AMERICAN UNIVERSITY OF BEIRUT

TOXOPLASMA GONDII TYPE II PRU AND ITS DERIVATIVE PRU*ΔKU80*: INVESTIGATION OF SIMILARITIES AND DIFFERENCES

by
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AN ABSTRACT OF THE THESIS OF

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Title: *Toxoplasma gondii* type II strains Pru and Pru*ΔKU80*: Investigation of Similarities and Differences

Toxoplasma gondii, the causative agent of toxoplasmosis, is an obligate intracellular parasite. The host-induced immune response to the infection dictates the ability of this parasite to switch from the acute (tachyzoite stages) to the chronic phase (bradyzoite stages) of infection, where it encysts in the brain under the tight control of the host immune system. The immune response against *T. gondii* is IFN-ɣ driven where the parasite control occurs *via* the production of iNOS which distates whether these parasites are cleared at the level of the peritoneum or the brain.

Pru*ΔKU80* has not been studied in comparison to its parental Pru strain and it is of importance to know whether these two type II strains exhibit the same properties at the level of cysts induction in the brain and immune response.

We have compared the transcription levels of BAG-1 during chronic infection to decide which mouse strain is the best murine model for parasite induction by Pru and Pru*ΔKU80* strains. Then we tested the differences in the capacity of these parasites to replicate inside macrophages during the acute and chronic stages of the infection by comparing SAG-1 transcription levels. We finally studied the immune response by these type II parasites in the mouse model of choice during acute and chronic infection through the comparison of IFN-g, iNOS and IL-10 transcription levels as well as IL-12 secreted levels.

We found that among the three mouse strains (Swiss Webster, Balb-c and Black-6), Swiss Webster mice showed the highest amounts of cysts when infected with Pru strain that caused stronger expression levels of BAG-1 compared to the Pru*ΔKU80* strain. We investigated the immune response in the brain of chronically infected mice and showed that no significant difference in IFN-g and iNOS levels was observed in either Pru or Pru*ΔKU80* infected mice. Further investigation showed that the Pru parasites escape the peritoneum and organs to reach the brain where they encyst where as Pru*ΔKU80* parasites leave the peritoneum to the spleen where most of them are killed by iNOS, before reaching the brain leading to the formation of lower number of cysts.

Pru and PruΔ*KU80* parasites exhibit different patterns of cyst induction corresponding to a difference in the immune response induced by each strain thus making the PruΔ*KU80*, the choice of strain for knockouts, questionable when studying genes that are immunity related.

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CHAPTER I

INTRODUCTION

A. Discovery and Origins

In 1908, *Toxoplasma gondii* (*T. gondii*) was first described in a hamster-like rodent called *Ctenodactylus gundi* by Nicolle and Manceaux while studying *Leishmania* at the Pasteur Institute in Tunis [1]. Around the same period of time, Alfonso Splendore, an Italian physician and bacteriologist, discovered *T. gondii* in a rabbit in Brazil [2]. "Toxo" is a Greek term standing for "arc" or "bow"; whereas "plasma" means "Life"; therefore, "*Toxoplasma*" was given its name because of its crescent shape. The first viable specimen of *T. gondii* had been isolated [3], whereas the first human case was described in 1938 in a neonate girl who suffered from acute encephalomyelitis and died at the age of one month in a hospital in New York [4]. Thereafter, many other cases of toxoplasmosis were described and a serological test called "dye test" was developed by Sabin and Feldman, in 1948 to detect this parasite [5]. Fifty years later, the attention had been shifting towards the immune response against *T. gondii* especially in light of the discovery of the lymphoid cell mediated-immunogenicity against it. A further emphasis on the role of T-cells in the infection by *T. gondii* came from athymic nude mice that, when infected with this parasite, failed to produce an immune response that could protect them from the parasite [6]. Interferon gamma (IFN-γ) produced by $CD8^+$ T lymphocytes was shown to be a key player in the immune response against *T. gondii in vivo* [7]. This IFN-γ driven response is stimulated by IL-12 primarily produced by dendritic cells found in spleen areas rich in high T cells populations [8]. The 80's and 90's had witnessed unpreceeded attempts to determine

the differences between the isolated strains of *T. gondii* from both animals and humans at the genetic level [9]. However, the parasite's genome was completely sequenced in 2005 [10] all at the aim of better understanding the mechanisms of the disease and ultimately designing better treatment and or possibly prevention [9].

B. Taxonomy

Along with *Toxoplasma gondii*, the causative agent of toxoplasmosis, the phylum *Apicomplexa* includes many human disease-causing organisms such as *Plasmodium* spp., causing the malaria disease which is the first parasitic cause of death among patients, and *Cryptosporidium* spp causing cryptosporidiosis. This phylum also includes other animal pathogens such as *Eimeria* spp., *Theileria* spp., *Babesia* spp., and *Neospora caninum* (Table 1).

Members of the phylum *Apicomplexa* have complex life cycles involving multiple host organisms in which they undergo several developmental stages restricted to one host or to another. Another important similarity is being essentially intracellular parasites, a property that confers to them a major benefit of evading the host immune system [11]. In particular, *T. gondii* infects mainly macrophages and dendritic cells, using them as a vehicle to spread to the different organs, altering and down regulating the secretion of pro inflammatory cytokines such as IL-12 and activating the anti-apoptotic machinery securing their spread [11, 12].

Class	Order	Species	Parasitosis
Hematozoa	Haemosporida	*Plasmodium*	Malaria
	Piroplasmida	*Babesia*	Animal and human babesiosis
		Theileria	Animal coccidiosis
Coccidia	Eucoccidia	*Eimeria tenella*	Animal Coccidiosis
		Sarcocystis	Animal and human infection cyst forms
		Cryptosporidium	Animal and human infection diarrhea
		Toxoplasma	Animal and human toxoplasmosis infection by cysts and oocysts
Perkinsea	Perkinsida	*Perkinsus sp.*	Oyster parasite

Table 1. Some members of the apicomplexan phylum, their classification, diseases and infectious stages pertaining to infection.

C. Invasion of Target Host Cell

Despite some differences between the members of the phylum *Apicomplexa*, such as the target-host cell type which differs from one member to another, all share a conserved mode of invasion that involves the sequential discharge of an apical complex, from which the phylum derived its name. This complex includes many organelles namely the micronemes, rhoptries and dense granules, whose discharge determines a successful infection by *T. gondii* (Figure1) [13, 14]. Micronemal proteins confer gliding, motility and attachment of the parasite on the target host cell. Then collaboration between some micronemal proteins and neck rhoptry proteins builds up a moving junction that propels the parasite inside a parasitophorous vacuole. This vacuole protein involves mainly the bulb

rhoptry protein content and its maturation largely depends on the dense granule content (Figure 1) [13, 15-21].

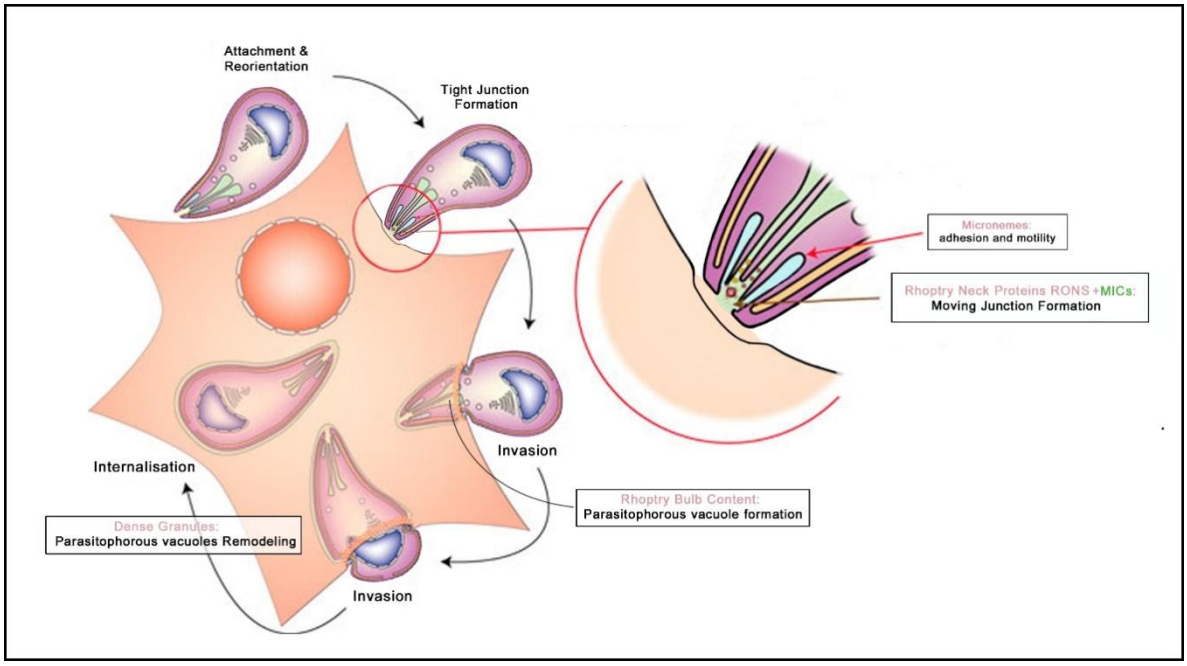

Figure 1. Schematic representation of *Toxoplasma* invasion. (1) Release of Micronemes'content in order to ensure host cell attachment. (2) Formation of the Moving Junction by the release of RONs and MIC proteins. (3) ROPs secretion leading to the formation of the Parasitophorous vacuole (PV). (4) Exocytosis of the dense granules into the PV leading to its maturation [22].

D. Stages of *Toxoplasma gondii*

Despite the discovery of *Toxoplasma gondii* as early as 1908, the full life cycle was not unraveled until 1970, where only the stages associated with the asexual life cycle were known, namely the bradyzoite and the tachyzoite stages [23]. The sexual life cycle and its stages were described in the intestines of obligate felid hosts [24]. Thus upon its complete life cycle, *T. gondii* can exhibit three infectious stages that are morphologically distinct: tachyzoites, bradyzoites, and sporozoites.

1. Tachyzoites

The term “tachyzoite” was coined by Frenkel inspired from the Greek word “Tacos” for speed [25] replacing the previous nomenclature “trophozoites” of the 2 μm crescent shaped parasites that were initially discovered by Nicolle and Manceaux in 1908. Also known as endozoites, tachyzoites are the proliferative intracellular forms of the parasite; they are pointed from the anterior end and rounded from the posterior end. Following the invasion of the host cell, each tachyzoite divides into two daughter parasites inside the mother parasite by a process known as endodyogeny (Figure 2a) [26]. These parasites keep on dividing until the host cell can no longer tolerate their increasing number and burst releasing them to infect other neighboring cells [27].

Structurally, the tachyzoite is made up of several organelles along with inclusions and a pellicle which is an outer covering. Within the pellicle are microtubules, polar and apical rings, rough and smooth endoplasmic reticula, mitochondrion, conoid, rhoptries, micronemes, dense granules, micropore, and Golgi complex. They also have an apicoplast, an organelle that resembles a plastid, inherited from plants, and is surrounded by four membranes, as for amylopectin granules, they sometimes are present but mostly absent. In addition to all these, there is a nucleus in the center with a central nucleolus surrounded by clumps of chromatin (Figure 2B, C, D) [28].

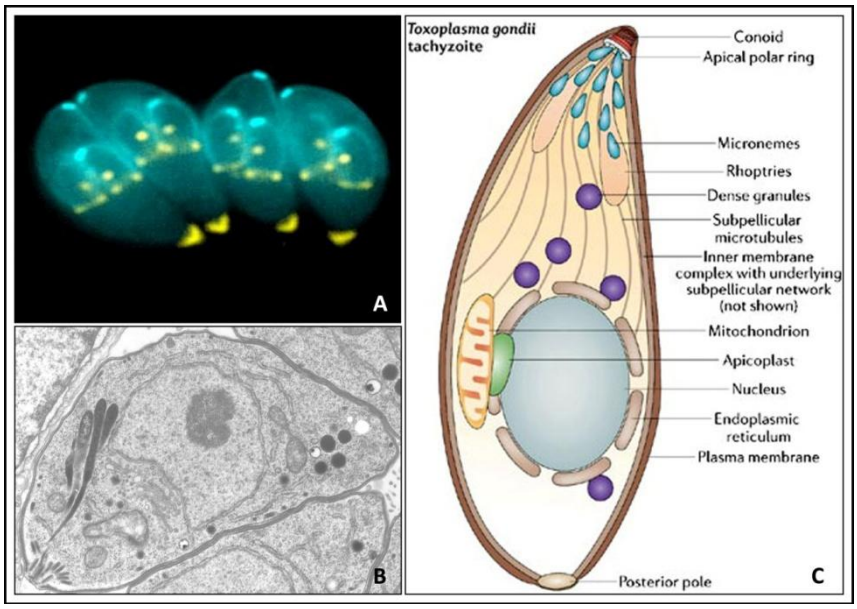

Figure 2. *Toxoplasma gondii* Tachyzoites. A. Endodyogeny process. Daughter *T. gondii* parasites forming inside the mother parasite (PLoS Pathogens Issue Image, 2006). B. Clicher of Jean-François Dubremetz: An intracellular *T. gondii* tachyzoite inside the parasitophorous vacuole membrane (PVM), showing the apical cytoskeleton (AC) and neighboring micronemes (M), rhoptry bulbs (ROP) and rhoptry necks (RON). Other components of the parasite, such as the nucleus (N), the Golgi apparatus (G) and the plastid that is specific to the Apicomplexa phylum, the 'Apicoplast' (A), are shown. The scale bar represents 0.5 µm. C. Schematic drawing showing the ultrastructure of *T. gondii* tachyzoite (Expert Reviews in Molecular Medicine-2001 Cambridge University Press).

2. Bradyzoites

In 1973, Frenkel was the one to propose the term "bradyzoite" to describe *Toxoplasma gondii* in its slow growing or non-proliferative developmental stage where "Brady" was inspired from the Greek language and means "slow". From that time, the bradyzoites stages are known to exist encysted in tissues. However, and in order to avoid any potential confusion between cysts of bradyzoites forming in tissues and oocyst stages shed in cats' feces, it was suggested that bradyzoite cysts should be called tissue cysts [29].

The first key difference of these parasite stages from the tachyzoites is the resistance of their protecting cyst to gastric enzymes. This property underlines the importance of these encysted forms in the continuity of *T. gondii*'s life cycle [30].

Upon the discovery of *T. gondii* cysts in cat feces, they were called oocysts [31]. An in-depth study of tissue cysts and bradyzoites development arose where Dubey and Frenkel made the first initiative and described the ontology and morphology of these forms (Figure 3) [31]. This study showed that three days following inoculation with tachyzoites, the mice start developing tissue cysts. Contrarily, in cats, the study took a different turn where the prepatent period for occysts shedding by cats was short and ranged between 3 and 10 days when these final hosts ingested tissue cysts or bradyzoites. This period increased to around 18 days when the cats were given tachyzoites and oocysts for ingestion irrespective of the inoculum size [32].

