

PARASITES AND PARASITIC DISEASES

Toxoplasma gondii

BIOLOGY AND ROLE
IN HEALTH AND
DISEASE

Erica S. Martins-Duarte, PhD • Daniel Adesse, PhD

Editors

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PARASITES AND PARASITIC DISEASES

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TOXOPLASMA GONDII

BIOLOGY AND ROLE IN HEALTH AND DISEASE

ERICA S. MARTINS-DUARTE

AND

DANIEL ADESSE

EDITORS



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DOI: <https://doi.org/10.52305/ZQKT5678>

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Additional color graphics may be available in the e-book version of this book.

Library of Congress Cataloging-in-Publication Data

ISBN: 978-1-68507-291-9 (e-book)

Published by Nova Science Publishers, Inc. † New York

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PREFACE

Toxoplasma gondii is a globally spread protozoan parasite responsible for causing toxoplasmosis. Infection by *T. gondii* shows high seroprevalence in humans, especially in South America. Written by specialists, this book brings important contributions and information on parasite biology, including its life cycle, ultrastructural features of tachyzoite, bradyzoite and their main specific organelles, mechanisms of host cell invasion, parasite replication and epigenetics regulation. Moreover, the book will encompass mechanisms of pathogenesis and host immune response to the infection, including purinergic signaling, and mechanisms of immune evasion by the parasite. Finally, this book also addresses relevant aspects of disease, information about the current chemotherapy of toxoplasmosis and perspectives for a future chronic disease treatment, epidemiology, and methods for visualization of the parasite by optical and electron microscopy.

Chapter 1 - The protozoa *Toxoplasma gondii* is the etiological agent of toxoplasmosis. The worldwide prevalence of *T. gondii* can be explained by its capacity to infect any warm-blood host. It is a heteroxenous parasite infecting the definitive host (felids) and several intermediate hosts, including humans. During its life cycle, the parasite differentiates into sexual and asexual forms. The sexual phase happens only on the felids, generating the production of oocysts that contaminate the environment, and the asexual phase corresponds to the multiplication of parasites in the intermediate hosts. The disease is usually asymptomatic in a healthy host, but severe clinical manifestations occur in immunodeficient hosts or when the parasite is transmitted to susceptible hosts or the fetus during pregnancy. *T. gondii* belongs to the phylum Apicomplexa presenting an apical complex composed of specific secretory organelles. Those organelles have an active role in the invasion of host cells. In this chapter, the authors provide a brief history of how the parasite was discovered as well as an overview of the important aspects of the parasite's infection during its life cycle.

Chapter 2 - Among the three infective stages of *Toxoplasma gondii*- tachyzoites, bradyzoites and sporozoites- the ultrastructure of tachyzoites, the stage characteristic of the acute phase of toxoplasmosis, is the best known and was the first to be described. The multiple instruments and techniques involving electron microscopy were, and still are, key tools in reaching the present knowledge on it. Starting with the first images obtained by Meyer and De Mendonça, a considerable number of information on *T. gondii* ultrastructure was achieved and compiled in a classic review by Dubey et al. The ultrastructure of *T. gondii* developmental stages can be considered a 'work in progress' since this early picture has been

refined since then, both concerning the ultrastructure and the functional dynamics of this organism.

Chapter 3 - *Toxoplasma gondii* is a successful intracellular parasite for developing effective motility mechanisms, invasion, intracellular growth, and egress to infect other cells, avoiding all of these steps being destroyed by the host. Regarding the mechanisms of entrance into the cells, the most studied is one directed by the parasite through a process known as an active invasion. This process depends on gliding motility and comprises the secretion of molecules from the parasite's secretory organelles, such as the micronemes and rhoptries, which will intermediate the invasion. This mediation occurs by the moving junction creation, a transient structure containing proteins secreted by *T. gondii*, and other molecules' participation from the host cell. The junction protects *T. gondii* in the parasitophorous vacuole in the process of assembling, excluding its membrane molecules that would address it to the fusion with the host lysosomal system. If the parasite's interiorization into the cell is active, it could develop normal replication by endodyogeny, forming rosettes throughout the lytic cycle. The parasite may enter the cell by mechanisms triggered by the host cell, such as phagocytosis and distinct endocytic processes, such as macropinocytosis and clathrin-mediated endocytosis, where some participation of the parasite also occurs. The fate of parasites that have been internalized the cell through endocytic processes, such as by phagocytosis, is, in general, the elimination by different intracellular mechanisms. Thus, the infection is not successful. After the parasite entry, the parasitophorous vacuole establishes close to the host cell organelles contributing to its growth. Inside the vacuole, *T. gondii* can secrete molecules that prevent the host elimination mechanisms, such as the occurrence of a classic autophagy pathway or the vacuolar membrane's disruption, thus giving course to infection in the cells. Such molecules may also turn the PV membrane-permeable through cytoplasmic molecules.

