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CYTOKINE PROFILING IN CHRONIC NEUROTOXOPLASMOSIS CAUSED BY THE KNOCK-OUT PARASITE FOR THE BRADYZOITE MARKER *P18* IN THE C57BL/6J MOUSE MODEL

by
KATIA SAWAYA, MD

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KATIA SAWAYA

Approved by:

Dr. Hiba El Hajj, Assistant Professor — Advisor
Department of Internal Medicine

Dr. Alexander Abdelnoor, Acting Chairperson & Professor — Member of Committee
Department of Experimental Pathology, Immunology & Microbiology

Dr. Hassan Zaraket, Assistant Professor — Member of Committee
Department of Experimental Pathology, Immunology & Microbiology

Dr. Ghassan Awar, Assistant Professor of Clinical Specialty — Member of Committee
Department of Internal Medicine

Date of thesis defense: April 20 2016

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AN ABSTRACT OF THE THESIS OF

Katia Sawaya for Master of Science
Major: Microbiology and Immunology

Title: Cytokine Profiling in Chronic Neurotoxoplasmosis caused by the knock-Out Parasite for the Bradyzoite Marker P18 in the C57BL/6J Mouse Model

Descriptive Statement: *Toxoplasma gondii* is an obligate intracellular parasite that forms chronic life-long bradyzoite cysts in the brain of infected humans. Our lab generated the Pru∆*KU80*∆*P18* strain in which the bradyzoite marker *P18* was deleted and found that this strain displays a higher capacity of cyst formation when compared to the wild type strain Pru∆*KU80.* Since the host immune response is the key player of controlling the switch between acute and chronic toxoplasmosis, we have investigated this immune response, through cytokine profiling, against the *P18* knock-out strain using the C57BL/6J susceptible mouse model.

Introduction: *T. gondii* is capable of establishing an acute and/or latent chronic infection in a wide variety of hosts. IFN-γ is key driver that tightly controls the chronic cerebral infection, and activates microglial cells as well as other brain reaching wandering cells such as macrophages to produce nitric oxide (NO). NO, along with pro- and anti-inflammatory cytokines, triggers the conversion to bradyzoite forms and is critically important to control infection in C57BL/6 mice. We investigated the cyst forming capacity and the immune response upon infection with Pru∆*KU80*∆*P18* and Pru∆*KU80* strains in the brains of the susceptible C57BL/6J mice by measuring transcription levels of BAG-1, a bradyzoite marker in the brains of mice infected with either strain and comparing these levels as well as the transcription levels of various cytokines involved in the immune response against *T. gondii*.

Methods: C57BL/6 mice were infected with Pru∆*KU80*∆*P18* and Pru∆*KU80* strains and were treated with sulfadiazine to overcome the acute phase of toxoplasmosis. Blood was collected on day 7 post infection to verify the acute infection by the western blotting. Mice were sacrificed on weekly basis from week 2 until week 5. Total mRNA was extracted from the brains, and different immunomodulatory cytokines and chemokines (MCP-1, IL-12, IFN-γ, TNFα, iNOS, IL-1β, IL-6, and IL-10) were measured in addition to the bradyzoite marker BAG-1 using Syber Green Real Time PCR.

Results: We have seen a clear difference in the amount of encysted bradyzoites in the brains of mice infected with Pru∆*KU80*∆*P18* and Pru∆*KU80* as reflected by the transcription levels of BAG-1 on day 28 post-infection We also have shown that Pru∆*KU80*∆*P18* stimulates higher levels of IFN-γ reaching a maximum at week 2 presumably leading to the recruitment of more IL-12 producing macrophages. This was consistent with MCP-1 levels that were also higher and

reached a maximum on week 3 post-infection in the *P18* knock strain. Since Nitric Oxide (NO) plays a vital role in controlling toxoplasmosis progression, and since inducible NOS (iNOS) levels are upregulated in brains of infected mice with type II *T.gondii*, we have shown that PruΔ*KU80*Δ*P18* leads to higher levels of iNOS at all time points reaching a maximum at day 35, eventually controlling the infection. We also have seen that IL-10 increases over weeks presumably as an anti-inflammatory cytokine alleviating the disease burden. Pro-inflammatory cytokine transcription levels of IL-1β and IL-6 showed opposite results to those of IL-10 at days 28 and 35 post-infection being higher in the brains of mice infected with PRUΔ*KU80.*

Conclusion: The current study highlights the role of the bradyzoite marker P18 in shaping the brain immune response, allowing the understanding of key molecular mechanisms related to the neurotoxoplasmosis.

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CHAPTER I
INTRODUCTION

A. History

In 1908, Nicolle and Manceaux came across a parasite they mistook for *Leishmania*, while experimenting on a rodent, *Ctenodactylus gundi*, in the laboratory of Charles Nicolle in Tunis (Nicolle and Manceaux, 1908) (Kean, 1972) (Ferguson, 2009) (Dubey, 2009). Little after, they had stumbled upon a major new parasite vastly distributed in all kinds of animals including birds and humans. They realized their major discovery, and in 1909 they named it *Toxoplasma gondii*, after its morphology, "Toxo" from the latin meaning arc, and "plasma" from the latin meaning life. During the same year of 1908, another scientist, Splendore, found the same parasite in a rabbit in Brazil, also mistaking it at first for *Leishmania* (Splendore, 1908) (Cross, 1947) (Dubey, 2014).

Findings of *T. gondii* continued to appear for several years in different hosts, but the first isolation of viable *T. gondii* from an animal host was at the hands of Sabin and Olitsky in 1937. They also showed that this parasite was obligate intracellular and that transmission could happen through inoculation of brain homogenates of an infected animal into a healthy one; they further went on, to deduce through experiments carried out on mice that the parasite can be transmitted through ingestion of infected tissues (Dubey, 2009).

This work was later followed by an isolation of *T. gondii* from human beings by Wolf et al. in 1939. This isolation came following a post-mortem examination of the nervous system of a child who died from encephalomyelitis at day 31 postnatally.

Granulomatous lesions were detected in the brain that contained an organism resembling *T. gondii*(Wolf 1939).

In 1991, Frenkle et al. successfully presented a vaccine, designated T-263 that prevents shedding of oocysts (infective stages of *T. gondii* if ingested) from its primary host, the domesticated cat (Frenkel, 1991). Another important hallmark came to be in 2005 after sequencing the whole *T. gondii* genome by Khan et al. (Khan et al., 2005).

Since then, many studies continued to be carried out on *T. gondii* spanning across its various aspects like morphology, life cycle, transmission, diseases, immunity, diagnosis, treatment, and potential vaccination, highlighting a world of knowledge on the biology of this parasite and its caused-disease.

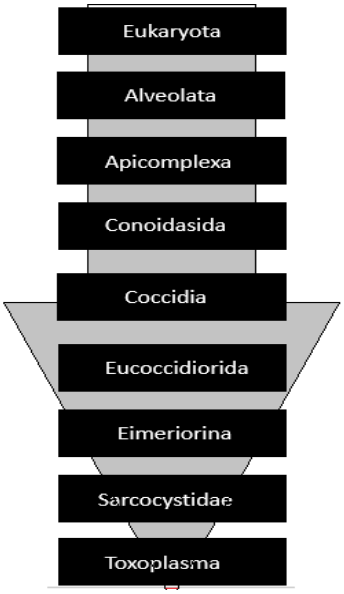

Figure 1. Taxonomy from the NCBI taxonomy database (http://eol.org/pages/34045746/hierarchy_entries/51390122/overview)

B. Taxonomy

Toxoplasma gondii is a single-celled obligate intracellular parasite. It belongs to the domain Eukaryota, kingdom Alveolata, phylum Apicomplexa (Levine, 1977) (Figure 1). Furthermore, *T. gondii* belongs to the class coccidia, the order Eucoccidiorida, and genus *Toxoplasma* which contains only one species "*gondii*".

Along with *Plasmodium spp.* which claims over a million lives each year (Manguin et al. 2010), *Toxoplasma gondii* is one of the most important apicomplexan parasites, affecting around 30% of the world's population (Dubey, 2009). There are common several physical features among the apicomplexan parasites, the most important being the apical complex structure formed of a conoid with polar rings (Lee et al., 2000). In addition, there are secretary organelles named rhoptries, micronemes, and dense granules, which are tightly involved in the host-cell invasion mechanism.

C. Host-Cell Invasion

Host cell invasion is a conserved mechanism among all apicomplexan parasites. In *Toxoplasma gondii*, it involves an initial step of attachment to the host cell, and involving mostly the parasite micronemal proteins (MICs) with the host cell plasma membrane (Figure 2). A second step is the formation of a moving junction from micronemal and rhoprty neck proteins (RONs), propelling the parasite inside a protective parasitophorous vacuole formed mostly by parasitic rhoptry proteins (ROPs) and where the parasite actively replicates, involving host and parasite proteins as well as scavenging nutrients from the host cell, until lysing the host cell (Figure 2) (Soldati, Dubremetz, and Lebrun, 2001; Hunter and Sibley, 2012).

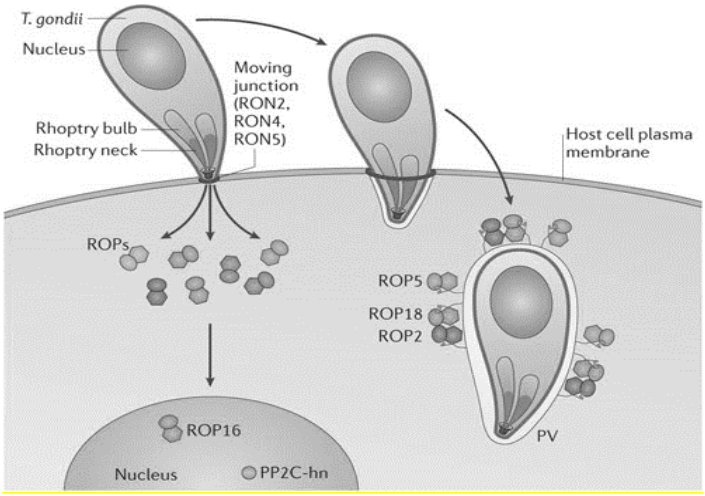

Figure 2. T. gondii Invasion of host cells. Hunter, Christopher A., and L. David Sibley. " Nature Reviews Microbiology 10.11 (2012): 766-778.

D. Stages responsible for acute and chronic toxoplasmosis

1. The Tachyzoite:

Previously known as the trophozoite from "trophicos", meaning feeding in greek, the tachyzoite named as such by Frankel in 1973 is the fast replicating stage of *T. gondii* where "Tachos" means fast and "zoite" means life. This form is responsible for the acute phase of the infection. This was the form of the parasite that Nicolle and Manceaux came across in 1908 and described as crescent shaped (Gustafson et al., 1954). The tachyzoite, lacking any specialized locomotive structures, presents the apical structure formed of the conoid, rhoptries, micronemes, and dense granules (Dubey and Linday, 1998), in addition to the normal organelles of a eukaryotic cell but also an inherited plant structure called apicoplast involved in fatty acid synthesis (Figure 3).

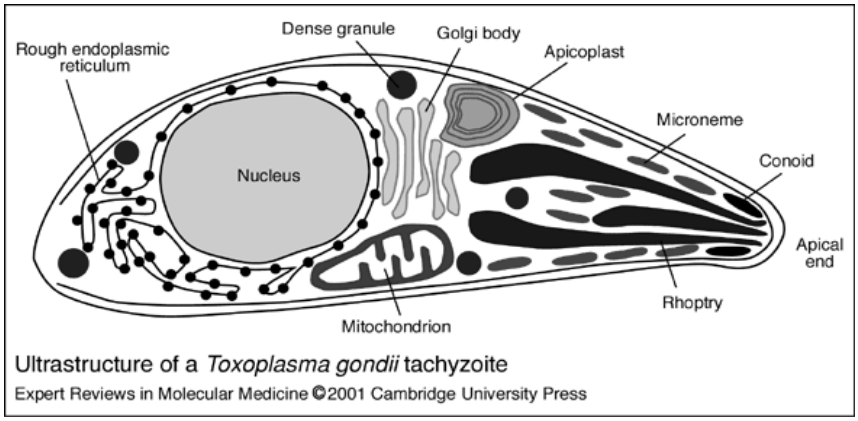

Figure 3. Ultrastructure of T. gondii Ajioka, Expert Review in Molecular Medicine. http:/www. ermm. cbcu. cam. ac. uk. Department of Pathology. University of Cambridge. Tennis Court Road. Cambridge. UK(2001).

Once inside the host cells of an intermediate host, the tachyzoites divide asexually by repeated endodyogeny, a process first described by Goldman et al. in 1958, in which two daughter parasites form inside the mother parasite killing it.

2. The Bradyzoite:

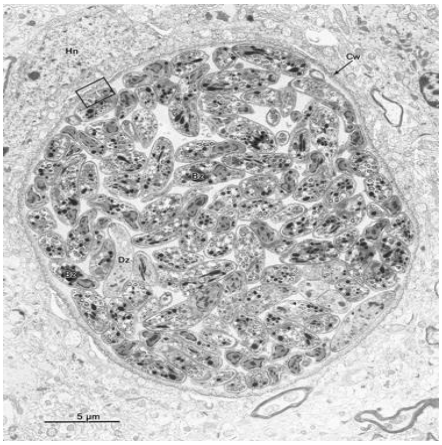

Figure 4. Bradyzoite cyst in the brain of a mouse infected with the VEG strain type II strain of T. gondii (Dubey et al., Clin Microbiol Rev 1998

The Bradyzoite is the slow multiplying form of the parasite. The term was also suggested by Frenkel in 1973 to describe the encysted stage in the intermediate host tissues. The cyst was first recognized by Levaditi, Schoen, and Sanchis Bayarri in 1928 and the first cytological description was by Frenkel and Friedlander in 1951. Later on, when Dubey et al. discovered the oocyst they suggested that the cysts be called tissue cysts to avoid confusion. These tissue cysts have been described to have high affinity for skeletal muscles, and the brain (Figure 4) (Dubey 1998).