Further investigation showed that bradyzoites and tachyzoites differ in several features (table 2; Figure 4) among which is the position of the nucleus being posterior in bradyzoites, compared to being central in tachyzoites, and the abundant amylopectin bradyzoite content that is almost absent in tachyzoites [9]. Moreover, rhoptries in tachyzoites show an intricate labyrinthine form in comparison to bradyzoites that show a more coiled and looping form and appear electron dense in electron micrographs [28]. Furthermore, micronemes are finer and much more numerous in bradyzoites in comparison to tachyzoites. As for lipid bodies, they are completely absent in bradyzoites and not very often present in tachyzoites [33].

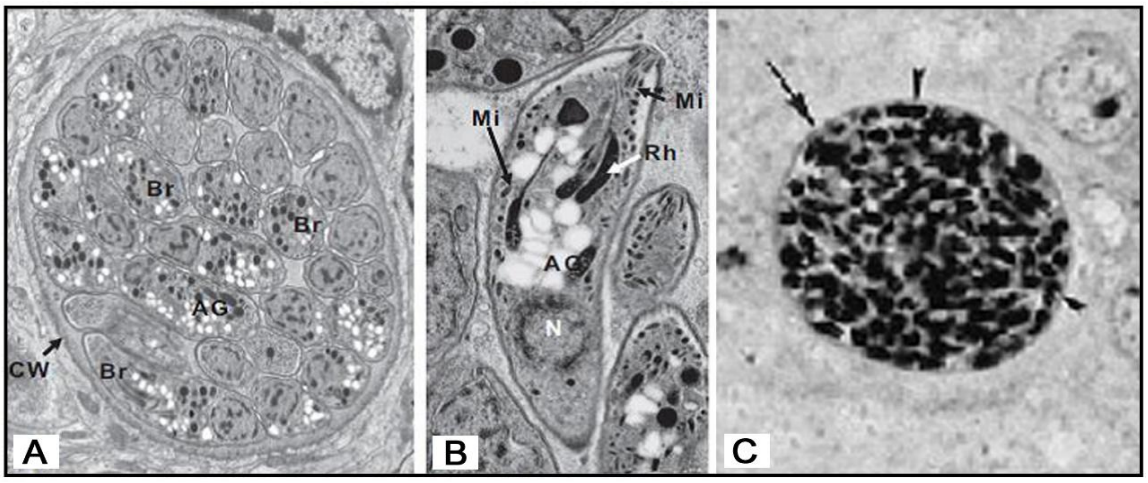

Figure 3. *Toxoplasma gondii* Bradyzoites. A. Transmission electron micrographs of bradyzoites (Br) within a tissue cyst. Note the presence of cyst wall (CW) and numerous amylopectin granules (AG) in the cytoplasm of bradyzoites. B. Higher magnification of ultrastructural morphology of bradyzoites. (C) Tissue cysts of *T. gondii* in mouse brains with numerous bradyzoites (arrow heads) enclosed in a cyst wall (arrow) [28].

Tachyzoites	Bradyzoites
Fast replicating forms	Slowly replicating forms
Intracellular and divide by endodyogeny	
More centrally located nucleus	Posteriorly located nucleus
Labyrinthine rhoptries	Solid rhoptries
Absent or few amylopectin granules	Numerous amylopectin granules
Occasionally presence of lipid bodies	Absence of lipid bodies
More susceptible to destruction by proteolytic enzymes	Less susceptible to destruction by proteolytic enzymes

Table 2. Comparison between tachyzoites and bradyzoites [28].

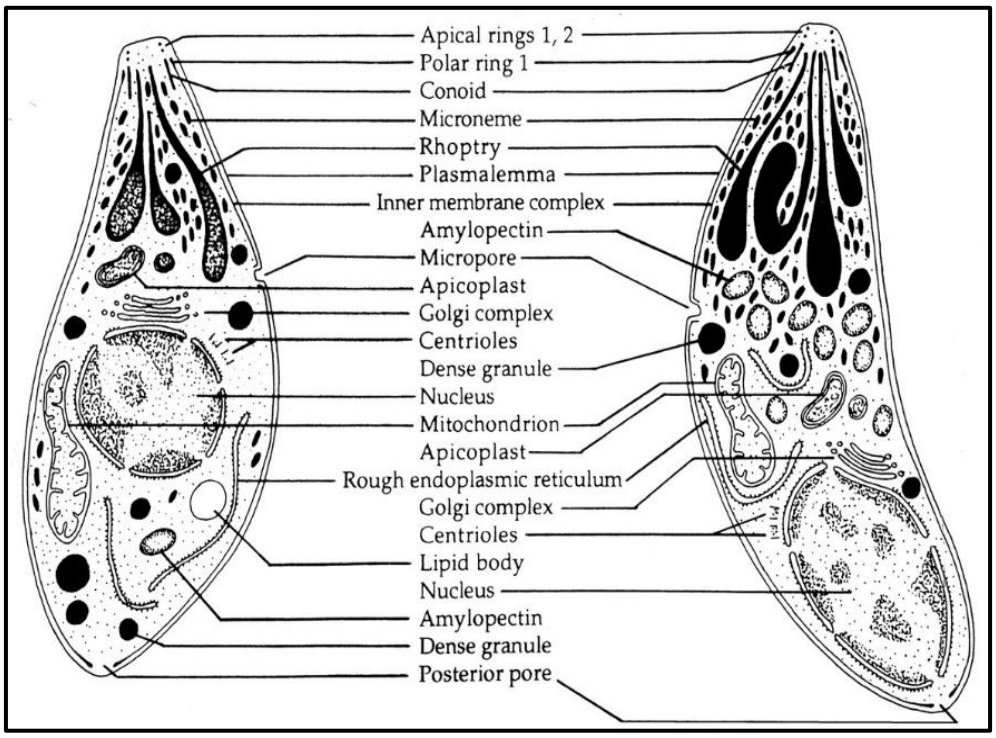

Figure 4. Tachyzoite versus Bradyzoite ultrastructure [28].

The tissue cysts vary in both size and bradyzoite content. Smaller sizes are associated with younger tissue cysts with a diameter of 5 μm and may contain as little as two bradyzoites, unlike older tissue cysts which may reach a diameter of 50 to 70 μm in the brain with around 1000 bradyzoites per cyst. However, tissue cysts can also occur in muscle tissues, in such cases the diameter can reach 100 μm and its shape takes a more elongated form, and the bradyzoite content is definitely larger [33]. Upon the infection by the virulent tachyzoites, the immune system initiates a response against *T. gondii* acute infection associated with the spread of these stages, resulting in their conversion to bradyzoites, which are responsible for the chronic phase of the infection [32]. More importantly, these bradyzoites, although asymptomatic in immunocompetent individuals,

they are of exceptional importance in immunocompromised patients where they become life threatening after their reactivation into tachyzoites [34].

3. Sporozoites

Sexual reproduction of *Toxoplasma gondii* occurs in the intestines of cats, where it leads to the production of oocysts in feces [35, 36]. The oocyst is resistant to environmental conditions, mainly due to the double layered wall that surrounds its contents. The content of a cyst is called the "sporont" and consists of a cytoplasm surrounding a nucleoplasm.

At the time of excretion, the occysts are not sporulated (Figure 5) but when the environmental conditions of aeration and temperature are favorable, sporulation can take place within 1 to 5 days outside the cat's body [28]. The process of sprorulation starts with the division of the nucleus twice giving rise to 4 nuclei, after which the cytoplasm follows by dividing and resulting in the formation of two spherical sporoblasts. Each sporoblast will then have 2 nuclei (figure 5). Elongation of the sporoblasts and formation of the sporocysts occur as sporulation continues. Within each sporocyst, four sporozoites are formed by the division of each nucleus into two along with cytoplasmic cleavage. Thus, the resulting sporulated oocyst contains two sporocysts, and each sporocyst contains four infective sporozoites, all ready to infect an intermediate host. Upon ingestion of contaminated food and by the action of the gastric digestive enzymes, these oocysts rupture, liberating the eight sporozoites which rapidly transform into tachyzoites inside the host [37].

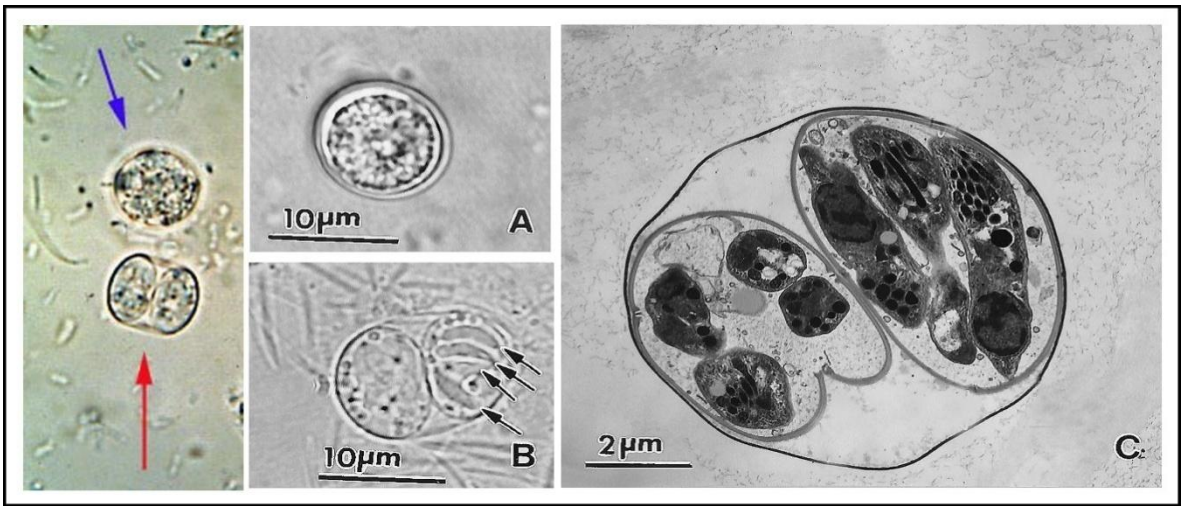

Figure 5. *Toxoplasma gondii* oocysts. An unsporulated oocyst (blue arrow) and a sporulated oocyst (red arrow) seen at high power (40 X) (University of Pennsylvania School of Veterinary Medicine, 2008). (A) Unsporulated oocyst. Note the central mass (sporont) occupying most of the oocyst. (B) Sporulated oocyst with two sporocysts. Four sporozoites (arrows) are visible in one of the sporocysts. (C) Transmission electron micrograph of a sporulated oocyst. Note the thin oocyst wall (large arrow), two sporocysts (arrowheads), and sporozoites, one of which is cut longitudinally (small arrows) [28].

Oocysts are shed in as much as one billion oocysts from infected cats, and are highly resistant to chemical and physical means of inactivation commonly used in sewage and waste-water treatment [38]. As for their survival and infectivity, they can last for years in fresh water [39] and a minimum of 2 years in salt water [40].

Sporozoites show structurally more similarities to tachyzoites than to bradyzoites (Table 3), yet they have more abundant micronemes and rhoptries as well as amylopectin granules. However, crystalloid and other refractile bodies are absent [28, 41].

	Tachyzoites	Bradyzoites	Sporozoites
Number of rhoptries	Similar		
Appearance of rhoptries	Uniformly labyrinthine	Uniformly dense, some of which are folded back on themselves	Both labrynthine and uniformly dense
Number of micronemes	Few	Many	Intermediate
Dense granules	More numerous	Less numerous	More numerous
Amylopectin granules	Few and small or absent	Numerous and relatively large	Numerous and relatively large
Lipid bodies	Rare	Absent	Numerous

Table 3. Ultrastructural comparison of tachyzoites, bradyzoites and sporozoites [28].

E. Life Cycle

Toxoplasma gondii is an obligate intracellular parasite that can infect all warm blooded animals, and has a prevalence rate of 30% among humans worldwide [42]. Its complete life cycle consists of an asexual part in an intermediate host that can be any warm blooded animal including humans, and a sexual part occurring in the feline intestinal epithelium (Figure 6) [43]. Intermediate hosts can acquire a *T. gondii* infection following the ingestion of undercooked or raw meat contaminated with tissue cysts, or the ingestion of vegetables contaminated with sporulated oocysts, as reviewed in [44].

1. Sexual Life Cycle

The sexual life cycle of *Toxoplasma gondii* commences when a member of the felidae family feeds on preys harboring tissue cysts. The digestive proteolytic enzymes of the cat break down the cyst wall releasing the encysted bradyzoites that will reach the enterocytes of the ileum where the sexual life cycle starts.

The incubation period of the parasite is about 2 days after which gamonts start to appear within the enterocytes and persist there for 3 to 15 days through a process known as gametogony [23]. Male and female gametes, microgametes and macrogametes respectively, develop after gametogenesis. Around 21 microgametes are harbored within one male microgamont, these microgametes have an organelle on the top end that is specialized in perforation as well as a flagellum to swim towards a mature female macrogamete and penetrate it, in order to fertilize it. These macrogametes are rich in organelles [45].