Chapter 4 - *Toxoplasma gondii* is an intracellular protozoan parasite. A key event for the longtime persistence of the parasite in a host is its ability to convert from tachyzoites, the fast-dividing form, into bradyzoites, the slow-dividing form, under the pressure of host immune response. *T. gondii* cysts are formed mainly in the Central Nervous System (CNS) during the transition from acute to the chronic phase of infection. Bradyzoites secrete glycoproteins to form the cyst wall and the intracystic matrix, markedly modifying the parasitophorous vacuole. In this chapter, the authors review the main ultrastructural features of the cyst and the process of cystogenesis, as well as the strains and mechanisms capable to favor or induce cyst formation *in vitro* and *in vivo*.

Chapter 5 - Cell division is fundamental to survival. It allows species to prevail, and its regulatory mechanisms underlie a plethora of fundamental biological processes ranging from evolution to disease. For apicomplexan parasites, cell division also constitutes a mechanism of pathogenesis. New cells emerge, invasion competent, from the infected host cell, ready to invade neighboring healthy cells. The asexual generation of progeny entails the multiplication and segregation of genetic material from a mother cell and its packaging into a variable number of daughter cells. How different cell types regulate the temporal and physical coordination of these processes, and their output, varies widely in nature. In this chapter, the authors will describe how *Toxoplasma gondii*, the so-called "model" apicomplexan organizes cell division, as an example of the remarkable adaptability and divergence of cell division in this phylum. The authors will describe the asexual cell division mechanism used by one of the clinically relevant life forms of the parasite; the tachyzoite. This process, known as

endodyogeny, has been described in exquisite structural detail, and more recently, new insight into the regulatory mechanisms has emerged. This chapter will focus on the factors triggering the onset of cell division and on the structural aspects underlying the sequential and hierarchical building of a new *T. gondii* cell.

Chapter 6 - How chromatin structure changes, allowing genetic information get accessed by different proteins is a key question to understand gene expression in all eukaryotes. In the *Toxoplasma* genome, an extensive repertoire of chromatin remodelers and histone modifiers is present, representing essential pieces to understand epigenetic regulation in this parasite. In addition to unique transcriptional factors, this epigenetic machinery represents a complex regulatory mechanism that must be further explored. Epigenetic regulation is represented essentially by three pillars: histone post-translation modification (PTMs), non-coding RNAs and DNA methylation. Mapping of all the histone PTMs in *Toxoplasma* revealed shared and unique modifications, including at least ten different types of modifications. The numerous potential combinations of PTMs are called the “histone code” and can trigger or inactivate several essential processes by either interfering with DNA accessibility or by recruiting chromatin remodeling complexes. Among the identified modifications, histone acetylation was remarkably abundant. Reversible acetylation/deacetylation has been explored as drug targets due to their role in gene expression control. So far, the available data reveals that *T. gondii* possesses a complex and robust histone code and understanding the code and the enzymes responsible for the modifications could reveal new key elements to elucidate essential processes and identify promising new targets to more efficient treatments.

Chapter 7 - *Toxoplasma gondii*, the causative agent of toxoplasmosis, is an obligate intracellular parasite with global distribution. The isolated strains of this parasite have a population structure with clonal and genetic diverse lineages reflecting their location and capacity to infect different warm-blooded vertebrate hosts resulting in distinct virulences. *T. gondii* actively penetrates virtually any nucleated cell and establishes an intracellular replicative niche named the parasitophorous vacuole. During host cell invasion, the parasite evades many microbicidal systems of the host cell. Some of these evasion mechanisms are based on virulence factors secreted from specialized secretory organelles, which, depending on the strain virulence and the host, are important for circumventing the host cell-autonomous immunity. *T. gondii* alters host cell signaling, evades the production of reactive oxygen species and nitric oxide, IRG and GBPs (GTPases that compose the microbicidal defense system in rodents) and autophagy. These microbicidal systems and the molecular mechanisms that regulate their evasion will be presented.