While the structural differences between tachyzoites and bradyzoites are subtle, we can still tell them apart (Table 1). Bradyzoites contain a larger number of amylopectin granules in their cytoplasm. Periodic acid –Schiff stain is used to detect cytoplasmic polysaccharides such as amylopectin. Tachyzoite nuclei are centrally located, unlike the bradyzoites that have them further down the posterior end. The bradyzoites are less metabolically active than the tachyzoites, and contain no lipid bodies as opposed to tachyzoites. Even though bradyzoites are capable of replication by endodyogeny like tachyzoites, they do it at a much slower rate. While tachyzoites are the hallmark of the acute infection, the bradyzoites are the cyst stages characterizing the chronic phase of the infection (Dubey, 1998).

Furthermore, the switch between these stages highly reflects the progress of toxoplasmosis between its acute and chronic phases, and is tightly governed by the host immune response (Dubey, 1998).

Tachyzoites	Bradyzoites
slender crescent shaped	more slender
nucleus more central	nuclues situated towards the posterior
labyrinthine rhoptries	electron dense rhoptries
usually no amylopectin granules	contain several amylopectin granules
more susceptible to destruction by proteolytic enzymes	less susceptible to destruction by proteolytic enzymes

Table 1. Differences between tachyzoite and bradyzoite stages of Toxoplasma gondii

As early as 1956, Frenkel showed through conducting experiments on hamsters chronically infected with *T. gondii* that not only a transition from the tachyzoite stage to the bradyzoite stage was possible, but that reactivation following cyst rupture and transition from the bradyzoite stage back to the tachyzoite stage occurs in cases of immunosuppression (Frenkel, 1957). This was later confirmed in immunosuppressed human patients (Figure 5).

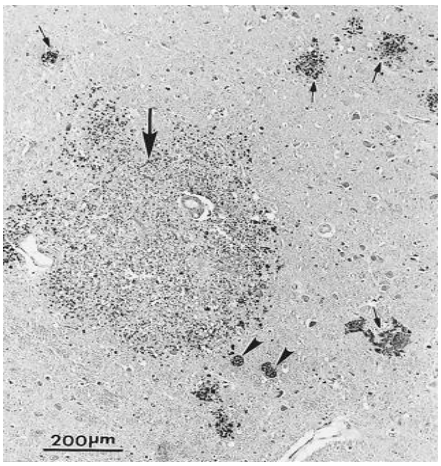

Figure 5. Section of the cerebrum of a patient with AIDS. Dubey et al. (Clin. Microbiol. Rev. April 1998vol. 11 no. 2 267-299)

E. **Life Cycle**

T. gondii has a complex life cycle, harboring a sexual stage in the primary or final host, the Felidae, and an asexual stage in the secondary or intermediate host, including humans (Figure 6). The sexual stage of the life cycle starts when a domesticated house cat, ingests by carnivorism the parasite mostly in its bradyzoite stage, bradyzoites are liberated and reach the intestines of the cat, where they invade the enterocytes and establish their sexual cycle. Merozoites are produced by schizogony, mature and develop into gametocytes, then gametes. After fertilization of a macrogamete by a microgamete, a zygote is formed in the intestinal lining cells of the infected feline definite host. Intestinal epithelial cells then rupture and oocysts are shed into the intestinal lumen as immature oocysts within 3-7 days following ingestion of the infected prey. Upon defecation, unsporulated oocysts are released in nature where sporulation occurs. After 4 to 10 days post excretion and under appropriate environmental conditions, a sporulated oocyst containing 2 sporocysts harboring 4 sporozoites each forms and represents the infective form, ready to start a new cycle (J. Dubey, 1998).

Upon oral ingestion of food contaminated with sporulated oocysts from the environment by any warm-blooded animal, the asexual or intermediate life cycle begins (Figure 6). The digestive enzymes of the gastro-intestinal tract lead to the liberation of sporozoites which will rapidly transform into tachyzoites in the blood. These tachyzoites are responsible for the acute phase of infection and are capable of invading any nucleated cell as well as crossing the blood-placental barrier. They hijack dendritic cells and macrophages recruited to the site of infection and use them as Trojan horses to disseminate to distal sites (Lambert, Henrik, et al., 2006). Approximately 5 days after

ingestion, and under the immune response, these tachyzoites transformed into bradyzoites that encyst in the brain and skeletal muscles. These formed tissue cysts stay dormant as long as the host is immunocompetent (J. P. Dubey et al., 1998) (J. P. Dubey, 2007). In immunosuppressed patients, these bradyzoites may become life threatening if they reactivate back into tachyzoites (Mele et al. 2002).

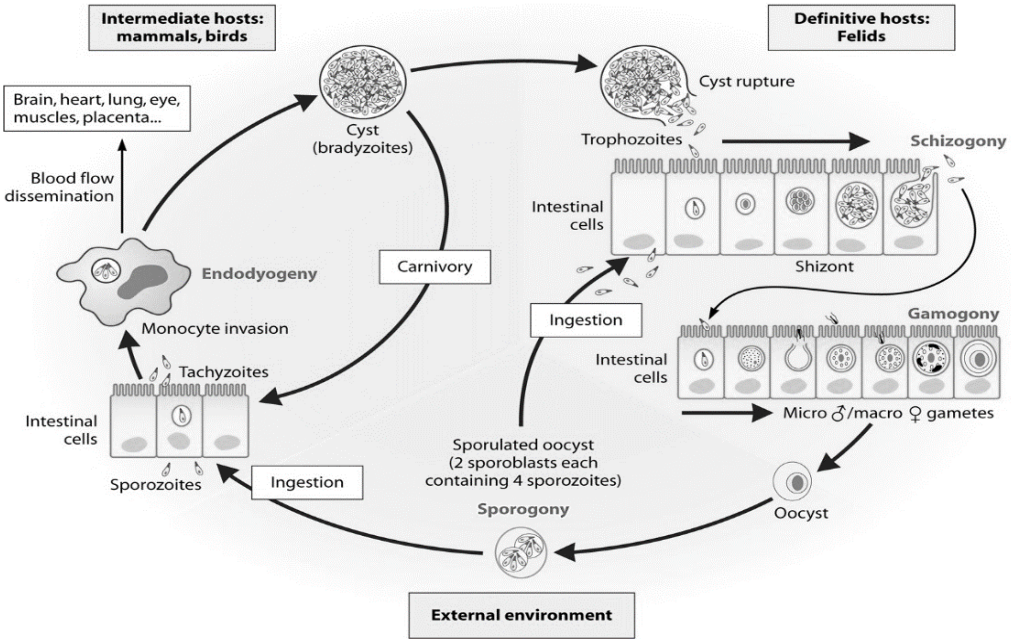

Figure 6. Toxoplasma gondii life cycle (Robert-Gangneux, and Dardé, Clin. Microbiol. Rev. 2012)

F. Epidemiology and Transmission

T. gondii has been described in 350 species so far (Robert-Gangneux et al., 2012). It is thought that around one third of the world's human population is infected with *Toxoplasma gondii* (Dubey et al., 2009). Depending on the environmental conditions of any specific area seroprevalence can range from zero to 100% (Tenter et al., 2000). Seroprevalence increases in climates that are tropical and decreases in dry and cold climates. In Lebanon, seroprevalence determined on 1371 sera from hospital laboratories and 2145 sera from private laboratories were 55% and 67%, respectively based on serum IgG levels of patients (Bouhamdan et al., 2010).

The high prevelance of *Toxoplasma gondii* is partly due to its prolonged ability to be transmitted from host to host in various forms depending on the circumstances of transmission (Figure 7). Ingestion of intermediate host meat contaminated with *T. gondii* tissue bradyzoite cysts seems to be the most common mode of transmission (Cook et al., 2000).

On the other hand, oocysts shed as unsporulated in cats' feces at first, sporulate later to become infective and represent the other way of the parasite transmission. Despite that cats' shedding of oocysts being of short duration, 2 to 3 weeks, (Dabritz et al., 2007) (Dumètre et al., 2003), nevertheless, a single cat can shed over a 100 million oocysts (Gangneux et al., 2012), which makes the environmental burden very high (Dubey et al., 2011).

Moreover, in primo-infected pregnant women, *T. gondii* is vertically transmitted in its tachyzoite through the placental barrier to the fetus (Wong and Remington, 1994). As pregnancy progresses, the placenta becomes more permissive to

the passage of tachyzoites replicating at the placental site, thus the risk of congenital toxoplasmosis increases from around 25% during the first trimester to 65% during the third trimester. However, severity of the congenital disease on the fetus is inversely related to the age of gestation at which the gravid mother acquires the parasite (McAuley, 2014).

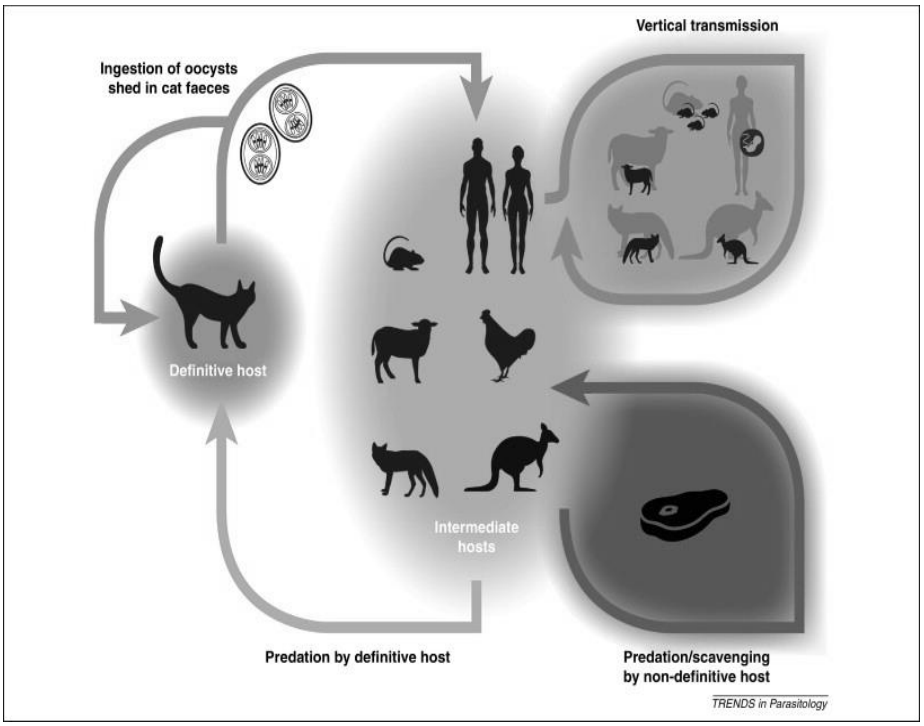

Figure 7. Transmission of T. gondii. Worth et al. Trends in parasitology 29.4 (2013): 150-155.

G. *Toxoplasma gondii* Pathogenesis

The type and severity of diseases caused by *T. gondii* vary according to the immunological state of the host as well as the type of the parasitic strain.

1. In immunocompetent patients:

It has been believed that only 20% of immunocompetent individuals that acquire the parasite exhibit signs of illness, mainly presented in the form of lymphadenopathy, and in rarer instances as chorioretinitis, myocarditis, or polymyositis (Montoya, 2002). Nowadays, scientific discoveries point out that the parasite might be associated with increased risk of psychiatric disorders in cases previously assumed asymptomatic carriers. For instance, *T. gondii* seroprevalence has been examined in association with disorders such as mania, schizophrenia, and bipolar disorder. An association has been found between these disorders and seropositivity (Fuller and Yolken, 2003) (Hamdani et al., 2013) (Dickerson et al., 2014).

2. In immunosuppressed patients

T. gondii infection in immunocompromised patients can be life-threatening. The disease outcome is the result of reactivation of bradyzoites back to the fast replicating tachyzoites. This mainly manifests as toxoplasmic encephalitis (TE) that can lead to serious complications such as dementia, seizures, and possibly death. Patients with AIDs who are harboring the parasite usually start showing signs of TE when their $CD4^+$ T cell counts falls under 100 cells/µl (Robert-Gangneux and Dardé, 2012). Treatment with highly active antiretroviral therapy (HAART) received by HIV

patients results in a dramatic drop in the development of TE (Abgrall et al., 2001). Furthermore, other manifestations of toxoplasmosis can appear due to the reactivation of a latent infection and include chorioretinits, pneumomitis, myocarditis and others which are less frequent than TE (Table 2).

Common Symptoms	Rare Symptoms
Encephalitis	Fever
Lymphadenopathy	Chorioretinitis
Pneumonitis	Splenomegaly
Myocarditis	Ploymyositis
Hepatitis	

Table 2. Symptoms associated with the reactivation of toxoplasmosis in immunocompromised patients

3. In primo-infected pregnant women

Primo-infection of seronegative pregnant women with *T. gondii* leads to congenital toxoplasmosis. The severity of the infection on the fetus depends on the pregnancy trimester during which infection occurs (Desmonts and Jacques, 1974). Virulence is higher during the first two semesters of the pregnancy and can lead to severe congenital abnormalities and even intrauterine fetal death. Congenital toxoplasmosis can present itself in a wide range of manifestations, involving several organs and resulting in disease entities such as myocarditis, hepatosplenomegaly, pneumonitis (McAuley, 2014), hearing loss (Salviz et al., 2013) and others. The symptoms are mostly pertaining to the CNS of the infant. The classic triad of congenital toxoplasmosis is chorioretinitis, intracranial calcifications, and hydrocephalus (McAuley, 2014). Other CNS symptoms include microcephaly (Schmidt et al., 2006). Even though most infants, around 85%, exhibit symptoms directly after birth or within the first month (Bollani et al., 2013) and may continue to do so until 5 years.

H. Significance of *T. gondii* as an Experimental Model

T. gondii is the major and tractable experimental model for the study of apicomplexan biology and parasitism because of many advantages: its different pathogenic stages are easily cultured, propagated and quantitated *in vitro*. Furthermore, *Toxoplasma* genome is fully sequenced and allows all possible manipulations/predictions of genes. More importantly, mouse models for studying host/parasite interactions during infection, as well as latent versus acute phases are well documented. Moreover, *T. gondii* can be easily crossed and transfected both transiently and stably *in vitro* and in diverse animal models (Hager et al., 1999) (Kim & Weiss,

2004). Finally, *T. gondii* is a very malleable model for establishing knock-outs, conditional knock-outs and more recently, the Crispr-Cas9 technology has been successful in this parasite (Shen et al., 2014). This genetic flexibility allows a better understanding of gene functions in *Toxoplasma* in particular, and in other related apicomplexans, namely *Plasmodium, Eimeria* and others.