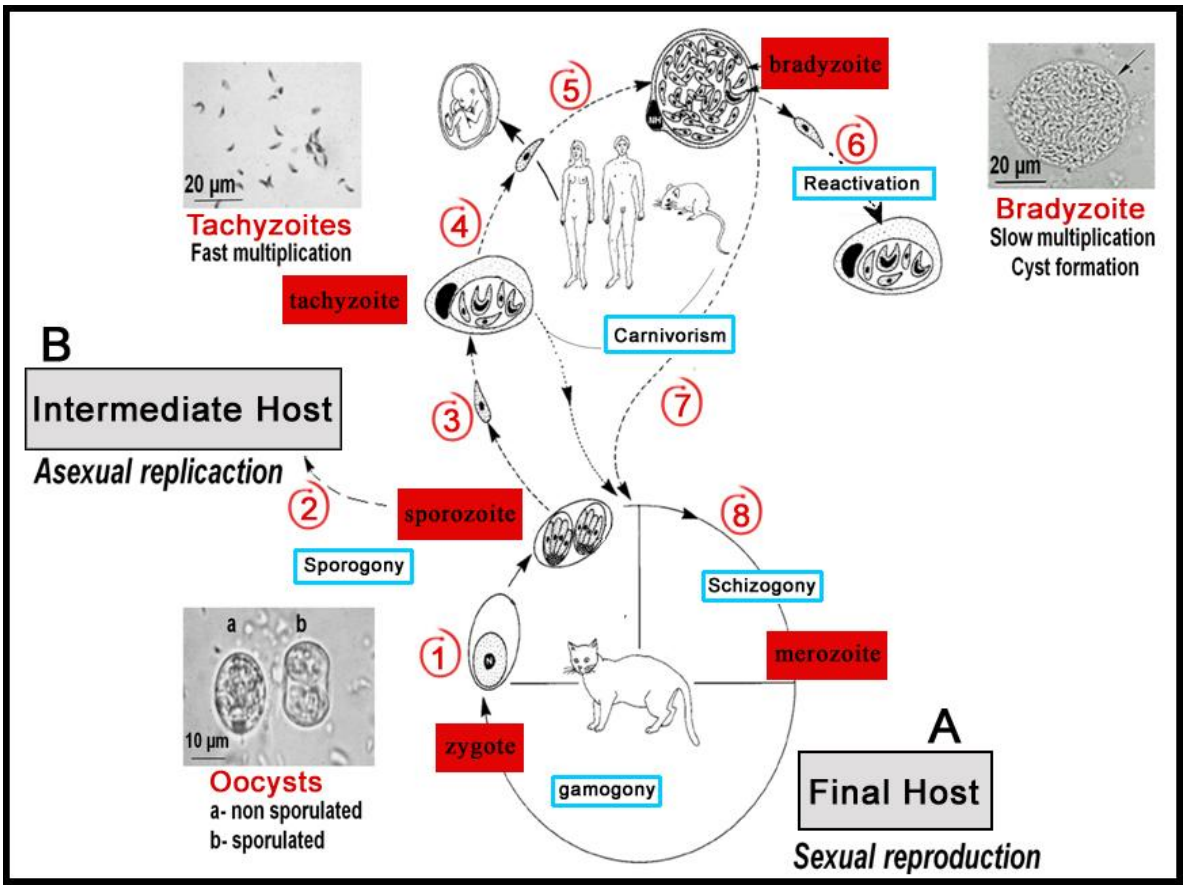

Figure 6**.** Life cycle of *T. gondii*. **A**. The sexual reproduction in feline (Final hosts). **B.** Asexual replication in intermediate hosts (including birds, rodents and humans). Unsporulated oocysts are shed in the cat's feces ①. Oocysts sporulate within 1-5 days in the environment and become infective. Intermediate hosts in nature become infected after ingesting any organic or inorganic material contaminated with sporulated oocysts. ② Oocysts rupture and liberate sporozoites which transform into tachyzoites shortly after ingestion ③. These tachyzoites are capable of infecting all types of nucleated cells and even cross the placental barrier and infect the fetus ④. Under the control of the immune system, these tachyzoites will transform into encysted bradyzoites in the brain, and the skeletal muscles ⑤. In immunocompromised patients, these bradyzoites can reactivate into tachyzoites ⑥. Cats become infected after predation of intermediate hosts harboring tissue cysts ⑦. Cats may also become infected directly by ingestion of sporulated oocysts ⑧.

Upon fertilization, a zygote is formed in the intestine of the final host. Oocysts are released upon rupture of epithelial cells as immature unsporulated oocysts defecated to the outside environment. Under the right environmental conditions, sporulation takes place and

a sporulated oocyst harboring 2 sporocysts each containing four sporozoites becomes the infective stage for intermediate hosts [28, 46].

2. Asexual Life Cycle

The asexual part of the life cycle begins when any warm blooded animal ingests food contaminated with sporulated oocysts (Figure 6). Sporozoites are then released after the action of the intermediate host digestive enzymes and transform to tachyzoites in the bloodstream shortly after release. These tachyzoites can then invade any nucleated cell and can even cross the blood-placental barrier. Tachyzoites are the key players of the acute phase of the infection. 5 days following ingestion of oocysts, tachyzoites start transforming into bradyzoites and encyst in the brain and skeletal muscles under the immune control. These bradyzoites can either remain dormant for years under the immune system control, or can begin a new cycle when this host is preyed upon by a felid [28]. An alarming danger of tissue cysts in intermediate hosts particularly humans, is their ability to reactivate back to tachyzoites in immunocompromised patients, such as HIV or bone marrow/organ transplant patients, where they become life threatening [47].

F. Modes of Transmission

Toxoplasma gondii complete life cycle was not fully understood until 1970 and only after that time the modes of transmission started to become better comprehended. However, being such a universal parasite capable of infecting virtually any warm blooded animal including marsupials, birds and even marine mammals, makes the possibility of human infection an undeniable certainty [48]. Regardless of the host, being intermediate or

definitive, the modes of transmission of *T. gondii* ensuring its survival and continuity [42] are classified as horizontal or vertical:

Horizontal transmission occurs *via* ingestion of food contaminated with cat feces harboring sporulated oocysts or through the ingestion of tissue cysts in raw or undercooked meat.

Vertical transmission happens mainly when a primo-infected mother transmits tachyzoites to her unborn fetus through the placenta.

A study conducted at the American University of Beirut reported that seropositivity against *T. gondii* was 62.2% for IgG and 6.8% for IgM [49]. Their results show that *T. gondii* infection is of high prevalence among the Lebanese population, raising awareness in case of immunosuppression where reactivation can become fatal.

G. Diagnosis

Diagnosis of infection with *T. gondii* is particularly hard, especially when there is need to determine whether the infection is acute or chronic. However, the methods used in diagnosis are mainly serologic tests, ultrasound scans and amniocentesis [50].

IgG and IgM specific serological tests results are hard to interpret when there is need to differentiate between acute and chronic infections especially that titers of IgM start rising on day 5 of acute infection and reach a maximum in about a month or two yet in many cases persist for years. As for IgG antibodies, they are normally detectable within a week or two following acute infection but they only peak in about 12 weeks up to 6 months and remain detectable for life [51]. When a patient tests positive for both immunoglobulins,

interpretation becomes hard and the positive results might be due to a recent infection or IgM titer persisting from a previous infection [52].

H. Pathogenesis of *Toxoplasma gondii*

Toxoplasma gondii infections are becoming increasingly interesting with the paralleling of myriad medical conditions with them. Some of these cases include the association of elevated IgG levels in pregnant women with prenatal anxiety and depression [53], others associated *T. gondii* infection with behavioral disorders [54], yet others took it to a whole new level where they related seropositivity to *T. gondii* with mental health problems like schizophrenia, depression and even suicide attempts [55]. However, congenital toxoplasmosis, neurotoxoplasmosis and reactivation, as well as psychiatric disorders associated with *T. gondii* are the three areas of active medical and basic research on the pathogenesis of this parasite.

1. Congenital Toxoplasmosis

The passage of *Toxoplasma gondii* from the mother to her unborn child through the placenta results in a wide spectrum of complications that are referred to as congenital toxoplasmosis. The first case of human congenital toxoplasmosis was documented by Wolf and colleagues in 1939 [4], then later it was found that it also can occur in other animal species such as sheep, goats and rodents. However, the severity of toxoplasmosis is highly dependent on the time at which the pregnant female acquires the infection. In 1974, Desmonts and Couvreur published a study involving 183 women acquiring *T. gondii* infection during pregnancy [56]. 11 had abortions with 7 of the cases being still birth or

post-natal death. Of the rest, 59 infants suffered from symptoms leading to 2 deaths and 7 with severe diseases involving ocular and cerebral complications. The remaining had mild infections of less severe nature[56]. This led to conclude that the semester of pregnancy at which a female acquires an infection for the first timedictates the severity of congenital toxoplasmosis. The most severe infection occurs during the first trimester [56] and often ends with spontaneous abortion [33]. During the second and third trimesters, the baby often develops chorioretinitis and symptoms of neurologic nature such as retardation, seizures, microcephaly, and hearing loss [50].

2. Toxoplasmosis in Adults

Adult toxoplasmosis can be associated with a variety of symptoms. In 1940 Pinkerton and Weinman published their findings about a 22 years old adult male who died in 1937 in Lima in Peru from a *Bartonella* spp. infection and fever and whose post-mortem analysis revealed *Toxoplasma gondii* in his spleen, heart and other tissues [57]. A year later, the same group reported another incident of two adult males, 43 and 50 years old, who died in Missouri and this team was able to recover *T. gondii* from their tissues and their blood. Among their symptoms there was rash, fever and malaise [58].

Those two case reports were the first of their kind in reporting acute adult toxoplasmosis without neurological involvement.

a. Lymphadenopathy

Toxoplasmosis in adults is associated with signs that are frequently observed. Siim noticed that lymphadenopathy was frequent in adults [59]. Siim's findings were then

confirmed in 1958 by Beverley and Beattie when they reported 30 similar cases [60]. Later outbreaks in Canada [61] and the United States of America [62] further confirmed that indeed lymphadenopathy is a frequent sign of acquired adult toxoplasmosis.

b. Ocular Disease

At the time of its discovery and up until 1950, doctors and scientists did not have a holistic understanding of toxoplasmosis, and until that date, ocular toxoplasmosis cases were all thought to arise following congenital transmission [63]. In 1952, a step closer in understanding ocular toxoplasmosis was taken by Wilder who succeeded in identifying *T. gondii* in the retinas of the eyes of patients after they had been enucleated [64]. In 1995 there was a toxoplasmosis waterborne outbreak in Canada and among the 95 patients that developed acute toxoplasmosis, 20 were diagnosed with ocular toxoplasmosis [65, 66].

c. Neurotoxoplasmosis

Neurotoxplasmosis gets its importance from the diseases caused after acquiring immunodeficiency. The causes of immunodeficiency are many including the Human Immunodeficiency Virus (HIV), malignancies as well as chemotherapy for cancer patients [67]. Thus in such cases, latent or dormant toxoplasmosis becomes a vicious disease that takes the opportunity of a weak immune system to kill the patient. In 1983, Luft and colleagues reported encephalitis induced as a result of acute of toxoplasmosis [68].

An increased interest in understanding the host-*Toxoplasma* interaction has been taking place ever since a positive association between seropositivity for *T. gondii* and brain cancer incidence was made; it was reported that a potential increase in incidence of brain

cancer by *Toxoplasma* is related to the ability of the parasite to interfere with the brain cells miRNAome [69]. Symptoms of neurotoxoplasmosis include headaches, confusion, and poor co-ordination, and seizures. At advanced stages, it could also manifest as respiratory problems, high grade fever, blurred and obstructed vision [70].

3. Psychiatric Disorders Associated with Toxoplasma gondii

Recently, studies on chronic infections of *Toxoplasma gondii* are shifting in the direction of psychiatric disorders in relation to chronic toxoplasmosis. However inconclusive, many of these studies are pointing in the direction of schizophrenia given than both chronic toxoplasmosis and schizophrenia are being coupled with an increased production of dopamine [71]. Furthermore, a survey revealed that exposure to cats was much more common among schizophrenics as compared to non-schisophrenics [72]. Ingram *et al.* showed that mice infected with *T. gondii* attained an odd behavior where these mice were not showing any signs of fear from the smell of cats [73].

I. Strains Genetic Overview and Virulence of *Toxoplasma gondii* Strains

The nucleus, mitochondria and the apicoplast are the three compartments that contain the whole genomic DNA of *Toxoplasma gondii*. The entire genome is 87 Mbp for the nuclear component, 6 Kbp for the mitochondrial component and a 35 Kbp circular extra-chromosomal DNA component in the apicoplast. Genetic analysis of the nuclear genome of *T. gondii* revealed that it is haploid throughout most of the life cycle except during a short diploid phase that takes place inside the cat intestines right before meiosis. During this diploid stage, the genome consists of 11 chromosomes, designated by roman

numerals, which range between 1.8 Mbp up to more than 10 Mbp [74]. Research published in 2001 by Ajioka and colleagues showed that the coding capacity of the organellar genome is very limited what prompted the belief that the nucleus codes for proteins responsible for functions of the organelles [75]. With the advancement of microarray technology, all predicted genes of *T. gondii* (based on ToxoDB 4th release) represented in probe sets for Affymetrix microarray were developed and available for commercial use [76, 77]. Afterwards, the entire sequenced *T. gondii* genome became freely available on www.ToxoDB.org following its full sequencing by the J. Craig Venter Institute.

1. Virulence

Toxoplasma gondii was perceived to be pathogenic mostly in immunocompromised patients unlike what happens with healthy people and non-pregnant females whose infections go almost unnoticed. However, following the discovery of pathogenic strains of *T. gondii*, this opportunistic parasite was no more taken for granted but questioned [78].

Virulence of *T. gondii* is not dictated by only the parasite's behavior. In fact, the host immune system is a key player alongside the parasite's success in invading the host. Thus the parameters of virulence of *T. gondii* are also associated with its capacity to modulate transcription of host signaling factors that are immunity related [12, 79, 80].

In literature, mostly mice have been the subject to experiments with *T. gondii*. Therefore, the amount of tachyzoites required to infect and kill a mouse has been the common measure of the parasite's virulence [81]. In humans, virulence of *T. gondii* is mostly associated with the location of the involved organ and parasitemia [78]. Moreover,

having said that the success of a *T. gondii* infection is related to factors pertaining to both the host and the parasite [78], it is worth mentioning that the nature of the immune response triggered is also crucial [12, 78]. Furthermore, in the light of discoveries made at the level of *T. gondii* effector molecules, SAG-1 protein is thought to have a role in the virulence of *T. gondii*, but has been proven to be dispensable [78]. The final image of the virulence of *T. gondii* is not yet drawn, but the involvement of recently discovered genes is being considered to be of major importance in relation to the severity of the infection [78].

Isolated strains of *T. gondii* fall under three lineages called types I, II or III (Table 4) [82], that differ by less than 1% at the genetic level [79]. Although *Toxoplasma* population is divided among these three types, isolates from patients majorly belong to the type II strain [83]. In mice, the phenotypic differences are tremendous. Accordingly, type I strains are fatal with a lethal Dose 50 (LD50) = 1 in different laboratory mouse models. Infections with parasites belonging to types II and III are in general controlled and persist for life, with a murine LD50 =10^2 and 10^3 respectively [84].

Strains	Type I	Type II	Type III
Genetic	**98 % similar**		
Virulence	**High**	**Intermediate**	**low**
Examples	**RH**	**ME49, Pru (Prugnaud), Pru KU80**	**NED**
Characteristics	**● unable to make cysts of bradyzoites because it lyses cells before making cysts ● grows faster than types II & III ● completely lyses a flask of cultured cells much faster than types II & III ● Extracellular parasites remain infectious for a longer time compared with the types II & III**	**● able to make bradyzoites cysts ● grow slower than type I ● completely lyses a flask of cultured cells much slower than types I ● Extracellular parasites remain infectious for a shorter time compared with the types I**	**● able to make bradyzoites cysts ● grow slower than type I ● completely lyses a flask of cultured cells much slower than types I ● Extracellular parasites remain infectious for a shorter time compared with the types I**

Table 4. Comparison between the 3 different strains of *T. gondii* [81].