Chapter 8 - Purinergic signaling was first characterized as the one mechanisms responsible for neurotransmission in non-noradrenergic, non-cholinergic nerves. Since then, two families of purinergic receptors were cloned in most tissue and organs: the P1 family (receptors for adenosine) and the P2 family (receptors for purine or pyrimidine nucleotides). Purinergic receptor activation is involved in intracellular signaling cascades that trigger cellular events such as calcium, sodium, and potassium currents, transcription factor activation, induction of motility, cell growth, cell death, production of inflammatory mediators, and microbicidal activity. The contribution of purinergic signaling to the parasite-host interaction was investigated during infection by pathogens such as bacteria, viruses, and protozoa. In the last 10 years, the activation of purinergic signaling has been proposed as an essential component of the immune response to infection by *Toxoplasma gondii* in both cellular and systemic contexts of the infection. In this chapter, the authors discuss the

participation of P2 receptors in the control of *Toxoplasma gondii* infection in hematopoietic and non-hematopoietic cells (*in vitro*), in human toxoplasmosis, and in murine models of experimental toxoplasmosis (*in vivo*).

Chapter 9 - In this chapter, the authors will approach several known mechanisms used by *Toxoplasma gondii* to disseminate throughout the host. Three major mechanisms are discussed: i) paracellular gliding movement; ii) leukocytes hitchhiking parasites and iii) Trojan horse dissemination. The authors show herein accumulated evidence of the involvement of the host's metalloproteinases of extracellular matrix and serine proteases from the uPA/uPAR pathway, gathered by the authors' group in the last decades. The authors also show data of *in situ* expression of matrix metalloproteinases and extracellular matrix metalloproteinase inducer, CD147, at the site of entry and early dissemination of *T. gondii* on acute experimental murine oral infection. The presence of circulating zymogen and active forms of MMP-2 and MMP-9 after oral infection of C57BL/6 and C57BL/6 IFN γ ^{-/-} with *T. gondii* was analyzed. Both forms of MMP-9 and MMP-2 were significantly increased in the serum after 7 days of infection. However, only MMP-9 was detected at the site of entry in infected mice, the ileum, while expression of MMP-2 was faintly detected in the absence of IFN γ . *T. gondii* infection induced the expression of MMP-9 at the lungs during its early dissemination. Expression of CD147 at the ileum and lungs was also increased upon infection. Interestingly, the authors also detected CD147 at the brain of infected mice, at the proximity of parasite foci. To the authors' knowledge, this is the first demonstration of MMP-9 and CD147 expression during *in vivo* oral infection with *T. gondii* at sites of early dissemination of the parasite and even at the brain. These host molecules are known to be involved in the metastasis of several types of cancer and are used as biomarkers of poor diagnosis of cancer progression. The authors suggest that *T. gondii* infection-prone infected cells modify their pericellular space to facilitate parasite dissemination throughout the host, taking advantage of cells from the immune system to reach the brain and retina, crossing crucial hosts' barriers.

Chapter 10 - *Toxoplasma gondii* is a worldwide-occurring parasite that infects all nucleated cells and numerous hosts. Among the different tissues infected by this parasite, muscle tissue requires special attention, as it is critical for *T. gondii* host permanence and transmission. However, beyond simply comprising a route for tissue cyst consumption, muscle cell interaction with this parasite is relevant for tachyzoite to bradyzoite stage conversion. On the other hand, *T. gondii* infection can cause myositis, both in immunocompetent or immunocompromised hosts, as well as malformations in congenitally infected embryos and post-natal myogenesis and tissue regeneration down-regulation or interference. The muscle-*T. gondii* interaction is unique, and this chapter aims to describe this important biological event in a context of intracellular *T. gondii* parasite development.

