(Cyp2j6)*	-3.4	-3.7	NS
	NM_026172	2,4-dienoyl CoA reductase 1, mitochondrial *(Decr1)*	-3.1	-2.0	NS
	NM_016764	Peroxiredoxin 4 *(Prdx4)*	-3.0	-2.8	-2.2
	NM_008160	Glutathione peroxidase 1 *(Gpx1)*	-3.0	NS	NS
	NM_020282	NAD(P)H dehydrogenase, quinone 2 *(Nqo2)*	-3.0	-2.4	NS
	NM_008278	Hydroxyprostaglandin dehydrogenase 15 (NAD) *(Hpgd)*	-3.0	-3.1	NS
	NM_027534	3-ketodihydrosphingosine reductase *(Kdsr)*	-2.9	-2.4	NS
	ENSMUST00000023559	Enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzy *(Ehhadh)*	-2.8	-2.5	-2.2
	NM_008212	Hydroxyacyl-Coenzyme A dehydrogenase *(Hadh)*	-2.7	-2.1	NS
	NM_146090	Zinc binding alcohol dehydrogenase, domain containing 2 *(Zadh2)*	-2.7	-2.4	NS
	NM_030017	Retinol dehydro;genase 12 *(Rdh12)*	-2.7	-2.3	NS
Signal Transduction	NM_009909	Interleukin 8 receptor, beta *(Il8rb)*	*26.1*	*15.3*	*6.5*
	NM_010259	Linker for activation of T cells *(Lat)*	*19.9*	*30.9*	*55.1*
	NM_181754	G protein-coupled receptor 141 (*Gpr141)*	*15.3*	*24.2*	*20.1*
	NM_028808	Purinergic receptor P2Y, G-protein coupled 13 *(P2ry13)*	*9.2*	*9.2*	*4.1*
	NM_009283	Signal transducer and activator of transcription 1 *(Stat1)*	*9.2*	*9.3*	*8.3*
	NM_010553	Interleukin 18 receptor accessory protein *(Il18rap)*	*7.5*	*9.2*	*7.7*
	NM_016846	Ral guanine nucleotide dissociation stimulator,-like 1 *(Rgl1)*	*7.1*	*7.4*	*4.9*
	NM_030720	G protein-coupled receptor 84 *(Gpr84)*	*6.9*	*4.1*	*3.7*
	NM_009915	Chemokine (C-C motif) receptor 2 *(Ccr2)*	*6.0*	*7.4*	*9.5*

Table 3-5. Continued

Functional Class	Accession no.	Gene Name *(Gene symbol)*	Fold Change in Transcription vs. NEG mice		
			GT1	PTG	CTG
	NM_028595	Membrane-spanning 4-domains, subfamily A, member 6C *(Ms4a6c)*	*5.8*	*5.5*	*5.6*
	NM_026835	Membrane-spanning 4-domains, subfamily A, member 6D *(Ms4a6d)*	*5.8*	*4.7*	*4.6*
	NM_008365	Interleukin 18 receptor 1 *(Il18r1)*	*4.9*	*8.4*	*8.2*
	NM_017466	CCr12	*4.9*	*3.0*	*2.7*
	NM_008152	G-protein coupled receptor 65 *(Gpr65)*	*4.8*	*4.4*	*3.6*
	NM_020260	CDC42 GTPase-activating protein *(Cdgap)*	*4.7*	*3.4*	*3.0*
	NM_019963	Signal transducer and activator of transcription 2 *(Stat2)*	*4.5*	*4.7*	*3.1*
	NM_011332	Chemokine (C-C motif) ligand 17 *(CCL17)*	*3.9*	*2.0*	*NS*
	NM_010851	Myeloid differentiation primary response gene 88 *(Myd88)*	*3.8*	*3.6*	*2.4*
	NM_010431	Hypoxia inducible factor 1, alpha subunit *(Hif1a)*	*3.8*	*2.9*	*2.1*
	NM_030701	G protein-coupled receptor 109A *(Gpr109a)*	*3.7*	*2.0*	*2.5*
	NM_153067	MAS-related GPR, member A3 *(Mrgpra3)*	*3.5*	*2.3*	*NS*
	NM_008042	Formyl peptide receptor 3 *(Fpr3)*	*3.4*	*3.4*	*2.9*
	NM_008315	5-hydroxytryptamine (serotonin) receptor 7 *(Htr7)*	*3.4*	*2.7*	*NS*
	NM_009539	Zeta-chain (TCR) associated protein kinase *(Zap70)*	*3.4*	*4.7*	*4.3*
	NM_025278	Guanine nucleotide binding protein (G protein), gamma 12 (*Gng12)*	*3.3*	*2.7*	*2.2*
	NM_001099624	Rap guanine nucleotide exchange factor (GEF) 2 *(Rapgef2)*	*3.1*	*2.9*	*2.3*
	NM_009910	Chemokine (C-X-C motif) receptor 3 *(Cxcr3)*	*3.1*	*4.2*	*6.0*
	NM_145630	Pyruvate dehydrogenase kinase, isoenzyme 3 *(Pdk3)*	*3.0*	*2.8*	*3.0*
	NM_009917	Chemokine (C-C motif) receptor 5 *(Ccr5)*	*3.0*	*3.5*	*3.6*
	BC119501	G protein-coupled receptor 15 *(Gpr15)*	*3.0*	*3.0*	*2.7*
	NM_010177	Fas ligand 1 *(Fasl)*	*3.0*	*3.9*	*4.4*
	NM_010177	Fas ligand *(Fas)*	*3.0*	*3.9*	*4.4*
	ENSMUST00000058535	G protein-coupled receptor 149 *(Gpr149)*	*2.8*	*2.1*	*2.5*
	NM_146934	Olfactory receptor 46 *(Olfr46)*	*2.7*	*2.9*	*NS*
	NM_053240	Vomeronasal 1 receptor, B10 *(V1rb10)*	*2.7*	*2.4*	*NS*
	NM_053227	Vomeronasal 1 receptor, B4 *(V1rb4)*	*2.7*	*2.0*	*NS*
	NM_146718	Olfactory receptor 430 *(Olfr430)*	*2.6*	*3.4*	*2.6*
	NM_013875	Phosphodiesterase 7B *(Pde7b)*	*2.6*	*3.0*	*2.7*
	NM_008364	Interleukin 1 receptor accessory protein *(Il1rap)*	*2.6*	*NS*	*NS*
	NM_030682	Toll-like receptor 1 *(Tlr1)*	*2.5*	*2.3*	*2.1*
	NM_008561	Melanocortin 3 receptor *(Mc3r)*	*2.4*	*NS*	*NS*
	ENSMUST00000020547	Vomeronasal 2, receptor 81 *(Vmn2r81)*	*2.4*	*2.2*	*NS*
	NM_007429	Angiotensin II receptor, type 2 *(Agtr2)*	*2.4*	*NS*	*NS*
	NM_183168	Pyrimidinergic receptor P2Y, G-protein coupled, 6 *(P2ry6)*	*2.3*	*2.6*	*4.5*
	NM_205820	Toll-like receptor 13 *(Tlr13)*	*2.3*	*2.0*	*3.0*
	NM_133193	Interleukin 1 receptor-like 2 *(Il1rl2)*	*2.3*	*2.1*	*2.2*
	NM_029522	G-protein signalling modulator 2 *(Gpsm2)*	*2.3*	*2.5*	*2.9*

Table 3-5. Continued

Functional Class	Accession no.	Gene Name *(Gene symbol)*	Fold Change in Transcription vs. NEG mice		
			GT1	PTG	CTG
	NM_198961	Vomeronasal 2, receptor 43 *(Vmn2r43)*	*2.2*	*2.6*	*NS*
	NM_146265	Olfactory receptor 1506 *(Olfr1506)*	*2.2*	*2.6*	*NS*
	NM_019925	G protein-coupled receptor 132 *(Gpr132)*	*2.2*	*2.3*	*NS*
	NM_007577	Complement component 5a receptor 1 *(C5ar1)*	*2.2*	*2.0*	*2.0*
	NM_029523	DEP domain containing 1a *(Depdc1a)*	*2.2*	*2.7*	*2.6*
	NM_146383	Olfactory receptor 460 *(Olfr460)*	*2.2*	*NS*	*NS*
	NM_009421	Tnf receptor-associated factor 1 *(Traf1)*	*2.2*	*2.1*	*NS*
	NM_021297	Toll-like receptor 4 *(Tlr4)*	*2.2*	*NS*	*2.1*
	NM_001081211	Platelet-activating factor receptor *(Ptafr)*	*2.2*	*2.4*	*2.2*
	NM_019408	Nuclear factor of kappa light polypeptide *(Nfkb2)*	*2.1*	*NS*	*NS*
	NM_008519	Leukotriene B4 receptor 1 *(Ltb4r1)*	*2.1*	*NS*	*3.3*
	NM_001011863	Olfactory receptor 406 *(Olfr406)*	*2.1*	*2.4*	*NS*
	NM_146879	Olfactory receptor 330 (Olfr330)	*2.1*	*2.4*	*NS*
	NM_025658	Membrane-spanning 4-domains, subfamily A, member 4D *(Ms4a4d)*	*2.1*	*2.0*	*NS*
	NM_010341	Neuromedin U receptor 1 *(Nmur1)*	*2.1*	*NS*	*NS*
	NM_198895	Active BCR-related gene *(Abr)*	*2.1*	*2.0*	*NS*
	NM_010185	Fc receptor, IgE, high affinity I, gamma polypeptide *(Fcer1g)*	*2.1*	*2.0*	*2.2*
	NM_178589	Tumor necrosis factor receptor superfamily, member 21*(Tnfrsf21)*	*2.1*	*NS*	*2.4*
	NM_207573	Olfactory receptor 1380 *(Olfr1380)*	*2.1*	*2.0*	*NS*
	NM_029094	Phosphatidylinositol 3-kinase, catalytic, beta polypeptide *(Pik3cb)*	*2.1*	*NS*	*NS*
	NM_213659	Signal transducer and activator of transcription 3 *(Stat3)*	*2.0*	*2.1*	*2.0*
	NM_147104	Olfactory receptor 550 *(Olfr550)*	*2.0*	*2.3*	*NS*
	NM_207253	Olfactory receptor 1371*(Olfr1371)*	*2.0*	*NS*	*NS*
	NM_028724	Ras and Rab interactor 2 *(Rin2)*	*2.0*	*NS*	*NS*
	NM_010437	Human immunodeficiency virus type I enhancer binding protein 2 *(Hivep2)*	*2.0*	*NS*	*NS*
	NM_008159	G protein-coupled receptor 33 *(Gpr33)*	*2.0*	*2.0*	*2.6*
	NM_001011867	Olfactory receptor 538 *(Olfr538)*	*2.0*	*2.1*	*NS*
	NM_010170	Coagulation factor II (thrombin) receptor-like 2 *(F2rl2)*	*2.0*	*NS*	*2.1*
	NM_146593	Olfactory receptor 1111 *(Olfr1111)*	-24.3	-23.2	-9.5
	NM_011158	Protein kinase, cAMP dependent regulatory *(Prkar2b)*	-17.0	-13.1	-2.1
	NM_023653	Wingless-related MMTV integration site 2 *(Wnt2)*	-14.5	-11.1	-2.3
	NM_008839	Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide *(Pik3ca)*	-12.2	-12.7	-2.7
	NM_130862	Brain-specific angiogenesis inhibitor 1-associated protein *(Baiap2)*	-10.4	-6.1	-2.0
	NM_007489	Aryl hydrocarbon receptor nuclear translocator-like *(Arntl)*	-9.2	-8.0	-6.0

Table 3-5. Continued

Functional Class	Accession no.	Gene Name *(Gene symbol)*	Fold Change in Transcription vs. NEG mice		
			GT1	PTG	CTG
	NM_001011530	Olfactory receptor 723 *(Olfr723)*	-9.1	-8.4	-7.3
	NM_146606	Olfactory receptor 24 *(Olfr24)*	-7.8	-3.8	NS
	NM_053190	Sphingosine-1-phosphate receptor 5 *(S1pr5)*	-7.4	-6.7	-5.1
	NM_007551	chemochine (C-X-C motif) receptor 5 *(Cxcr5)*	-7.1	-4.9	-2.7
	NM_001039692	Rho GTPase activating protein 12 *(Arhgap12)*	-6.5	-5.7	-4.6
	NM_023121	Guanine nucleotide binding protein (G protein) *(Gngt2)*	-6.0	-5.3	-3.5
	NM_139138	EGF-like module containing, mucin-like, hormone receptor-like 4 *(Emr4)*	-5.7	-5.4	-5.1
	NM_022420	G protein-coupled receptor, family C, group 5, member B *(Gprc5b)*	-5.6	-3.8	-2.5
	BC087872	Guanine nucleotide binding protein (G protein), gamma 5 *(Gng5)*	-4.8	-4.9	NS
	NM_009707	Rho GTPase activating protein 6 *(Arhgap6)*	-4.6	-4.4	-3.1
	NM_009706	Rho GTPase activating protein 5 *(Arhgap5)*	-3.9	-2.7	NS
	NM_007995	Ficolin A *(Fcna)*	-3.9	-3.8	NS
	NM_001081298	Latrophilin 2 (Lphn2)	-3.8	-3.8	NS
	NM_172671	Leucine-rich repeat-containing G protein-coupled receptor 4 *(Lgr4)*	-3.7	-3.9	-3.2
	NM_001033251	G protein-coupled receptor 174 *(Gpr174)*	-3.7	-2.8	NS
	NM_013516	Membrane-spanning 4-domains, subfamily A, member 2 *(Ms4a2)*	-3.6	-2.9	NS
	NM_010184	Fc receptor, IgE, high affinity I, alpha polypeptide *(Fcer1a)*	-3.6	-3.3	-2.5
	NM_001122998	Tiam2 *(Tiam2)*	-3.0	-2.4	NS
	NM_001122759	Phosphodiesterase 7A *(Pde7a)*	-2.9	-2.8	NS
	NM_001008548	Phosphodiesterase 2A, cGMP-stimulated *(Pde2a)*	-2.8	-2.6	NS
	NM_018869	G protein-coupled receptor kinase 5 *(Grk5)*	-2.7	-2.1	NS
	AK129042	Protein phosphatase 2, regulatory subunit B *(Ppp2r5c)*	-2.6	NS	-3.2
	NM_008137	Guanine nucleotide binding protein, alpha 14 *(Gna14)*	-2.5	-2.4	NS
	NM_133222	EGF, latrophilin seven transmembrane domain containing 1 *(Eltd1)*	-2.5	-2.6	NS
	NM_001011852	Olfactory receptor 1029 *(Olfr1029)*	-2.5	-3.1	-2.3
	NM_007641	Membrane-spanning 4-domains, subfamily A, member 1 *(Ms4a1)*	-2.5	-2.6	NS
	NM_007904	Endothelin receptor type B *(Ednrb)*	-2.4	-2.4	NS
	NM_010332	Endothelin receptor type A *(Ednra)*	-2.4	-2.0	NS
	NM_146903	Olfactory receptor 871 *(Olfr871)*	-2.4	-2.2	-2.0
	NM_019917	Vomeronasal 2, receptor 26 *(Vmn2r26)*	-2.4	-2.4	NS