2. New Genetic Model of Type II: Pru∆KU80

The two recombination pathways used by most eukaryotes to repair a double strand break (DSB) are the homologous recombination pathways and the non-homologous end-joining (NHEJ) pathway [85]. Repair of a DSB by the homologous recombination pathways requires mechanisms that rely on recognizing extreme homology in DNA

sequences unlike the NHEJ pathway that instead, relies on the direct ligation of broken DNA strands ends. An early step in the NHEJ pathway involves the formation of a dimer between KU70 and KU80 proteins which joins the broken DNA ends at a DSB [86, 87]. This NHEJ pathway is preferentially used in many eukaryotes for DSB repair. Thus exogenous targeting DNA gets integrated anywhere into the genome irrespective of the homology on the DNA sequence [85]. *Toxoplasma gondii* belongs to this category and have a preference for the NHEJ pathway [88-91]. A breakthrough in the development of the understanding of the role of many genes in the *T. gondii* genome goes back to Fox and her colleagues who managed to knockout *KU80 (Δku80)* in *T. gondii* parasites belonging to lineage II, by relying on the NHEJ pathway [92]. These knockout parasites were void of the dominant pathway that mediates the random integration of targeting episomes. Thus the efficiency of double-crossover homologous recombination was increased at targeted loci [92]. The value of such knockouts was immediately tested and revealed by the same team through successfully deletion of four genes encoding parasite antigens, namely *GRA4*, *GRA6*, *ROP7*, and *tgd057*.

J. Significance of *Toxoplasma gondii* as an Experimental Model

Many features make *Toxoplasma gondii* a major and amenable experimental model for studying the biology of *Apicomplexa*ns and their associated parasitic diseases. It is very easy to culture, propagate and quantify *T. gondii in vitro*. Moreover, manipulation of the genome of *T. gondii* is very well established along with the mouse models for studying this parasite interaction with its host during an infection [93, 94]. In addition, crossing and transiently or permanently transfecting *T. gondii in vitro* is relatively easy. Moreover,

studying phenotypes of genes is extremely successful and methods to generate knock out parasites and select them are available. In case of lethality in case of an essential gene, conditional knock-outs were obtained either using Tetracycline or the newly used technology of Crispr/Cas9 [95, 96].

K. Selective Bradyzoite and Tachyzoite Markers

The expression profiles of the molecular markers associated with the different developmental stages of *Toxoplasma gondii* are different and have been studied. *T. gondii* not only undergoes stage conversion, but also undergoes morphological and biomolecular changes represented by the expression of stage-specific antigens [97].

1. Tachyzoite Specific Surface Antigen-SAG1: a marker for acute Toxoplasmosis

Tachyzoites are responsible for the acute stage of the *Toxoplasma gondii* infection and are capable of activating the host immune system in an early response that leads to the elimination of most of the parasites but also initiates the response against one of its surface antigens SAG-1 [98] resulting in the production of the majority of antibodies against it [99]. Although, the surface of *T. gondii* expresses many surface proteins, yet the most important one is the SAG-1, which by itself accounts for 5% of all proteins of *T. gondii*.

The complete *T. gondii SAG-1* gene was first cloned by Burge and colleagues in 1988 and was found to have a 1.1 kb length. It encodes for 336 amino acids and translates into a 30 kDa protein and for this reason SAG-1 protein is also known as P30 [100]. SAG-1 protein undergoes many post translational modifications mainly glycosylation resulting in a mature GPI-surface anchored protein [100, 101].

SAG-1 protein stimulates specific humoral and cellular immune responses. Different T lymphocytes are involved in this process which is coordinated by cytokines such as IFN-γ and IL-12, thus playing a pivotal role in providing protective immunity against *T. gondii* [7]. These T lymphocytes include $CD4^+$ and $CD8^+$ T cells; however $CD8^+$ T cells make the primary subpopulation of cells that targets *T. gondii* infections in the host cells and they provide antigen-specific cytotoxicity. Various *T. gondii* antigens are known, nonetheless SAG-1 immunogenicity and reactivity with immune components makes it the best choice for diagnosis of an acute infection as well as vaccine research [102, 103]. The high IgG, IgM and IgA titers induced by SAG-1 [100, 104], give it a very important role in diagnostic procedures, especially that it is the marker of choice in Western blotting for acute infection verification in research labs, this method being the golden standard among researchers [105, 106].

2. Bradyzoite Surface Marker-BAG1

Tachyzoites and bradyzoites have different metabolic profiles associated with the difference in the morphology of these two developmental stages of *T. gondii*. These differences in the metabolic activity are associated with differences at the level of expression of metabolically active molecules and heat shock proteins expressed by both tachyzoites and bradyzoites [33]. Bradyzoite Antigen-1 (BAG-1) is used as one of the bradyzoite specific markers [107].

BAG-1 is a bradyzoite specific antigen of 28 kDa. Because of its homology to small heat shock proteins, especially those of plants, it was previously also known as hsp30 [108, 109]. Upon conversion of tachyzoites to bradyzoites, both BAG1 mRNA and protein

are upregulated, suggesting that the BAG-1 expression undergoes transcriptional regulation in this process. BAG-1 protein is encoded by the most abundant gene specific for bradyzoites, *bag1* [110], and its expression is detectable as early as 24 hours post induction of tachyzoites to convert to bradyzoites under stressful conditions [33]. Knockout of *bag1* gene shows a major effect on the reduction of cysts burden in the brains of mice indicating an effect of BAG-1 on the efficiency of cyst formation *in vivo* [111, 112].

L. The Immune Response to *Toxoplasma gondii*

Invasion of host cells by *Toxoplasma gondii* is a very active process driven by the parasite [113] towards the formation of a parasitophorous vacuole [114]. Many secretory proteins are effector molecules capable of making the host cell a suitable environment for the survival and growth of the parasite [11]. With more research done on these molecules, such as rhoptry and dense granule proteins, it is becoming more evident that these proteins are capable of manipulating mechanisms of resistance in the pro-inflammatory pathway of the host (Figure 7) [12].

Microorganisms express molecules that the host does not possess, and these molecules, known as pathogen associated molecular patterns or PAMPs, are recognized by Toll-like receptors (TLRs) which make a family of innate immune receptors [115]. *T.* gondii possesses many of these molecular structures that are recognized by Toll-like receptors on the surface of macrophages and dendritic cells [116]. Profilin is one important *T. gondii* molecule that binds specifically to TLR11. Type II *T. gondii* activates TLR11 mainly, which in turn regulates IL-12 production in response to the parasite [117] either in a myeloid differentiation factor 88 (MyD88) dependent or MyD88 independent manner.

MyD88 is an essential adaptor protein in the signaling pathway of TLRs *via* TLR11 activation [118], and it induces the early production of IL-12 by macrophages and dendritic cells [119]. IL-12, a pro-inflammatory cytokine, is used by the host as a first step towards controlling a *T. gondii* attack [120, 121], assisted by IL-1β and Il-18, induces natural killer (NK) cells and T cells to secrete IFN-γ [122], provoking intracellular elimination of *T. gondii*, thus highlighting the crucial role of the innate immune system in the control of *T. gondii* infection.

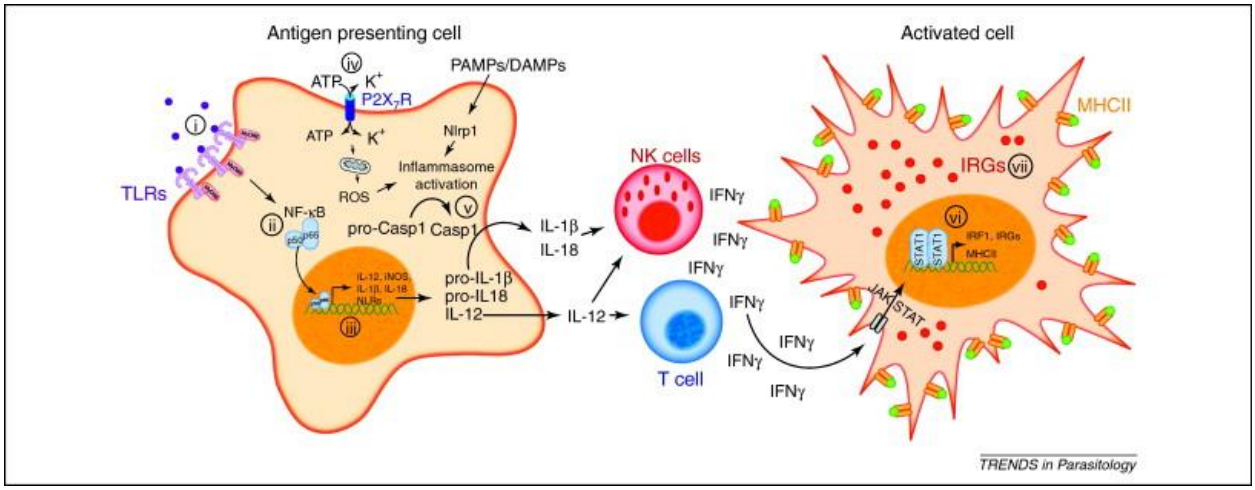

Figure 7: Host cell responses that can be modulated by *Toxoplasma gondii* [12].

1. Effectors of the Immune Response against type II Toxoplasma gondii

a. <u>Pro-inflammatory Response</u>

Immune responses vary from one *Toxoplasma gondii* strain to another, and type II strains can activate a very effective early response. The dense granule protein GRA-15, when expressed as an active isoform, as is the case in the type II strains, causes excessive activation of NF-κB in the infected cells [123], the antigen presenting cells, that leads to an

early surge in IL-12, IL-18 and IL-1β which in turn cause an overproduction of INF-γ by T helper and Natural Killer cells (Figure 8) [78].

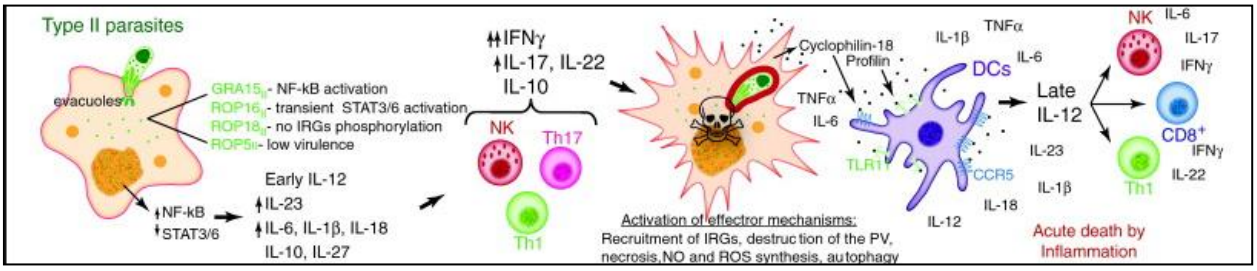

Figure 8: How *Toxoplasma* type II strain modulates host immune pathways [12].

i. Interleukin-12 (IL-12)

Toxoplasma gondii infection is distinguished by a vigorous immune response mediated by helper type 1 T cells (Th-1) as well as high levels of INF-γ which are essential for acute and chronic infection stages control in the murine model [124, 125]. Such an immune environment induced by the parasite inside the host moderates several T cell subtypes activation focusing the response on Th1 cells [126]. It is noteworthy that $CD8\alpha^+$ dendritic cells are the major cell species that produce IL-12, furthermore, a subset of dendritic cells in the spleen known as splenic $CD8\alpha^+$ dendritic cells induce a very strong early surge of IL-12 a few hours after infection unlike macrophages and other dendritic cells that require INF-γ priming [8] and these dendritic cells are responsible for the initial production of IL-12. Early IL-12 by dendritic cells activates natural killer, T helper cells & neutrophils to produce INF-γ that then activates macrophages and other non $CD8\alpha^+$ dendritic cells to produce IL-12 and further enhance the inflammatory response [127].

ii. Interferon gamma (IFN-γ)

Toxoplasma gondii infection is an IFN-γ driven infection. IFN-γ is the dominant factor that enhances the ability of macrophages to destroy *T. gondii* [127] because this parasite is among the intracellular pathogens that are IFN-γ controlled through activation of effector mechanisms. IFNγ effects are many on infected cells (figure 9) resulting in parasite replication reduction through the induction of the expression of inhibitory proteins such as indoleamine 2,3-dioxygenase (IDO) and inducible nitric oxide synthase (iNOS) as well as immunity-related GTPases (IRGs) and guanylate-binding proteins (GBPs) [127]. IDO causes the depletion of tryptophane through converting it into *N*-formylkynurenine. Tryptophane is an essential amino acid required for *T. gondii* survival while being unable to synthesize it. Thus the tryptophan uptake by the parasite is only from the infected host cell and its absence makes the parasite vulnerable to starvation [128]. The highly toxic nitric oxide (NO) is generated by inducible nitric oxide synthase (iNOS) downstream of IFN-γ which interacts with the parasite directly in the parasitophorous vacuole. The IRGs and GBPs are in charge of parasitophorous vacuole destruction [129]. The parasitophorous vacuole is a special organelle that protects the parasites from destruction by endolysosomal mechanisms that once damaged render the parasites vulnerable to fast elimination from the infected cells cytoplasm [127].

iii. Inducible Nitric Oxide Synthase (iNOS)

The iNOS produced in response to IFN-γ stimulation depletes arginine as it produces NO thus restricting the parasite replication; arginine is another essential amino acid for the growth and replication of *T. gondii* (figure 9) [130]. iNOS induced arginine

starvation was primarily investigated as a differentiation trigger of tachyzoites to bradyzoites, yet its role as a host defense mechanism cannot be ignored *in vivo* [131]. It is worth mentioning, though, that iNOS is very important in exerting stress on tachyzoites to convert to bradyzoites in tissues such as the brain and to keep them under stress to prevent reactivation in the case of brain. The role of NO in controlling the chronic stage of *T. gondii* infection suggests that tachyzoite and bradyzoite elimination mechanisms may be different [127]. Studies have shown that the parasitc control in the brain is under direct control of iNOS, NOS2 specifically, in TNF-α dependent and independent mechanisms [125]. Furthermore, iNOS-deficient mice have been shown to be more prone for reactivation in latent infections, thus spotting the light on the importance of NO in the control of persistent infection [132].