Chapter 11 - The research for a treatment for toxoplasmosis started after *Toxoplasma gondii* was recognized as a human pathogen in 1939. Several studies with different sulfonamides identified sulfadiazine as one of the most effective in mice models of toxoplasmosis. Soon later, studies with a series of 2:4-diaminopyrimidines identified pyrimethamine (Daraprim®) as the most effective drug for the treatment of toxoplasmosis so far studied. However, the most important discovery in the search for an effective treatment for toxoplasmosis came from the demonstration of the synergistic effect of sulfadiazine and pyrimethamine against *T. gondii*, providing then the first effective therapy for the treatment of this disease in humans in 1954. Currently, even after the introduction of other drug options

for the treatment of toxoplasmosis, the combination of sulfadiazine and pyrimethamine remains the first-option therapy for this disease. This chapter will present the drugs and discuss the current therapies recommended for the treatment of different manifestations of toxoplasmosis.

Chapter 12 - It is estimated that one-third of the world population is chronically infected with *Toxoplasma gondii*. Even after 80 years of the conclusive identification of *T. gondii* as an important human pathogen, there is no available treatment for the chronic phase of toxoplasmosis. In general, chronic infection is considered harmless and without risk for most immunocompetent individuals, however, it is a major concern in immunocompromised patients due to the potential emergence of serious outcomes, such as toxoplasmic encephalitis. Indeed, recent evidence point that, even in immunocompetent individuals, the parasite persistence in the host organism leads to relevant negative changes in the central nervous system (CNS) functioning. The growing concern that both immunocompromised and immunocompetent individuals may suffer the deleterious effects of long-lasting *T. gondii* infection, makes the search and discovery of active drugs for the treatment of chronic toxoplasmosis necessary. This chapter will present the advances and improvements in experimental models *in vitro* and *in vivo* used in the discovery of compounds with activity against encysted bradyzoites, and highlight targets and compounds recently identified as promising for the development of future treatment for latent toxoplasmosis.

Chapter 13 - In this chapter, the authors will present data about the prevalence of toxoplasmosis in South America. Toxoplasmosis is an important public health problem worldwide, affecting one-third of the world population. A high prevalence of infection by *Toxoplasma gondii* is found in South America. Besides numerous outbreaks of acute toxoplasmosis also occur in South America. In this region, the infection incidence is higher in low socioeconomic status and education individuals and may be related to greater exposure to the parasite, reduced access to health services, and delays in acquiring appropriate treatment for infection control.

Chapter 14 - The availability of combined antiretroviral therapy reduced the incidence of cerebral toxoplasmosis in people living with HIV/AIDS (PLWHA). However, cerebral toxoplasmosis continues to be the most common cause of expansive brain lesions and to cause high morbidity and mortality in PLWHA, particularly from low and middle-income countries. The typical manifestations are focal sub-acute neurological deficits and ring-enhancing brain lesions with a predilection for the corticomedullary junction, basal ganglia and thalami. However, the spectrum of clinical and neuroradiological manifestations is broad. Pyrimethamine-based regimens and trimethoprim-sulfamethoxazole (TMP-SMX) present similar efficacy but TMP-SMX shows potential practical advantages. This chapter reviews current literature on cerebral toxoplasmosis and emphasizes on host immune responses to the parasite antigens; host immunosuppression in HIV and cerebral toxoplasmosis; advances in diagnostic methods; and the main issues about clinical and radiological manifestations and treatment.

Chapter 15 - Toxoplasmosis is the most important and frequent cause of blindness secondary to inflammatory retinal diseases. *T. gondii* infects up to a third of the world's population and is responsible for the majority of infectious uveitis (intraocular inflammation) cases. In some countries, up to 50% of all cases of posterior uveitis in a given population are attributable to toxoplasmosis. Toxoplasmic retinochoroiditis is often seen in settings of congenital or postnatally acquired disease as a result of acute infection or recurrence. The

retina is the primary site of *T. gondii* infection in the eye, but the choroid, vitreous and anterior chamber are also involved. The population structure of *T. gondii* is highly clonal. There are three predominant clonal lineages, namely I, II, and III found in Europe and North America, but the genetic makeup of *T. gondii* is more complex than this three-strain population, and unusual genotypes, known as atypical, may contribute to various clinical outcomes of toxoplasmosis in different localities. An effective vaccine against human *T. gondii* infection is a desirable but elusive target, and therefore, toxoplasmosis will remain an essential blinding disease for many years to come.