Table 3-5. Continued

Functional Class	Accession no.	Gene Name *(Gene symbol)*	Fold Change in Transcription vs. NEG mice		
			GT1	PTG	CTG
	NM_144800	Metastasis suppressor 1 *(Mtss1)*	-2.3	NS	NS
	NM_007901	Sphingosine-1-phosphate receptor 1 *(S1pr1)*	-2.3	-3.1	-2.4
	NM_022985	Zinc finger, AN1-type domain 6 *(Zfand6)*	-2.3	NS	NS
	NM_153820	Rho GTPase activating protein 15 *(Arhgap15)*	-2.3	-2.1	-2.0
	NM_011739	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase *(Ywhaq)*	-2.2	-2.2	NS
	NM_175531	MAS-related GPR, member B2 *(Mrgprb2)*	-2.2	-2.0	NS
	BC031465	Family with sequence similarity 13, member B *(Fam13b)*	-2.2	-2.7	-3.0
	NM_001034868	MAS-related GPR, member X2 *(Mrgprx2)*	-2.1	NS	NS
	NM_138956	Ras association (RalGDS/AF-6) domain family member 3 *(Rassf3)*	-2.1	NS	-2.0
	NM_183031	G protein-coupled receptor 183 *(Gpr183)*	-2.1	-2.2	-2.3
	NM_147070	Olfactory receptor 604 *(Olfr604)*	-2.1	NS	NS
	NM_133926	calcium/calmodulin-dependent protein kinase I *(Camk1)*	-2.1	NS	NS
	NM_172525	Rho GTPase activating protein 29 *(Arhgap29)*	-2.0	NS	NS
	XM_884524	MAS-related GPR, member B13 *(Mrgprb1)*	-2.0	NS	NS
Translation	NM_011710	Tryptophanyl-tRNA synthetase *(Wars)*	*6.6*	*6.9*	*12.6*
	NM_011163	Eukaryotic translation initiation factor 2-alpha kinase *(Eif2ak2)*	*5.4*	*5.0*	*4.6*
	NM_027350	Asparaginyl-tRNA synthetase *(Nars)*	*2.7*	*2.2*	*2.1*
	NM_025936	Arginyl-tRNA synthetase *(Rars)*	*2.5*	*NS*	*NS*
	NM_146217	Alanyl-tRNA synthetase *(Aars)*	*2.5*	*2.2*	*NS*
	NM_008214	Histidyl-tRNA synthetase *(Hars)*	*2.2*	*2.0*	*2.3*
	NM_133916	Eukaryotic translation initiation factor 3, subunit B *(Eif3b)*	*2.2*	*2.0*	*NS*
	NM_029735	Glutamyl-prolyl-tRNA synthetase *(Eprs)*	*2.1*	*NS*	*NS*
	ENSMUST00000055731	Eukaryotic translation initiation factor 5A-lik *(Eif5)*	2.7	2.5	2.4
	NM_148945	Ribosomal protein S6 kinase polypeptide 3 *(Rps6)*	2.1	2.0	2.3
	NM_207523	Ribosomal protein L23a (*Rpl23)*	-2.0	NS	NS
	AF260271	Ribosomal protein L9 *(Rpl9)*	-2.0	NS	NS
	NM_001031808	Ribosomal protein L41 *(Rpl41)*	-2.0	NS	NS
	NM_007990	Finkel-Biskis-Reilly murine sarcoma virus *(Fau)*	-2.0	NS	NS
	NM_080636	Histidyl-tRNA synthetase 2, mitochondrial (putative) *(HARS2)*	-2.0	NS	NS
	NM_011300	Ribosomal protein S7 *(Rps7)*	-2.0	NS	NS
	NM_016959	Ribosomal protein S3 *(Rps3)*	-2.1	-2.0	NS
	BC086914	Ribosomal protein l36 *(Rpl36)*	-2.1	-2.0	NS
	NM_009091	Ribosomal protein L15 *(Rps15)*	-2.1	-2.0	NS
	NM_009076	Ribosomal protein L12 *(Rpl12)*	-2.1	NS	NS
	NM_011029	Ribosomal protein SA *(Rpsa)*	-2.1	NS	NS
	BC082284	Ribosomal protein L27 (Rpl27)	-2.1	NS	NS

Table 3-5. Continued

Functional Class	Accession no.	Gene Name *(Gene symbol)*	Fold Change in Transcription vs. NEG mice		
			GT1	PTG	CTG
	NM_001039534	Phosphoseryl-tRNA kinase *(Pstk)*	-2.1	-2.2	-2.1
	NM_009079	Ribosomal protein L22 (Rpl22)	-2.1	-2.0	NS
	NM_011029	Ribosomal protein SA *(Rpsa)*	-2.1	NS	NS
	BC082284	Ribosomal protein L27 (Rpl27)	-2.1	NS	NS
	NM_001039534	Phosphoseryl-tRNA kinase *(Pstk)*	-2.1	-2.2	-2.1
	NM_016844	Ribosomal protein S28 *(Rps28)*	-2.1	NS	NS
	NM_053159	Mitochondrial ribosomal protein L3 *(Mrpl3)*	-2.1	-2.5	-2.1
	NM_175004	Peptidyl-tRNA hydrolase 2 (Ptrh2)	-2.1	-2.0	NS
	BC096413	Ribosomal protein L37A *(Rpl37a)*	-2.2	NS	-NS
	BC096413	Ribosomal protein L37 *(Rpl37)*	-2.2	NS	NS
	NM_011291	Ribosomal protein L7 *(Rpl7)*	-2.2	NS	NS
	NM_001033865	Ribosomal protein S27 *(Rps27)*	-2.2	-2.3	NS
	NM_001033865	Ribosomal protein S27A *(Rps27a)*	-2.2	-2.3	NS
	NM_016980	Ribosomal protein L5 *(Rpl5)*	-2.3	-2.1	-2.1
	NM_001005859	Ribosomal protein L34 *(Rpl34)*	-2.3	-2.0	NS
Transcription	NM_172648	Interferon activated gene 205 *(Ifi205)*	*26.4*	*21.8*	*21.4*
	NM_016850	Interferon regulatory factor 7 *(Irf7)*	*18.2*	*19.7*	*15.5*
	NM_008329	Interferon activated gene 204 *(Ifi204)*	*9.7*	*8.4*	*8.4*
	NM_009283	Signal transducer and activator of transcription 1 *(Stat1)*	*9.2*	*9.3*	*8.3*
	NM_008390	Interferon regulatory factor 1 *(Irf1)*	*8.3*	*7.6*	*7.7*
	NM_010118	Early growth response 2 *(Egr2)*	*7.3*	*3.0*	*2.6*
	NM_010751	MAX dimerization protein 1 *(Mxd1)*	*6.6*	*4.4*	*2.4*
	NM_019963	Signal transducer and activator of transcription 2 *(Stat2)*	*4.5*	*4.7*	*3.1*
	NM_009504	Vitamin D receptor *(Vdr)*	*4.4*	*2.2*	*2.3*
	NM_028083	Chromatin assembly factor 1 *(Chaf1b)*	*4.3*	*3.3*	*3.4*
	NM_010931	Ubiquitin-like, containing PHD and RING finger domains, 1 *(Uhrf)*	*4.1*	*3.6*	*2.3*
	NM_010431	Hypoxia inducible factor 1, alpha subunit *(Hif1a)*	*3.8*	*2.9*	*2.1*
	NM_008234	Helicase, lymphoid specific *(Hells)*	*3.7*	*3.7*	*3.1*
	NM_019507	T-box 21 *(Tbx21)*	*3.5*	*5.6*	*5.7*
	NM_025866	Cell division cycle associated 7 *(Cdca7)*	*3.4*	*2.0*	*NS*
	NM_021878	J umonji, AT rich interactive domain 2 *(Jarid2)*	*3.4*	*3.1*	*2.6*
	NM_007829	Fas death domain-associated protein *(Daxx)*	*3.2*	*2.8*	*2.2*
	NM_024184	Anti-silencing function 1 homolog B (S. cerevisiae) *(Asf1b)*	*3.1*	*3.9*	*3.6*
	NM_008320	Interferon regulatory factor 8 *(Irf8)*	*3.1*	*3.7*	*3.5*
	ENSMUST00000039876	MYC binding protein 2 *(Mycbp2)*	*3.0*	*2.6*	*2.7*
	NM_008566	Minichromosome maintenance deficient 5 *(Mcm5)*	*2.9*	*3.0*	*2.7*
	NM_023598	AT rich interactive domain 5B *(Arid5b)*	*2.9*	*2.5*	*2.2*

Table 3-5. Continued

Functional Class	Accession no.	Gene Name *(Gene symbol)*	Fold Change in Transcription vs. NEG mice		
			GT1	PTG	CTG
	NM_011119	Proliferation-associated 2G4 *(Pa2g4)*	*2.8*	*2.1*	*NS*
	NM_021326	Nucleolar and spindle associated protein 1 *(Sap30l)*	-2.0	NS	NS
	NM_175770	cAMP responsive element binding protein 3-like 1 *(Creb3l1)*	-2.0	-2.3	-2.0
	NM_011957	TAF10 RNA polymerase II, TATA box binding protein *(Taf10)*	-2.0	NS	NS
	NM_007970	Histone deacetylase 5 *(Hdac5)*	-2.0	NS	NS
	NM_020024	Myocyte enhancer factor 2D *(Mef2d)*	-2.0	-2.3	NS
	NM_001077696	Putative homeodomain transcription factor 2 *(Phtf2)*	-2.0	NS	NS
	NM_133665	AT rich interactive domain 4B (RBP1-like) *(Arid4b)*	-2.0	NS	NS
	NM_172992	HOP homeobox *(Hopx)*	-2.0	NS	NS
	NM_194262	Zinc finger protein 110 *(Zfp110)*	-2.0	NS	NS
	NM_175606	Zinc finger protein 161 *(Zfp161)*	-2.0	NS	NS
	NM_022981	Polymerase I and transcript release factor *(Ptrf)*	-2.1	NS	NS
	NM_009547	Zinc finger protein 606 *(Zfp606)*	-2.1	-2.2	NS
	NM_008986	Zinc finger protein 809 *(Zfp809)*	-2.1	-2.2	-2.0
	NM_026112	IKAROS family zinc finger 3 (Ikzf3)	-2.1	-2.2	NS
	NM_011771	Zinc finger protein 273 *(Zfp273)*	-2.1	NS	NS
	NM_175009	Transcription factor A, mitochondrial *(Tfam)*	-2.1	NS	NS
	NM_198322	Zinc finger protein 740 *(Zfp740)*	-2.1	NS	NS
	NM_009360	CCAAT/enhancer binding protein zeta *(Cebpz)*	-2.1	NS	NS
	NM_153194	Mortality factor 4 like 1 *(Morf4l1)*	-2.1	NS	NS
	NM_001024806	Polymerase (RNA) II (DNA directed) polypeptide K *(Polr2k)*	-2.1	NS	NS
Transport	NM_008491	Lipocalin 2 *(Lcn2)*	*24.1*	*8.7*	*7.1*
	NM_023044	Solute carrier family 15, member 3 *(Slc15a3)*	*12.9*	*9.9*	*7.2*
	NM_011990	Solute carrier family 7 *(Slc7a11)*	*11.6*	*2.9*	*0.0*
	NM_013683	Transporter 1 *(Tap1)*	*9.6*	*10.4*	*9.0*
	NM_054098	STEAP family member 4 *(Steap4)*	*7.6*	*5.1*	*2.5*
	NM_016917	Solute carrier family 40 (iron-regulated transporter) *(Slc40a1)*	*6.9*	*3.9*	*2.5*
	NM_011400	Solute carrier family 2 (facilitated glucose transporter) *(Slc2a1)*	*6.0*	*4.3*	*3.5*
	NM_011400	Solute carrier family 16 (Slc16a3)	*6.0*	*4.3*	*3.5*
	NM_023908	Solute carrier organic anion transporter family (Slco3a1)	*5.9*	*6.3*	*6.0*
	NM_011227	RAB20, member RAS oncogene family *(Rab20)*	*5.7*	*3.9*	*2.6*
	NM_009721	ATPase, Na+/K+ transporting, beta 1 polypeptide *(Atp1b1)*	*5.3*	*6.7*	*6.9*
	NM_053108	Glutaredoxin *(Glrx)*	*4.9*	*3.3*	*3.0*
	NM_015774	ERO1-like *(Ero1l)*	*4.3*	*3.4*	*2.2*
	NM_016972	Solute carrier family 7 *(Slc7a8)*	*4.2*	*3.9*	*3.5*
	NM_172621	Chloride intracellular channel 5 *(Clic5)*	*4.2*	*4.3*	*4.9*
	NM_172621	Nucleoporin 93 *(Nup93)*	*4.2*	*4.3*	*4.9*

Table 3-5. Continued

Functional Class	Accession no.	Gene Name *(Gene symbol)*	Fold Change in Transcription vs. NEG mice		
			GT1	PTG	CTG
	NM_213616	ATPase, Ca++ transporting, plasma membrane 4 *(Atp2b4)*	*4.0*	*5.4*	*4.3*
	NM_011930	Chloride channel 7 *(Clcn7)*	*4.0*	*4.9*	*3.7*
	NM_013885	Chloride channel 7 *(Clic4)*	*3.6*	*3.1*	*2.6*
	NM_011530	Transporter 2 *(Tap2)*	*3.3*	*3.6*	*2.9*
	NM_024281	Ribosome binding protein 1 *(Rrbp1)*	*3.3*	*2.7*	*2.4*
	NM_009729	ATPase, H+ transporting *(Atp6vOc)*	*3.1*	*2.7*	*2.7*
	NM_010688	LIM and SH3 protein 1 *(Lasp1)*	*3.1*	*2.9*	*3.4*
	NM_011404	Solute carrier family 7 *(Slc7a5)*	*3.1*	*2.1*	*NS*
	NM_026331	Solute carrier family 25, member 37 *(Slc25a37)*	-2.0	2.0	NS
	NM_178746	Solute carrier family 38, member 9 *(Slc38a9)*	-2.0	2.0	NS
	ENSMUST00000066921	Solute carrier family 25, member 40 *(Slc25a40)*	-2.0	-2.1	NS
	ENSMUST00000036862	Component of oligomeric golgi complex 5 *(Cog5)*	-2.0	NS	NS
	NM_011895	Solute carrier family335, member 1 *(Slc35a1)*	-2.1	NS	NS
	NM_030057	Trafficking protein particle complex 6B *(Trappc6b)*	-2.1	NS	NS
	NM_023202	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex *(Ndufa7)*	-2.1	-2.1	NS
	NM_133891	Solute carrier family 44, member 1 *(Slc44a1)*	-2.2	-2.0	NS
	NM_153163	Ca2+-dependent activator protein for secretion 2 *(Cadps2)*	-2.2	-2.7	NS
	NM_008771	Purinergic receptor P2X, ligand-gated ion channel *(P2rx1)*	-2.2	-2.8	-2.1
	NM_053198	Sideroflexin 4 *(Sfxn4)*	-2.3	-2.2	-2.3
	NM_026684	NADH dehydrogenase (ubiquinone) 1 beta subcomplex *(Ndufb10)*	-2.3	-2.7	NS
	ENSMUST00000082411	NADH dehydrogenase subunit 3 *(ND3)*	-2.3	NS	NS
	ENSMUST00000082419	NADH dehydrogenase subunit 6 *(ND6)*	-2.3	NS	NS
	NM_001111	Cmah	-2.4	-2.5	NS
	BC002089	Sec61 beta subunit *(SEC61b)*	-2.4	-2.5	-2.9
	ENSMUST00000100262	Solute carrier family 38, member 1 *(Slc38a1)*	-2.4	-2.2	-2.1
	NM_020258	Solute carrier family 37, member 2 *(SLC37a2)*	-2.5	NS	-2.0
	NM_138756	Solute carrier family 25, member 36 *(Slc25a36)*	-2.5	-2.6	-2.7
	NM_026693	Gamma-aminobutyric acid (GABA-A) receptor-associated protein *(Gabarapl2)*	-2.5	-2.0	NS
	NM_013790	ATP-binding cassette, sub-family C (CFTR/MRP), member 5 *(Abcc5)*	-2.5	-2.2	NS
	NM_147218	ATP-binding cassette, sub-family A (ABC1), member 6 *(Abca6)*	-2.6	-2.6	-3.0
	NM_025571	Mitochondria-associated protein involved in granulocyte *(Magmas)*	-2.6	-2.8	-3.3
	NM_011515	Vesicle-associated membrane protein 7 *(Vamp7)*	-2.7	-2.5	NS
	NM_026879	Chromatin modifying protein 2B *(Chmp2b)*	-2.7	-2.4	NS