I. *Toxoplasma gondii* Surface Markers

1. Tachyzoite Surface Antigen-1 (SAG-1)

Toxoplasma gondii displays a wide array of surface antigens that are mostly stage specific (Manger et al.,1998) and each having a role in adhesion, cell penetration, or parasite survival through evasion of host cell immune response (Carruthers and Boothroyd, 2007) (Cong et al., 2013). The surface of the parasite is dominated by a Surface Antigen Glycoprotein-1, called SAG-1, belonging to a family of Glycosyl-Phosphatidyl-Inositol (GPI)-linked proteins of which over 160 members have been discovered and that bifurcate into 2 branches the SAG-1-sequence like branch and the SAG-2-sequence like branch (Crawford et al., 2009) (Cong et al., 2013). Being the most prominent surface antigen of the parasite's tachyzoite stage, SAG-1 plays a major antigenic immune-stimulatory role, mainly during the acute phase of the infection. It is an important target for both the humoral and the cell-mediated acute immune response (Wang and Yin, 2014). The cell-mediated immune response, however, is the dominant one in the case of *T. gondii* infection and SAG-1-specific CD8+ T cells can be observed in mice as an aftermath of acute toxoplasmosis (Wang and Yin, 2014).

2. The Bradyzoite Antigen-1(BAG-1) marker

Among many bradyzoite markers (Table 3), specific heat shock proteins were characterized, among which is the Bradyzoite Antigen-1 BAG-1. BAG-1 is expressed by the *hsp30/bag1* gene. It has homology to small heat shock proteins and therefore has also been called hsp30 (Bohne et al., 1995; Parmley et al., 1995). Both BAG-1 mRNA and protein (a 28 kDa cytoplasmic antigen) are up-regulated during bradyzoite formation, suggesting transcriptional regulation of its expression. In fact, BAG-1 is one of the most abundant bradyzoite specific genes found in the *T. gondii* bradyzoite. Its expression is detectable within 24 hours post stress induction on *T. gondii* parasites and hence its characterization as a stress respondent would naturally accompany the transition from tachyzoites to bradyzoites under stress conditions (Knoll et al., 2014).

3. The Bradyzoite Markers: p36, p34, p21 and p18

For quite a long while, research in the field of toxoplasmosis remained centered around the readily available tachyzoite developmental stage. However with the emerging interest in stage conversion, many studies were directed towards the characterization of stage specific developmentally regulated molecules. Antibodies against stage-specific markers for bradyzoites and tachyzoites were developed (Tomavo et al., 1991). These antibodies were selected by differential immunofluorescence assays aiming to isolate them from hybridomas produced against these organisms. Antigenically reactive markers to these antibodies were found on human isolates of *T. gondii*, as well as on laboratory strain bradyzoites obtained from either brain cysts or *in vitro*-grown parasites (Tomavo et al., 1991). These monoclonal antibodies are T_8 $4A_{12}$

recognizing a 36 kDa protein called P36, T_8 $2C_2$ recognizing a 34 kDa protein called P34, T_8 $4G_{10}$ recognizing a 21 kDa protein called P21 and T_8 $3B_1$ recognizing an 18 kDa protein called P18 (Tomavo et al., 1991). These proteins represent four pellicular antigens, three of which are exposed on the surface of the organism (Tomavo et al., 1991).

4. The bradyzoite marker SAG-4 or P18:

Among the three bradyzoite markers (P34, P21 and P18), only the gene encoding for P18 was sequenced and published (Ödberg-Ferragut et al., 1996). Real time using P18-specific primers demonstrated the stage specific expression of this gene only in the bradyzoites transcripts. The sequenced gene showed no substantial homology to any of the known genes, hence it was recognized as novel and in accordance to the nomenclature proposed by Sibley, the *P18* gene was given the name *SAG4* (Ödberg-Ferragut et al., 1996). Insights towards a functional characterization of P18 were still absent until our laboratory established the Knockout of *P18* in the Pru Δ*KU80* type II strain (PRU Δ*KU80*Δ*P18*).

Name of Antigen	Size in kDa	Localization	Proposed Function	Cloned *T. gondii* database No.
BAG1 (hsp30/BAG5)	28	Cytoplasm	Small heat-shock protein	TGME49_259020
BSR4 (p36)	36	Surface	SAG1 family Ag	TGME49_320180
SAG4 (p18)	18	Surface	Under Investigation	TGME49_280570
p21	21	Surface	Surface Ag	N/A
p34		Surface	N/A	N/A

Table 3. Common Bradyzoite Markers

J. *Toxoplasma gondii* Strains

T. gondii displays very low genetic divergence and its strains can be grouped into 3 stable clonal lineages or major genotypes (called types I, II, and III) (Table 4). These lineages only exhibit about 1-2% genomic differences (Robert-Gangneux, 2012). While type I strain has been demonstrated to be the most virulent in mice resulting in parasitemia and ultimately death, type II strain has a higher capacity for forming tissue cysts in the brain and is more readily reactivated in cases of immunosuppression. Type III strain is considered avirulent in mice and is found almost as frequently in animals as type II. In humans, the most common strain remains type II and is the most frequently isolate from cases of immunosuppressed individuals as well as those with congenital toxoplasmosis (Howe and Sibley, 1995).

Strain	Characteristics	Examples
I	Most virulent, least common strain in animals.	RH strain
II	Less virulent, asymptomatic to mild disease in immunocompetent hosts, high capacity for cyst formation, most common in animals and humans.	ME49 and PRU strains
III	Less virulent, asymptomatic to mild disease in immunocompetent hosts, common in animals, least common in humans.	NED strain

Table 4. The 3 common lineages of T. gondii

K. A New genetic model of type II strains: PRUΔ*KU80*

Double Strand Break (DSB) repair in most eukaryotes occurs primarily *via* two different recombination pathways (Haber, 2000). The homologous recombination pathways repair a DSB using mechanisms that recognize highly homologous DNA sequences, while the non-homologous end-joining (NHEJ) pathway does not rely on DNA sequence homology. Instead, NHEJ involves direct ligation of the ends of broken DNA strands. KU70 and KU80 proteins form a heterodimer that tightly binds the DNA ends at the DSB, an early and essential step of NHEJ (Walker et al., 2001; Wu et al., 2008). Many eukaryotes preferentially use the NHEJ pathway to repair a DSB, and exogenous targeting DNA can be integrated anywhere into the genome independent of DNA sequence homology (Haber, 2000). The NHEJ pathway also appears to be preferentially used by *T. gondii* based on the high rates of non-homologous recombination and low gene targeting frequencies observed experimentally (Donald & Roos, 1998; Fox & Bzik, 2002; Gubbels et al., 2008; Morrissette et al., 1997). Because KU80 has been implicated in the DNA double-strand break repair mechanism associated with the DNA kinase repair model, by binding to the free ends of the broken DNA strands (Taccioli et al., 1994), Fox et al. decided to develop *KU80* knockouts (Δ*KU80*) in type II *T. gondii* (Fox et al., 2009), making it easier to further genetically manipulate this strain of the parasite by eliminating the *KU80* housekeeping gene. These Δ*KU80* are void of the dominant pathway mediating random integration of targeting episomes and hence allow for a 97% increase in efficiency of double-crossover homologous recombination at targeted loci (Fox et al., 2009). Fox et al. targeted the deletion of four parasite antigen genes (*GRA4*, *GRA6*, *ROP7*, and *tgd057*) that encode characterized $CD8^+$ T cell epitopes that elicit corresponding antigen-specific

$CD8^+$ T cell populations associated with control of infection (Fox et al., 2011), thus highlighting the importance and value of such knock-outs.

1. PRUΔKU80ΔP18:

Using the PRUΔ*KU80* that has been shown to be very efficient to knock out genes, especially those in type II stains, our laboratory investigated the function of the bradyzoite surface marker P18. Our laboratory has used the vector (P2854) containing the selectable marker cassette hypoxanthine-xanthine-guanine-phosphoribosyl-transferase (HXGPRT) and cloned the 5' and 3' flanking regions of *P18.* This vector was introduced by electroporation to the PRUΔ*KU80* strain which favors its integration by double crossing over and homologous recombination. Our laboratory successfully generated and cloned the PRUΔ*KU80*Δ*P18* knock-out parasites and investigated their phenotype *in vivo* (Tawil, Diss. 2014). Furthermore, deleting *P18* leads to the formation of more bradyzoite cysts in the brains of Swiss Webster mice. However, these bradyzoites reactivate much later than the wild type strain (Tawil, Diss. 2014). In more details, after induction of immunosuppression using dexamethasone, mice had better survival capacity when then were infected with the PRUΔ*KU80*Δ*P18.* Since the host immune response is the key driver of the back and forth switch between tachyzoite and bradyzoite stages, additional studies investigating the host immunological response upon *P18* deletion were needed, underlying the importance of the current study. This will allow a better understanding on how *P18* expression affects the host immune response and therefore the cyst burden and the disease outcome.

L. Immunology of *Toxoplasma gondii*

1. Introduction:

The success of the parasite resides in its ability to establish a balance within the host immune response, such that the infection does not lead to the death of the host, and by extension that of the parasite, but allows it to persist in such a way that it increases its chances of propagating in nature. This can happen only with the delicate balance between an immune response strong enough to drive the parasite into latency but week enough not to cause permanent tissue damage or even death to the host.

With the aid of its surface antigens and secretory proteins, *T. gondii* starts invading host cells and creating a desirable environment for its long-term survival within parasitophorous vacuoles. The role of effector proteins secreted during the establishment of the host-cell infections are still being studied and are becoming better understood today in light of their effect in manipulating the medium of the host cell and its pro-inflammatory and anti-inflammatory processes. Among these initial modulators are rhoptries and dense granular proteins.

T. gondii, like other microorganisms, possesses a variety of molecules foreign to the host's body known as Pathogen Associated Molecular Patterns (PAMP). These PAMPs can be recognized by Toll-like receptors on the surface of a wide variety of host cells. The interaction between PAMPs and TLRs is one of the major triggers of the innate immune response (Takeda, Tsuneyasu, and Akira, 2003). In this regard, *T. gondii* can trigger cytokine secretion by immune cells through either myeloid differentiation factor 88 (MyD88) dependent or MyD88 independent signaling pathways (Figure 8). MyD88 is an important adapter molecule in the cellular signaling

cascade initiated by PAMP-TLR interaction that triggers cellular cytokine secretion (Scanga et al., 2002).

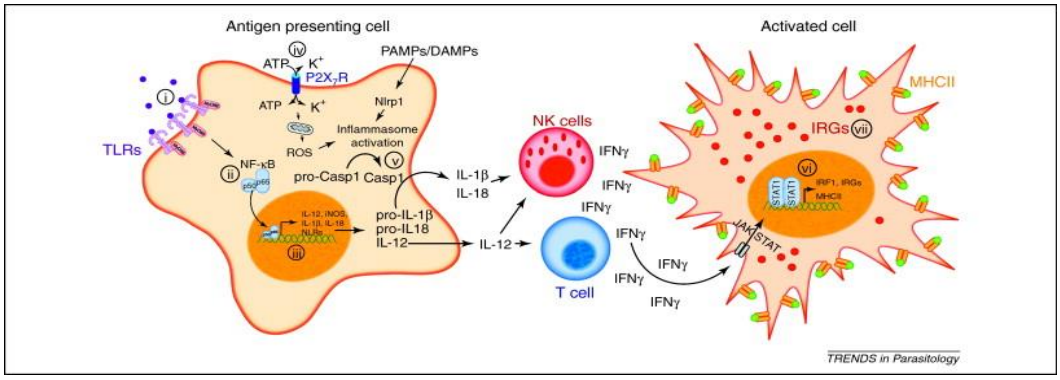

Figure 8. Host cell responses that can be modulated by Toxoplasma gondii (Melo et al., Trends in parasitology 2011)

T. gondii possesses a range of molecules that behave as PAMPs one of which is cyclophillin which is a profillin-like molecule that interacts with TLR-11 on macrophages and dendritic cells prompting them to secrete IL-12 (Figure 9) (Melo et al., 2011).

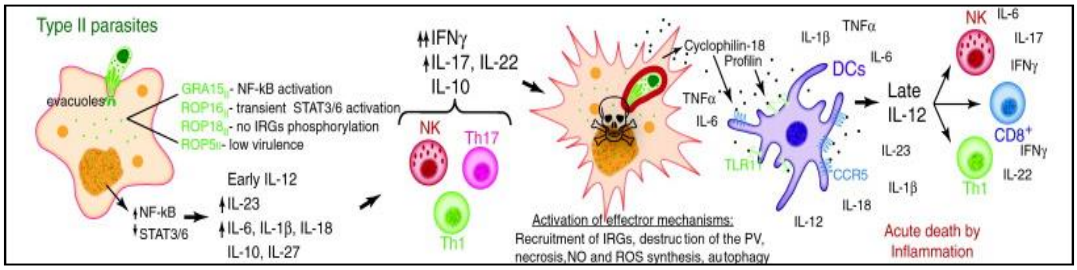

Figure 9. Effector mechanisms in Type II toxoplasmosis (Melo et al., Trends in parasitology 2011)

T. gondii stimulates the innate immune response with the ultimate player being NO (NO) which has a direct highly toxic effect on the parasite, interacting with it through the parasitophorous vacuole, as well as the ability to control the acute stage of the infection through triggering its switch from the tachyzoite to the bradyzoite stages. At the basis of this switch possibly lies parasitic deprivation of L-arginine, required for the metabolism of the parasite (Figure 10). Inducible NO synthase enzyme promotes the synthesis of NO (NO) from host cell L-arginine leading to its depletion (Melo et al., 2011).