Chapter 16 - From simple slide observation to cutting edge methods (e.g., electron tomography and super-resolution microscopy), Imaging and Image Analysis are important steps in any research project. The use of light and electron microscopy techniques have been used extensively to study *Toxoplasma gondii* cell biology and its interaction with the host cell, leading to recent discoveries and characterizations. Here the authors will discuss these techniques and how they have been applied in the *Toxoplasma* field. The authors will also discuss sample preparation and Image Analysis set-ups. The authors will conclude by presenting newly developed approaches that they think are the new areas that will be important for novel discoveries.

Chapter 1

***TOXOPLASMA GONDII*: INTRODUCTION TO PARASITE LIFE CYCLE AND DISEASE**

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ABSTRACT

The protozoa *Toxoplasma gondii* is the etiological agent of toxoplasmosis. The worldwide prevalence of *T. gondii* can be explained by its capacity to infect any warm-blood host. It is a heteroxenous parasite infecting the definitive host (felids) and several intermediate hosts, including humans. During its life cycle, the parasite differentiates into sexual and asexual forms. The sexual phase happens only on the felids, generating the production of oocysts that contaminate the environment, and the asexual phase corresponds to the multiplication of parasites in the intermediate hosts. The disease is usually asymptomatic in a healthy host, but severe clinical manifestations occur in immunodeficient hosts or when the parasite is transmitted to susceptible hosts or the fetus during pregnancy. *T. gondii* belongs to the phylum Apicomplexa presenting an apical complex composed of specific secretory organelles. Those organelles have an active role in the invasion of host cells. In this chapter, we provide a brief history of how the parasite was discovered as well as an overview of the important aspects of the parasite's infection during its life cycle.

1. BRIEF HISTORY OF PARASITE DISCOVERY

The years between 1910 and 1987 were marked by the description of important protozoa causing human diseases, thanks to the examination of tissues from sick animals using optical microscopy for diagnosis purposes (Souza and Belfort Jr. 2014). That was also the case of the

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parasite *Toxoplasma gondii* identified for the first time in 1908 by scientists working with *Leishmania* or viruses after examination of different tissue sections from infected animals. However, the parasite was not named *T. gondii* “at first glance”. The history of *T. gondii* started when two different groups described it as a similar organism found in rabbit, *T. cuniculi*, from Brazil (Splendore 1908) and hamster-like rodents, *Ctenodactylus gundi*, from North Africa (Nicolle and Manceaux 1908). It was of special interest because the publications offered proof that the organism was present on two different continents. Splendore published a report on the Journal of Scientific Society of São Paulo introducing a new organism without naming it, while Nicolle and Manceaux described it as *Leishmania* (or similar organism) during investigations in the laboratory of Charles Nicolle at the Pasteur Institute in Tunis. Only after a while, the researchers realized it was indeed a new organism, and new reports confirming the discovery of a novel pathogenic protozoa species were published (Splendore 1908; Nicolle and Manceaux 1908; Ferreira and Vitor 2014). For years, there were discussions on who should have the merit for the first description. Currently, there is an agreement that both groups deserve recognition for the discovery. This decision was made during an international Symposium held in Rio de Janeiro, September of 2008 (Souza and Belfort Jr. 2014).

The current genus name of the etiological agent of toxoplasmosis was based on its bow-like shape (from Greek: Toxo=bow or arc; plasma=creature or shape), as proposed by Nicolle and Manceaux (1909). They introduced the genus *Toxoplasma*, and *T. gondii* became the species of the genus. The given name is probably due to a typo, where the “u” of gundi was replaced by “o”. They also demonstrated that the multiplication of parasites was possible only within living cells, describing the intracellular feature of *T. gondii*. For the next few years after the first description, the parasite was also found in other hosts as dogs (Mello 1910), pigeons (Carini 1911), mice (Sangiorgi 1913), and mainly birds (Dubey 2002). The initial reports on humans date from the 1920s when the parasite was identified in blood, ocular and cerebral tissue of infected children with clinical symptoms (Castellani 1914; Janku 1923; Torres 1927). In 1937, Wolf and Cowen described a lethal case of toxoplasmosis in a newborn (Wolf and Cowen 1937). Viable *T. gondii* was first obtained from animals by Sabin and Olitsky (1937), who described that neutralizing antibodies, developed during the disease in some animals, could be used for *Toxoplasma* identification. Some years later, Wolf and Cowen (1937) isolated, for the first time, *T. gondii* from human infants and demonstrated that it was experimentally transmitted to animals. It also seems to be the first example with proof of congenital toxoplasmosis.