Table 3-5. Continued

Functional Class	Accession no.	Gene Name *(Gene symbol)*	Fold Change in Transcription vs. NEG mice		
			GT1	PTG	CTG
Apoptosis	NM_009909	Interleukin 8 receptor, beta *(Il8rb)*	*26.1*	*15.3*	*6.5*
	NM_010370	Granzyme A *(Gzma)*	*24.8*	*41.2*	*34.5*
	NM_013542	Granzyme B *(Gzmb)*	*21.9*	*49.8*	*16.0*
	NM_001001495	TNFAIP3 interacting protein 3 (Tnfaip3)	*8.3*	*4.8*	*5.0*
	NM_008655	Growth arrest and DNA-damage-inducible 45 beta *(Gadd45b)*	*6.5*	*3.3*	*2.2*
	NM_007523	BCL2-antagonist/killer 1 *(Bak1)*	*4.5*	*4.6*	*5.3*
	NM_001037713	XIAP associated factor 1 *(Xaf1)*	*4.4*	*3.7*	*3.0*
	NM_007611	Caspase 7 (Casp7)	*4.1*	*3.4*	*3.3*
	NM_019955	Receptor-interacting serine-threonine kinase 3 *(Ripk3)*	*3.9*	*3.9*	*5.7*
	NM_007987	Fas ligand *(Fas)*	*3.5*	*2.5*	*2.1*
	BC046640	Transmembrane protein 173*(Tmem173)*	*3.4*	*3.2*	*3.5*
	NM_009773	Budding uninhibited by benzimidazoles 1 homolog, beta *(Bub1b)*	*3.4*	*3.6*	*3.6*
	NM_197889	Interferon zeta *(Ifnz)*	*3.2*	*3.8*	*NS*
	NM_007829	Fas death domain-associated protein *(Daxx)*	*3.2*	*2.8*	*2.2*
	NM_011817	Growth arrest and DNA-damage-inducible 45 gamma *(Gadd45g)*	*3.0*	*3.9*	*3.0*
	NM_026669	Transmembrane BAX inhibitor motif containing 6 *(Tmbim6)*	*3.0*	*2.9*	*3.0*
	NM_009425	Tumor necrosis factor (ligand) superfamily, member 10 *(Tnfsf10)*	*3.0*	*4.2*	*4.1*
	NM_010177	Fas ligand (Fasl)	*3.0*	*3.9*	*4.4*
	NM_145827	NLR family, pyrin domain containing 3 (NLrp3)	*2.9*	*2.0*	*NS*
	NM_010019	Death-associated protein kinase 2 (Dapk2)	*2.7*	*3.3*	*3.5*
	NM_009760	BCL2/adenovirus E1B interacting protein 3 *(Bnip3)*	*2.5*	*NS*	*NS*
	BC092213	Cytochrome c, somatic (Cycs)	*2.5*	*2.6*	*2.6*
	NM_009402	Peptidoglycan recognition protein 1*(Pglyrp1)*	*2.4*	*2.1*	*2.2*
	NM_009742	B-cell leukemia/lymphoma 2 related protein A1a *(Bcl2a1a)*	*2.4*	*NS*	*NS*
	NM_009743	BCL2-like 1 *(Bcl2l1)*	*2.4*	*2.3*	*2.1*
	NM_207680	BCL2-like 11 *(Bcl2l11)*	*2.3*	*2.1*	*2.1*
	NM_001040654	Cyclin-dependent kinase inhibitor 2A *(Cdkn2a)*	-2.2	-2.2	-2.1
	NM_008771	Purinergic receptor P2X *(P2rx1)*	-2.2	-2.8	-2.1
	NM_144931	NEDD8 activating enzyme E1 subunit 1 *(Nae1)*	-2.3	-2.4	NS
	NM_010872	NLR family, apoptosis inhibitory protein 2 *(Naip2)*	-2.3	-2.1	NS
	BC059254	PH domain and leucine rich repeat protein phosphatase *(Phlpp)*	-2.3	NS	NS
	NM_008612	Menage a trois 1 *(Mnat1)*	-2.4	NS	NS
	NM_007465	Baculoviral IAP repeat-containing 2 *(Birc2)*	-2.5	-2.2	-2.5
	NM_020558	TBC1 domain family, member 2B *(C1d)*	-2.7	-2.1	NS
	NM_021897	Transformation related protein 53 inducible nuclear protein *(Trp53inp1)*	-2.7	-2.2	NS

Table 3-5. Continued

Functional Class	Accession no.	Gene Name *(Gene symbol)*	Fold Change in Transcription vs. NEG mice		
			GT1	PTG	CTG
	NM_019745	Programmed cell death 10 *(Pdcd10)*	-2.8	-3.1	NS
	NM_134131	Tumor necrosis factor, alpha-induced protein 8 *(Tnfaip8)*	-2.8	-3.0	-2.1
	NM_010121	Eukaryotic translation initiation factor 2 alpha kinase *(Eif2ak3)*	-2.8	-2.2	NS
	NM_010241	Thymoma viral proto-oncogene 1 interacting protein *(Aktip)*	-2.8	-2.7	-2.0
	NM_008160	Glutathione peroxidase 1*(Gpx1)*	-3.0	NS	NS
	NM_144792	Sphingomyelin synthase 1*(Sgms1)*	-3.1	-2.9	NS
	NM_030750	Sphingosine-1-phosphate phosphatase 1*(Sgpp1)*	-3.1	-2.5	NS
	NM_008580	Mitogen-activated protein kinase kinase kinase 5 *(Map3k5)*	-3.2	-2.8	-2.2
	NM_029653	Death associated protein kinase 1*(Dapk1)*	-3.8	-3.8	NS
	NM_008670	NLR family, apoptosis inhibitory protein 1 *(naip1)*	-3.9	-3.5	NS
	ENSMUST00000027373	Protein phosphatase 1F *(Ppm1f)*	-4.0	-3.3	-3.3
	NM_010871	Baculoviral IAP repeat-containing 1f *(Birc1f)*	-4.1	-3.1	NS
	NM_010870	NLR family, apoptosis inhibitory protein 5 *(Naip5)*	-4.6	-3.6	-2.2
	NM_146042	Ring finger protein 144B *(Rnf144b)*	-4.8	-5.9	-4.1
	NM_011050	Programmed cell death 4 *(Pdcd4)*	-7.4	-5.6	-2.9
	NM_010258	GATA binding protein 6 *(Gata6)*	-7.8	-7.9	-2.7
	NM_009690	CD5 antigen-like *(Cd51)*	-19.0	-2.2	4.3
Cell Cycle	NM_009828	Cyclin A2 *(Ccna2)*	*5.9*	*6.4*	*5.7*
	NM_001037713	XIAP associated factor 1 *(Xaf1)*	*4.4*	*3.7*	*3.0*
	BC085238	Cyclin B1*(CCnb1)*	*4.4*	*6.0*	*4.8*
	NM_028083	Chromatin assembly factor 1, subunit B (p60) *(Chaf1b)*	*4.3*	*3.3*	*3.4*
	NM_145150	Protein regulator of cytokinesis 1*(Prc1)*	*4.2*	*5.6*	*5.0*
	NM_010615	Kinesin family member 11 *(Kif11)*	*4.0*	*4.9*	*3.8*
	NM_008563	Minichromosome maintenance deficient 3 *(Mcm3)*	*4.0*	*3.8*	*2.7*
	NM_027954	Synaptonemal complex central element protein 2 *(Syce2)*	*3.9*	*2.5*	*2.6*
	NM_008564	Minichromosome maintenance deficient 2 mitotin *(Mcm2)*	*3.8*	*3.1*	*2.6*
	NM_008234	Helicase, lymphoid specific *(Hells)*	*3.7*	*3.7*	*3.1*
	NM_001113179	Budding uninhibited by benzimidazoles 1 homolog *(Bub1)*	*3.6*	*4.6*	*4.1*
	NM_133762	Non-SMC condensin II complex, subunit G2 *(Ncapg2)*	*3.6*	*5.2*	*4.4*
	NM_009773	Budding uninhibited by benzimidazoles 1 homolog *(Bub1b)*	*3.4*	*3.6*	*3.6*
	NM_007669	Cyclin-dependent kinase inhibitor 1A *(Cdkn1)*	*3.4*	*3.0*	*3.3*
	NM_144553	Discs, large (Drosophila) homolog-associated protein 5 *(Dlgap5)*	*3.2*	*3.5*	*3.2*
	NM_009829	Ubiquitin-like, containing PHD and RING finger domains, 1 *(Uhrf1)*	*3.2*	*2.7*	*2.4*

Table 3-5. Continued

Functional Class	Accession no.	Gene Name *(Gene symbol)*	Fold Change in Transcription vs. NEG mice		
			GT1	PTG	CTG
	NM_009829	Cyclin D2 *(CCnd2)*	*3.2*	*2.7*	*2.4*
	NM_028760	Centrosomal protein 55 *(Cep55)*	*3.0*	*3.6*	*3.6*
	NM_010892	NIMA (never in mitosis gene a)-related expressed kinase 2 *(Nek2)*	*3.0*	*3.4*	*3.0*
	NM_175155	SAM and SH3 domain containing 1 *(Sash1)*	*2.9*	*3.1*	*2.9*
	NM_144818	Non-SMC condensin I complex, subunit H *(Ncaph)*	*2.8*	*3.2*	*3.1*
	NM_001025577	Avian musculoaponeurotic fibrosarcoma *(Maf)*	*2.7*	*2.0*	*NS*
	NM_175155	SAM and SH3 domain containing 1 *(Sash1)*	*2.9*	*3.1*	*2.9*
	NM_144818	Non-SMC condensin I complex, subunit H *(Ncaph)*	*2.8*	*3.2*	*3.1*
	NM_001025577	Avian musculoaponeurotic fibrosarcoma *(Maf)*	*2.7*	*2.0*	*NS*
	NM_133851	Nucleolar and spindle associated protein 1 *(Nusap1)*	*2.7*	*3.0*	*3.8*
	NM_011497	Aurora kinase A *(Aurka)*	*2.6*	*3.0*	*3.1*
	NM_001083188	Ligase I *(Lig I)*	*2.6*	*2.8*	*2.6*
	NM_026560	Cell division cycle associated 8 *(Cdca8)*	*2.6*	*2.8*	*3.0*
	NM_009828	Cyclin A2 *(Ccna2)*	*5.9*	*6.4*	*5.7*
	NM_008885	Peripheral myelin protein 22 *(Pmp22)*	-8.4	-5.9	NS
	NM_011050	Programmed cell death 4 *(Pdcd4)*	-7.4	-5.6	-2.9
	NM_007964	Ecotropic viral integration site 5*(Evi5)*	-7.0	-6.1	-3.3
	NM_181394	Anaphase promoting complex subunit 13 *(Anapc13)*	-4.5	-3.4	-2.5
	NM_001081222	Establishment of cohesion 1 homolog 1 *(Esco1)*	-3.4	-2.8	NS
	NM_026902	Malignant T cell amplified sequence 1 *(Mcts1)*	-3.1	-2.9	-2.0
	NM_019405	Centrin 2 (Cetn2)	-3.0	-2.6	NS
	NM_178667	Transcription factor Dp 2 (Tfdp2)	-2.9	-2.5	NS
	NM_001024622	PEST proteolytic signal containing nuclear protein *(Pcnp)*	-2.8	-2.1	-2.1
	NM_009826	RB1-inducible coiled-coil 1 *(Rb1cc1)*	-2.7	-2.4	NS
	NM_007635	Cyclin G2 (*Ccng2)*	-2.6	-2.3	NS
	NM_007635	Wilms' tumour 1-associating protein *(Wtap)*	-2.6	-2.3	NS
	NM_145541	RAS-related protein-1a *(Rap1a)*	-2.5	-2.4	-2.0
	NM_008612	Menage a trois 1 *(Mnat1)*	-2.4	-1.9	NS
	NM_026832	Cell growth regulator with ring finger domain 1 *(Cgrrf1)*	-2.3	-2.0	NS
	NM_144931	NEDD8 activating enzyme E1 subunit 1 *(Nae1)*	-2.3	-2.4	NS
	NM_009875	Cyclin-dependent kinase inhibitor 1B (Cdkn1b)	-2.2	NS	NS
	NM_183186	Forkhead box N3 (Foxn3)	-2.2	NS	NS
	NM_001040654	Cyclin-dependent kinase inhibitor 2A *(Cdkn2a)*	-2.2	-2.2	-2.1
	NM_001114328	Cell cycle progression 1 *(Ccpg1)*	-2.2	-2.1	NS
	NM_007631	Cyclin D1 *(Ccnd1)*	-2.1	-2.1	NS

Table 3-5. Continued

Functional Class	Accession no.	Gene Name *(Gene symbol)*	Fold Change in Transcription vs. NEG mice		
			GT1	PTG	CTG
	AB041558	Myeloid/lymphoid or mixed-lineage leukemia 5 *(Mll5)*	-2.1	NS	NS
	NM_025389	Anaphase promoting complex subunit 11 *(Anapc11)*	-2.1	NS	NS
	NM_001031808	Mitochondrial ribosomal protein L41 *(Mrpl41)*	-2.0	NS	NS

Table 3-5. *T. gondii* microarray experiment was carried out as previously described above. Altered transcripts are categorized based on functional group. Genes listed correspond to gene profiles shown in Figure 6 A-I. Values represent fold change differences in signal intensity levels compared to NEG samples. Genes were considered induced if significantly up regulated, ≥ 2 fold and repressed if significantly down regulated, ≤ -2 fold. NS indicates not significantly expressed. *italicized* font indicates

Uninfected cells served as control (Figure 3-5.J-L). Bar graphs show percent expression of duplicate samples stained for each treatment (Figure 3-5)

Discussion

In this study, we examined gene expression in mice infected with three distinct *T. gondii* strains. These data suggest that even though virulent, intermediately virulent and non-virulent strains elicit an overall similar pattern of response in mice, there are specific differences of host gene expressions from the infection of the three genetically different *T. gondii* strains. Day 5 post infection was selected for microarray analysis based on the tissue burden assay (Figure. 2-2). It is likely that at day 5 post infection, significant shifts in host response to infection occur and Type II and III strains start to switch from tachyzoite to bradyzoite growth. It is shown that Type I strain reached significantly higher spleen tissue burden compared to that of Type II and III strains which had comparable parasite loads (Figure. 3-3). Using the genes highly upregulated or downregulated, RT-PCR confirmed our microarray and trends in gene expression were comparable. Oddly, those genes that were mildly upregulated during non virulent infection were suppressed in our RTPCR data (Table 3-3). One possible explanation could be that the low parasite numbers during non virulent infection could significantly change the ratio of host cells present and sampled. Interestingly, gene expression results showed that Type I and II consistently induced similar trends of gene expression among biological processes with the latter having slightly lower levels, while Type III response was much lower (Figure. 3-2 A-I). In addition, both Type I and II strains caused a strong polarized effect on host gene expression, in that host genes were either highly induced or more suppressed than that by Type III strain. These results are surprising, as mouse infections with *Toxoplasma* Type II and III strains result in parasite persistence that is associated with cyst formation and chronic infection within the hosts,

and it was expected that Type II and III would share more similarities in affecting gene expressing in mice, not that of the Type I and II strains. These results suggest parasite genetic background play an important role in host response. A recent study characterizing the strain specific host cell response to *T. gondii* using neuroepithelial cells showed that Type I strain caused a wide transcriptional response encompassing > 1,000 genes, while cells infected with Type II and III strains results in the alteration of 78 and 344 genes, respectively (Xiao J, 2010). This study suggests that Type II elicits a weaker response to infection in contrast to Type III. This conclusion is quite different from results observed in our present study. These differences are possibly attributed to the human neuroepithelial cells used in the above mentioned study versus the diverse cell types present in peritoneal lavage of mice collected during our study.