NO is released by macrophage under the effect of another important cytokine, IFN-γ, which in turn is released by cells stimulated with IL-12. So while these 3 cytokines play the major role in inducing an immune response sufficient to control an acute infection, and keep it under control, IL-10 is the major anti-inflammatory cytokine that keeps the immune response in check (Munoz, Liesenfeld, and Heimesaat, 2011).

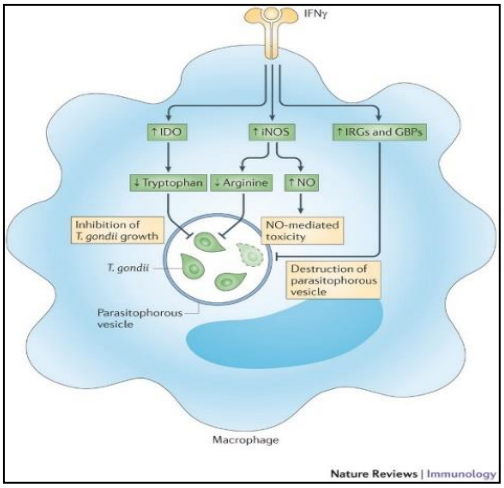

Figure 10. Effector mechanisms of IFNγ-mediated parasite elimination in infected cells. Yarovinsky, Nature Reviews Immunology 14.2 (2014): 109-121.

2. *Host immune Response during acute toxoplasmosis in Type II Toxoplasma gondii infection*

After ingestion of tissues containing viable bradyzoites within cysts or of sporozoites inside oocysts, the parasite is released in the alimentary tract and reaches the intestinal cells which it then starts invading to initiate replication in the form of tachyzoites. Enterocytes start secreting chemotactic factors such as monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory proteins and others as well as interleukins such as IL-12, IL-15, to a lesser extent IL-10 and others (Miller et al., 2009).

GRA-15, a dense granular protein in type II *Toxoplasma gondii* activates the NF-κB inside the infected host cells, macrophages and dendritic cells, further stimulating the release of IL-12 as well as the pro-inflammatory cytokine IL-1β which in turn cause an overproduction of IFN-γ by T helper and Natural Killer (NK) cells (Figure 9). (Melo et al., 2011)

IL-12 is an important cytokine that activates different cells of the immune response into secreting IFN-γ, such as neutrophils and NK cells and T lymphocytes (Gazzinelli et al., 1994) (Hunter et al., 1994). While IL-15 is directed at NK cells also leading to stimulation of these cells to secrete IFN-γ. The released chemokines then attract firstly neutrophils to the site of infection and secondly macrophages and dendritic cells that are hijacked by the parasite and used as Trojan horses in order to disseminate from the primary site of infection to the rest of the body (Lambert et al., 2009) (Ueno and Lodoen, 2015).

In cases of intraperitoneal injection of mice with tachyzoites, it usually takes around 6-8 hours for the neutrophils to arrive at the site of the infection, under the effect

of chemokines released from infected necrotized tissues (Bliss, et al., 1999). Upon interaction with the parasite through their Toll Like Receptors (TLRs), these cells start secreting cytokines such as IL-12, TNF-α, IFN-γ, and others (Bennouna et al., 2003) (Denkers et al., 2004) (Bliss et al., 1999) (Figure 8). When macrophages and dendritic cells arrive, some are hijacked by the parasites, while some of them interact as well with the parasite through their TLRs to release IL-12 (Melo et al., 2011). This interaction occurs through their surface TLR-2 and TLR-4 with parasitic GPI and LPS respectively as well as TLR-11 which interacts with parasitic profillin (Yarovinsky, 2008)(Kumar et al., 2009) (Figure 11). The release of IL-12 activates T lymphocyte proliferation and polarizes the immune system towards a strong Th-1 response (Denkers et al., 2004). Dendritic cells in addition to being crucial sources of IL-12 (Miller et al., 2009) are also APCs, they migrate to secondary lymphoid tissues and present *T. gondii* antigens to T lymphocytes priming them and leading to their activation with the production of IL-12 (Tait and Hunter, 2009). Though T lymphocytes do play an important role controlling the infection through their cytotoxic effect on infected cells (Montoya et al.,1996) especially cytotoxic $CD8^+$ T cells by secretion of perforin and lysis of the infected cells (Blanchard, Dunay, and D. Schlüter, 2015), the major role of T lymphocytes is demonstrated in the secretion of IFN-γ (Figure 11), especially in the setting of the brain (Munoz, Liesenfeld, and Heimesaat, 2011) (Parlog, Schlüter, and Dunay, 2015) (Denkers and Gazzinelli, 1998) which stimulates the release of NO by macrophages and microglia, as well as TNF-α, and other proinflammatory cytokines such as IL-1β and IL-6 (Weiss and Kami, 2011).

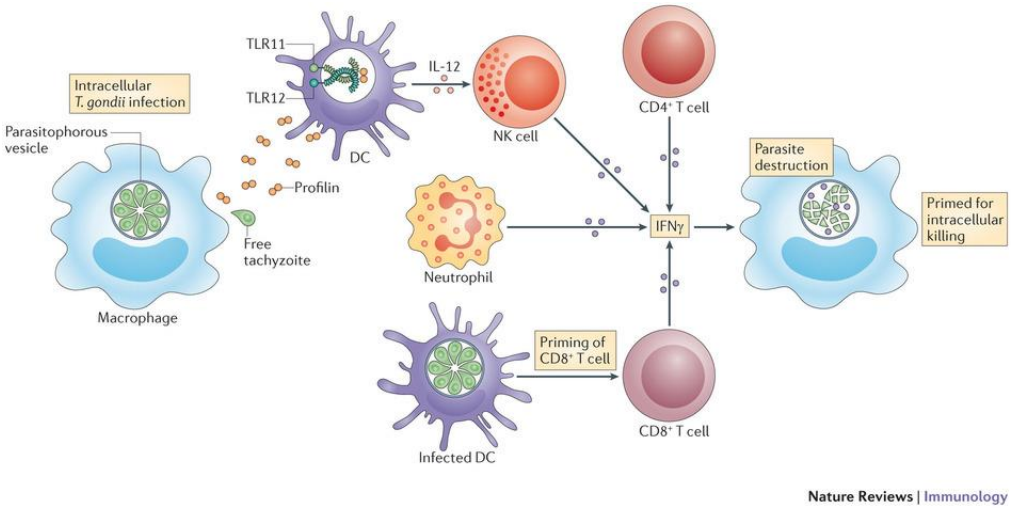

Figure 11. Interferon-γ (IFNγ) is crucial for survival during Toxoplasma gondii infection. (Yarovinsky et al., Nature Reviews Immunology 2014).

It is noteworthy to mention that the role played by IFN-γ secreted from T lymphocytes and NK cells extends beyond stimulating the secretion of NO as it also stimulates the expression of indoleamine 2,3-dioxygenase (IDO) which leads to depletion of host cell tryptophane, an essential requirement for *T. gondii* metabolism and survival pushing the parasite into a state of "starvation" , an inhibitory protein. Adding to that it stimulates the expression of immunity-related GTPases (IRGs) and guanylate-binding proteins (GBPs) (Figure 10). The IRGs and GBPs play a role in the destruction of the parasitophorous vacuole, the organelle engulfing intracellular parasites and protecting them from destruction through resisting binding to intacellular lysosomes (Melo, Jensen and Saeij, 2011).

In addition to the above-mentioned inflammatory process, there should exist an anti-inflammatory process through which the parasite can ensure the survival of the host. This can be achieved by allowing the host to avoid excessive tissue necrosis and damage as a result of inflammasome-inducing pro-inflammatory cytokines (Melo et al.,

2011). This is especially important at the site of the brain, and mice deficient in IL-10 succumb to death (Wilson et al., 2005). One of the major players in down-regulating the immune response, thus protecting the host and the parasite alike, is IL-10 (Figure 12). The major source of IL-10 remains the same IFNγ-secreting Th1 $CD4^+$ lymphocytes (Melo et al., 2011).

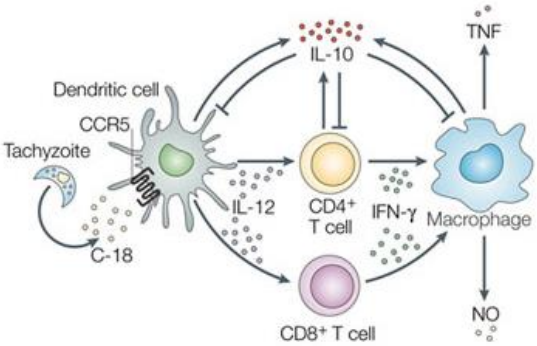

Figure 12. IL 10, an important immunosuppressor (Aliberti et al., Nature Reviews Immunology 2005)

3. Host brain immune Response during the chronic phase of toxoplasmosis

Upon reaching the brain, carried in macrophages and dendritic cells, the parasite is able to establish an infection in all three types of resident brain cell, the neurons, microglia, and astrocytes, in addition to immune cells migrating to the brain as a site of infection (Blanchard et al., 2015) (Figure 13). Neurons, however, act as a safest niche for the successful establishment of chronic infection, possibly due to their diminished ability to process and present antigens, therefore behaving as a safe medium for bradyzoites to hide (Blanchard et al., 2015). MCP-1 secreted by astrocytes serves as a chemoattractant for wandering monocytes to enter the brain (Munoz et al., 2011). Collectively, the parasitic infection in the brain is kept under control by Th-1

proinflammatory cytokines such as IL-1β and IL-6 secreted by T lymphocytes that have migrated to the brain either through crossing the BBB or as more recently suggested through lymphatics (Louveau et al, 2015), in addition to IFN-ɣ and TNF-α, that activate IL-12 secreting microglia and macrophages to produce NO (Parlog et al., 2015). Another important sources of brain IFN-ɣ comes also from NK cells that have just as well migrated to the brain (Blanchard et al., 2015). In addition to IFN-ɣ, NK cells and $CD4^+$ T lymphocytes are important in producing IL-10, which has an important role in dampening the immune response in order to avoid excessive damage by the immune response to the host's tissues (Hunter et al., 1994) (Neyer et al., 1997) (Miller et al., 2008). Infiltrating macrophages have also been found to be a major source of IL-10 in the brain, demonstrating an essential role in inhibiting the effects of excessive inflammation in TE (Wilson et al., 2005). *T. gondii* has also been suggested to mediate the anti-inflammatory effect of IL-10 as a counter-regulatory immune mechanism by hijacking the STAT3 pathway of infected cells (Munoz et al., 2011).

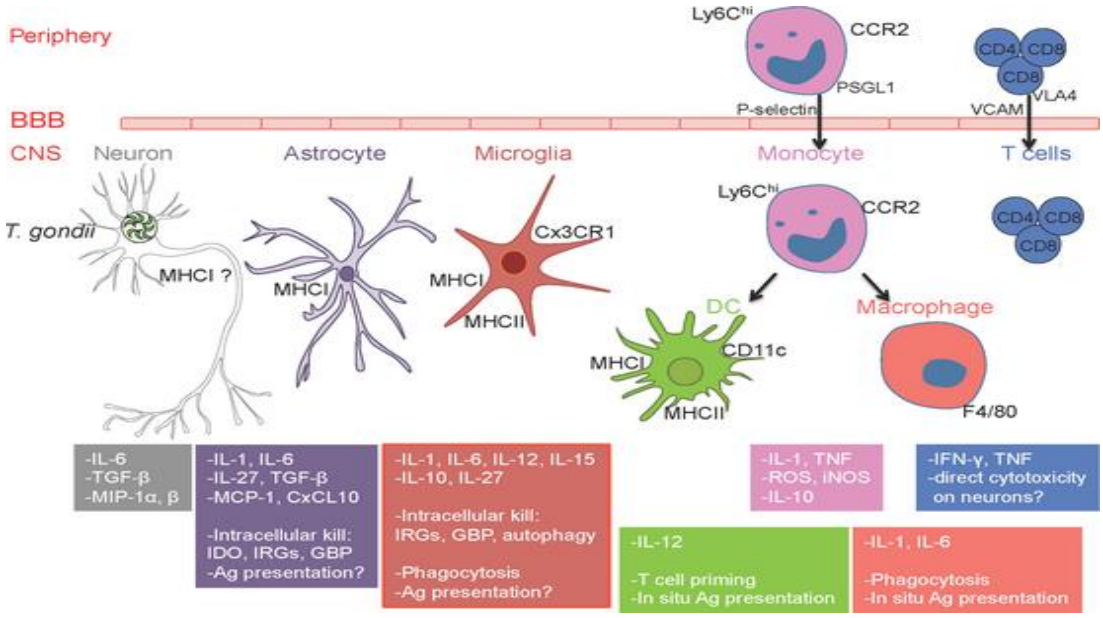

Figure 13. Immune response to T. gondii in the infected brain by resident and recruited immune cells. Blanchard et al., Parasite immunology 37.3 (2015): 150-158.

In our experiment we decided to focus our investigation on the following cytokines (Table 5), being the major players in the immune response against *T. gondii*.

Cytokine	Role
MCP-1	chemokines that regulate the recruitment of of monocytes, macrophages, and dendritic cells to the site of infection. Mainly secreted by activated neutrophils and brain astrocytes
IL12	Secreted mainly by macrophages and dendritic cells as well as microglia. Polarizes immune response to Th1
IFNγ	Promotes expression of iNOS and therefore secretion of NO in macrophages and microglia. Main source is activated T and NK cells
TNFα	Co-stimulates macrophages with IFNγ to secrete NO
IL1β and IL6	Pro-inflammatory cytokines secreted mainly by activated T lymphocytes
IL10	Anti-inflammatory cytokine that downregulates the immune response. Main source is $CD4^+$ T lymphocytes
iNOS	Inducible nitric oxide synthase enzyme responsible for production of nitric oxide by macrophages and microglia

Table 5. Summary of important cytokines involved in toxoplasmosis immune response

M. Cytokine Summary

1. NO (NO)

Produced by macrophages and brain microglia through the conversion of L-arginine under the effect of inducible NO synthase (iNOS) enzyme (Aktan, 2004). It has been found to be one of the most effective element of the innate immune response in the control of *T. gondii* infection (Suzuki et al., 2007). Its role exceeds direct parasitic destruction through its microbicidal effect into triggering conversion of tachyzoites into

bradyzoites, essentially through depriving the intracellular parasites from fulfilling their L-arginine metabolic needs (Weiss and Kami, 2011).