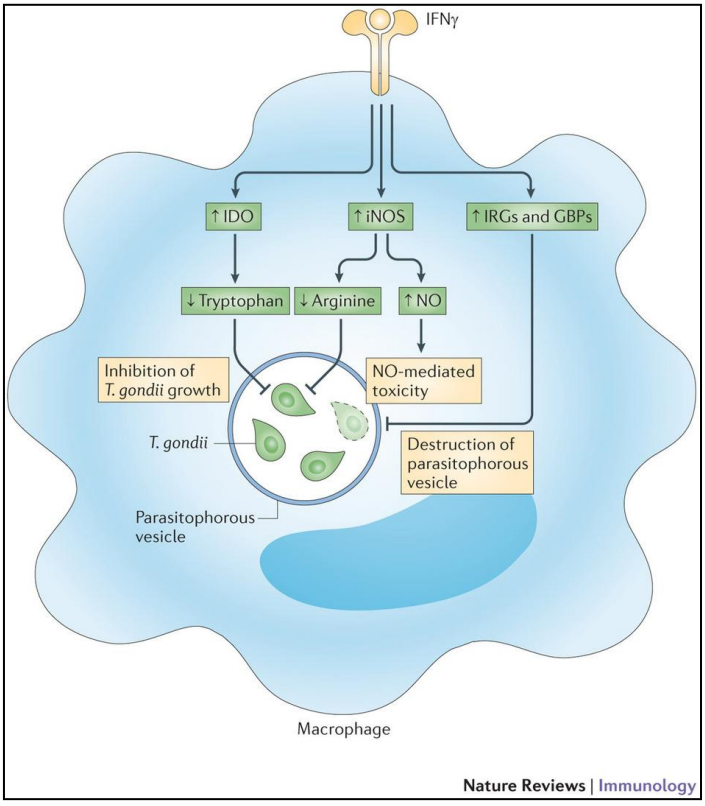

Figure 9: Effector mechanisms of IFNγ-mediated parasite elimination in infected cells [127].

b. Anti-inflammatory response

The excess production of pro-inflammatory cytokines, such as IL-1b and IL-18 along with others, ends up damaging the host cell [12] through pyroptosis *via* inflammasome activation. Thus for the survival of both the host and the parasite, a very delicate balance should be maintained between pro- and anti-inflammatory signals, and recent studies have shown that many *Toxoplasma* effectors are involved in controlling parasite burden and disease *via* manipulating inflammatory pathways [133, 134].

i. Interleukin 10 (IL-10)

Type II infections are very potent at the immune level, and can lead to IL-12/IFN-γ mediated immunopathology, thus negative regulators of the immune response are required to maintain a balanced immune reaction that inhibits the parasite and does not harm the host. IL-10 is one of those negative regulators. IL-10 deficiency in mice led to their death during the acute stage of the *Toxoplasma* infection, because of a severe immunopathology *via* Th1 immune responses in liver [135] and intestines [136]. Thus INF-γ mediated immune response is down-regulated by IL-10 which is very important in preventing the pathology due to the immune response. Moreover, IL-10 is also implicated in the chronic stage of toxoplasmosis where IL-10 deficient mice treated with sulfadiazine early after infection helped these animals survive the acute infection but then developed lethal immune responses and inflammation in the brains of these animals during the chronic infection [137]. The traditional IFNγ secreting T-bet^{+}Foxp3^{-} Th1 cells are the main IL-10 producers in mice infected with *T. gondii*. Not only these IL-10^{+}IFNγ^{+}CD4^{+} T-cells have the capacity to prevent intracellular replication of tachyzoites in macrophages, they also strongly suppress IL-12 production by antigen presenting cells [138]. However, IL-10 has no effect on macrophages thus only increases to prevent tissue pathology but not to compromise immune effectiveness, because IL-10 increase does not compromise the production of NO [139].

c. Other Immune Modulators

Th-17 cells, another T helper cells subpopulation, respond to *Toxoplasma* in two ways in terms of host survival, either decreasing the parasite burden on the host through IL-

17 signaling, or IL-22 induced production by IL-23 of $CD4^+$ T cells that leads to the destruction of the host tissue [140].

Toxoplasma gondii infection and the mice and human immune system are an example of how the coevolution of parasite and mammalian cells alongside affected the talk between these two entities, and how each of them recognizes specifically which mechanism to alter and what functions to modify where in the case of *T. gondii* to survive in the host and in the case of the host, to eliminate the parasite.

CHAPTER II

MATERIALS AND METHODS

A. Culture:

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

Toxoplasma gondii type II strains Pru and Pru *Δku80* tachyzoites, kindly provided by Drs Maryse Lebrun and Jean Francois Dubremetz from France, 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).

2. Primary Macrophages

Black-6 mice were injected intraperitoneally with 1 mL thioglycollate (38.5 g/L). Primary elicited macrophages were then extracted by peritoneal lavage and grown in RPMI media (Lonza), supplemented with 10% fetal bovine serum (FBS), 20 mM HEPES (Sigma), 2 mM L-glutamine (Gibco), 1% penicillin–streptomycin and kanamycin (Lonza). Where activation of macrophages is specified, the procedure was performed using 0.04% lipopolysaccharide of $2.5*10^4$ ng/mL concentration for 6 hours prior to infection with type II *T.gondii* strains.

B. *In vitro* Infection of Macrophages

Upon confluency, primary macrophages were infected with Pru or Pru *Δku80* strains at the ratio of 1:1 parasite to macrophage. Infected cells were then left in culture for

4 days whereby at days 2 and 4 post infection (p.i.), supernatants were collected for Enzyme-Linked ImmunoSorbent Assay (ELISA) analysis whereas cells were scraped for total RNA extraction.

C. *In vivo* Experiments

1. Acute infection of mice

Swiss Webster male mice at the age of 4 months were used to study the immune response following acute infection. 5 mice of each strain were intraperitoneally infected with 1 million of either Pru or Pru *ΔKU80* parasites. 4 days p.i., mice were sacrificed, blood collection was sampled using retro-orbital puncture (Eye prick) and serum was used for ELISA experiments. Peritoneal lavage was also performed for RNA extraction. Spleens and brains were then collected to test tissue parasite burden and the corresponding elicited immune responses. Organs were then homogenized using (BioSpec) and were used for RNA extraction.

2. Chronic infection of mice

3 strains of male mice (Black-6, Swiss Webster, and Balb-c) were used. 3 to 4 months old mice were intraperitoneally infected with 100 parasites of Pru or Pru *ΔKU80*. At day 4 p.i., 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 10). 7 days p.i., blood was sampled using retro-orbital puncture and almost 100 µL of blood were obtained from the medial

cathenus of each injected mouse to verify acute infection by western blotting (detailed later). Seropositive mice against the acute infection were then sacrificed at late week four (28 days p.i.) and their brains were harvested and homogenized for RNA extraction, whereas seronegative mice were discarded from our study.

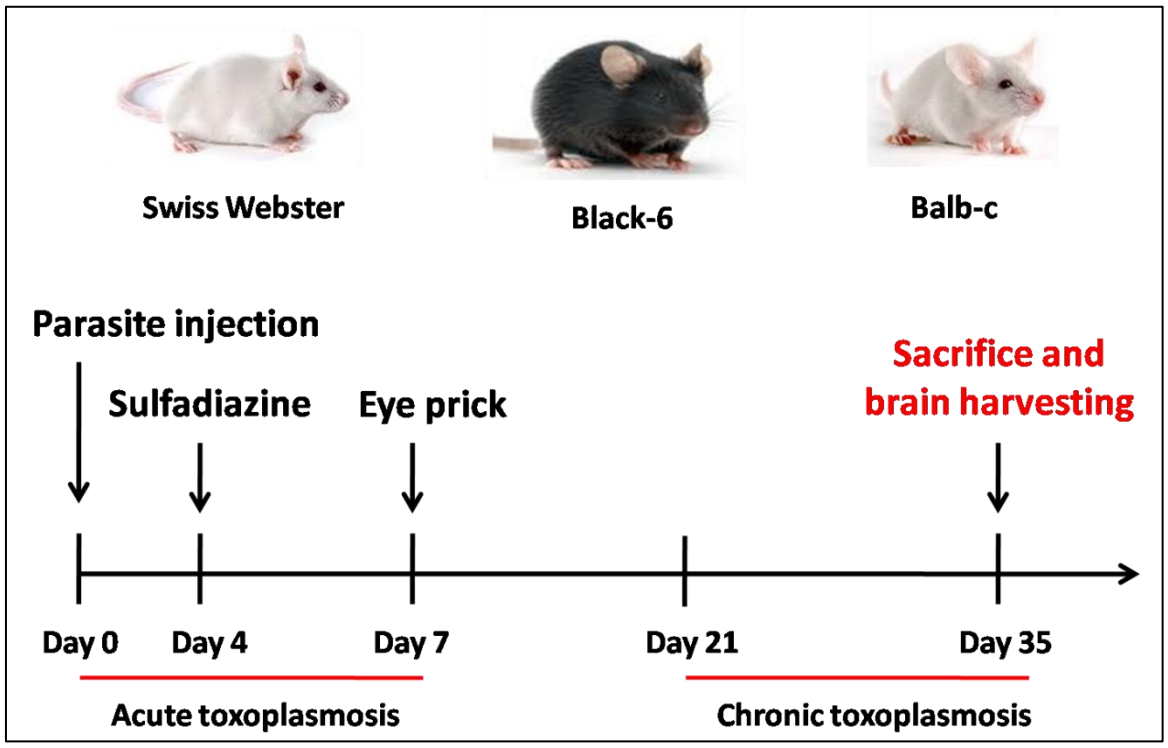

Figure 10: Timeline of the chronic infection.

3. Verification of the Number of Injected Parasites: "Plage de Lyse" or Plaque Assay

After rupture of the HFFs infected with Pru and *PruΔku80,* tachyzoites were collected, centrifuged at 1500 r.p.m for 5 minutes, washed and resuspended in serum-free medium at the concentration of 100 parasites in 200 µl per mouse for the chronic infection experiment and 1 million parasites in 200 µl per mouse for the acute infection experiment.

To ensure that the same amount of tachyzoites is injected to mice, the “plages de lyse” or plaque assay was applied. Briefly, a volume corresponding to a known number of parasites is taken from the formerly mentioned suspension. HFF cells were cultured in a 24 well plate until they become confluent. After counting 100 or 1 million parasites per injection per mouse, and after injection, a 5 fold dilution for the first dose and 20 to 100 fold dilution for the second dose were performed and used to infect preseeded confluent HFF; As a result shores/plaques of lysis (appearance of numerous tachyzoites in distinct regions) will appear, where each patch/plaque corresponds to a an initial invasion by a single parasite. Staring day 4 p.i., the number of patches per well was counted. The number of visualized patches multiplied by 5 or 20/100 corresponds to the number of injected parasites per mouse (figure 11).

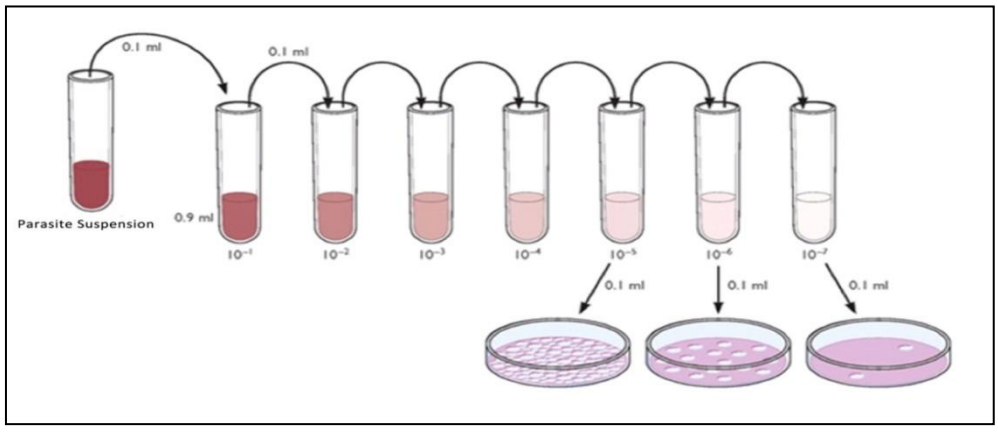

Figure 11: Plaque assay. Serial dilution of the parasite suspension which is then used to infect a confluent flask of HFF cells. The number of patches seen is multiplied by the dilution factor to know the exact number of parasites in the suspension that was initially used in an experiment. Adapted from http://virusabc.weebly.com/isolation-and-cultivation-of-viruses.html.

4. Verification of Acute Infection by Protein Gel Electrophoresis and Western Blotting

A sample of freshly collected tachyzoites of Pru *Δku80* obtained from a T25 flask was boiled in Laemmli SDS-PAGE sample buffer and separated on 12% polyachrylamide gels. Proteins were transferred to nitrocellulose membranes (BIO RAD Cat# 162-0112) at 30 Volts overnight using a BioRad transfer unit. Nitrocellulose membranes were tested for successful transfer using Ponceau Red and cut into strips that were saturated/blocked for 1 h in 5% non-fat dry milk in wash buffer (15 mM Tris-HCl (pH 8), 150 mM NaCl, and 0.05% Tween 20). On average, 100µl of blood collected from eye pricks were centrifuged at 13000 r.p.m for 15 minutes. Then strips were incubated with sera (10µl in 1ml of 5 % non-fat milk in wash buffer) from different mice overnight at 4^0C, to check for seropositivity. After washing, the strips were then incubated with HRP (Horseradish peroxidase)-conjugated secondary antibodies (anti-mouse, 1:5000 dilution, Santa cruz, sc-2031, lot: B2212) and revealed with luminol-based chemiluminescent substrate (Santa cruz, sc-048) which binds to the secondary antibody and produces illuminescence detected by autoradiography. Any mouse that didn't show seropositivity was discarded from the study.

D. Total RNA Extraction

Total RNA was extracted using the TRIzol extraction method. Briefly, 1ml of Trizol was added to the cell pellets or organs homogenate. Mixture was left for 5min at room temperature and followed by the addition of 200 µl of chloroform. Then the mixture was vortexed for 1 min, followed by incubation for 1 min at room temperature,then centrifuged at 15000g for 10 min. After obtaining a clear aqueous-organic interphase, the upper phase containing the total RNA was gently taken, transferred into a new Eppendorf

tube and precipitated by adding 0.7V isopropanol. After centrifugation at 15000g for 10 min, the pellet was washed twice with excess pure ethanol and centrifuged for 10 min at 15000g. RNA pellet was then spun down to remove excess ethanol and allowed to air-dry for few minutes to remove any excess of ethanol. The obtained precipitated RNA was resuspended in 30 µl of RNAse/DNAse Free water. RNA concentration was then quantified using the nanodrop (ND-1000) Spectrophotometer at an absorbance of 260 nm.

E. cDNA Synthesis

After quantification, 3 µg of total RNA were used to prepare cDNA in a total volume of 20 µL. Components of the cDNA synthesis kit provided by Thermo (#K1622) were added in the same order indicated in the table below (table 5) as per the manufacturer's recommendations. The mixture was gently mixed and incubated for 5 min at 25^0C followed by 60 min at 42^0C, then for 5 min at 70^0C and the reaction was stopped at 4^0C.

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 5: Components of cDNA preparation process and volumes involved.