The cells responsible for gene expression during *T. gondii* infection in our study were monitored. FACS analysis showed that there is no significant difference between macrophages and dendritic cells among all three treatments (Figure 3-4 and Figure 3-5). Interestingly, percent of neutrophils expressed only differ among virulent GT1 and non virulent CTG infection. Taken together, gene expression profiles obtained from in vivo infections are not influenced by specific cells but expression is based on parasite genotype. Due to the inadequate replications and shortcomings of experimental design, further conclusions could not be made.

An overactive host immune response has been shown in mice infected with *T. gondii* virulent strains compared to infection with non virulent strains. This over reaction of Th1 proinflammatory response plays a significant part to mortality in mice infected with virulent Type I strain (Mordue, 2001). We expected that genes related to the immune response group would have higher expression levels during virulent *T. gondii* infection compared to intermediate and non virulent infection. Analysis of genes belonging to functional groups of immune and

inflammatory responses showed that genes highly expressed were cytokines, chemokines and upstream factors responsible for induction of proinflammatory response (CCL22, CCL17, CCL12, CCL7 and CCL2) (Table 3-5). In addition, NOS, Myd88 and TNF were up-regulated in all three types of infections. Compared to uninfected mice, NOS was 13 and 12 fold higher in Type I and II infection, respectively. In contrast, this same gene was only 4 fold higher during Type III infection. Myd88 was up-regulated 4 fold in both Types I and II while up-regulated by 2.4 fold in Type III infection. Lastly TNF was up-regulated ~5 fold during Type I and II infections and up-regulated at a lower level (3 fold) during Type III infection (Table 3-5). IL-27 was significantly expressed (3 fold higher) during all *T. gondii* infection compared to uninfected mice. This cytokine has been shown to regulate T helper cell response during parasite infection, regulating the expansion of the Th1 response (15). Taken together, expression profiles based on biological processes showed that Type I infection modulated a larger number of gene expression in mice, closely followed by the Type II infection, and Type III infection had the least effect on mice. The proinflammatory cytokines induced during *T. gondii* infection may serve as an important mediator for defense, but due to the uncontrolled response when infected with the Type I strain, more damage is caused to the host.

Macrophages are among the immune cells recruited and activated upon infection with *T. gondii* (Denkers, 2003). Our results showed an increased suppression of a macrophage receptor gene (MARCO) in those mice infected with the non virulent strain. This same gene was suppressed during Type I and II strain infection when compared to negative control mice (TABLE 3-3). MARCO has been shown to play a role in determining infection outcome in mice infected with *Leshmania major*. Gomes et al showed that MARCO expression increased in spleens of mice infected with *L. major* compared to saline injected mice (negative control)

leading to increased uptake of parasite, which eventually neutralized the infection (Gome, 2009) This phenomenon may suggest that the Type I and II *T. gondii* strains may be more efficiently internalized by macrophages than that of the Type III strain. Another interesting finding was the enhanced suppression of proteoglycan 4 (Prg4) with Type I infection. Prg4 expression was suppressed by 139.5, 86.7 and 1.9 folds in Type I, II and III *T. gondii* strains, respectively. Prg4 functions in lubrication and cytoprotection in the synovial fluid. The protein has large mucin-like repeats which, substituted with O-linked oligosaccharides, impart lubricating and cytoprotective properties (Ikegawa, 2000). However, the role of Prg4 in *T. gondii* infection is not clear.

Two serine protease inhibitors (Serpinb2 and Serpinb10) were also highly suppressed in mice infected with the Type I and II *T. gondii* strains. Serpinb2 was suppressed by 61.4, 31.7 and 1.4 folds in Type I, II and III infection, respectively. While Serpinb10 was suppressed by 83.5, 94.2 and 6.7 folds in Types I, II and III, respectively. It was suggested that SERPINs could inhibit host cell apoptosis (Ahn, 2009). Some SERPINs have been shown to inhibit cathespins and lysosomal enzymes. A previous study showed that, when *T. gondii* serine protease activity was inhibited via two serine protease inhibitor 3,4-dichloroisocoumarin and 4-(2-aminoethyl)-benzenesulfonyl fluoride, the parasite was unable to successfully invade host cells (Conseil, 1999). When *T. gondii* was treated with chymotrypsin-like serine protease inhibitors Z-Gly-Gly-Phe-CMK and TPCK, its replication was inhibited and the viability decreased (Shaw, 2002). Lagal et al showed that when TgSUB1 (a serine protease secreted from microneme) was disrupted, *T. gondii* showed decreased attachment and invasion (Lagal, 2010). It has been reported that ROP18, a serine protease, is highly expressed in Type I *T. gondii* strains and is responsible for acute virulence in mice (Taylor, 2006). Taken together, these data demonstrate that proteases are important for survival and transmission of *T. gondii*. Suppression of host

protease inhibitors maybe an offensive mechanism developed by highly virulent *T. gondii* strains. This current study indicated that Type I *T. gondii* strain had the strongest effect to up- or down regulate mouse gene expression, closely followed by the Type II strain, whereas Type III strain had mild effect on mouse gene expression. As reported in previous study, over reaction of Th1 pro-inflammatory response was associated with the death of mice infected with Type I strain (Mordue, 2001). In the perspective of evolution, Type II strain may have the adaptive advantage over Type I and III strains. It is likely that high virulence of Type I strain may kill its mouse host quickly, posing a negative impact to parasite transmission. Though both Type II and III strains can readily establish chronic infection in mice, our current study showed that the former can induce much stronger immune and inflammatory responses than the latter. This may allow Type II strain to out compete Type III strain in the context of within-host competition using host immune and inflammatory responses to exclude the competitor. This may explain the dominance of Type II lineage worldwide (Ajzenberg, 2002; Howe, 1995). Though there is no direct evidence to support this hypothesis, it is possible to test it experimentally in future studies. In this study we showed that distinct parasite strains caused different responses in its host. The differential host-parasite interaction may ultimately dictate the pathogenesis of *T.gondii* infection. Future work should be aimed towards identifying genetic regulators in the host to understand the mechanisms of *T. gondii* pathogenesis.

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CHAPTER IV. FUTURE DIRECTIONS

Introduction

In this dissertation, research confirming parasite replication in tissue among distinct *T. gondii* strains and the effect parasite genetic background has on host gene expression are presented. Here, we show that uncontrolled growth is a common feature of all virulent strains.. In addition, it is shown that Type I and II have similar expression profiles during acute infection while Type III elicits a decreased response in mice. Taken together, understanding both parasite and host gene expression during infection could lead to the identification of key elements involved in *T. gondii* virulence and resistance in host.

Identification of Mechanisms of Host Resistance

In my work presented in Chapter III, microarray data identified several host defense genes that were significantly suppressed by virulent *T. gondii* GT1 strain. To elucidate the role of these genes in the pathogenesis of *T. gondii* infection, further studies are needed. The use of gene specific knock out mice would provide mechanistic insight of the roles of these genes. For example, serine protease inhibitors (*Serpinb2, Serpinb10*), were significantly repressed during Type I and II in vivo infection. Using knockout mice, we could monitor this protein's role in protection and pathogenesis. Genetically engineered knock out mice could also answer a critical question in the field about whether the numerous *Toxoplasma* regulated host transcription factors identified using *in vitro* experimental systems are actually activated in vivo. These types of experiments would add biological and mechanistic significance to our microarray data.

In addition, we can use mice of different genetic backgrounds to identify the role that host genetics play on host resistance. Using C57Bl/6 and DBA/2 mice, the former are resistant, while the latter are susceptilble to *T. gondii* infection, we can study a population of progeny from

genetic crossing of these two inbred strains to study gene expression network and pathways.. Taking advantage of the in vivo methods and microarray technology described in this dissertation we can infect the progeny with the Type I virulent strain and perform microarray analysis to observe alterations in gene expression. Using expression quantative trait loci mapping, we can identify the genomic loci responsible for expression of thousands of genes. Ultimately, we can characterize candidate genes by genetic knockout mice experiments.

Comparison of *T. gondii* gene expression profiles during in vivo infection

By and large, the original goal of this project was to identify upstream genetic elements that regulate virulence in *T. gondii* and confer resistance in host. Experiments using microarray technology to elucidate genetic factors regulating virulence in *T. gondii* presented technical difficulties. Due the uncontrolled growth phenotype characteristic of virulent strains, gene expression comparison to non virulent strains becomes inaccurate. In order to overcome this feat, we can increase inoculum dose of parasite in mice. Preliminary results testing this method, proved that indeed parasite concentration was increased. The consequence of increasing the parasite concentration had major effects on gene expression profiles, therefore limited conclusions could be made. Moving forward, we will optimize inoculum dose needed to obtain accurate conclusions. Taking advantage of the alternative method for determining virulence described in chapter II, we can confirm optimal infectious dose by comparing parasite load ratios between Type I and III strains in peritoneal lavage. Results from this study will lay framework for identification of key genes controlling virulence in *T. gondii*.

References

List of References

Ahn, H.J., Kim, J.Y., Ryu, K. and Nam, H.W., 2009, STAT6 activation by Toxoplasma gondii infection induces the expression of Th2 C-C chemokine ligands and B clade serine protease inhibitors in macrophage. Parasitology Research 105, 1445-1453.

Ajioka, J.a.S., D. , 2007, Toxoplasma Molecular and Cellular Biology. Horizon Bioscience.

Ajzenberg, D., Cogne, N., Paris, L., Bessieres, M. H., Thulliez,P. Fillisetti, D., Pelloux, H., Marty, P., Darde, M. L. , 2002, Genotype of 86 *Toxoplasma gondii* isolates associated with human congential toxoplasmosis and correlation with clinical findings Journal of Infectious disease 186, 684-689.

Aliberti, J., 2005, Host persistence: exploitation of anti-inflammatry pathways by Toxoplasma gondii. Nat Rev Immunol 5, 162-170.

Andrade, R.M., Wessendarp, M., Gubbles, M.J., Striepen, B. and Subauste, C.S.,, 2006, CD40 induces macrophage anti-Toxoplasma gondii activity by triggering autophagy-dependent fusion of pathogen- containing vacuoles and lysosomes. J Clin Invest. 116, 2366-2377.

Barragan A, S.L., 2002, Transepithelial migration of Toxoplasma gondii is linked to parasite motility and virulence. Journal of Experimental Medicine 95, 1625-1633.

Black, M.W.a.B., J. C, 2000, Lytic Cycle of Toxoplasma gondii. Microbiol. Mol. Biol. Rev. 64, 607.

Bliss, S.K., Gavrilescu, L.C., Alcaraz, A. and Denkers, E.Y., 2001, Neutrophil depletion during Toxoplasma gondii infection leads to impaired immunity and lethal systemic pathology. Infect Immun. 69, 4898-4905.

Bohne, W., Heesemann, J. and Gross, U., 1994, Reduced replication of Toxoplasma gondii is necessary for induction of bradyzoite- specific antigens: a possible role for nitric oxide in triggering stage conversion. Infect Immun. 62, 1761-1767.

Boyle, J.P., Rajasekar, B., Saeij, J.P., Ajioka, J.W., Berriman, M., Paulsen, I., Roos, D.S., Sibley, L.D., White, M.W. and Boothroyd, J.C. , 2006, Just one cross appears capable of dramatically altering the population biology of a eukaryotic pathogen like Toxoplasma gondii. Proc. Nat. Acad. Sci. U S A 103, 10514-10519.

Bradley, P.J., Ward, C., Cheng, S.J., Alexander, D.L., Coller, S., Coombs, G.H., Dunn, J.D., Ferguson, D.J., Sanderson, S.J., Wastling, J.M. and Boothroyd, J.C., 2005, Proteomic analysis of rhoptry organelles reveals many novel constituents for host-parasite interaction in Toxoplasma gondii. J. Biol. Chem 280, 34245-342458.

Carme B, B.F., Ajzenberg D, Bouyne R, Aznar C, Demar M, Bichat S, Louvel D, Bourbigot AM, Peneau C, Neron P, Dardé ML., 2002, Severe acquired toxoplasmosis in immunocompetent adult patients in French Guiana. Journal of Clinical Microbiology 40, 4037-4044.

Conseil, V., Soete, M. and Dubremetz, J.F., 1999, Serine protease inhibitors block invasion of host cells by Toxoplasma gondii. Antimicrobial Agents Chemotherepy 43, 1358-1361.

Corad, P., Miller, MA., Kreuder, C., James, ER., Mazet, J., Dabritz, H., Jessup, DA., Gulland, F. and Grigg, ME, 2005, Transmission of Toxoplasma: clues from the study of sea otter as sentinels of Toxoplasma gondii flow into the marine environment. Int J Parasitol 35, 1155-1168.

Darde, M.L., 2004, Genetic analysis of the diversity in Toxoplasma gondii. Ann Ist Super Sanita 40, 57-63.

Del Rio, L., Bennouna, S., Salinas, J. and Denkers, E.Y., 2001, CXCR2 deficiency confers impaired neutrophil recruitment and increased susceptibility during Toxoplasma gondii infection. J Immunol 167, 6503-6509.