In 1941, Sabin described a report of two cases of encephalitis in children caused by *Toxoplasma*. In case 1, Sabin described the history of R. H., a 6-year-old boy admitted at the Cincinnati General Hospital on Oct. 31st, 1939, after an incident with a baseball bat. He was asymptomatic and suddenly developed convulsions some days later, but the accident had no relation with the disease. Unfortunately, the clinical symptoms got worse, and he died on the thirtieth day of infection. This case is historically interesting because the patient's spinal fluid was used to isolate a virulent and fatal strain that became the famous RH strain.

Almost at the same time, Pinkerton and Weinman (1944) reported the first case of a fatal disease in an adult after a postmortem examination of tissues from a patient who died after a brief illness, complicated by coexistent bartonellosis. Posteriorly, Pinkerton and Henderson (1941) encountered two more fatal adult cases of an acute illness in the United States of America, and Guimarães (1943) described an adult case in an 18-year-old Brazilian, with

clear evidence of complications due to the illness. Guimarães and Meyer established a methodology for the cultivation of *T. gondii* in tissue culture *in vitro* (Guimaraes and Meyer 1942) at the Federal University of Rio de Janeiro, providing one of the many contributions of Brazilian science to biology knowledge of *T. gondii* (de Souza, DaMatta, and Attias 2009). It is necessary to highlight that some landmarks in the history of *T. gondii* have been revised elsewhere (Dubey 2009; Weiss and Dubey 2009; Halonen and Weiss 2013; Lopes et al. 2014), and Dubey provided a very well organized table with a list of several historical publications (Dubey 2008).

Curiously, until the 1950s, human toxoplasmosis was thought to be rare, in striking contrast to the widespread occurrence of the parasite throughout the animal kingdom in all parts of the world (Kass et al. 1952). Probably this might be a consequence of the diagnosis that was mainly based on morphology, leading to misinterpretation, in addition to the inconspicuous symptoms that rarely permit clinical diagnosis. Certainly, this instigated scientists to study this new disease in humans, and consequently, Sabin and Feldman (1948) developed a serological test, a dye-test, that triggered a breakthrough in the field of toxoplasmosis and opened doors for extensive epidemiological studies. The test is highly sensitive and specific and helped to confirm that *T. gondii* infections are widely prevalent in humans in many countries (Feldman 1974). Although Sabin-Feldman is a reference test, it uses live parasites and standardization had to be improved (Reiter-Owona et al. 1999). For several years, other diagnostic tests have been developed, involving detection of antibodies, direct agglutination test (DAT), and detection of *T. gondii* DNA (Dubey 2008). Currently, the epidemiological investigation provides evidence of the widespread distribution of toxoplasmosis in animals worldwide. Identification of strain genotypes has been of great value for understanding the epidemiology of toxoplasmosis in different regions (Robert-Gangneux and Darde 2012).

Initial studies of genotype analysis of *T. gondii* have revealed highly clonal population structure in North America and Europe, comprising three distinct genotypes named types 1, 2, and 3 (Dardé 2008; Sibley et al. 2009; Behnke 2016). The type 1 strains are uniformly lethal in laboratory mice, whereas type 2 is intermediate, and type 3 strains are avirulent, causing little or no mortality in laboratory mice (Sibley and Boothroyd 1992, Behnke 2016). In North America and Europe, most of the *T. gondii* isolates fall into one of the three main lineages, however, a fourth clonal lineage (Type 12) is now recognized as prevalent in wild animals in North America (Khan et al. 2011). A high genetic variation is found in South America (Behneke 2016) and Asia (Gerhold et al. 2017; Bernstein et al. 2018; Pena et al. 2018; Silva et al. 2017), as the genotypes are different from the main lineages described in Europe and North America (Hosseini et al. 2018; Dardé 2004). The diversity of strains seems to have an impact on the severity of the disease (Hutson et al. 2015).