2. IL-12

Is supplied mainly by dendritic cells, in addition to macrophages and brain microglia and others such as neutrophils (Gazzinelli et al., 1994) (Bliss, Zhang, and Denkers, 1999). It is secreted in response to parasitic PAMPs interactions with TLRs on these cells (Yarovinsky, 2008) and is a participant in the polarization of the immune response to a strong Th-1 response (Miller et al., 2009) as well as for inducing IFN-γ secretion by T lymphocytes and NK cells (Suzuki et al., 2007).

3. IFN-γ

Is possibly one of the most studied effectors of the immune response to *T. gondii* due to its essential role in stimulating the production of NO from macrophages and microglia (Pelloux, 1996) (Yarovinsky, 2014). IFN-γ is mostly secreted by NK cells and T lymphocytes (Suzuki et al., 2007) (Munoz, Liesenfeld, and Heimesaat, 2011).

4. TNF-α

Secreted by neutrophils and T lymphocytes, it is responsible for co-stimulating macrophages to produce NO along with IFN-γ. It has an important protective role against the development of toxoplasmic encephalitis in the infected host (Deckert-Schlüter, 1998).

5. IL-1β and IL-6

These proinflammatory cytokines are mainly secreted by $CD4^+$ T lymphocytes systemically and within the brain in the presence of toxoplasmosis (Nagineni, Detrick, and Hooks, 2000). They are pyrogenic and regulate the acute phase of reaction of the immune response (Chao et al., 1994). IL-6 has been found to play a role in controlling the parasite burden as well as the amount of cysts found in the brain of the infected host (Jebbari et al., 1996). Furthermore, IL-1β was shown to be required for stimulating the production of IFN-γ by IL-12 stimulated NK cells (Hunter, Chizzonite, and Remington, 1995).

6. IL-10

IL-10, being an immunosuppressant is crucial for the control of the pro-inflammatory response directed at toxoplasmosis (Khan, Tadashi, Kasper, 1995). In cases of IL-10 deficiency, murine hosts have been found to succumb to death to extensive inflammation and necrosis (Gazzinelli, 1996).

7. MCP-1/CCL-2

MCP-1 is one of the most important chemokines that regulate the recruitment of monocytes, macrophages, and dendritic cells to the site of infection. Along with other chemokines, it is secreted in *T. gondii* infections primarily by activated neutrophils (Del Rio et al., 2004) and by astrocytes in the brain.

CHAPTER II

Materials and methods

A. Experimental Design

The following diagram represents the timeline of our experiment including major hallmarks (Figure 14). On Day 0, mice were injected with either strain of *Toxoplasma gondii*. On Day 4, all mice were provided with sulfadiazine in their drinking water in order to help them survive the acute phase of toxoplasmosis. On Day 7, we obtained blood samples from the periorbital sinus of each mouse in order to verify the infection using the sera in the western blot technique. We started sacrificing the mice on day 14 post-infection, 4 from the group infected with the *P18* knock-out strain and 4 infected with the original strain. We repeated these sacrifices in 7-day-intervals.

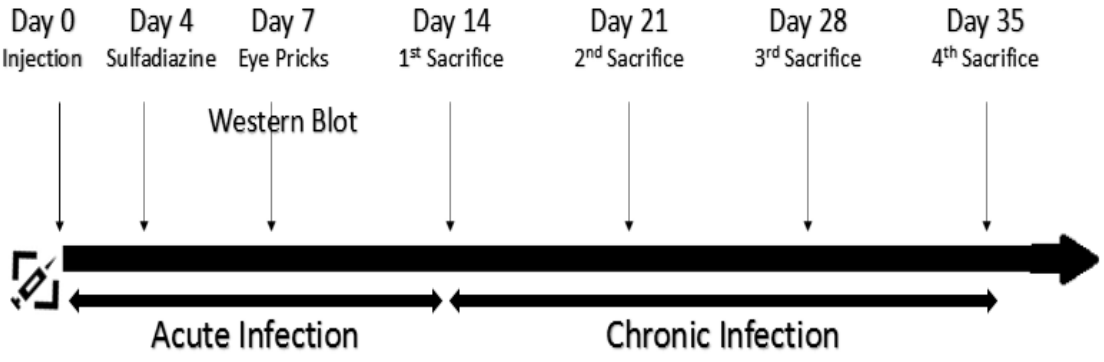

Figure 14. Experiment Timeline

B. Ethical Statement

The Institutional Animal Care and Utilization Committee of the American University of Beirut approved the murine protocols (#15-10-350). The mice were subjected to a 12-hour light and dark cycles. Mice were anesthetized using isoflurane and sacrificed by cervical dislocation.

C. Maintenance of Parasites in Human Foreskin Fibroblasts (HFF) culture:

Toxoplasma gondii tachyzoites from type II strains PruΔ*KU80* (kindly provided by Drs Maryse Lebrun and Jean Francois Dubremetz from France) and PruΔ*KU80*Δ*P18* (generated in our lab), were maintained by serial passages in Human Foreskin Fibroblasts (HFFs) grown in Dulbecco's modified Eagle medium (DMEM) (Lonza) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine 1% penicillin –streptomycin and kanamycin (Lonza).

Parasites were quantified using a haemocytometer. And caculations were performed to aquire 250 tachyzoites per 200 µl of PBS per injection per mouse.

D. The C57BL/6 mouse model

32 females C57BL/6 mice of 10-12 weeks old were used in our experiments. Mice were divided into two groups and were intraperitoneally infected with 250 parasites of either PruΔ*KU80* or PruΔ*KU80*Δ*P18* parasites. At day 4 post infection (post-infection), sulfadiazine was added to the drinking water at the concentration of 300 mg/L for two weeks of the experimental duration. Sulfadiazine

slows the rate of replication of tachyzoites and helps the mice to overcome death that may occur during the acute phase of infection thus switching into the chronic phase (Figure 14). 7 days post-infection, blood was sampled using retro-orbital puncture and blood was obtained from the medial cathenus of each injected mouse to verify acute infection by western blotting (section below). Seropositive mice against the acute infection were sacrificed on a weekly basis starting day 14 post-infection Brains were collected to quantify parasite load and the corresponding elicited immune responses. Brains were homogenized using (BioSpec) and were used for RNA extraction.

E. **Verification of Infection by Western Blot**

On day 7 post-infection, approximately 1 ml of blood from the retro-orbital sinus of each mouse were collected.

PRUΔ*KU80* tachyzoites were obtained from a culture T25 flask for boiling in Laemmli SDS-PAGE sample buffer and running on previously prepared 12% polyacrylamide gels covered with 4% stacking gel in order to separate the protein bands, which were then transferred onto a nitrocellulose membrane overnight using a BioRad transfer unit at 30V.

5% non-fat dry milk in wash buffer composed of 15 mM Tris HCL (pH 8), 150 mM NaCl and 0.05% Tween 20 was utilized for blocking for 1 hour. Blood samples obtained from mice were centrifuged at 13,000 r.p.m for 15 min and 10µl of obtained sera were used in 1 ml 5% non-fat milk dissolved in wash buffer to incubate the strips and reveal seropositivity. Incubation was carried out overnight at 4^0C. The strips were then washed and incubated with anti-mouse HRP (Horseradish peroxidase)-conjugated

secondary antibodies (1:5000 dilution, Santa cruz, sc-2031, lot: B2212). A luminol chemiluminescent (Santa cruz, sc-048) was used to confirm seropositivity. This chemiluminescent substrate binds to the secondary antibodies and producing autoradiography detectable luminescence.

F. **Brain Harvesting and RNA Extraction**

14 days post-infection., we started sacrificing mice after deep anesthesia with isoflurane followed by cervical dislocation and their brains were harvested for mRNA extraction.

500µl of TRIZOL© were added to each brain followed by homogenization and the addition of another 500µl of TRIZOL©. The samples were then left for 5 minutes at room temperature. 200µl of chloroform were then added to the samples which were vortexed for 1 minute followed by spinning at 15,000g for 10 minutes at 4^0C.

The upper phase of the solution was then placed in new Eppendorf tubes and 500µl of isopropanol were added to each tube, followed by another spin at 15,000g for 10 minutes at 4^0C.

The supernatant this time was discarded, the pellet air-dried, and resuspended in RNAse-free H2O. The absorbance was measured at 260nm using a nano drop spectrophotometer (ND-1000).

G. cDNA synthesis

cDNA synthesis kit provided by Thermo (#K1622) was utilized and the protocol was followed according to the manufacturer's recommendations. 1µl of random primer was added to each sample containing 2µg of extracted mRNA diluted in 11µl RNAse-free H2O and the samples were incubated for 5 minutes at 65^0C.

This was followed by the addition of 7µl of a master mix to each sample, containing 4 µl of 5x Reaction Buffer, 1 µl of Ribolock RNase Inhibitor (20U/µl), and 2 µl of 10 Mm dNTP Mix. The 20 µl mixture was pulse spun for 30 seconds and incubated in a PCR machine at 25^0C for 5 minutes followed by 60 minutes at 42^0C and finally for 5 minutes at 70^0C before it was stopped at 4^0C.

The following table summarizes the contents of the reaction:

RNase/DNase free water containing 5 µg of template RNA	11 µL
Random Primers	1 µL
5X Reaction Buffer	4 µL
Ribolock RNase Inhibitor(20U/µl)	1 µL
10 Mm dNTP Mix	2 µL
RevertAid M-MuLV RT (200U/µl)	1 µL
Total Volume	20 µL

Table 6. cDNA components utilized for synthesis and respective volumes

H. Quantitative Real-Time Polymerase Chain Reaction (qRt-PCR)

qRT-PCR was performed using SYBR Green as dye and a BIORAD machine(CFX96 Optics Module, Serial No. 785BR04788) Glyceraldehyde-3-Phosphate dehydrogenase (GAPDH) was used as the reference gene for normalization of Ct values. Mouse GAPDH primers (Table 7) were used to detect and quantify the GAPDH transcription levels.

150 ng of cDNA (1.5 µl) was used for the mixture of each sample, then we added 5 µl of SYBR Green dye, 0.25 µl respective forward primer (15 µM) and 0.25 µl respective reverse primer (15 µM) along with 4.5µl RNAse-free H2O. Total volume of each sample became 11.5 µl.

Procedure protocol involved a DNA denaturation cycle at 95^0C for 3 minutes followed by 35 cycles of denaturation at 95^0C for 15 seconds, annealing for 60 seconds at a primer-dependent temperature and extension at 72^0C for 30 seconds. Each sample was quantified in duplicate reactions.

Relative quantification was performed on the results obtained from Rt-PCR. This is mainly done by calculating ΔCt (Ct (Cytokine) – Ct(GAPDH)). Then ΔΔCt was calculated as ΔCt (Cytokine in infected mice)– ΔCt (Cytokine in mice infected with PRUΔ*KU80* on day 14 post-infection). We proceeded according to the Livak method ($2^{-\Delta\Delta Ct}$) to obtain the percentage of expression (Schmittgen and Livak, 2008).

Primer	Sequence 5'→3'
Mouse GAPDH Forward Primer	5'-CATggCCTTCCgTgTTCCTA-3'
Mouse GAPDH Reverse Primer	5'-CCTgCTTCACCACCTTCTTgAT-3'
Mouse BAG-1 Forward Primer	5´-gCggAgAAAgTggACgATgATgg-3´
Mouse BAG-1 Reverse Primer	5´-gTCgggCTTgTAATTACTCggg-3´
Mouse iNOS Forward Primer	5'-CTT TGC CAC GGA CGA GAC-3'
Mouse iNOS Reverse Primer	5'-TCA TTG TAC TCT GAG GGC TGAC-3'
Mouse IL-12 Forward Primer	5'-TCA AACCAG ACC CAC CGA A-3'
Mouse IL-12 Reverse Primer	5'-GCT GACCTC CAC CTG CTG A-3'
Mouse IL-10 Forward Primer	5'- GTG ATGCCC CAA GCT GAG A-3'
Mouse IL-10 Reverse Primer	5'-CAC GGC CTT GCT CTT GTT TT-3'
Mouse IFN-γ Forward Primer	5'-TgAACgCTACACACTgCATCTTgg-3'
Mouse IFN-γ Reverse Primer	5'-CgACTCCTTTTCCgCTTCCTgAg-3'
Mouse TNF-α Forward Primer	5'-CCA CGT CGT AGC AAA CCA CC-3'
Mouse TNF-α Reverse Primer	5'- GGT GAG GAG CAC GTA GTC GG-3'
Mouse IL-6 Forward Primer	5'-CCA TCC AGT TGC CTT CTT GGG-3'
Mouse IL-6 Reverse Primer	5'-GGT CTG TTG GGA GTG GTA TCCT-3'
Mouse IL-1β Forward Primer	5'-CATCCAgCTTCAAATCTCgCAg-3'
Mouse IL-1β Reverse Primer	5'-CACACACCAgCAgCATTATCATC-3'

Table 7. Primers used in Rt-PCR

CHAPTER III
RESULTS

A. PRU*ΔKU80* and PRU*ΔKU80ΔP18* parasites successfully establish acute toxoplasmosis in C57BL/6 mice

After intraperitoneal injection of 250 parasites of the PRUΔ*KU80* or PRUΔ*KU80*Δ*P18* strains in C57BL/6 (16 mice per parasite strain), the mouse immune system mounts a response against the tachyzoites of *T. gondii* clearing some of them and pushing some into the bradyzoite stage that hides in tissue cysts creating the chronic infection later on (Dubey, 1998). During the first week of infection, a resultant of this immune response is the development of antibodies (IgM) directed against parasitic proteins (Robert-Gangneux, and Dardé, 2012). In order to confirm infection in our mice, we collected blood samples on day 7 post-infection from each mouse by eye pricks and sera were used to recognize tachyzoite antigens by western blots, confirming the presence of mouse antibodies and therefore seropositivity. This later is demonstrated by the emergence of a complex banding pattern from antibodies present in the sera of infected mice against tachyzoite proteins. Seronegative mice were excluded from the study.