Denkers, E.Y., Butcher, B.A., Del Rio, L. and Benouna, S., 2004, Neutrophils, dendritic cells and Toxoplasma International Journal of Parisitology 34, 411-421.

Denkers, E.Y., Kim, L. and Butcher, B.A., 2003, In the belly of the beast: subversion of macrophage proinflammatory signalling cascades during Toxoplasma gondii infection. Cell Microbiol 5, 75-83.

Denkers, E.Y.a.G., R.T. , 1998, Regulation and function of T-cell-mediated immunity during Toxoplasma gondii infection. Clin Mirobiol Rev. 11, 569-588.

Denkers, G.L.C.a.E.Y., 2001, IFN- Overproduction and High Level Apoptosis Are Associated with High but Not Low Virulence Toxoplasma gondii Infection. Journal of Immunology 167, 902-909.

Diana, J., Vincent, C., Peyron, F., Picot, S., Schmitt, D. and Persat, F., 2005, Toxoplasma gondii regulates recruitment and migration of human dendritic cells via different soluable secreted factors. Clin Exp Immunol. 141, 475-484.

Djurkovic-Djakovic, O., Nikolic, A., Bobic, B., Klun, I. and Alesksic, A. , 2005, Stage conversion of Toxoplasma gondii RH parasites in mice by treatment with atovaquone and pyrrolidine dithiocarbamate. Microbes Infect. 7, 49-54.

Dobbin, C.A., Smith N.C. and Johnson, A.M., 2002, Heat shock protein 70 is a potential virulence factor in murine Toxoplasma infection via immunomodulation of host NF-kB and nitric oxide. Journal of Immunology 169, 958-965.

Draghici, S., Khatri P, Bhavsar P, Shah A, Krawetz S.A. and Tainsky M.A, 2003, Onto-Tools, the toolkit of the modern biologist: Onto-Express, Onto-Compare, Onto-Design and Onto-Translate. Nucleic Acid Research 31, 3775-3781.

Dubey, J.P., 1998, Advances in the life cycle of Toxoplasma gondii. Int J. Parisitol 2, 1019-1024.

Dubey, J.P., 2009, Toxoplasmosis of Animals and Humans, Second Edition. Taylor and Francis Group, Boca Raton, 313 p.

Dubey, J.P., Applewhaite, L., Sundar, N., Velmurugan, G.V., Bandini, L.A., Kwok, O.C.H., Hill, R., and Su, C. , 2007, Molecular and biological characterization of *Toxoplasma gondii* isolates from free-range chickens from Guyana, South America, identified several unique and common parasite genotypes. Parasitol. 134, 1559-1565.

Dubey, J.P., Quirk, T., Pitt, A., Sundar, N., Velmurugan, G.V., Kwok, O. C H., Leclair, D., Hill, R., and Su, C., 2008, Isolation and genetic characterization of *Toxoplasma gondii* from raccoons (*Procyon lotor*), Cats (*Felis domesticus*), Striped skunk (*Mephitis mephitis*), Black Bear (*Ursus americanus*) and Cougar (*Puma concolor*) from Canada. J Parasitol. 94, 42-45.

Fischer, H.G., Bonifas, U. and Reichmann, G. , 2000, Phenotype and function of rain dendritic cells emerging during chronic infection of mice with Toxoplasma gondii J Immunol 164, 4826-4834.

Gavrilescu, L.C., Denkers, E. Y., 2001, IFN gamma overproduction and high level apoptosis are associated with high but not low virulence *Toxoplasma gondii* infection. J. Immunol. 167, 902-909.

Gome, I.N., Palma, L.C., Campos, G.O., Lima, J.G.B., De Almeida, T.F., De Menezes, J.P.B., Ferreira, C.A.G., Dos Santos, R.R., Buck, G.A., Manque, P.A.M., Ozaki, L.S., Probst,

C.M., De Freitas, L.A.R., Krieger, M.A. and Veras, P.S.T., 2009, The scavenger receptor MARCO is involved in *Leishmania major* infection by CBA/J macrophages. Parasite Immunology 31, 188-198

Grigg ME, B.J., 2001, Rapid identification of virulent type I strains of the protozoan pathogen Toxoplasma gondii by PCR-restriction fragment length polymorphism analysis at the B1 gene. Journal of Clinical Microbiolgy 39, 398-400.

Howe, D.K., B.C. Summers and L. D. Sibley, 1996, Acute virulence in mice is associated with markers on chromosome VIII in *Toxoplasma gondii*. Infection and Immunity 64, 5193-5198.

Howe, D.K., Honore, S., Derouin, F., Sibley, L.D., 1997, Determination of genotypes of *Toxoplasma gondii* strains isolated from patients with toxoplasmosis. J. Clin. Microbiol. 35, 1411-1414.

Howe, D.K.a.S., L.D. , 1995, Toxoplasma gondii comprises three clonal lineages: correlation of parasite genotype with human disease. . Journal of Infectious disease 172, 1561-1566.

Ikegawa, S., Sano, M., Koshizuka, Y., and Nakamura, Y., 2000, Isolation, characterization and mapping of the mouse and human PRG4 (proteoglycan 4) genes. Cytogenet Cell Genet. 90, 291-297.

Jebbari, H., Roberts, C.W., Ferguson, D.J., Bluthmann, H. and Alexander, J., 1998, A protective role for IL-6 during early infection with Toxoplasma gondii. Parasite Immunol 20, 231-239.

Jerome, M.E., Radke, J. R., Bohne, W., Roos, D.S., White, M. W., 1998d, *Toxoplasma gondii* bradyzoites form spontaneously during sporozoite-initiated development. . Infect. Immun. 66, 4838–4844.

Joynson, D.H.a.W., T. J. , 2001, Toxoplasmosis: a comprehensive clinical guide Cambridge University Press,.

Kaufman, H., Remington, J., Jacobs, L., 1958, Toxoplasmosis: the nature of virulence. Am J Ophthalmol 46, 255-260.

Kaufman He, M.M., Remington Js, Jacobs L, 1959, Strain differences of Toxoplasma gondil. Journal of Parisitology 45, 189-190.

Khan, A., Fux, B., Su, C., Dubey, J.P., Darde, M.L., Ajioka, J.W,, Rosenthal, B.M., Sibley, L.D., 2007, Recent transcontinental sweep of *Toxoplasma gondii* driven by a single monomorphic chromosome. Proc. Nat. Acad. Sci. U S A 104, 14872- 14877.

Khan, A., Taylor, S., Su, C., Mackey, A.J., Boyle, J., Cole, R., Glover, D., Tang, K., Paulsen, I.T., Berriman, M., Boothroyd, J.C., Pfefferkorn, E.R., Dubey, J.P., Ajioka, J.W., Roos, D.S., Wootton, J.C., Sibley, L.D., 2005, Composite genome map and recombination parameters derived from three archetypal lineages of *Toxoplasma gondii*. Nucleic Acids Res. 33, 2980-2992.

Khan, I.A., Murphy, P.M., Casciotti, L., Schwartzman, J.D., Collins, J., Gao, J.L. and Yeaman, G.R. , 2001, Mice lacking the chemokine receptor CCR1 show increased susceptibility to Toxoplasma gondii infection. J Immunol 166, 1939-1937.

Lagal, V., Binder, E.M., Huynh, M.H., Kafsack, B., Harris, P., Diez, R., Chen, D., Cole, R., Carruthers, V. and Kami, K., 2010, Toxoplasma gondii protease TgSUB1 is required for cell sruface processing of micronemal adhesive complexes and efficient adhesion of tachyzoites. Cellular Microbiology 12, 1792-1808.

Lee, C.W., Sukhumavasi, W. and Denkers, E.Y., 2007, Phosphoinositide-3-kinase-dependent, MyD88-independent induction of CC-type chemokines characterizes the macrophage response to Toxoplasma gondii strains with high virulence. Infect Immun. 75(12), 5788-5797.

Lyons, R.E., McLeod, R. and Roberts, C.W. , 2002, *Toxoplasma gondii* tachyzoite-bradyzoite interconversion. Trends Parasitol 18, 198-201.

Mehlhorn, H.a.F., JK., 1980, Ultrastructural comparison f cysts and zoites of Toxoplasma gondii, Sarcocystis muris and Hammondia Hammondi in skeletal muscle of mice. J Parasitol. 66, 59-67.

Miller, C.M., Boulter, N.R., Ikin, R.J. and Smith, N.C. , 2009, The immunobiology of the innate repsonse to Toxoplasma gondii. Int J Parasitol 39, 23-39.

Modlin, I.M., Gustafsson, B.I., Drozdov, I., Nadler, B., Pfranger, R. and Kidd, M., 2009, Principal Component Analysis, Hierarchical Clustering, and Decision Tree Assessment of Plasma mRNA and Hormone Levels as an Early Detection Strategy for Small Intestinal Neuroendocrine (Carcinoid) Tumors. Ann of Surg. Oncol. 16, 487.

Mordue, D.G., F. Monroy, M. LaRegina, C.A Dinarello, L. D. Sibley 2001, Acute toxoplasmosis leads to lethal overproduction of Th1 cytokines. J. Immunol. 167, 4574-4584.

Parmley, S., Yang, S. Harth, G., Sibley, LD., Sucharczuk, A. and Remington, JS. , 1994, Molecular characterization of a 65 kilodalton Toxoplasma gondii antigen expressed abundantly in the matrix f tissue cysts. Mol Biochem Parasitol 66, 283-296.

Pollard AM, S.S., Mordue DG, Knoll LJ, 2009, A transmembrane domain-containing surface protein from Toxoplasma gondii augments replication in activated immune cells and establishment of a chronic infection. Infect Immun. 77, 3731-3739.

Radke, J.R., White, M.E., Donald, R., Eibs, A., Jerome, M., Behnke, M., Liberator, P. , 2006, Changes in the expression of human cell division autoantigen-1 influence *Toxoplasma gondii* growth and development. Plos Pathog 2, 964-974.

Robben, P.M., Mordue, D.G., Truescott, S.M., Takeda, K., Akira ,S. and Sibley LD, 2004, Production of IL-12 by macrophages infected with Toxoplasma gondii depends on the parasite genotype. Journal of Immunology 172, 3686-3694.

Saeij, J.P., Boyle, J.P., Boothroyd, J.C., 2005, Differences among the three major strains of Toxoplasma gondii and their specific interactions with the infected host. Trends Parasitol 21, 476-481.

Saeij, J.P., Boyle, J.P., Coller, S., Taylor, S., Sibley, L.D,, Brooke- Powell, E.T., Ajioka, J.W., Boothroyd, J.C., 2006, Polymorhic secreted kinases are key virulence factors in toxoplasmosis. Science 1780-1783.

Saeij, J.P., Boyle, J.P., Grigg, M.E., Arrizabalag, G., Boothroyd, J.C. , 2004, Bioluminescence imaging of *Toxoplasma gondii* infection in living mice reveals dramatic differences between strains. Infect. Immun. 73, 695-702.

Schmittgen, T.D., Livak, K.J., 2008, Analyzing real-time PCR data by the comparative CT method. Nat Protoc 3, 1101-1108.

Shaw, M.K., Roos, D.S. and Tilney, L.G., 2002, Cysteine and serine protease inhibitors block intracellular development and disrupt the secretory pathway of *Toxoplasma gondii* Microbes and Infection 4, 119-132.

Sibley, L.D., 2011, Invasion and intracellular survival by protozoan parasites. Immunol Rev. 240, 72-91.

Sibley, L.D., Boothroyd, J.C., 1992a, Virulent strains of *Toxoplasma gondii* comprise a singel clonal lineage Nature 359, 82-85.

Sibley, L.D., Boothroyd, J.C., 1992b, Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage Nature 359, 82-85.

Soete, M., Camus, D. and Dubremetz, JF. , 1994, Experimental induction of bradyzoite-specific antigen expression and cyst formation by the RH strain of Toxoplasma gondii in vitro. Exp Parasitol 78, 361-370.

Soete, M., Fortier, B., Camus, D. and Dubremetz, J.F. , 1993, Toxoplasma gondii: kinetics of bradyzoite- tachyzoite interconversion in vitro. Exp Parasitol 76, 259-264.

Straw, A.D., MacDonald, A.S., Denkers, E.Y. and Pearce, E.J., 2003, CD154 plays a centrol role in regulating dendritic cell activation during infections that induce Th1 or Th2 responses. J Immunol 170, 727-734.

Su, C., Evans, D., Cole, R.H., Kissinger, J.C., Ajioka, J.W., Sibley, L.D., 2003, Recent expansion of *Toxoplasma* through oral transmission. Science 299, 414-416.

Su, C., Howe, D.K., Dubey, J.P., Ajioka, J.W., Sibley, L.D., 2002, Identification of quantitative trait loci controlling acute virulence in Toxoplasma gondii. Proc. Nat. Acad. Sci. U S A 99, 10753-10758.

Su, C., Shwab, E.K., Zhou, P., Zhu, X.Q., Dubey, J.P., 2010, Moving towards an intergrated approach to molecular detection and identification of *Toxoplasma gondii*. Parasitol 137, 1-11.

Su, C., Zhang, X., Dubey, J.P., 2006, Genotyping of *Toxoplasma* gondii by multilocus PCR-RFLP markers: a high resolution and simple method for identification of parasites. Int J Parisitol 36, 841-848.

Taylor, S., Barragan, A., Su, C., Fux, B., Fentress, S.J., Tang, K., Beatty, W.L., Hajj, H.E., Jerome, M., Behnke, M.S., White, M., Wootton, J.C., Sibley, L.D., 2006, A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen Toxoplasma gondii. Science 314, 1776-1780.

Tenter AM, H.A., Weiss LM, 2000, Toxoplasma gondii: From animals to humans. Int J Parasitol 30, 217–1258.

Weiss, L.a.K., K. , 2000, The development and biology of bradyzoites of Toxoplasma gondii. Front Biosci 5, D391-405.

Xiao J, J.-B.L., Talobt Jr C and Robert H. Yolken, 2010, Differential effects of three canonical Toxoplasma strains on gene expression in human neuroepithelial cells. Infect Immun. doi:10.1128/IAI.00947-10.

Yap, G.S., Ling, Y. and Zhao, Y., 2007, Autophagic elimination of intracellular parasites: convergent induction by IFN-gamma and CD40 ligation? Autophagy 2, 163-165.

Yap, G.S.a.S., A. , 1999, Cell-mediated immunity to Toxoplasma gondii: initiation, regulation and effector function. Immunobiol 20, 240-247.

Zhao, Y.O., Khaminets, A., Hunn, J.P. and Howard, J.C. , 2009, Disruption of the Toxoplasma gondii parasitophorous vacole by IFNγ– Inducible Immunity-Related GTPases (IRG Proteins) triggers necrotic cell death. Plos Pathog 5, 1371.

Appendix I: Genotyping natural isolates

Appendix I is a collection of three co-authored publications.