F. Quantitative Real Time PCR (q RT PCR)

Syber green based qRT PCR was performed using the BIORAD machine (CFX96 Optics Module, Serial No. 785BR04788). SAG-1 primers (table 6) were chosen to detect and quantify the tachyzoite specific marker SAG-1 in both wild type Pru and Pru *Δku80* strains in the brain, spleen and periteoneal lavage of mice at day 4 p.i. as well as in the primary macrophages [141]. BAG-1 primers (table 6) were chosen to detect and quantify the bradyzoite specific marker BAG-1 in both wild type Pru and Pru *Δku80* strains in the brain, as described by Walker et al.[141]. iNOS, IL-12, IL-10 and IFN-γ primers (table 6) were used to study the immune resonse produced by Pru or Pru *Δku80* strains, in the brain, spleen and peritoneal lavage of mice as well as in the primary macrophages. Primers for the reference gene are directed against the mouse Glyceraldehyde-3-Phosphate dehydrogenase GAPDH (table 6) and they were used to detect and quantify GAPDH transcripts, to which all the expression results of other candidate genes were normalized as described by Walker et al.[141].

In qRT-PCR, individual reactions were prepared with 0.25 μM of each of the reverse and the forward primers, 150 ng of cDNA and SYBR Green PCR Master Mix to a final volume of 11 μl. PCR reaction consisted of a DNA denaturation step at 95°C for 3min, followed by 35 cycles (denaturation at 95°C for 15 sec, annealing at each primer's specific temperature for 60 sec, extension at 72°C for 30 sec). For each experiment, reactions were performed in duplicates and expression of individual genes was normalized to GAPDH Ct values. The Threshold cycle (Ct) corresponds to the cycle at which there is a significant detectable increase in fluorescence. ΔCt ($Ct_{\text{candidate gene}} - Ct_{\text{GAPDH}}$) was then calculated. Thereafter, ΔΔCt was calculated according to the Livak method: $2^{-\Delta\Delta Ct}$ to obtain

the percentage of expression. This method is widely used for relative gene expression analysis[142].

Primer	Sequence 5'→3'
Mouse GAPDH Forward Primer	5'-CATggCCTTCCgTgTTCCTA-3'
Mouse GAPDH Reverse Primer	5'-CCTgCTTCACCACCTTCTTgAT-3'
Mouse SAG-1 Forward Primer	5'-ACT CAC CCA ACA ggC AAA TC 3'
Mouse SAG-1 Reverse Primer	5'-gAg ACT AgC AgA ATC CCC Cg-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'

Table 6: Forward and reverse primers used in this study.

G. ELISA Experiments on Sera of Mice

The Sera of mice infected with 1 million parasites were obtained following eye bleeding at day 4 p.i. before the mice were sacrificed. These blood samples were then probed with BD biosciences ELISA kits specific for mouse IL-12 (p40) (cat# 555165) according to the manufacturer's instructions).

H. Ethic Statement

All murine protocols were approved by the Institutional Animal Care and Utilization Committee (IACUC number: #1312273) of the American University of Beirut.

All animals were housed in specific facility with a 12h ON/OFF light cycle. Animals were sacrificed by cervical dislocation after deep anesthesia with isoflurane.

I. Statistics

All *in vivo* experiments were analyzed on Microsoft Excel, presented as averages with standard deviations. Statistics were also analyzed using Microsoft Excel's two-tailed Student's t-tests to determine the statistical significance of differences observed between indicated groups for parametric comparisons. Statistical significance is reported as * for P value between 0.05 and 0.01, ** for P value between 0.01 and 0.001, and *** for P value less than 0.001.

CHAPTER III

RESULTS

A. Pru and Pru*ΔKU80* Type II Parasites Successfully Establish Acute Toxoplasmosis in Balb-c, Swiss Webster, and Black-6 Mice

During the acute phase of toxoplasmosis, tachyzoite multiplication is inhibited by the immune response. The outcome of this immunologic response to the tachyzoite results in the development of bradyzoites, the hallmark of chronic infection. Therefore, we have tested whether all mice developped immunity against the infected parasites. 100 tachyzoites of either Pru or pru*ΔKU80* strains were intraperitoneally injected into 5 Swiss Webster, Balb-c or black-6 mice. After verification of the number of injected parasites by plaque assay to ensure an equal number of injected parasites, we followed the timeline indicated in figure 10. Briefly, on day 4 p.i., mice were treated with sulfadiazine over the course of two weeks to help them overcome the acute phase of toxoplasmosis and successfully establish the chronic phase of infection. On day 7 p.i., blood sample collection using retro-orbital puncture from the Medial Cathenus of each infected mouse was performed, and served for verifying the successful acute infection by western blotting (Figure 12). In case of seropositivity against *T. gondii* infections, it was documented since as early as 1983 [143] that a complex pattern of immunoreactivity in which most of the antigens of tachyzoites (with molecular weights of 75, 70, 65, 54, 38, 31, 29, 28, 26, 24 and 6 kDa) react with serum IgG from acutely infected mammals, in our case mice. The majority of sera from all tested mice showed a complex profile of bands with a major band showing at 30 kDa

corresponding to the SAG-1 (P30) antigen; our negative control serum was taken from a none infected mouse and didn't show any reactivity with tachyzoites. We obtained few seronegative mice; these were discarded from the study.

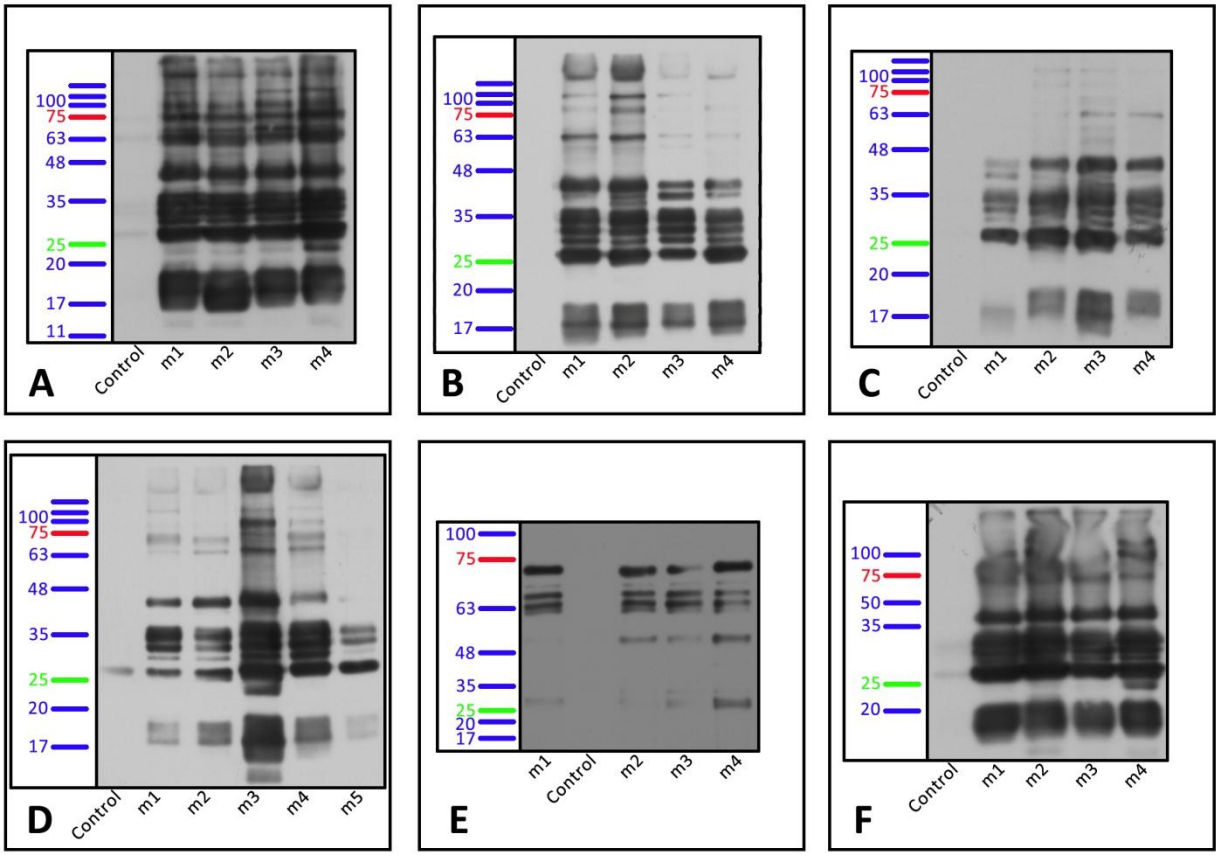

Fig 12. Verification of acute infection by western blotting. A) Swiss Webster mice infected with Pru strain and B) Pru*ΔKU80* strain. C) Black-6 mice infected with Pru and D) Pru*ΔKU80* strains. E) Balb-c mice infected with Pru and F) Pru*ΔKU80* strains. All negative control mice belong to the same strain as the experimental mice except that they were not infected with *T. gondii.*

B. Pru and Pru*ΔKU80* Type II Parasites Display a Different Phenotype upon the Chronic Phase of Toxoplasmosis and Form a Different Pattern of Cysts in Different Mice Brains

Our initial work in the lab started with a high interest in purifying and identifying new bradyzoite markers for later immunopurification and mass spectrometry analysis. This led us to first test which mouse strain is capable of producing a larger number of cysts, and which type II *Toxoplasma* strain is leading to such a higher cyst yield. We have tested 3 mouse strains (Balb-c, Swiss Webster and Black-6) and the two type II *Toxoplasma* strain Pru and its derivative Pru*ΔKU80*. Four weeks p.i., mice were sacrificed according to the IACUC standards at the American University of Beirut, and their brains were harvested for total RNA extraction. After cDNA synthesis, the bradyzoite marker BAG-1 expression levels were tested by qRT-PCR to see the capacity of each parasite strain to form cysts in the brains of the mice.

We first compared the capacity of the same type II *Toxoplasma* strain to produce cysts in the different mouse strains. Our results show that the Pru was able to form more cysts in the brain of Swiss Webster mice, where the relative expression levels of BAG-1 were hundred folds of those in Balb-c and black-6 mice (figure 13 a).

As for Pru*ΔKU80*, the highest levels of BAG-1 were obtained in the brains of Swiss Webster mice, yet closely comparable to Balb-c mice but 2 folds higher than those in black-6 mice (figure 13 b).

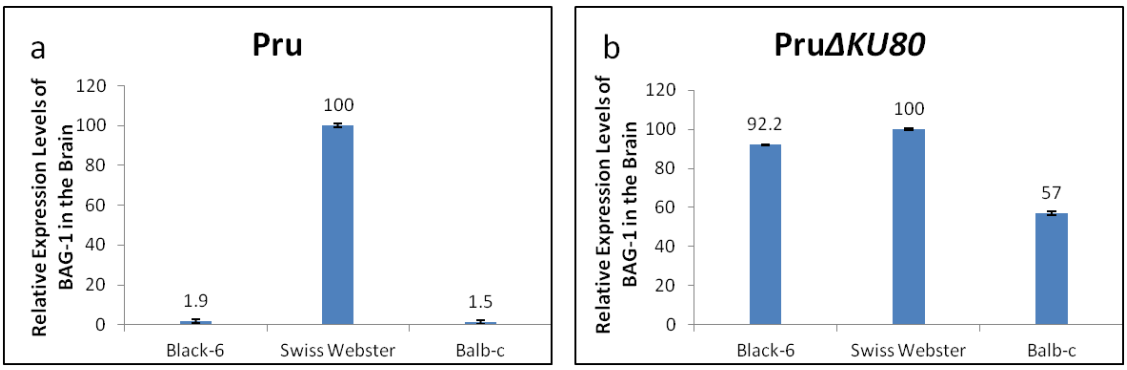

Figure 13: Relative Expression levels of BAG-1 in the brains of Black-6, Swiss Webster and Balb-c mice infected with (a) Pru parasites and (b) Pru*ΔKU80* parasites.

We then, compared in each mouse model, the capacity of both *Toxoplasma* strains to produce cysts. Our results showed that Pru parasites were capable of inducing more bradyzoite expression in brains of all tested mouse strains, when compared to Pru*ΔKU80* (figure 14a, b, c). Moreover, both strains led to comparable differences in their capacity of forming cysts in both Black-6 and Balb-c mice (Figure 14a, c). Conversely, the greatest difference was obtained in Swiss Webster mice (figure 14b). These results implied our choice of mice for the acute infection where we performed all experiments of acute infection in Swiss Webster mice and led us to decorticate the difference in immune profiles elicited by both strains since their capacity of bradyzoite formation differed in all mouse strains.

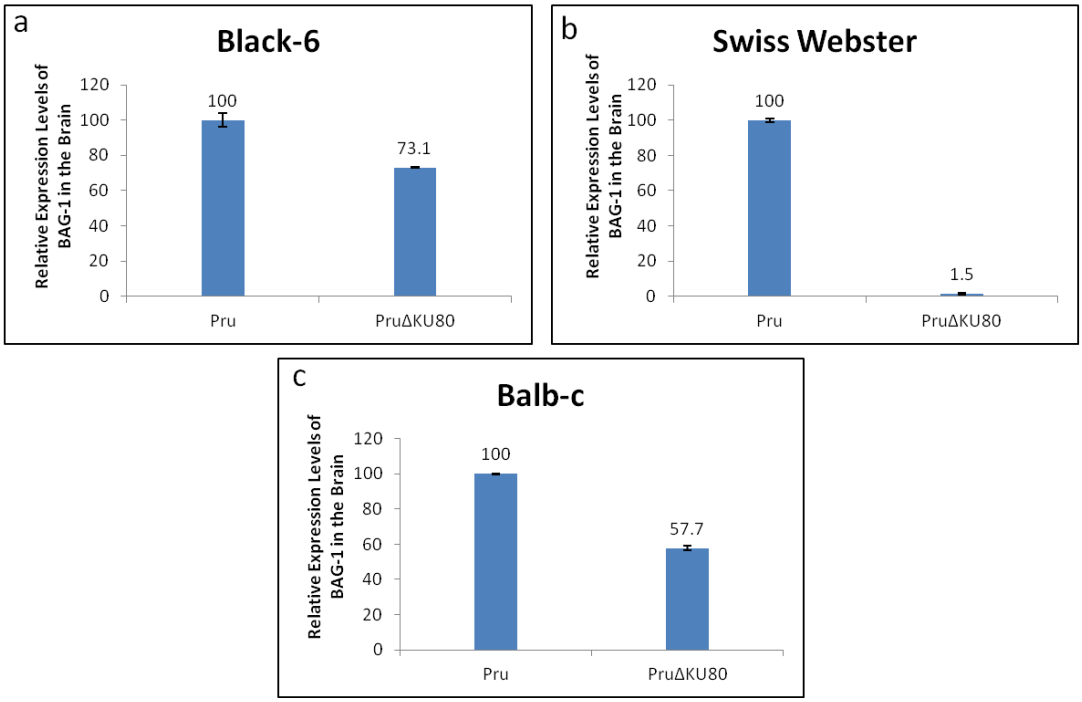

Figure 14: Relative Expression levels of BAG-1 induced by Pru compared to Pru*ΔKU80* parasites in the brains of (a) Black-6, (b) Swiss Webster and (c) Balb-c mice.