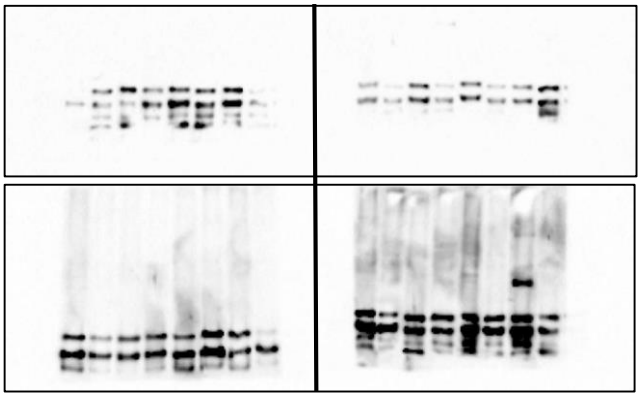

Figure 15. Verification of T. gondii infection by western blot. Each strip corresponds to one mouse's seropositivity test

Upper left group of strips correspond to mice sacrificed on day 14 post-infection. Upper right group of strips correspond to mice sacrificed on day 21 post-infection. Lower left group of strips correspond to mice sacrificed on day 28 post-infection. Lower right group of strips correspond to mice sacrificed on day 35 post-infection. In all gels, from left to right, the first 4 strips belong to sera from mice infected with PRUΔ*KU80,* and the remaining four belong to sera from mice infected with PRUΔ*KU80*Δ*P18.*

B. C57BL/6 mice infected with PRUΔ*KU*80Δ*P18* harbor more cysts than those infected with PRUΔ*KU*80 parasites

BAG-1 is the most abundant bradyzoite marker that has been associated with the latent cyst stages of *T.gondii* specifically as an indicator of chronic infection occurrence in the brains of infected mice (Knoll et al., 2014)

Our lab has previously investigated the effect of knocking out *P18* in the brains of Swiss Webster mice. The results showed that the brains of mice infected with PRUΔ*KU*80Δ*P18* harbor more cysts than those infected with PRUΔ*KU80* (Tawil, Diss. 2014). Since the best animal model to reproduce human TE is the C57BL/6 specially the immune response against it, we started by comparing the capacity of this mouse model of producing cysts in case of the *P18* knock-out. To do so, we first measured the transcription levels of the bradyzoite surface marker BAG-1 in order to insure the

presence of bradyzoites in the brain as well as to compare the capacity of cyst formation in mice infected with the PRUΔ*KU80* strain vs. those infected with the respective *P18* knock-out strain.

Since the peak of cyst amount is obtained between 3 to 4 weeks post-infection with type II *T. gondii*, we measured the transcription levels of BAG-1 on Day 28 post-infection of mice.

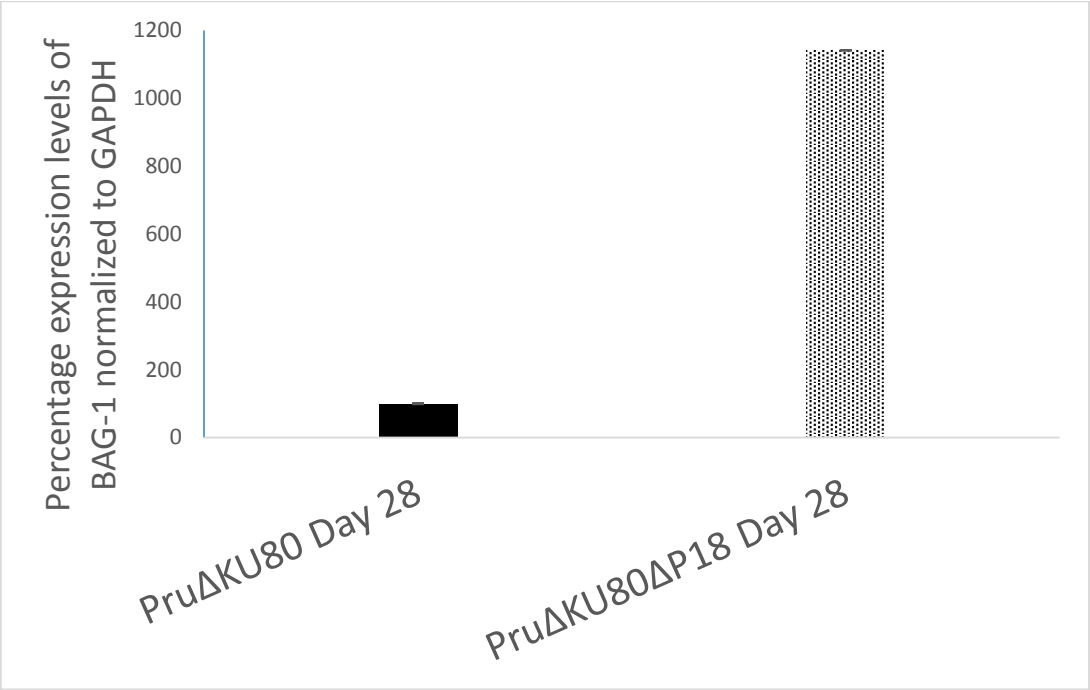

Figure 16. BAG-1 transcription levels measured on day 28 post-infection in the brains of mice infected with PRUΔKU80ΔP18 (dotted) and PRUΔKU80 (black) (n=4 mice each).

Similar to our lab's findings in the Swiss mouse model (Tawil, Diss 2014), a higher level of transcription of BAG-1 was observed in the brains of mice infected with the knock-out strain. This is reflective of a higher number of bradyzoites and therefore a higher cyst burden in the brains of these mice.

C. PRUΔ*KU*80Δ*P18* recruits more macrophages and dendritic cells than PRUΔ*KU*80 to the brain of C57BL/6

MCP-1 is one of the most important chemotactic factors responsible for attracting macrophages and dendritic cells (Del Rio et al., 2004), representing the main players in spreading the parasite, in addition to their other roles in the immune response such as the secretion of IL-12 and NO by macrophages (Yarovinsky et al., 2014). Measuring transcription levels of MCP-1 gives us important insights on the number of recruited cells in response to the infection with either strains of parasites.

Our results indicate that over-all MCP-1 transcription levels were higher in the brains of mice infected with the PRU*ΔKU80ΔP18,* reaching peak levels on Day 21 (week 3) post-infection (Figure 17), most likely suggesting is a higher number of infected wandering macrophages and dendritic cells reaching the brain.

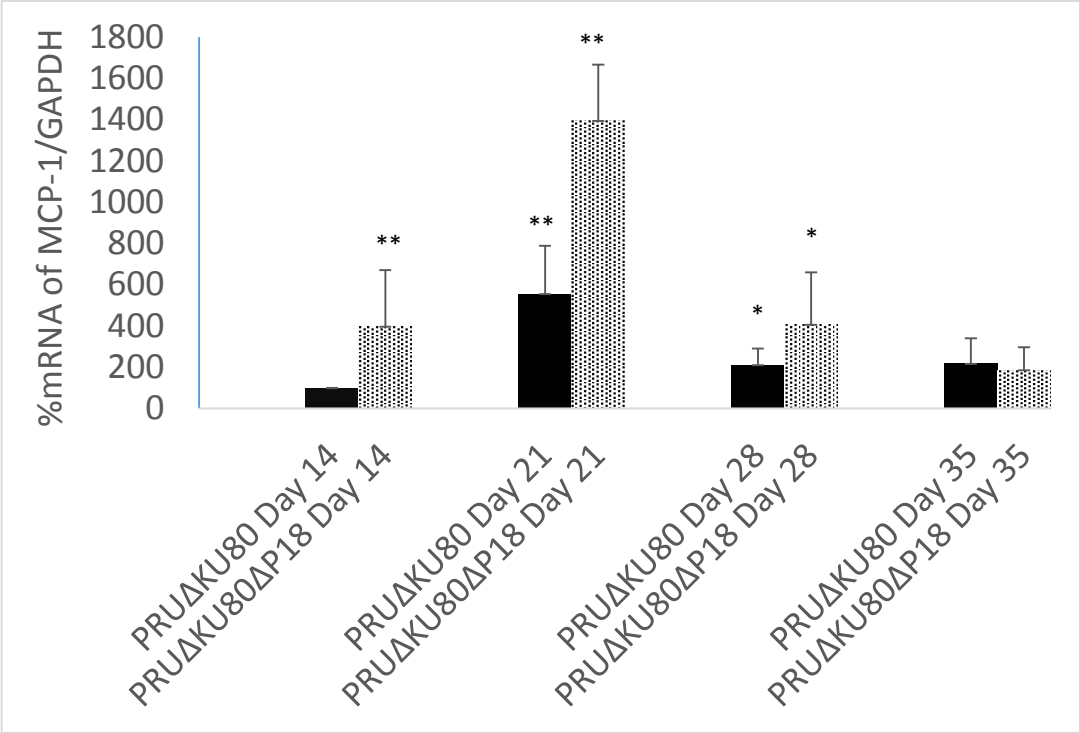

*Figure 17. MCP-1 transcription levels on days 14, 21, 28,35 post-infection Brains of mice infected with PruΔKU80 (black) and those of mice infected with PRUΔKU80ΔP18 (diagonal) (n=4 mice) each. P value: between 0.01-0.05 corresponds to *, between 0.01-0.001 corresponds to ** and less than 0.001 corresponds to *** using student's T-test.*

D. PRUΔ*KU*80Δ*P18* induce higher levels of transcription of IL-12 when compared to PRUΔ*KU*80

As previously mentioned, the major source of IL-12 in *T. gondii* infection in the brain comes from dendritic cells, wandering macrophages and resident microglia that come in contact with the parasitic PAMPs through their TLR receptors (Yarovinsky, 2008).

We assessed whether knocking out *P18* affects IL-12 production. Our results show that higher levels of IL-12 transcription in the brains of mice infected with the PRU*ΔKU80ΔP18* strain were obtained on days 14 and 21 post-infection, but being significantly higher on day 28 post-infection ($P \leq 0.05$) (Figure 18). This could be due to a higher incidence of interaction between parasites and APCs, supporting the claim

that more parasites are initially being transported to the brain by macrophages and dendritic cells as a result of better camouflage. On day 35 post-infection these levels became higher in the brains of mice infected with the PRUΔ*KU80* strains. This presumably reflects a faster secretion of IL-12 upon infection with the knock-out *P18* versus a delayed one that shows up only 5 weeks post-infection upon infection with the wild type PRU*ΔKU80* parasites, a result that can be due to the number of parasites of each strains reaching the brain at a different time point (4 weeks for PRU*ΔKU80ΔP18* and 5 weeks for PRU*ΔKU80)*.

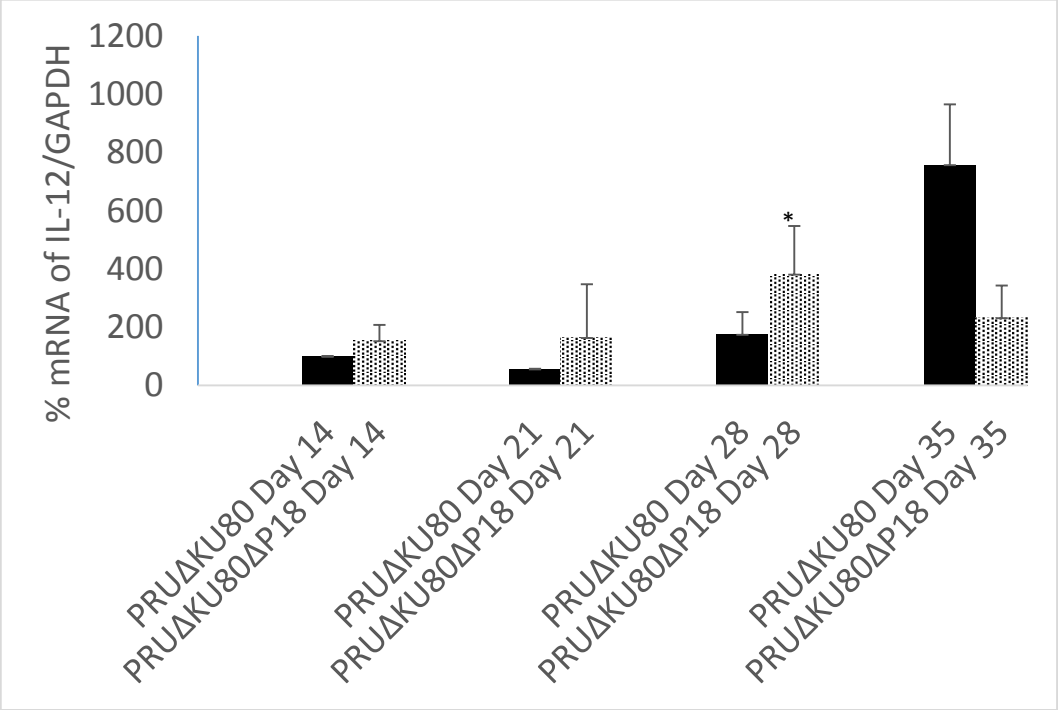

*Figure 18. IL-12 transcription levels on days 14, 21, 28, 35 post-infection Brains of mice infected with PruΔKU80 (black) and those of mice infected with PRUΔKU80ΔP18 (dotted) (n=4 mice) each. P value: between 0.01-0.05 corresponds to *, between 0.01-0.001 corresponds to ** and less than 0.001 corresponds to *** using student's T-test.*

E. PRUΔ*KU*80Δ*P18* induce significantly higher levels of transcription of IFN-γ when compared to PRUΔ*KU*80

IFN-γ is possibly the most studied of all secreted or transcribed cytokines in case of *T. gondii* infections, due to its significant various roles in controlling the acute infection, stimulating the switch to the chronic infection through its effect on inducing NO secretion and maintaining the infection in its chronic state (Yarovinsky, 2014).