1. Dubey JP, Applewhaite L, Sundar N, Velmurugan GV, Bandini LA, Kwok, C.H, **Hill, R**, Su C. 2007. Molecular and biological characterization of *Toxoplasma gondii* isolates from free-range chickens from Guyana, South America identified several unique and common parasite genotypes. Parasitology. 134:1559-1565

The prevalence of *Toxoplasma gondii* in free-ranging chickens *(Gallus domesticus*) is a good indicator of the prevalence of *T. gondii* oocysts in the soil because chickens feed from the ground. The prevalence of *T. gondii* in 76 free-range chickens from Guyana, South America was determined. Antibodies to T. gondii were assayed by the modified agglutination test (MAT), and found in 50 (65.8%) of 76 chickens with titres of 1 : 5 in four, 1 : 10 in one, 1 : 20 in five, 1 : 40 in seven, 1 : 80 in six, 1 : 160 in eight, 1 : 320 in four, 1 : 640 or higher in 15. Hearts and brains of 26 chickens with titres of<1: 5 were pooled in 5 batches and bioassayed in mice. Hearts and brains of 50 chickens with titres of 1 : 5 or higher were bioassayed in mice. *Toxoplasma gondii* was isolated by bioassay in mice from 35 chickens with MATtitres of 1 : 20 or higher. All mice inoculated with tissues of 30 infected chickens remained asymptomatic. *Toxoplasma gondii* isolates from 35 chickens were genotyped using 11 PCR-RFLP markers including SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, a new SAG2, and Apico. A total of 9 genotypes were identified, with 5 genotypes (nos 1, 4, 5, 6 and 7) unique to Guyana, 2 genotypes (nos 2 and 3) previously identified in chickens from Brazil, 1 genotype (no. 8) previously identified in chickens from Brazil, Costa Rica and Nicaragua, and 1 genotype (no. 9) belonging to the clonal type III lineage that exists globally. Infection with 2 genotypes was found from 1 chicken (Table A1-1). This is the first report of genetic characterization of *T. gondii* isolates from any host from Guyana

Table A1-1. Summary of genotyping of *Toxoplasma gondii* isolates from chicken from Guyana

	Genetic markers											
Genotype	SAG1	SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	new SAG2	Apico	Isolate ID
Reference	I	I	I	I	I	I	I	I	I	I	I	RH88
Reference	II or III[a]	II	II	II	II	II	II	II	II	II	II	PTG
Reference	II or III	III	III	III	III	III	III	III	III	III	III	CTG
Reference	I	II	III	II	II	II	u-1	I	u-2	II	I	COUGAR
Reference	u-1	I	III	III	III	u-1	I	I	III	II	I	MAS
Reference	I	III	III	III	III	I	I	I	u-1	III	I	TgCatBr5
No. 1	I	III	I	I	III	II	III	III	I	III	III	TgCkGy2,3,5,6,9,12, 16,19,20,25,28,32
No. 2	I	III	I	III	III	III	III	III	I	III	III	TgCkGy7,11,13,31
No. 3	I	III	I	III	III	III	III	III	I	III	I	TgCkGy8,10,14, 15,35
No. 4	I	III	III	I	III	III	III	III	III	III	III	TgCkGy17,18
No. 5	I	III	III	I	III	III	III	III	I	III	III	TgCkGy1,4,29,30,33
No. 6	I	III	III	III	III	I	III	III	III	III	III	TgCkGy34
No. 7	I	III	III	III	III	III	III	III	III	III	III	TgCkGy21,22
No. 8	I	III	III	III	III	III	III	III	III	III	I	TgCkGy23,24
No. 9	II or III	III	III	III	III	III	III	III	III	III	III	TgCkGy26,27
Mixed	I	III	I and III	III	III	III	III	III	I and III	III	I	TgCkGy36

[a] Cannot distinguish II or III.

2. Dubey, J.P., López-Torres, H.Y., Sundar, N., Velmurugan, G.V., Ajzenberg, D., Kwok, P. CH., **Hill, R.,** Dardé, M.L., and Su, C. 2007. Mouse-Virulent *Toxoplasma gondii* Isolated from Feral Cats on Mona Island, Puerto Rico. Journal of Parasitology. 93: 1365-1369

Cats are essential in the life cycle of *Toxoplasma gondii* because they are the only hosts that can excrete the environmentally resistant oocysts. Samples of serum, feces, and tissues from cats from Mona, a remote island off the coast of Puerto Rico, were examined for *T. gondii* infection. Antibodies to *T. gondii* were assayed by the modified agglutination test and found in 16 of 19 (84.2%) of cats, with titers of 1:10 in 2, 1:80 in 1, 1:160 in 4, 1:320 in 3, and 1:1,280 or higher in 6. Tissues of 19 of the 20 cats were bioassayed in mice for *T. gondii* infection. *Toxoplasma gondii* was isolated from tissues of 12 cats: from the hearts of 9, skeletal muscle of 10, and brain of 1 cat. All infected mice from 10 of 12 isolates died of acute toxoplasmosis during primary infection. Genotyping of these 12 *T. gondii* isolates (designated (TgCatPr 1-12) by 10 multilocus PCR-RFLP markers, i.e., SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and an apicoplast marker Apico, and the 6 multilocus microsatellite markers TUB2, W35, TgM-A, B18, B17, and M33, revealed 7 genotypes; 5 isolates had Type I alleles at all loci except at 1 microsatellite locus, and the remainder were atypical (Table A1-2). The latter isolates of *T. gondii* were different biologically and phenotypically from the feline isolates from the rest of the Americas. One isolate (TgCatPr 12) was a mixed infection with 2 genotypes.

3. Dubey, J.P., Quirk, T., Pitt, J.A., Sundar, N., Velmurugan,G.V., Kwok, O.CH., Leclair, D., Hill, R. and Su, C. 2008. Isolation and Genetic Characterization of *Toxoplasma gondii* from Raccoons (*Procyon lotor*), Cats (*Felis domesticus*), Striped Skunk (*Mephitis mephitis*), Black Bear (*Ursus americanus*), And Cougar (*Puma concolor*) from Canada. Journal of Parasitology, 94:42-45.

Viable *Toxoplasma gondii* was isolated by bioassay in mice from tissues of 2 feral cats (*Felis domesticus*), 2 raccoons (*Procyon lotor*), a skunk (*Mephitis mephitis*) trapped in remote locations

in Manitoba, Canada, and a black bear (*Ursus americanus*) from Kuujjuaq, northern Quebec, Canada. Genotyping of these *T. gondii* isolates using polymorphisms at 10 nuclear markers including SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and an apicoplast marker Apico revealed 4 genotypes (Table A1-3). None of the isolates was clonal archetypal Types I, II, and III found in the United States. These results are in contrast with the Type II genotype that is widespread in domestic animals and humans throughout the United States and Europe. This is the first genotyping of *T. gondii* isolates from this part of North America.

Table A1-2. Genotyping of *Toxoplasma gondii* isolates from cats from Mona Island, Puerto

		Genetic markers																
Genotypes	*Toxoplasma gondii* isolate	SAG1	(5′ + 3′) SAG2†	SAG2‡	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	TUB2	W35	TgM-A	B18	B17	M33
Reference	GT1	I	I	I	I	I	I	I	I	I	I	I	1	1	1	1.3	1	1.2
Reference	PTG	II or III[a]	II	II	II	II	II	II	II	II	II	II	2.3	2.3	2	2	2.3	1.2
Reference	CTG	II or III	III	III	III	III	III	III	III	III	III	III	2.3	2.3	3	1.3	2.3	3
Reference	COUGAR	I	II	II	III	II	II	II	u-1	I	u-2	I	2.3	2.3	3	2	2.3	1.2
Reference	MAS	u-1	I	II	III	III	III	u-1	I	I	III	I	1	2.3	3	4	10	1.2
Reference	TgCatBr5	I	III	III	III	III	III	I	I	I	u-1	I	1	2.3	3	1.3	10	3
No. 1	TgCatPr1	I	I	I	I	I	I	I	I	I	I	ND	1	1	1	1.3	2.3	1.2
No. 1	TgCatPr2	I	I	I	I	I	I	I	I	I	I	ND	1	1	1	1.3	2.3	1.2
No. 1	TgCatPr3	I	I	I	I	I	I	I	I	I	I	ND	1	1	1	1.3	2.3	1.2
No. 1	TgCatPr4	I	I	I	I	I	I	I	I	I	I	ND	1	1	1	1.3	2.3	1.2
No. 2	TgCatPr5	II	II	II	I	II	II	II	II	III	II	ND	2.3	4	2	4	2.3	1.2
No. 3	TgCatPr6	I	II	II	I	I	I	III	II	III	I	ND	1	4	1	4	2.3	1.2
No. 4	TgCatPr7	II	II	II	I	II	II	II	II	III	II	ND	2.3	4	2	4	2.3	5
No. 5	TgCatPr8	I	III	III	III	III	I	I	III	III	I	ND	2.3	10	1	1.3	2.3	3
No. 6	TgCatPr9	I	III	III	I	III	III	III	III	III	III	I	2.3	2.3	3	9	1	3
No. 1	TgCatPr10	I	I	I	I	I	I	I	I	I	I	I	1	1	1	1.3	2.3	1.2
No. 2	TgCatPr11	II	II	II	I	II	II	II	II	III	II	ND	2.3	4	2	4	2.3	1.2
No. 7	TgCatPr12	II	I	II	I	II	II	II	nd	I, III	I, II	ND	1 and 2.3	1 and 4	1 and 2	1.3 and 4	2.3	1.2 and 5

[a] ND = no data, 1 2 means that types I and II share the allele, 1.3 means that types I and III share the allele; allele 2.3 means that types II and III share the allele.
† The SAG2 marker based on 5′- and 3′-end DNA sequence polymorphisms of SAG2 gene (Howe et al., 1997).
‡ The SAG2 marker developed recently based on 5′-end DNA sequence of SAG2 gene is able to identify additional alleles often seen in atypical *T. gondii* strains (Su et al., 2006).

Table A1-3. Genotyping of *Toxoplasma gondii* isolates from wild cats from Canada

Host	*T. gondii* isolate	SAG1	SAG2*	SAG2†	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico
Cat 1	TgCatCa1	II or III	III	III	I	III	III	II	I	III	III	III
Cat 2	TgCatCa2	u-1	II	II	II	II	II	II	II	I	II	I
Raccoon 3	TgRaCa2	u-1	II	II	II	II	II	II	II	I	II	I
Skunk	TgSkCa1	u-1	II	II	II	II	II	II	II	I	II	I
Black bear	TgBBeCa1	I	I	I	III	III	III	III	III	III	I	III
Cougar‡	TgCgCa2	I	II	II	III	II	II	II	u-1	I	u-2	I

* SAG2 marker based on 5′- and 3′-ends of the gene sequence (Howe et al., 1997).
† A new SAG2 marker based on the 5′-end of the gene sequence (Su et al., 2006).
‡ Sample B of Aramini et al. (1998).

Appendix II: Comparing gene expression profiles of different *T. gondii* strains *in vivo*

Introduction

Previous data has suggested that *T. gondii* virulence may be regulated by a few key genetic factors (Denkers 2007, Taylor 2006, Jerome 1998). In general, our goal is to understand these genetic elements that may be regulating virulence among different *T. gondii* strains. Therefore, it is necessary to observe key differences in gene expression of parasite during in vivo infection. Due to major phenotypic differences between Type I and III clonal strains, we hypothesized that gene expression differences should be greater among these two lineages in vivo. We aimed to identify significant differences among these strains using microarray technology. Our results showed that due to the increased replication rate of Type I parasites, the RNA concentrations between strains were not at comparable levels for further analysis. Here we describe troubleshooting methods to enrich non virulent parasites.

Materials and Methods

Cell culture of parasite strains and infection in mice. Human foreskin fibroblasts (HFF) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% of heat inactivated fetal bovine serum (Hyclone#SH30070.03 HI/IR), 1% of 100x Non Essential Amino Acids (NEAA-Fisher Scientific cat. no. - SH3023801), 0.4% of 1M HEPES buffer and 0.1 % of 10 mg/ml gentamicin (Invitrogen #15710-64) and maintained in T25 vented culture flasks at 37°C with 5% CO2. *T. gondii* strains GT1 (virulent) and CTG (non-virulent) were expanded and maintained in confluent HFF monolayer to reach consistent two-day passages prior to use for experiments.

Toxoplasma gondii tachyzoites of GT1 and CTG strains were harvested by filtering through 3 μm pore size polycarbonate filters (Whatman cat. no. 420400) and counted. Five hundred tachyzoites of each of the GT1 and CTG strains were inoculated into four 6-8 week old

female outbred CD-1 (ICR- Harlan Sprague) mice by intraperitoneal injection (IP).For increased inoculum assay, mice were infected with 10^4 of non-virulent CTG tachyzoites. At day 5 post infection, mice were anesthetized to the state of unconsciousness, followed by euthanization with cervical dislocation. Five mL of ice cold PBS was injected into peritoneal cavity and then peritoneal lavage was collected. Cells of peritoneal lavage were pelleted by centrifugation. Supernatant was removed and pellet was resuspended in 1ml RNAlater, mixed and incubated in 4ºC for 1 hour, then stored in -70ºC until RNA extraction for microarray experiments. The spleens were collected from mice, weighed, homogenized and diluted in PBS to determine parasite load by real-time PCR (RT-PCR).

Microarray analysis. Total RNA was extracted from cells collected from peritoneal lavage of mice infected with GT1 and CTG using Qiagen RNeasy Plus Mini Kit (Qiagen cat. no. 98 74131) following manufacturer's instruction. For parasite gene expression profiling, ToxoGeneChip array containing ~ 8000 features was used. Microarray was carried out at the University of Tennessee Affymetrix Core Facility. RNA samples were processed according to the Affymetrix Protocol for One-Cycle DNA Synthesis using a Message Amp II102 Biotin Enhanced Kit (Ambion PN#am1791, Austin, TX). 5 μg of fragmented cRNA was hybridized to the GeneChip, previously described. Arrays were washed and stained using the Affymetrix Hyb/Stain kit PN900720 and Affymetrix prepared wash solutions and an Affymetrix Fluidics 450 wash station. Arrays were immediately scanned using the Affymetrix 7G scanner. Array images were visually inspected for anomalies. The individual chip scans were quality checked for the presence of control genes and background signal values.

Ranking Analysis. The raw data from Affycore Analysis was extracted. Transcripts were sorted based on signal intensity values.

Filtration method. HFF cells were infected with Type III VEG parasites. Parasites were harvested as described above. In addition, we added and additional filtration step via 3µm polycarbonate filter (Whatman cat no. 420400). DNA was extracted using Qiagen DNeasy Blood and Tissue Kit (Qiagen cat no. 69504). The number of parasites was determined by qPCR of extracted DNA using a TaqMan probe targeting the ITS1 sequence (GenBank Accession# AY143141). The primers for PCR amplification were ITS1-Fx: GAAGGGGCTCAATTTCTGG and ITS1-Rx: TGTTCCTCAGATTTGTTGTTTGA, which amplifies a 117 bp sequence. The ITS1 probe was 5'-/56-FAM/CGTGTCTCTGTTGGGATACTGATTTCCAGG/3BHQ-1/-3', with the 5' end labeled with FAM and the 3' end labeled with Black Hole Quencher-1 (BHQ-1) (Integrated DNA Technologies, Inc.)