C. Pru Parasites Escape from the Peritoneum to Encyst in the Brain

Our results have clearly shown that there is a difference in the capacity of both *T. gondii* strains in forming brain cysts. This difference may be due to either a clearance of the parasites in the peritoneal macrophages, or a faster transport to the brain where they can be stimulating a different immunodulation of the microglia leading to their encystment. In order to validate which of these hypotheses is true, we investigated the immune response in brains of mice chronically infected with Pru and Pru*ΔKU80* in the three mouse strains.

Since IFN-γ is the dominant factor that enhances the ability of macrophages to destroy *T. gondii* (Yarovinsky, 2014) and its activation will provoke the regulation of toxoplasmocidal mediators such as iNOS, we measured the expression levels of IFN-ɣ and iNOS in the brains of all infected mice. Consistently with the higher amount of cysts

obtained for Pru parasites in both Black-6 (figures 15a and 15d respectively) and Balb-c mice (figures 15c and 15e respectively), Our results showed that Pru causes less production of IFN-ɣ and iNOS than Pru *ΔKU80*. This result may suggest a less toxoplasmocidal effect on Pru in the brain microglia as compared to Pru*ΔKU80*. Strikingly, and despite the enormous difference of cysts amount between the parasite strains in the brains of Swiss mice, IFN-ɣ and iNOS expression levels were very similar (figures 15b and 15e respectively) for both Pru and Pru*ΔKU80* strains. This result may be explained by a difference capacity of replication in microglia or even an earlier event of immunomodulation during the acute phase of infection.

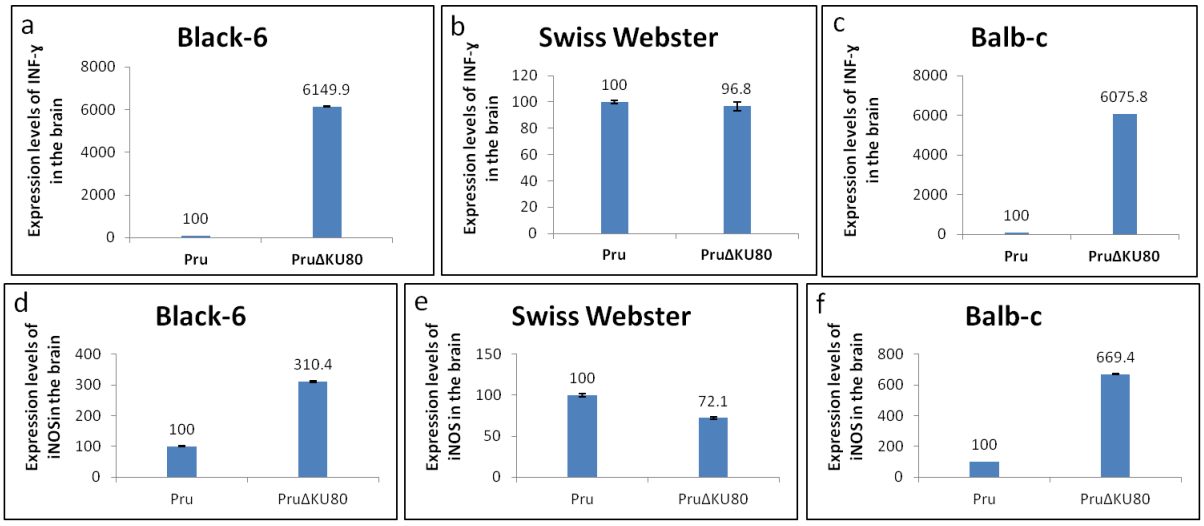

Figure 15: Expression levels of IFN-ɣ (a,b,c) and iNOS (c,d,e) in the brains of Black-6, Swiss Webster and Balb-c mice chronically infected with pru and Pru*ΔKU80*.

D. Pru and Pru*ΔKU80* Parasites Display a Different *in vitro* Tachyzoite Replication Capacity in Primary Elicited Murine Macrophages

Following our obtained results, we further investigated the replication capacity of Pru and its derived Pru*ΔKU80 in vitro* in primary elicited macrophages (PEM).

For that purpose, PEM were obtained from black-6 mice 4 days post-intraperitoneal injection after stimulation by thioglycollate. These macrophages were then infected with Pru and Pru*ΔKU80* strains of parasites at the ratio of 1 parasite/1 cell and the capacity of replication of these two strains was assessed at days 2 and 4 p.i. Using the tachyzoite marker SAG-1, we have first assessed the capacity of each strain separately to replicate inside PEM. Our results showed that there is a higher expression of SAG-1 in both strains at d4 as compared to d2 p.i. clearly showing a successful replication in PEM (Figure 16 a, b).

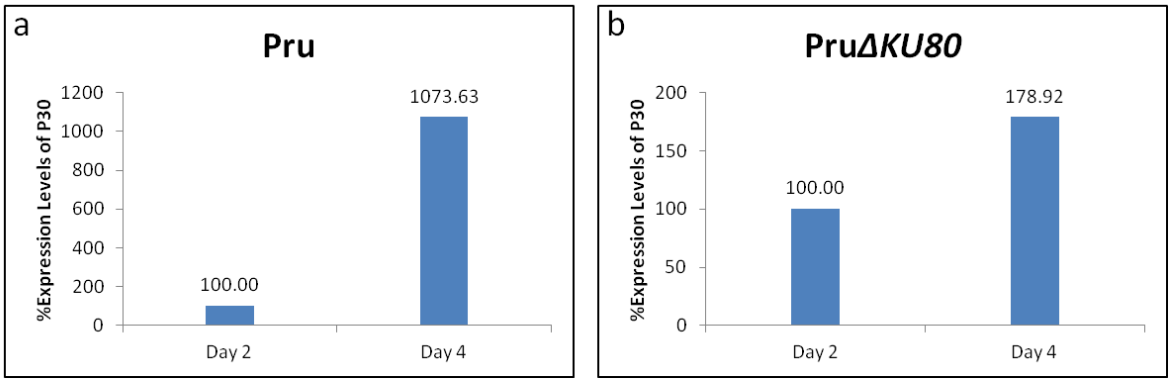

Figure 16: SAG-1 (P30) Expression levels in primary macrophages at days 2 and 4 p.i. with (a) Pru and (b) Pru*ΔKU80* strains of *T. gondii.*

But since the replication rate of Pru was much higher than that of Pru*ΔKU80*, this prompted us to investigate the difference in the capacity of these two parasite strains to induce SAG-1 expression levels at the same time points. At d2 p.i., Pru parasites have a two folds lower capacity of replication in PEM as compared to Pru*ΔKU80* (figure 17a). Conversely, on d4 p.i., SAG-1 expression levels were around 3 folds higher in PEM infected with Pru strain as compared to those infected with Pru*ΔKU80* strain (figure 17b). Furthermore, comparison of both strains over time showed that Pru parasites are responsible for the highest SAG-1 expression levels and this occurs at d4 p.i. (figure 18).

This result may imply a different immune response modulation of macrophages by these two strains leading to a better ability of these macrophages to clear Pru*ΔKU80*.

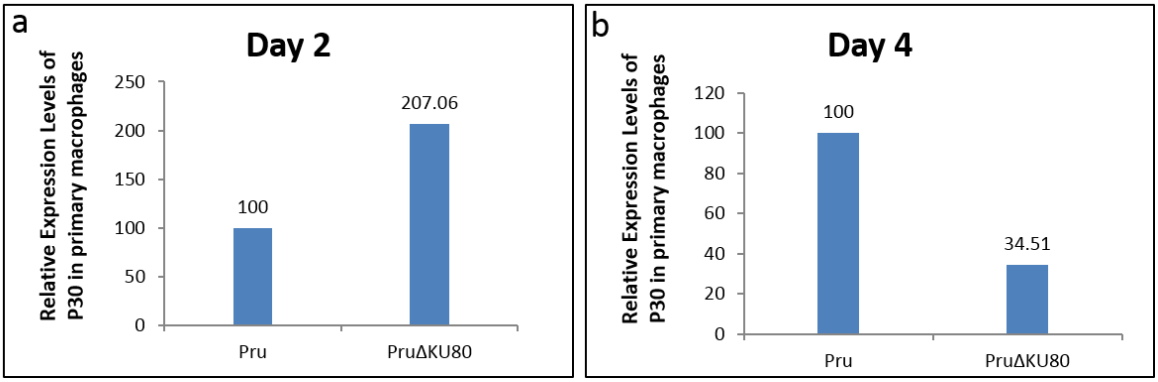

Figure 17: SAG-1 (P30) expression levels in primary macrophages infected with either Pru or Pru*ΔKU80* strains at (a) d2 or (b) d4 p.i.

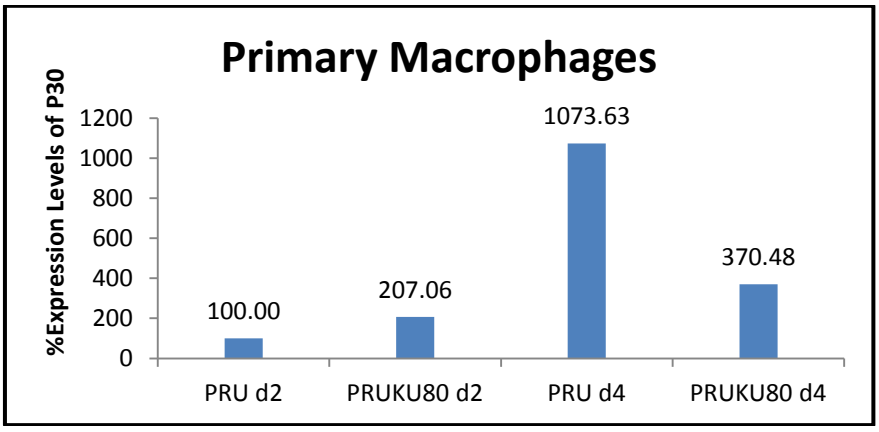

Figure 18: SAG-1 (P30) expression levels in primary macrophages by both Pru and Pru*ΔKU80* strains over time.

E. Pru Induces a Stronger Immune Response in the Peritoneal Macrophages

Having shown that both Pru and Pru*ΔKU80* can both successfully replicate *in vitro* in PEM, and that there is a difference pattern of replication over time, we went further in our analysis in checking the acute phase of infection *in vivo*. Since the peritoneal macrophages are the key vehicle cells and play a crucial role in spreading the infection to different organs, we assessed the capacity of replication and the immune modulation induced by both strains in the peritoneum of infected Swiss mice. Mice were infected intraperitoneally with 1 Million of Pru or Pru*ΔKU80* parasites. On d4 p.i., blood was collected by eye prick to test for IL-12 secreted levels by ELISA. Then mice were sacrificed, peritoneal lavage was performed, and spleens were harvested. SAG-1, IFN-ɣ, iNOS and IL-10 transcript levels were then measured by qRT-PCR.

We first assessed the differences between both strains at the level of the peritoneum. Our results have shown that SAG-1 expression levels were much higher in mice infected with Pru*ΔKU80* strain as compared to those infected with Pru strain (p value

= 0.041) (figure 19a). This result may either imply a higher number of peritoneal infected cells or a higher capacity of Pru*ΔKU80* to replicate inside these cells. For that purpose, and since *T. gondii* can infect any nucleated cell with a preference for macrophages and dendritic cells as a vehicle *in vivo*, we decided to check on the percentage of peritoneal macrophages, specially that when we perform peritoneal lavage, a big mixture of different peritoneal cells will be purified. This led us to test the levels of the Monocyte Chemoattractant Protein-1 (MCP-1/CCL2), being one of the key chemokines that regulate migration and infiltration of monocytes/macrophages. Strikingly, and despite the higher SAG-1 levels of Pru*ΔKU80* in peritoneal cells, MCP-1 levels were much higher following infection with Pru strain (Figure 19b). This result was consistent with higher levels of both IFN-ɣ (p value = 0.025) (Figure 19c), and iNOS (p value = 0.04) (Figure 19d). These results clearly show a higher number of recruited peritoneal macrophages upon infection with Pru strain and a higher stimulation of immune system at the level of the peritoneum, probably leading to a faster escape of Pru to the brain where it switches and forms latent bradyzoites. On the other hand, Pru*ΔKU80* parasites lower MCP-1, IFN-ɣ (p value = 0.025) and iNOS (p value = 0.04) (figures 19 b, c, and d) expression levels in the peritoneum could be explained by either a less vigorous immune response against these parasites or an earlier strong immune response that decreased at d4 and led to the escape of Pru*ΔKU80* to organs when most of the infection is cleared before reaching the brain where only it caused a low amount of cysts.

Since the Pru strain induced a much greater transcription of IFN-ɣ and iNOS, this could result in tissue immunopathology in mice, thus it was of great importance for us to

know if IL-10 is also increasing to lower the impact of the immune response. Consistently, IL-10 expression levels in the peritoneal lavage of mice infected with the Pru strain were much higher than those of IL-10 in case of infection with PruΔ*KU80* strain (p value = 0.04) (figure 19e).