We have measured the transcription levels of IFN-γ in the brains of mice infected with either strains of parasites and showed that IFN-γ transcription is higher in the brains of mice infected with the *P18* knock-out strain on days 14 and 21 post-infection, and peaking on day 21 post-infectionat. This is likely due to higher parasite burden in case of *P18* knock-out-strain infections (Figure 19). It is also an indication of the possibility of higher NO secretion by microglia and macrophages and by extension of a higher cyst burden in the brains of mice infected with the knock-out strain, firstly due to a higher parasite burden and secondly due to a more effective trigger towards the cyst stage transformation.

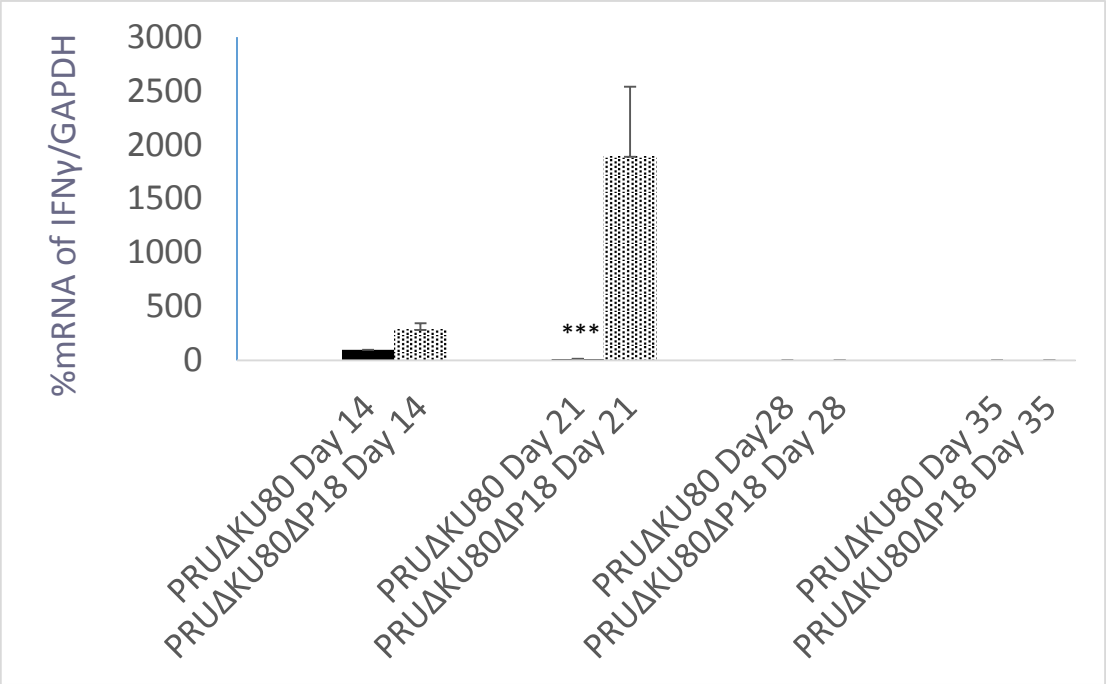

*Figure 19. Transcription levels of IFN-γ on days 14, 21, 28, 35 post-infection Brains of mice infected with PruΔKU80 (black) and those of mice infected with PRUΔKU80ΔP18 (dotted) (n=4 mice) each. P value: between 0.01-0.05 corresponds to *, between 0.01-0.001 corresponds to ** and less than 0.001 corresponds to *** using student's T-test.*

F. PRUΔ*KU*80Δ*P18* and PRUΔ*KU*80 display a gradual increase of TNF-α in the brain of C57BL/6 mice over weeks post-infection with significantly higher levels of transcription of TNF-α in case of the *P18* depleted strain

TNF-α works synergistically with IFN-γ to promote the secretion of NO by macrophages and microglia (Deckert-Schlüter, 1998). We have shown that IFN-γ levels are higher in the brains of mice infected with the *P18* knock-out strain on days 14 and 21 post-infection (Figure 19). When we assessed TNF-α transcription levels, we noticed a gradual increase in mice infected with either one of the two strains over weeks post-infection. Yet, TNF-α transcription levels were higher in the knock-out-infected mice brains on days 14 and 21 post-infection, while on days 28 and 35 post-infection ($P \leq 0.01$), the conditions shifted to higher levels of TNF-α transcription in mice infected with the wild type strain.

This may be indicative of a larger parasitic load interacting with the parasites, which later decreases in the *P18* knock-out infected mice brains upon better induction of more effective brain cyst formation as compared to the wild type strain.

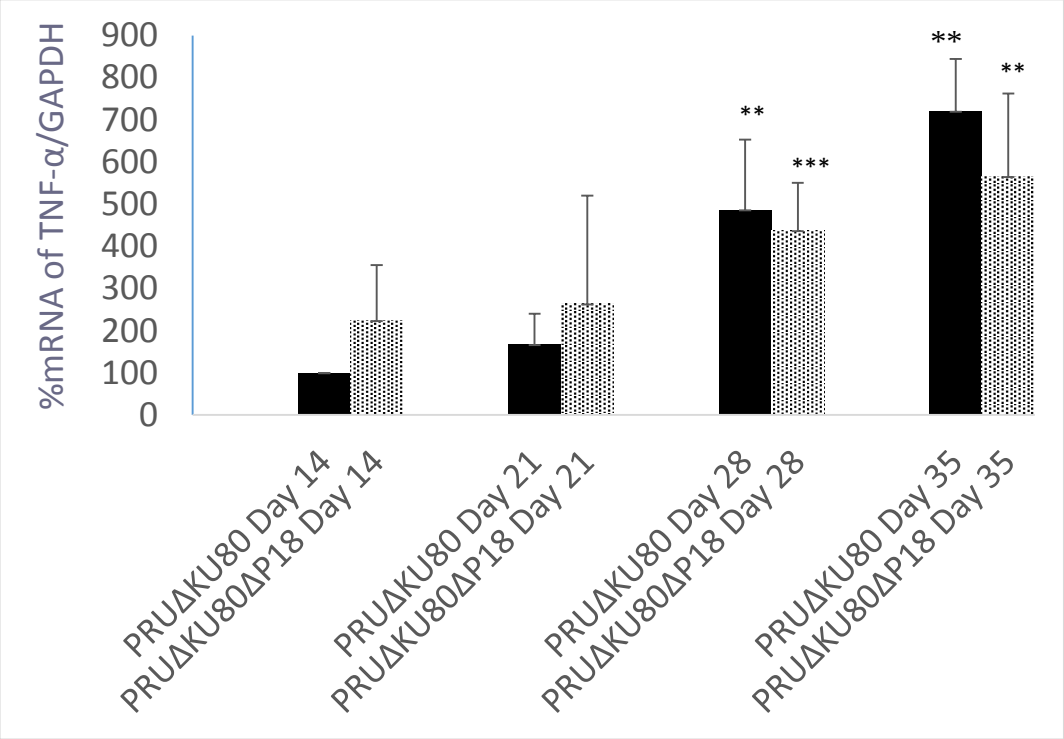

*Figure 20. Transcription levels of TNF-α on days 14, 21, 28, 35 post-infection Brains of mice infected with PruΔKU80 (black) and those of mice infected with PRUΔKU80ΔP18 (dotted) (n=4 mice) each. P value: between 0.01-0.05 corresponds to *, between 0.01-0.001 corresponds to ** and less than 0.001 corresponds to *** using student's T-test.*

G. PRU*ΔKU80ΔP18* and PRU*ΔKU80* display a different pattern of IL-6 transcript stimulation in the brain of C57BL/6 mice over weeks post-infection with peaks of IL-6 reached at d21 post-infection with PRU*ΔKU80ΔP18*

The pro-inflammatory IL-6 secreted by T lymphocytes is ultimately a product of toxoplasmic antigen priming of T lymphocytes by APCs (Nagineni, Detrick, and Hooks, 2000). We have shown that IL-6 transcription levels progressively increase

from day 14 till day 35 post-infection with the PRUΔ*KU80* strain. However, in the *P18* knock-out counterpart, the level of transcription of IL-6 reaches its peak on day 21 post-infection ($P \leq 0.01$) to diminish on the subsequent weeks.

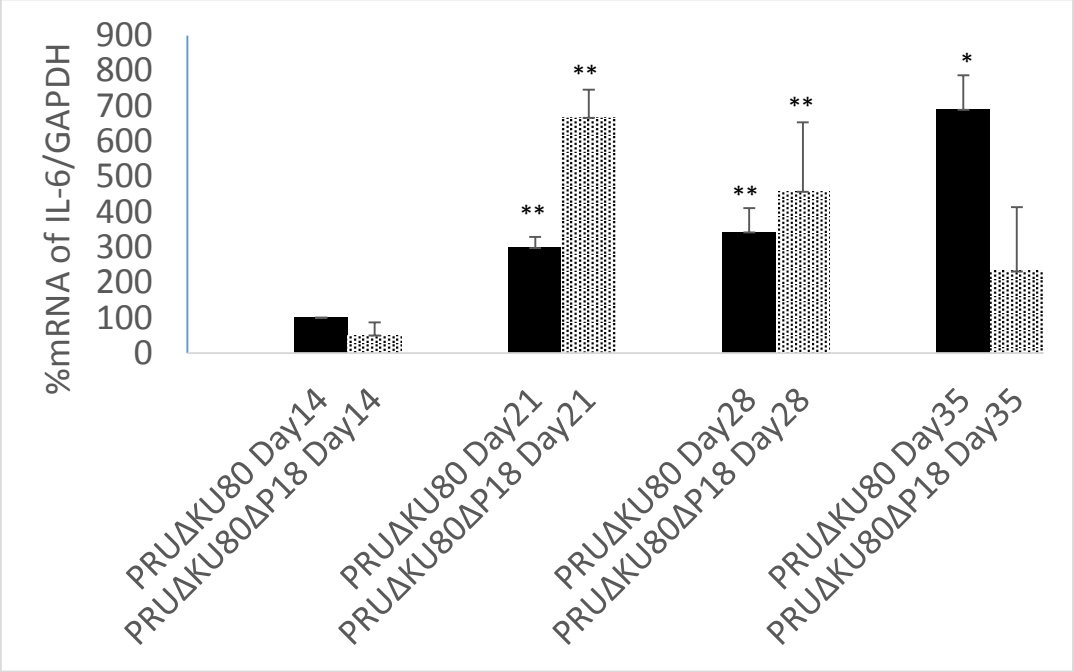

*Figure 21 Transcription levels of IL-6 on days 14, 21, 28, 35 post-infection Brains of mice infected with PruΔKU80 (black) and those of mice infected with PRUΔKU80ΔP18 (dotted) (n=4 mice) each. P value: between 0.01-0.05 corresponds to *, between 0.01-0.001 corresponds to ** and less than 0.001 corresponds to *** using student's T-test.*

This may suggest that P18 raises the level of IL-6 secretion because of a higher number of parasites reaching the brain upon deleting the gene. This might explain the higher levels of IL-6 secreted in the brain of mice infected with the Pru*ΔKU80ΔP18* on day 21 while this is not the case on day 35 post-infection (Figure 21) when these parasites could have switched to a bradyzoite cyst-hiding stance.

H. PRU*ΔKU80ΔP18* and PRU*ΔKU80* display a different pattern of IL-1β transcript levels regulation in the brain of C57BL/6 mice over weeks post-infection

IL-1β is another pro-inflammatory cytokine secreted mainly by $CD4^+$ T lymphocytes (Nagineni, Detrick, and Hooks, 2000). Initial measurements of IL-1β on day 14 post-infection were higher in the brains of mice infected with the knock-out strain. These results shift following day 21 post-infection to become higher in the brains of mice infected with the PRUΔ*KU80* strain as shown on day 28 post-infection ($P \leq 0.01$).

These results are consistent with those of IL-6 and other cytokines, shown to be initially higher in the brain of mice harboring the *P18* knock-out strain on days 14 and 21 post-infection and later dropping on days 28 and 35 post-infection below the transcription levels found in the brains of mice with the PRU*ΔKU80* strain infection (Figure 22).

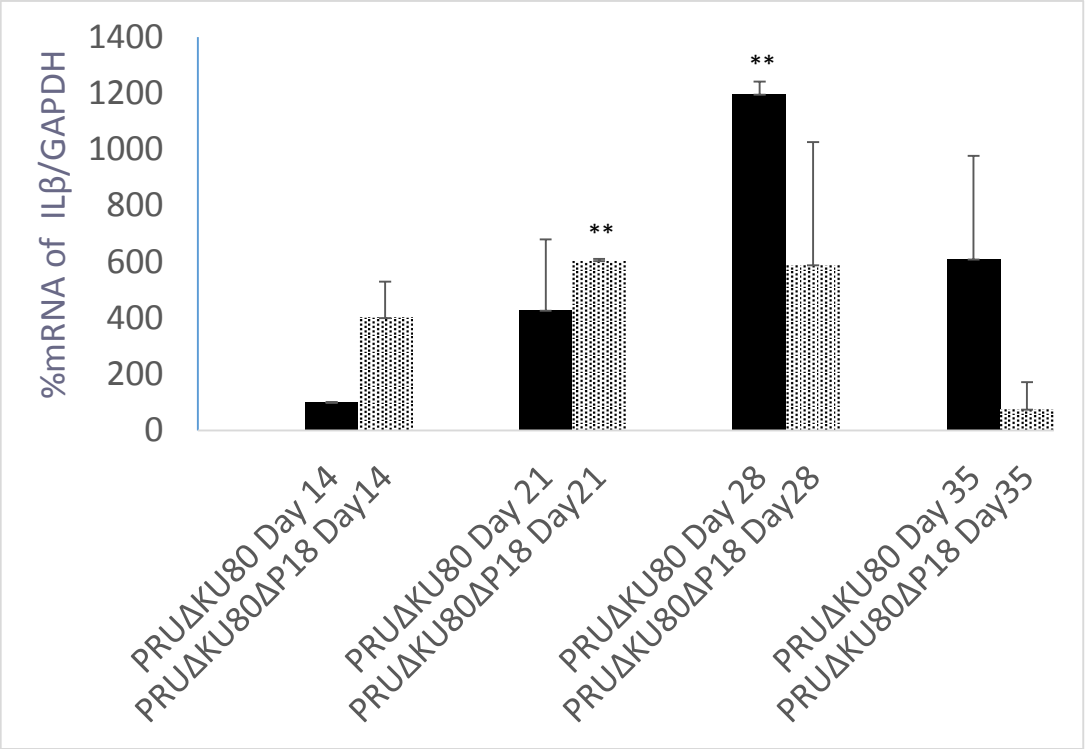

*Figure 22. Transcription levels of IL-1ϐ on days 14, 21, 28, 35 post-infection Brains of mice infected with PruΔKU80 (black) and those of mice infected with PRUΔKU80ΔP18 (dotted) (n=4 mice) each. P value: between 0.01-0.05 corresponds to *, between 0.01-0.001 corresponds to ** and less than 0.001 corresponds to *** using student's T-test.*

I. PRU*ΔKU80ΔP18* induces consistently higher levels of iNOS than PRU*ΔKU80* in the brain of C57BL/6 mice over weeks post-infection

iNOS is the enzyme behind the secretion of NO from L-arginine found in the cytoplasm of macrophages and microglia (Aktan, 2004). NO is essential in the shift from the tachyzoite to the bradyzoite stage (Melo et al., 2011), the control of the acute infection and the establishment of the chronic one.