Sonication method. In brief, ITS1 sequence (GenBank Accession# AY143141) was used to quantify parasite as described above, for parasite DNA present after sonication treatments. The number of host cells was determined by qPCR of extracted DNA using a TaqMan probe targeting the GAPDH sequence (GenBank Accession# NC_000012.11). The primers for PCR amplification were GAPDH- Fx: TGCACCACCAACTGCTAG and GAPDH-Rx: GGATGCAGGGATGATGTTC, which amplifies a 177 bp sequence. The GAPDH probe was 5'-/56-FAM/CAGAAGACTGTGGATGGCCCT/3BHQ-1/-3', with the 5' end labeled with FAM and the 3' end labeled with Black Hole Quencher-1 (BHQ-1) (Integrated DNA Technologies, Inc.). We determined the concentration of host cell (HFF) only, parasite only and host/ parasite combined after harvesting. Samples were sonicated at five different settings (No sonication, 10sec for 5, 5sec for 5, 10sec for 4, 5sec for 4 and 10sec for 3). to determine optimal sonication

using Sonicator Heat Systems ultrasonics inc. cell disrupter. Model W220F. For parasite/ host combined detection parasite were grown in macrophage J77A.1 cell line.

Results

Our results showed that during virulent infection genes upregulated were associated with tachyzoite stage, host cell adherence and invasion of the parasite. These same genes were undetectable during non virulent infection (Fig A2-1). These results further suggest, that patterns of expression may be controlled by a few "master" regulators. Unfortunately, the significant differences among virulent and non virulent strains in vivo growth rate presented technical difficulties in drawing accurate conclusions from this experiment.

In order to observe the effect increased filtration has on segregating parasite from host cells, we added an additional filtration step to common harvesting procedures. Our results showed that there was no significant difference in parasite numbers after additional filtration when compared to normal filtration methods (Fig A2-2).

As a secondary troubleshooting method we employed the use of sonication to increase parasite RNA. In biology, sonication is often used to disrupt cell membranes and release cellular contents. For this reason we aimed to use this technique to release intracellular parasites in order to enrich total parasite RNA extracted. Results showed that regardless of sonication treatment, there were no significant differences in parasite concentrations (Fig A2-3).

Type I		
Rank	**Gene**	**Signal Intensity**
1	GRA5	10476
2	SAG1	8543
3	GRA1	8382
4	GRA3	7877
5	GRA2	5038
6	SAG2	3457
7	MIC3	3387
8	20K cyclophilin	2891
9	GRA6	2660
10	GRA7	1975
11	MIC1	1302
12	MIC11	1314
13	MIC10	1251
14	ROP18	1197
15	Ser/Thr phosphatase	1072

Virulent (GT1)

Type III		
Rank	**Gene**	**Signal Intensity**
1	hypothetical protein	2388
2	hypothetical protein	577
3	hypothetical protein	443
4	subtilisin-like serine protease	423
5	hypothetical protein	227
6	hypothetical protein	234
7	conserved hypothetical protein	171
8	hypothetical protein	156
9	conserved hypothetical protein	155
10	hypothetical protein	158
11	hypothetical protein	157
12	hypothetical protein	142
13	Casein Kinase I	134
14	Conserved hypothetical protein	93
15	hypothetical protein	97

Non Virulent (CTG)

Figure A2-1. Parasite gene expression. CD-1 outbred mice were infected with 200 tachyzoites of either Type I (GT1) or Type III (CTG), sacrificed, peritoneal lavage collected and total RNA isolated on Day 5. Biotinylated cRNA was hybridized to cDNA microarrays and signal intensity values were obtained. Only genes highly ranked were selected and normalized to GAPDH internal control.

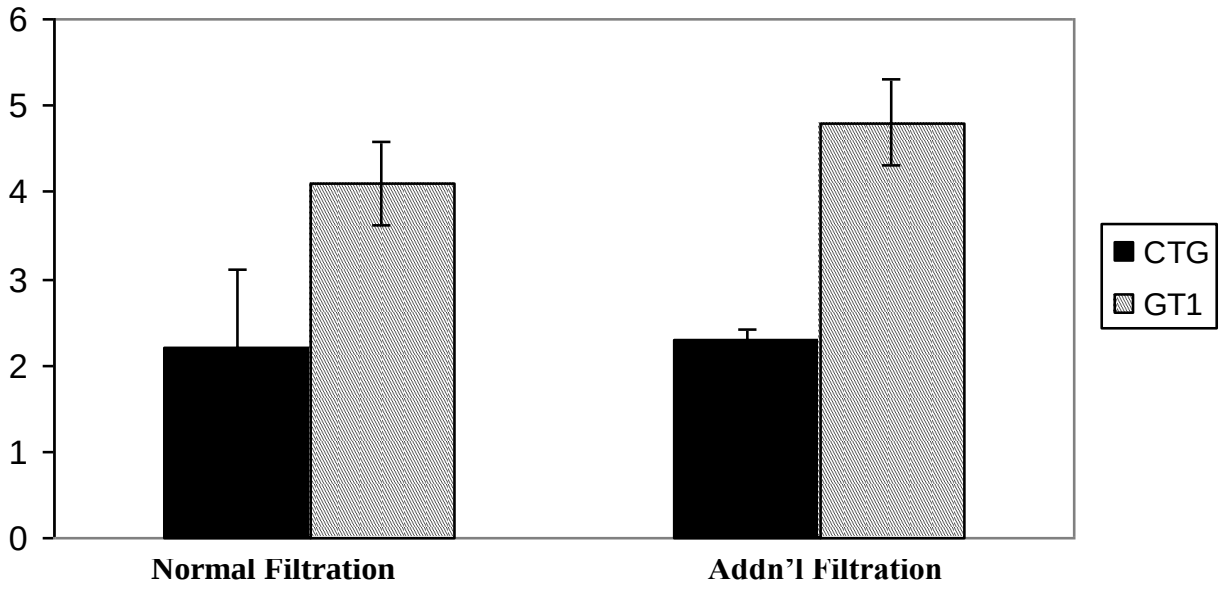

Fig A2-2. Filtration method. HFF cells were infected in vitro with Type III VEG. Harvested tachyzoites were either filtered under normal harvesting conditions or treated with an additional filtration step. $P = .05$.method.

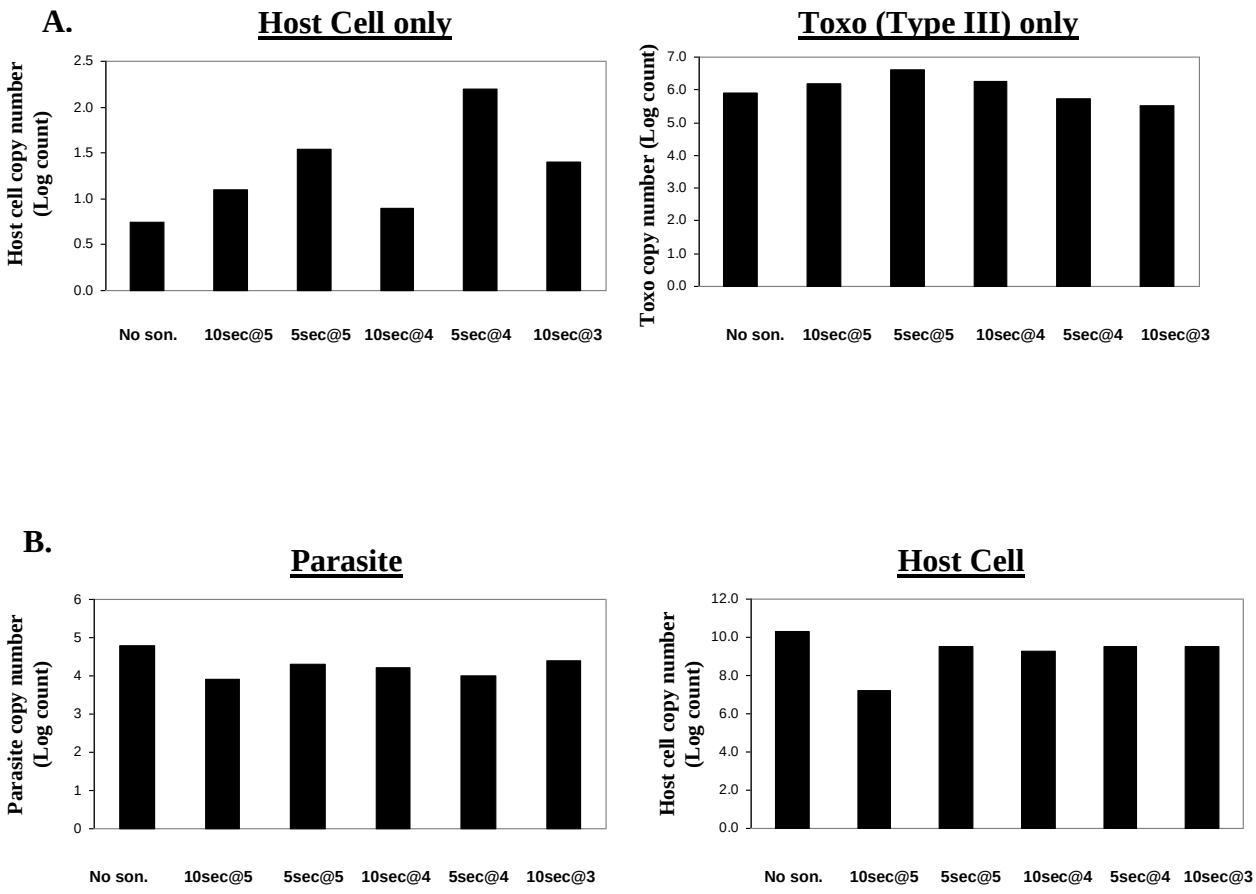

Figure A2-3. Sonication method. HFF and RAW cell lines were grown and maintained in vitro. Different sonication settings were used to observe effect on cell disruption. (A) Quantification of HFF host cells only and parasites harvested from host cells. (B) Host/ parasite detection. Parasites were not harvested until after sonication of mixed (parasite/ host) cells.

As a last attempt to enhance parasite RNA of non virulent strain, we increased the initial inoculum into mice. Typically, during in vivo infections, we inoculate approximately 200 – 1000 tachyzoites into mice. Due to the decreased levels of parasite RNA extracted from non virulent infected mice, we hypothesized that if we increase the initial dose, downstream parasite concentrations will also increase. CD-1 outbred mice were I.P. inoculated with 10^4 Type III (CTG) parasites. On day 5, mice were sacrificed and peritoneal lavage was collected. Total RNA was extracted and microarray was carried out as described above. For parasite gene expression profiling, ToxoGeneChip array containing ~ 8000 features was used. Our results demonstrated that indeed, parasite genes were detectable. Ranking genes based on signal intensity value showed, genes involved in invasion (tubulin beta chain) and active replication of tachyzoite (MIC10, SRS52A) form to be highly expressed (Fig A2-4).

Conclusions

Unfortunately, limited conclusions could be made from this dataset. The initial increase in parasite concentration significantly changed the parasite ratio between strains. Based on this change, we are uncertain if gene expression profiles observed are due to parasite genetic background or parasite concentration. Further work is aimed to overcome this obstacle.

Type III (10^4)		
Rank	**Gene**	**Signal Intensity**
1	Translationally-controlled tumor protein	17841
2	MIC10	17719
3	Histone Variant H3	16100
4	Hypothetical protein	13650
5	Elongation factor 1β	13627
6	TgMLC1 0	13279
7	Ribosomal protein L41	13171
8	Tubulin beta chain, putative	12249
9	Non-transmembrane antigen	10448
10	Cytochrome oxidase I	10357
11	Hypothetical protein	9624
12	ER retention receptor, putative	8608
13	ATP-dependent RNA helicase	8528
14	SRS52A	8156
15	Glycine-rich protein 2	7766

Figure A2-4. In vivo increased parasite infection. CD-1 outbred mice were infected with 200 tachyzoites of either Type I (GT1) or Type III (CTG), sacrificed, peritoneal lavage collected and total RNA isolated on Day 5. Biotinylated cRNA was hybridized to cDNA microarrays and signal intensity values were obtained. Only genes highly ranked were selected and normalized to GAPDH internal control.

Appendix III. Protocols

Challenge mice for *In vivo* competition assay

1. Grow non-virulent *T. gondii* strain CTG and virulent GT1 in 2 (1 flask/ strain) T25 HFF cell culture until HFF cells are nearly lysed (normally in 48 hrs)
2. Filter purify and harvest tachyzoites in 15-ml centrifuge tube
3. Make 1:10 dilution in D10 media. Determine parasite concentration using a hemacytometer
4. Add 500 µl of GT1 ($1x10^4$) to 4mL of D10 *
5. Add 500 µl of CTG ($5x10^4$) to 4.5mL D10/GT1 solution *
6. Infect mice with 200 µl of GT1:CTG (1:5) via peritoneal injection
7. Collect spleen, lung and peritoneal lavage @ days 4, 6 and 8
8. For controls, infect 4 mice with GT1 and CTG
    a. Infect 2 mice with GT1 (200 tachys) only
    b. Infect 2 mice with CTG (1000 tachys) only
    c. On day 8 collect spleen, lung and peritoneal lavage from all mice

Detecting *T. gondii* in mouse tissue samples

* All buffers, spin columns and collection tube are supplied by kit.

1. Infect 4 mice with 200 toxo and harvest spleen and lung at day 7
2. Weigh spleen and lung, freeze at -70C before use
3. For spleen, adjust volume to .02 g/ml using PBS (total volume will be between 5 to 20 ml). For lung, adjust volume to .01 g/ml using PBS. Take two aliquots (1^{st}-200µl and 2^{nd} -1mL) of tissue lysate, store at -20C before use.
4. For controls (positive and negative), use tissue samples without T.gondii infection.
5. Thaw tissue lysate (200µl). Spin at 12,000 rpm for 5 min. Remove supernatant and resuspend pellet in 180µl of Buffer ATL.
6. Add 20µl of proteinase K. Mix by vortexing and incubate @ 56ºC until completely lysed. Invert periodically to facilitate digestion of tissue. Lysis usually occurs within 1-3 hrs. Treat positive controls by same procedure.
7. **For positive controls**, harvest toxo (from four T25 flasks) and re-suspend in 1 ml of PBS. Make 2x 1:10 dilutions (100µl toxo + 900µl PBS) and count dilution 1:100. The concentration will be about 10^8 toxo/ml. Make a series of 1:10 dilution to give 10^7 to 10^4 toxo/ml. Spike the original and diluted toxo into 200µl of tissue lysate to make a series of concentration from10^7 toxo /ml to 10^3 toxo /ml. e.g.