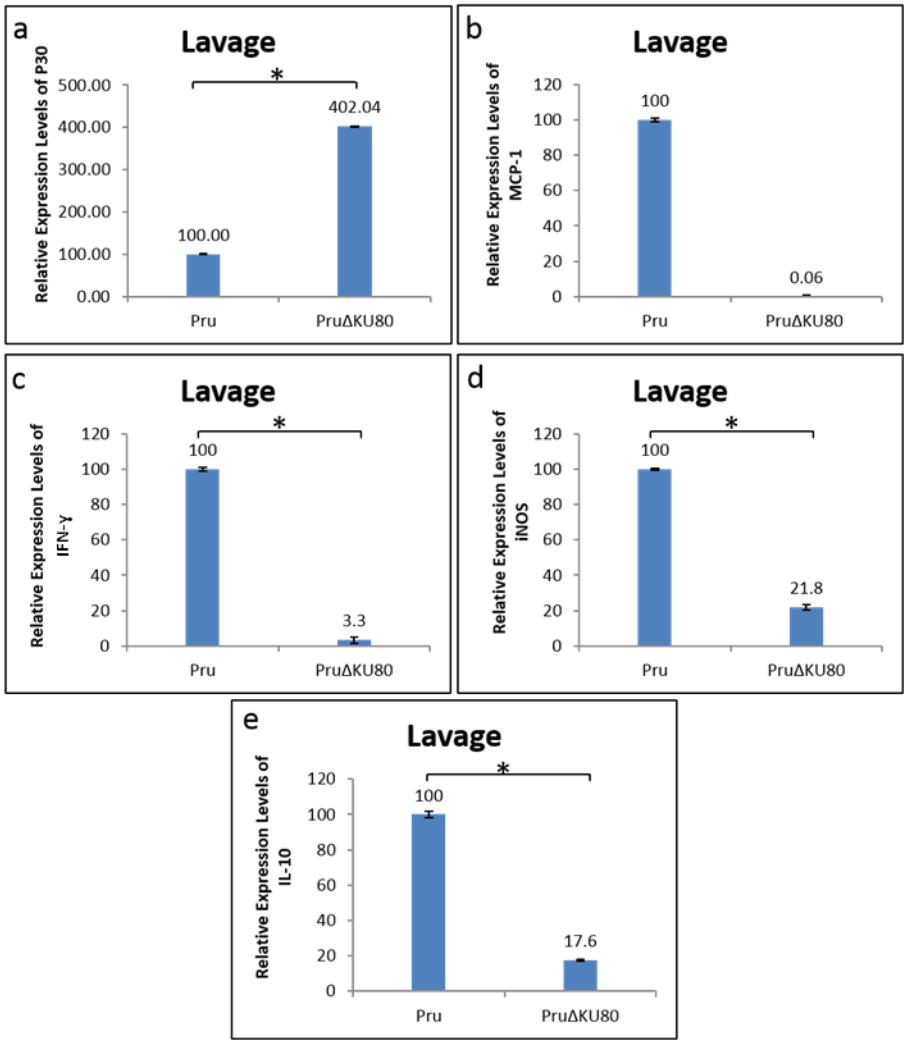

Figure 19: The expression levels of (a) SAG-1 (P30), (b) MCP1-, (c) IFN-ɣ, (d) iNOS and (e) IL-10 in the lavage of Swiss Webster mice infected with Pru and Pru*ΔKU80* parasites and sacrificed at day 4 p.i. n=5. P value: between 0.01-0.05 corresponds to *, between 0.01-0.001 corresponds to ** and less than 0.001 corresponds to *** using student's T-test.

F. Pru*ΔKU80* Induces a Stronger Immune Response in the Spleen

In order to explain our IL-10 obtained result, and in order to draw a clearer image of the difference of immune responses induced by Pru and PruΔ*KU80* strains, investigation of organ parasite burden and its corresponding immune response was tremendous. Since the spleen is a major organ involved in acute toxoplasmosis whereby spleen dendritic cells secrete high IL-12 and upon activation these cells migrate to areas highly populated with T cells and induce an even stronger response (Reis e Sousa et al., 1997), we decided to check on parasite burden and corresponding immune response in spleens of infected mice with either Pru or Pru*ΔKU80* parasites.

Importantly, PruΔ*KU80* strain induced a 300 fold increase of SAG-1 levels in the spleen as compared to the Pru strain (p value = $8.95*10^{-8}$) (figure 20a). Consistently, IFN-ɣ (figure 20b) and i-NOS (figure 20c) levels were also elevated in the case of infection with the PruΔ*KU80* strain compared to the spleens of mice infected with the Pru strain (p value of IFN-ɣ = $2.86*10^{-5}$; p value of iNOS = $1.12*10^{-6}$). This could suggest the clearance of most of Pru*ΔKU80* in the spleen before even reaching the brain to form cysts.

Conversely, IL-10 spleen expressed levels were increased in case of PruΔ*KU80* infection potentially to decrease the immunopathology of this organ (p value = 0.000375) (figure 20d).

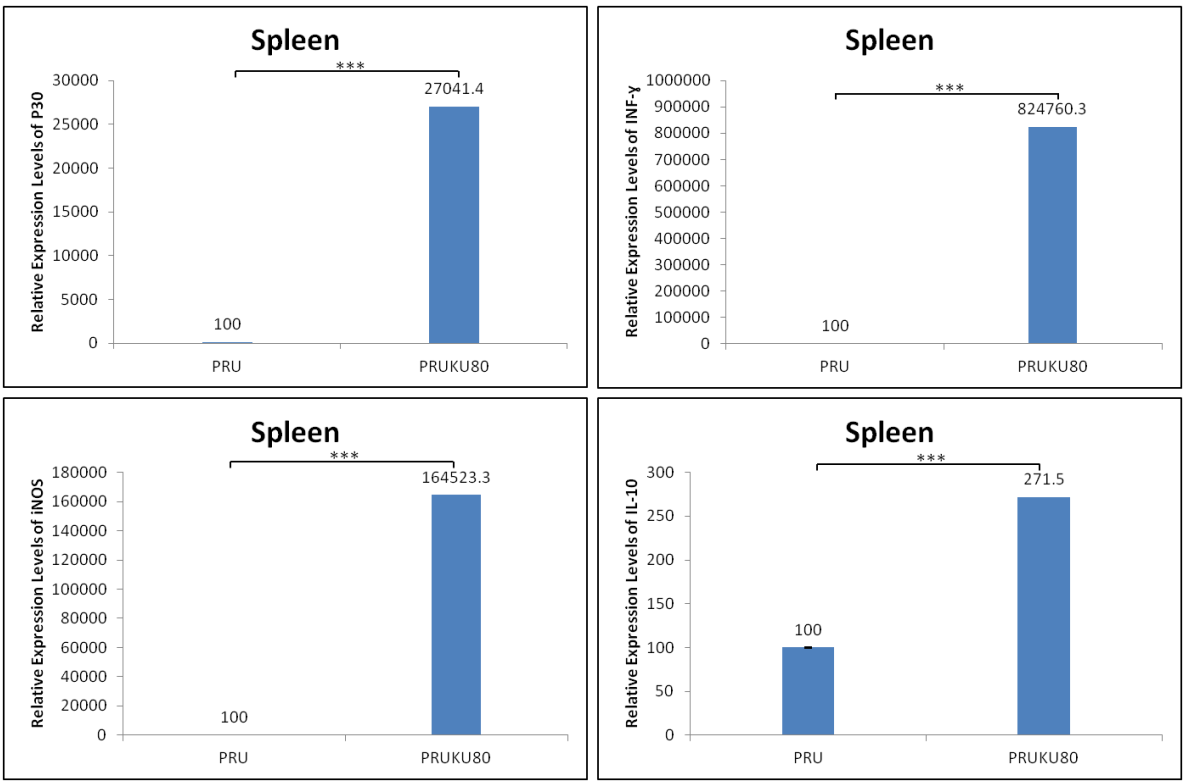

Figure 20: The expression levels of (a) SAG-1, (b) IFN-ɣ, (c) iNOS and (d) IL-10 in the spleens of Swiss Webster mice infected with Pru and Pru*ΔKU80* parasites and sacrificed at day 4 p.i. n=5. P value: between 0.01-0.05 corresponds to *, between 0.01-0.001 corresponds to ** and less than 0.001 corresponds to *** using student's T-test.

G. Pru and Pru*ΔKU80* Produce Similar Levels of Secreted IL-12

Toxoplasmosis events are characterized by an excess of production of IL-12 that might eventually lead to the control of the infection by promoting the ability of NK-cells and T-cells to make IFN- ɣ (Denkers *et al.*, 1993; Hunter *et al.*, 1994; Scharton-Kersten i 1996, 1998). This cytokine can be produced by a variety of cells of the immune system such as neutrophils, dendritic cells, and macrophages as well as inflammatory monocytes in response to *T. gondii* infection (Lieberman *et al.*, 2004).

In order to check for IL-12 levels, we performed ELISA experiments on the sera of mice infected with Pru and Pru*ΔKU80* strains (figure 21). Although our results showed that

IL-12 concentrations were higher in the sera of mice infected with Pru*ΔKU80* than those of mice infected with Pru, the levels of secreted IL-12 were very close (p value = 0.007). This result may be due to our observation at d4 p.i. which is considered a late time point following acute infection. An investigation of IL-12 secreted levels at earlier time points in mice may lead to a better understanding and better correlation of IL-12 with our IFN-ɣ results.

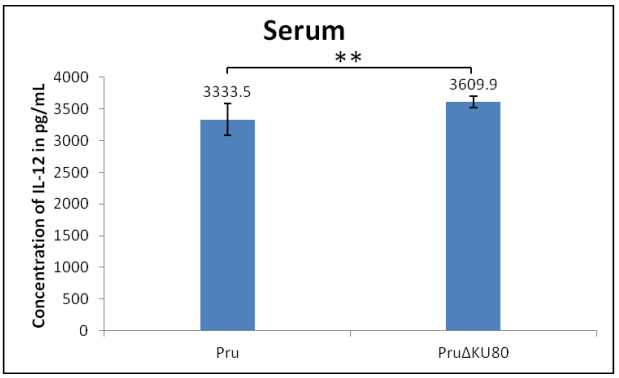

Figure 21: Concentration of secreted IL-12 in the sera of mice infected Pru compared to those infected with Pru*ΔKU80*. n=5. P value: between 0.01-0.05 corresponds to *, between 0.01-0.001 corresponds to ** and less than 0.001 corresponds to *** using student's T-test.

CHAPTER IV

DISCUSSION AND FUTURE PERSPECTIVES

For decades, tachyzoite-bradyzoite interconversion has been a crucial event in the life cycle of *T. gondii* and a major challenge in immunocompromised individuals. Reactivation of chronic toxoplasmosis can have severe and potentially lethal impacts upon immunosuppression. Since type II strains are the mostly isolated strains from patients [83], we focused on these strains to pursue our studies on bradyzoite markers. As a first step, we investigated the type II parasite strains, Pru and Pru*ΔKU80*, and the mice strains (Black-6, Swiss Webster and Balb-c) to see which model is the best for cysts induction in the brain during chronic toxoplasmosis. On another hand, *Toxoplasma* is an excellent model for studying host/parasite interaction and a model parasite to study other apicomplexan members, mainly *Plasmodium falciparum*. The Pru*ΔKU80* was generated from the Pru strain [92] and was shown to be an excellent genetic model for Knock-out genes and study their function. This reason was behind the Pru*ΔKU80* strain. Although both belong to type II strain, and one derives from the other, our results have clearly shown that Pru and Pru*ΔKU80* display a different capacity to form cysts in the brains of 3 different mouse strains (Balb-c, Swiss Webster and Black-6 mice). This led us to investigate similarities and differences between two largely adopted models and key laboratory tools for understanding both acute and chronic phases of toxoplasmosis at the cellular level, host/parasite immune modulation and even understanding not only toxoplasmosis but other apicomplexan pathologies. The intriguing results we obtained on cyst yields, led us to assess in depth the

differences between both strains and to verify the host immune modulation following infection.

Type II strains can activate a very effective early response, as reviewed in [12]. The dense granule protein GRA-15, when expressed as an active isoform, as is the case in the type II strains, causes excessive activation of NF-κB in the infected cells [123], the antigen presenting cells, that leads to an early surge in IL-12, IL-18 and IL-1β which in turn cause an overproduction of INF-γ by T helper and Natural Killer cells [78]. These high levels of INF-γ are essential for acute and chronic infection stages control in the murine model [124, 125]. IFNγ effects are many on infected cells (figure 9) resulting in parasite replication reduction through the induction of the expression of inhibitory proteins such iNOS (GBPs) [127].These well known studied and documented iummuno-modulators led us to seek for the reason behind such cyst formation differences between a type II Pru strain and its derivative Pru*ΔKU80*. This difference in cysts induction in the brain can be explained by a defect of Pru*ΔKU80* replication, a better clearance of the parasites at the level of the peritoneum and/or infected organs mainly the spleen where the infection spreads or a greater capacity of clearance of this strain in the brain by its microglia. IFN-γ is the dominant factor that enhances the ability of macrophages to destroy *T. gondii* [127] and its activation will provoke the regulation of toxoplasmocidal mediators such as iNOS. Consistently with the high levels of BAG-1 in Balb-c an Black-6 mice infected with Pru, IFN-ɣ and iNOS levels were higher in the brains of all infected mice. This result may suggest a less toxoplasmocidal effect on Pru in the brain microglia explaining the higher cyst amount. In Swiss mice, and despite the enormous difference of cysts in the brains,

IFN-ɣ and iNOS expression levels were very similar for both Pru and Pru*ΔKU80* strains, potentially suggesting a difference in microglia immunomodulation or even a consequence of an earlier event of immunomodulation and may be clearance of the parasites either in peritoneal or tissue macrophages/dendritic cells during the acute phase of infection.

We first examined the replication capacity of both strains in the peritoneal cells and showed that Pru*ΔKU80* parasites have a greater ability to replicate. But our results SAG-1 couldn't be conclusive if the replication difference was happening at the level of peritoneal macrophages. Knowing that the *T. gondii* parasites highjack the macrophages, we investigated MCP-1 levels. We showed that Pru parasites recruit more macrophages to the peritoneum. Whether these macrophages will exert a microbicidal effect or serve as vehicle was yet to be identified. We therefore tested for IFN-γ and iNOS and seen higher levels of both upon acute infection with Pru strain. These results suggest a higher immune modulation that may cause the escape of Pru parasites to the brain where they can hide after interconversion into bradyzoites. The lower MCP-1 expression levels consistent with the lower IFN-γ and iNOS levels in the lavage of Pru*ΔKU80* infected mice could be explained by a either a less vigorous immune response against them or an earlier response that led to their clearance in the peritoneum, their escape to organs where they can also be cleared before even reaching the brain. This latter option was consolidated by the higher amounts of both IFN-γ and iNOS in the spleens of mice infected with Pru*ΔKU80*, suggesting a role of the spleen macrophages in clearing the infection. Since IFNγ and IL-12 are inter-related cytokines and IL-12 is an upstream regulator of IFN-γ production by macrophages, and since this later is known to induce higher levels of IL-12 from Th1 cells [12], we

investigated the levels of IL-12 on both transcriptional and secreted levels. We faced some technical problems with IL-12 transcripts, however, secreted IL-12 in the sera of mice infected with Pru*ΔKU80* were slightly higher than those of mice infected with the Pru strain. Knowing that IL-12 can be involved upon early infection with *Toxoplasma* to activate macrophages [120], our results at day 4 may not reflect the potential IL-12 levels produced against Pru and Pru*ΔKU80*. Earlier time points should be investigated.

In conclusion, understanding the differences between the two type II strains Pru and Pru*ΔKU80* is of major benefits to the field of *Toxoplasma* research especially that these strains are extensively used in research and type II strains in general are abundantly isolated from patients. Understanding these key immunomodulation capacities of these parasites and more research on the immune modulation of these parasites could open the door for better tailoring treatments such as in the case of immunocompromised patients so that the chances of survival of terminally ill individuals are enhanced.

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