Our results show that the transcription levels of iNOS (inducible NO synthase) enzyme, reflecting NO secretions are over-all higher in the brains of mice infected with the *P18* knock-out strain. They keep rising till day 35 post-infection.

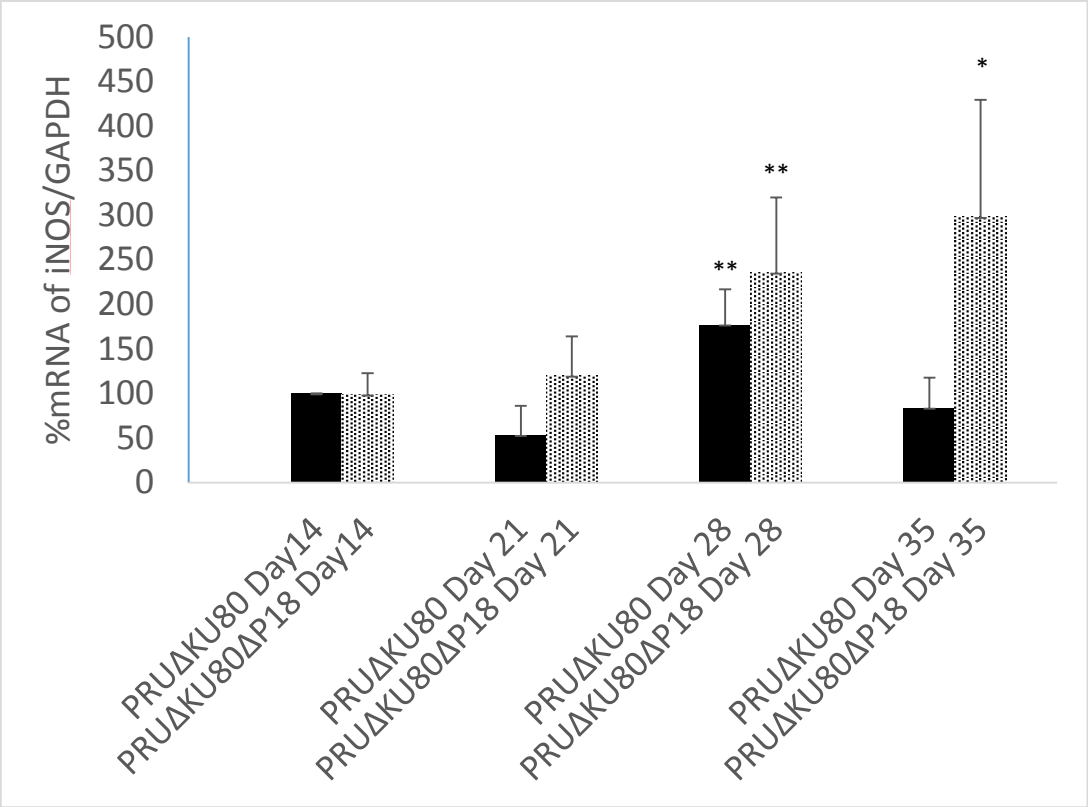

*Figure 23. Transcription levels of iNOS on days 14, 21, 28, 35 post-infection Brains of mice infected with PruΔKU80 (black) and those of mice infected with PRUΔKU80ΔP18 (dotted) (n=4 mice) each. P value: between 0.01-0.05 corresponds to *, between 0.01-0.001 corresponds to ** and less than 0.001 corresponds to *** using student's T-test.*

Bearing in mind that tachyzoite to bradyzoite conversion is present at the initial site of infection, before reaching the brain (Dubey, 1998), due to local effects of the immune system and specifically that of NO, parasites converting to the bradyzoite stage from the PRUΔ*KU80ΔP18* strain may be less effectively cleared out and therefore are reaching the brain in larger quantities. Assuming this is true then the surface marker P18 would be playing an antigenic role that permits better parasite detection and clearance by the host's immune system.

The higher levels of NO, as reflected by higher transcription levels of iNOS in the brains of mice infected with the *P18* knock-out strain (Figure 23) could also be

resulting from possible stimulation of a larger number of immune cells, by a bigger parasite load. This may be more effective in inducing the transformation into the bradyzoite stage inside cysts and poses a good justification for the results of previous histopathological examinations (obtained in our lab and not shown) showing a larger number of cysts found in the brains of mice infected with the knock-out strain.

J. PRU*ΔKU80ΔP18* induces higher levels of IL-10 than PRU*ΔKU80* in the brain of C57BL/6 mice

IL-10 is an anti-inflammatory cytokine that functions in dampening down the immune response and is essential for both host and parasite survival (Aliberti et al., 2005), its secretion comes highest as reflected by its transcriptions levels in accordance with highest NO levels as well as with the progression of the disease further into the chronic phase. Intracellular type II parasites residing within PVs have demonstrated a capacity to promote IL-10 secretion by hijacking the STAT3 pathway on which the host cells rely for the secretion of IL-10 (Melo, Jensen and Saeij, 2011).

We have seen that IL-10 transcription is higher in the brains of mice harboring the knock-out strain on day 35 post-infection This coincides with the highest transcription levels of iNOS. So higher levels of IL-10 in the brains of mice infected with the knock-out strain can also be due to a higher load of parasites promoting IL-10 secretion.

On days 14 and 21 post-infection, mice brains infected with either strain of the parasite showed no detectable levels of IL-10 mRNA transcripts. This may be

explained by a stimulated but delayed secretion of the anti-inflammatory IL-10 cytokine to alleviate the parasite burden in the brain.

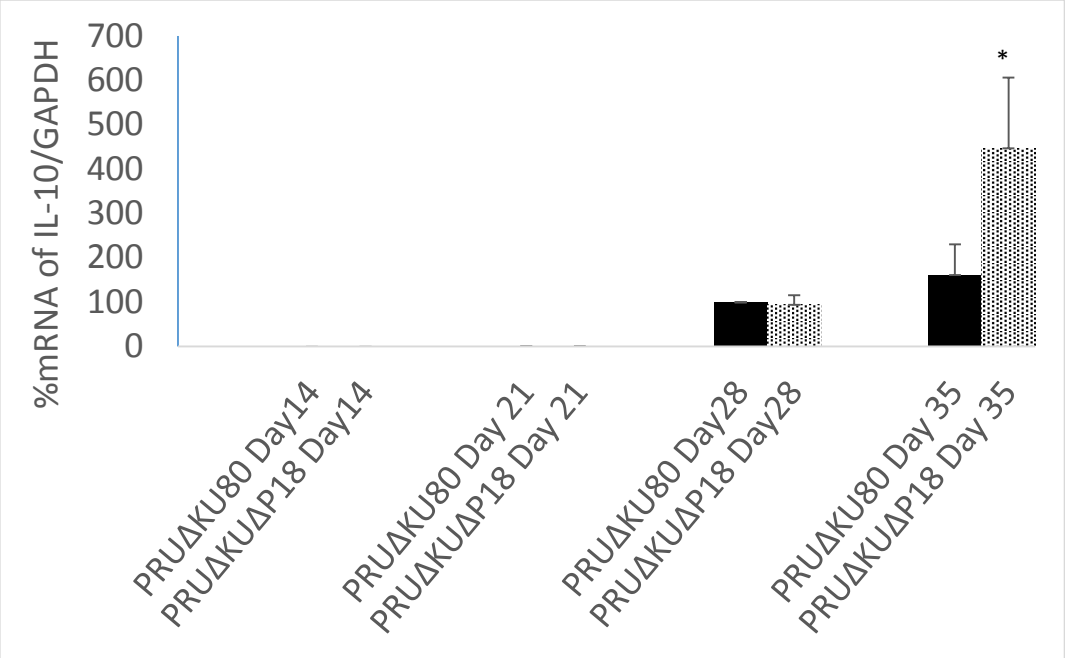

*Figure 24. Transcription levels of IL-10 on days 14, 21, 28, 35 post-infection Brains of mice infected with PruΔKU80 (black) and those of mice infected with PRUΔKU80ΔP18 (dotted) (n=4 mice) each. P value: between 0.01-0.05 corresponds to *, between 0.01-0.001 corresponds to ** and less than 0.001 corresponds to *** using student's T-test.*

CONCLUSION

Toxoplasma gondii is one of the most important parasites affecting almost one third of the world's population (Dubey, 2009). Its ability to switch between the tachyzoite and bradyzoite stages has made it a highly successful parasite in eluding the immune response and thereby establishing a chronic infection within its hosts and increasing its chances of propagation in nature. The switch between these two stages is yet to be fully understood.

Among the distinctive features between tachyzoites and bradyzoites are specific stage markers, each serving a role in the respective stage during which they are secreted. Understanding the roles and specific functions of these markers brings us closer to understanding these stages themselves and the switch that takes place between them, its driving factors, causes, mechanisms, and results.

P18, the bradyzoite surface marker coded for by the *P18* gene which was sequenced and published in 1996 (Ödberg-Ferragut et al., 1996) had yet unknown function, and our lab is trying to unravel its function through its deletion using the PRUΔ*KU80* type II strain. Our lab showed a higher capacity of forming brain cysts in the *P18* knock-out strain using the Swiss Webster mouse model. Furthermore, a failure of reactivation was observed upon immunosuppression with dexamethasone (Tawil, Diss. 2014).

Due the difference of the immune backgrounds in different mouse models, we first questioned whether the cyst phenotype was also obtained in the C57BL/6 mouse model. Our results on the transcription levels of BAG-1 have allowed us to

verify not only the presence of the parasite in the brains of our mice, but also the presence of a higher bradyzoite load in the brains of mice infected with the *P18* knock-out strain as compared to the brains of mice infected with the PRU*ΔKU80* strain (Figure 16).

Since the host immune system is the key factor the controls the back and forth switch between tachyzoites and bradyzoites, this prompted us to conduct the first immune studies in relation to the bradyzoite surface marker P18, by measuring and comparing the transcription levels of several important cytokines involved in the immune response. Since the immune response against *T.gondii* is centered mostly around macrophages and dendritic cells as well as microglia in the brain (Yarovinsky et al., 2014), we opted to center our studies around the cytokines that affect them and are secreted by them, alongside with MCP-1, an important chemoattractant of these cells.

Our results showed increased transcription levels of MCP-1 in the brains of mice infected with the *P18* knock-out strains, this was significantly obvious on days 14 and 21 post-infection ($P \leq 0.01$) (Figure 17) this was followed by significantly higher levels of IL-12 in these mice on day 28 p.i ($P \leq 0.05$) (Figure 18). MCP-1 is a recruiter of macrophages and dendritic cells and IL-12 is a product of their interaction with the parasites.

Under the effect of higher IFN-γ in the brains of mice harboring the P18 knock-out strain, visible mainly on day 21 post-infection (Figure 19), more NO seems to be produced by macrophages and microglia in the brain as reflected by higher iNOS transcription levels in these mice especially on days 28 ($P \leq 0.01$) and 35 ($P \leq 0.05$) post-infection (Figure 23). The remaining cytokine transcription levels fall into place to

support our conclusions. We can see that in the case of pro-inflammatory cytokines mainly released by $CD4^+$ T lymphocytes (Nagineni, Detrick, and Hooks, 2000) are usually significantly higher ($P \leq 0.01$) on days 21 post-infection in the brains of mice harboring the *P18* knock-out strain (Figures 21 and 22), a result that changes on day 28 post-infection to become significantly higher in those infected with the PRUΔ*KU80* strain ($P \leq 0.01$). On the other hand, we see that the transcription levels of the anti-inflammatory IL-10 is significantly higher ($P \leq 0.05$) in the brains of mice infected with the *P18* knock-out strain on day 35 post-infection (Figure 24)

In conclusion, we postulate that the surface marker P18 expressed on the surface of *T. gondii* bradyzoites by the *P18* gene plays an antigenic role that aids in further stimulation of the immune response. In that sense, the *P18* knock-out strain becomes superior at camouflaging itself from the immune response. The *P18* knock-out stains also seems to cause less stimulation of pro-inflammatory cytokines on the long run, either due to initially higher levels of NO forcing it more efficiently into earlier and more effective latency or due to the knock-out strain's ability to more readily convert from the tachyzoite into the bradyzoite stage and chronic latency.

Our results from the transcription levels of cytokines seem to support the theory that more parasites are reaching the brain and therefore are being prone to less destruction on their way from the primary site of infection. In addition we observe higher levels of IFN-γ and iNOS transcription in the brains of mice infected with the knock-out PRUΔ*KU80*Δ*P18* strain. The amount of IFN-γ and iNOS (Figures 19 and 23) positively correlates with the higher number of cysts that appear to be in the brains of mice infected with the knock-out strain (Figure 16).

This is not the first step in investigating P18, albeit it is the first on the immunological level. The findings have been encouraging in opening the door to examine and study more surface markers belonging to the P18 family and investigating their roles. Future investigations of P18 should focus on identifying receptors for these bradyzoite surface antigens on the cells of the immune system as well as examining the role of P18 during the acute phase of the infection, if present.

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