   Spike 20 µl of $1x10^8$ toxo/ml into 200µl lysate to make $1x10^7$ toxo/ml

   Spike 20 µl of $1x10^7$ toxo/ml into 200µl lysate to make $1x10^6$ toxo/ml

   Spike 20 µl of $1x10^6$ toxo/ml into 200µl lysate to make $1x10^5$ toxo/ml

   Spike 20 µl of $1x10^5$ toxo/ml into 200µl lysate to make $1x10^4$ toxo/ml

   Spike 20 µl of $1x10^4$ toxo/ml into 200µl lysate to make $1x10^3$ toxo/ml

* Pellet and resuspend as in step 5. Process as step 6

8. Once complete lysis, vortex for 15s. Add 200µl Buffer AL to the sample. Mix thoroughly by vortexing. Then add 200µl ethanol (96-100%). Mix again thoroughly.
9. Pipet the mixture into a DNeasy Mini spin column in a 2ml collection tube. Centrifuge at ≥ 6000xg (8000 rpm) for 1min. Discard flow through and collection tube.
10. Place the spin column in a new 2ml collection tube. Add 500µl Buffer AW1. Centrifuge for 2 min at ≥ 6000xg. Discard flow through and collection tube.
11. Place the spin column in a new 2ml collection tube. Add 500µl Buffer AW2. Centrifuge for 3 min at 20,000xg (14,000). Discard flow through and collection tube.
12. Transfer the spin column to a new 1.5ml or 2ml microcentrifuge tube (not provided by kit) and add 200µl Buffer AE for elution. Incubate for 1 min at room temperature. Centrifuge for 1 min at ≥ 6000xg.
    a. Repeat this step for maximum yield.

RTPCR: Cephaid Smartcycler

ITSI primers

ITS1-Fx: GAAGGGGCTCAATTTCTGG
ITS1-Rx: TGTTCCTCAGATTTGTTGTTTGA
ITS1 probe was 5'-/56-FAM/CGTGTCTCTGTTGGGATACTGATTTCCAGG/3BHQ-1/-3'

H_20	16.7µl
$MgCl_2$	2.5µl
dNTPs (2.5 µm)	2.5µl
BSA	1.0 µl
For Primer (50 µM)	.15µl
Rev Primer (50 µM)	.15µl
Probe (50 µM)	.3 µl
Taq Polymerase	.2 µl
Total	23.5 µl

Add 1.5 µl cDNA each rxn.

RT-PCR samples in smartcycler: 95ºC for 15 min, **94ºC for 10s, 55º for 30 s, 72º for 30 s, Repeat 40 cycles,** 72ºC for 5min, Melting curve: 72-95ºC, 72ºC for 5 min.

Once completed, analyze data from results table.

Isolation of total RNA from mice for Microarray Analysis

Materials: Qiagen RNeasy Plus Mini Kit

RNeasy Plus Mini Kit (50), Catalog no. 74134	
Number of preps	50
gDNA Elimiantor Mini Spin Colums (uncolored)	
(each in a 2 ml Collection Tube)	50
Rneasy Mini Spin columns (pink)	
(each in a 2ml Collection Tube)	50
Collection Tubes (1.5ml)	50
Collection Tubes (2ml)	50
Buffer RLT Plus*	45 ml
Buffer RW1*	45ml
Buffer RPE+ (concentrate)	11ml
RNase- Free Water	10ml

70% ethanol

Beta mercaptoethanol

*Contains a guanidine salt. Not compatible with disinfectants containing bleach. See page 6 for safety information.

+ Before using for the first time, add 4 volumes of ethanol (96-100%) as indicated on the bottle to obtain a working solution.

Procedure:

1. Grow parasite in T25 flask until nearly lysed (` 48 hr passage)
2. Infect four CD1 outbred mice I.P. with 500 tachyzoites
3. On day 5 collect peritoneal lavage in 5 ml of ice cold PBS
4. In lab, pellet sample at 13,000 rpm for 10 min. Resuspend in 1 ml RNA later.. Store @ -20°C until use.

Isolate total RNA:

5. Spin down the cells at 12,000 rpm for 10 min, remove supernatant
6. Resuspend in 1ml of PBS, take 200 µl to determine parasite load by Real-time PCR and genotype by PCR –RFLP (DNA will be extracted by Qiagen tissue and blood kit).
    a. For DNA extraction, Spin down cells at 12,000 rpm for 10 min.
    b. Resuspend in 1 ml PBS, pellet again at 12,000 rpm for 5 min
    c. Resuspend in 200 µl PBS, now samples are ready for extraction
7. Take remaining (800 µl) spin down the cells at 12,000 rpm for 10 min, remove supernatant.
8. Take aliquot of Buffer RLT Plus, add 10 µl beta- mercaptoethanol to 1ml RLT. Disrupt the cells by adding 600 µl Buffer RLT Plus. Pipet to mix. Ensure that no cell clumps are visible and cells are lysed.
9. Transfer the lysate to a gDNA Eliminator spin column placed in a 2 ml collection tube. Centrifuge for 30 s at 12,000 rpm. Discard the column, and save the flow through.
10. Add 1 volume (600 µl) of 70% ethanol to the flow through and mix well by pipetting. Do not centrifuge.
11. Transfer 600 µl of the sample to an RNeasy spin column placed in a 2 ml collection tube. Centrifuge for 15 s at 12,000 rpm. Discard the flow-through. Transfer the remaining 600 µl of samples to the spin column and centrifuge. Discard the flow through.

12. Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid and centrifuge for 15 s at 12,000 rpm to wash the spin column membrane. Discard the flow through. Be sure to empty the collection tube completely. Reuse the collection tube.
13. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid and centrifuge for 2 min at 12, 000 rpm to wash the spin column membrane. Discard the flow through. Reuse the collection tube.
14. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid and centrifuge for 2 min at 12, 000 rpm to wash and dry the spin column membrane. Make sure no residual ethanol is carried over.
15. Place the RNeasy spin column in a new 2 ml collection tube and centrifuge at 12,000 rpm for 1 min to eliminate any possible carryover of Buffer RPE.
16. Place the RNeasy spin column in a new 1.5 ml collection tube. Add 30 µl of RNase-free water directly to the spin column membrane. Close the lid and centrifuge at 12,000 rpm for 1 min to elute the RNA.
17. Add additional 20 µl of RNase- free water directly to the spin column membrane. Close the lid and centrifuge at 12,000 rpm for 1 min to elute the RNA.
18. Store RNA at -70°C before use.

Sonication/ Filtration of *T. gondii* Tachyzoites

Purpose: To test sonication/ filtration method and its efficiency to rid host cell contamination for microarray analysis

NOTE- test *in vitro* to confirm purification of parasite before *in vivo* work goes forth

GAPDH Primers- Roussel et. Al, Vaccine 25 (2007) 2919-2929

GAPDH-F: 5′-TGCACCACCAACTGCTTAG-3′ 19bp

GAPDH-R: 5′-GGA TGC AGG GAT GAT GTT C-3′ 19bp

5′-CAG AAG ACT GTG GAT GGC CCT-3 probe 21bp

Product size – 177 bp

ITSI primers

ITS1-Fx: GAAGGGGCTCAATTTCTGG

ITS1-Rx: TGTTCCTCAGATTTGTTGTTTGA

ITS1 probe was 5'-/56-FAM/CGTGTCTCTGTTGGGATACTGATTTCCAGG/3BHQ-1/-3'

Procedure: III parts

I. Host cell detection

1. Grow (2) HFF T175 and (2) J774A.1 T175 flasks until confluent monolayer
2. Remove media
3. Dilute Trypsin 1:1 with sterile PBS
4. Add 2 ml of Trypsin/ PBS to each flask
5. Incubate at 37ºC for 5min to detach cells from flask surface
6. Determine host cell concentration using hemacytometer
    i. Make 1:100 dilution for counting

7. Aliquot 5 ml (2 x 10^6 conc.) cells into six 15ml tubes for each cell line
    i. Make total volume of 70 ml of 2 x 10^6 cells for each cell line tested
8. Sonicate at 5 different settings to obtain optimal setting using: Sonicator Heat Systems ultrasonics inc. cell disrupter. Model W220F

- make sure tubes stay on ice during sonication, produces heat*

    i. 10 sec @ setting 3
    ii. 5 sec @ setting 4
    iii. 10 sec @ setting 4
    iv. 5 sec @ setting 5
    v. 10 sec @ setting 5

    } x 2

    vi. Non sonicated cells serve as control

8a. Pellet cells @ 1500rpm for 5 min

8b. Resupsend in 5 ml PBS

9. Remove 1 ml and extract DNA via Qiagen DNeasy Kit
    i. Elute in 100µl of elution buffer provided by kit
10. Run Real Time PCR – according to Su lab protocol
    i. Target: GAPDH
11. Determine host cell concentration
    i. Non sonicated cells will be determined as 1 and make a series of 1:10 dilution for standard curve

II. Parasite Detection

1. Infect 2 HFF T25 HFF flasks w/ VEG until nearly lysed (~48hrs)
2. Filter purify

3. Harvest parasite in 50ml tube
    i. Pellet cells @ 1500g for 5 min
    ii. Resuspend in 1 ml PBS
4. Determine parasite concentration using hemacytometer
5. Aliquot 5 ml (2×10^6) parasite into six 15 ml tubes
6. Follow sonication steps as described above in part I, step #8

6a. Pellet @ 1500g x 5 min

6b. Resuspend in 2 ml PBS

7. Remove 1 ml and extract DNA via Qiagen DNeasy Kit
    i. Elute in 100µl of elution buffer provided by kit
8. Run Real Time PCR- according to Su lab protocol
    i. Target: ITSI
9. Determine parasite concentration

III. <u>Toxo/ Host cell detection</u>

1. Infect (4) T25 J77 A.1 flasks w/ VEG ~ 24-36 hrs
2. Dilute Trypsin 1:1 with sterile PBS
3. Add ~500µl of Trypsin/ PBS to each flask
4. Incubate at 37ºC for 5min to detach cells from flask surface
5. Determine host cell and parasite concentration using hemacytometer
    i. Make 1:100 dilution for counting
    ii. If counting is challenging, mount slide, fix in methanol and Giemsa stain for differentiation
6. Sonicate at same settings as in Part I, step #8

7. Pellet @ 1000g x 5min
8. Remove supernatant
9. Resuspend in 5 ml PBS
10. Filter purify and harvest parasite in 50ml tube (Do not filter control)
    i. Pellet @ 1500g x 5 min
    ii. Resuspend in 2 ml PBS
11. Extract DNA according to Qiagen DNeasy Kit
    i. Elute in 100µl of elution buffer provided by kit
12. Run Real Time PCR
    i. Targets: ITSI and GAPDH to quantify both parasite and host cell concentrations
13. Determine optimal setting for destroying host cell but recovering the most parasite

2 Step qRT-PCR protocol: (DyNAmo Sybr Green Kit)

Purpose: To obtain mRNA transcript levels of specific genes from mice infected w/ *T. gondii.*

Materials:

Primers as described above

Finnzymes DyNAmo Sybr Green 2 step qRT- PCR kit

Cat No. F430S (CDNA syn 20rxn, qPCR 80rxn)

F430L (cDNA syn 100 rxn, qPCR 400 rxns)

Procedure:

1. Infect mice CD-1 outbred mice with 500 tachys of *Toxoplasma gondii*
2. On day 5 post infection, sacrifice mice and collect peritoneal lavage (in 5ml cold PBS), spleen and brain.
3. Store tissue samples in 1 ml RNAlater and store in -70 until use.
4. In lab, pellet the cells in peritoneal lavage by centrifuging at 15000 rpm for 5 min @ 4C. Keep 2 ml and remove extra supernatant.
5. Isolate total RNA according to protocol established by Rachel in accordance with Qiagen RNeasy Kit
6. Store in -70C until use.
7. Quantify RNA samples before use to ensure accurate RNA concentrations.
    a. Normalize all samples to one concentration as not to have more than 1μg/ rxn.

I. <u>cDNA synthesis-</u> all reagents are supplied by kit

H_20*	x µl
RT buffer	10µl
Random hexamer primer set	1µl
M-MuLV RNase RT	2 µl
RNA template*	x µl
Total	20 µl

- values will vary based on initial concentration of RNA

Run samples in mastercycler: 25ºC @ 10 min, 37ºC @ 30 min, 85ºC @ 5min, Hold @4C

(If not using samples immediately, store @ -20ºC until use)

II. <u>qPCR</u>

H_20	8.6 µl
Master Mix	10 µl
For Primer (50 µM)	.2 µl
Rev Primer (50 µM)	.2 µl
Total	19 µl

Add 1 µl cDNA from part I to each rxn.

RT-PCR samples in smartcycler: 95ºC for 15 min, **<u>94ºC for 10s, 55º for 30 s, 72º for 30 s,</u>**

<u>Repeat 40 cycles,</u> 72ºC for 5min, Melting curve: 72-95ºC, 72ºC for 5 min.

Once completed, analyze data from results table.

Cell Surface Staining

Purpose: To quantify cell population of mice infected with *Toxoplasma gondii* .

Materials: 96 well U-Bottom plate, Abs (Macrophage- CD11b/F18:FITC, DC's – CD11b:APC, Neutrophils-GR1:PE, Toxoplasma- Surface antigens: Secondary antibody (goat anti-mouse IgG-PerCP; Santa Cruz biotechnology cat no. sc-45092 $96)

Normal mouse serum 1/100 in PBS-0.1% BSA (FACS buffer)

Experiment:

1. Add $1x10^6$ cells to designated wells of a 96 well U Bottom plate
2. Centrifuge plate at 1500 rpm for 5 min at room temperature (~23ºC)
3. Flick off supernatant, and vortex plate (be sure that all cells are in suspension)
4. Dilute normal mouse serum 1/100 in PBS-0.1% BSA (Sangster Lab) and add 100 µl to each sample. Pipette each sample up and down to ensure the cells and thoroughly resuspended. Change tips b/n each sample.
5. Incubate plate on ice for 30 mins.
6. Centrifuge plate at 1500 rpm for 5 min at room temperature (~23ºC)
7. Flick off supernatant, and vortex plate (make sure that all cells are in suspension)
8. Dilute selected primary antibody 1/200 in PBS-.1%BSA **(For Toxo; mouse serum from infected mice)**
9. Add 100 µl of primary Ab to appropriate wells. Pipette each sample up and down to ensure the cells are thoroughly resuspended. Change tips b/n each sample.
10. Incubate plate on ice for 30 mins
11. Centrifuge plate at 1500 rpm for 5 min at room temperature (~23ºC)
12. Wash 2xs w/ PBS (vortex @ each wash to ensure complete cell suspension)

13. Dilute selected secondary Ab (goat anti-mouse IgM-PerCP) and Macrophage, DCs and neutrophil Abs to 1/200 and mix in cocktail. Add 100 µl to appropriate wells
14. Add 100 µl of PBS-.1% BSA to unstained wells
15. Wrap plate with foil and incubate on ice for 30 mins (Abs are light sensitive)
16. Centrifuge plate at 1500 rpm for 5 min at room temperature (~23ºC)
17. Flick off supernatant, and vortex plate (make sure that all cells are in suspension)
18. Resuspend cells in 100 µl of PBS -.1% BSA and transfer cells to appropriate tubes
19. Add 600 µl of PBS -.1% BSA to each tube (If not analyzing on same day- Add 50 µl paraformaldehyde to fix cells
20. Keep samples on ice and covered until ready to run using FACScan and Cellquest Software

** Cells are viable ~48 hrs after staining to obtain accurate read, anything later discard

VITA

Rachel D. Hill was born in Cincinnati, OH. She received her Bachelor of Science Degree in Biology/ Pre-Med from Knoxville College in 2005. While completing her Bachelors, she worked approximately one year and a half as a research technician at the University of Tennessee, Knoxville. In the fall of 2005, she entered graduate school at The University of Tennessee, Knoxville, Department of Microbiology. She received her Doctorate Degree in Microbiology in 2011.