The focus of the initial works was mainly on describing the presence of the parasite in several types of animals, and the Taxonomic position of *T. gondii* was undefined for years. In the old days, several species of *Toxoplasma* were named mainly following the host species in which they were detected (Dubey and Jones 2008). In the late 1930s, Sabin provided evidence that various isolates of animal and human origin were identical to *T. gondii* (Sabin 1939). However, until the 1960s, only asexual stages of *T. gondii* were known, and its classification was uncertain (Levine 1961). Coccidia is one of the first parasitic protozoa discovered but the importance of this parasite in cats and dogs was not known until the late 1960s (Dubey 2009). Hutchison, in 1965, first presented experimental evidence that the parasite *Toxoplasma* could

be isolated from the fecal material of infected cats (Hutchison 1965). This author also hypothesized that the infection in mice by the cat feces was due to nematode transmission (Hutchison 1967). However, only after the discovery of sexual stages in the 1970s the complete life cycle of *T. gondii* was elucidated, and the coccidian-nature of *T. gondii* was confirmed, revealing the heteroxenic nature of this parasite with the definitive and intermediate hosts (Hutchison et al. 1970; Frenkel, Dubey, and Miller 1970; Dubey, Miller, and Frenkel 1970; Sheffield and Melton 1970).

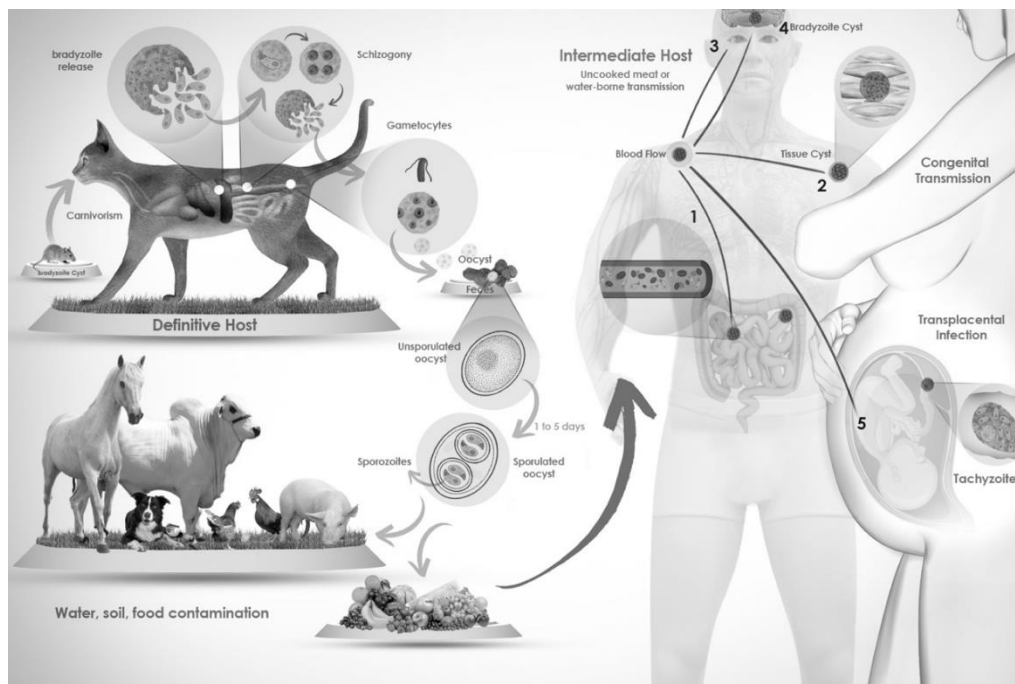


Figure 1. Life cycle of *T. gondii*. The sexual stage of the parasite life cycle occurs exclusively in the intestine of felines (the definitive host). Ingested tissue cysts, in the intestine, release bradyzoites that infect enterocytes. Rounds of schizogony occur inside the intestinal cells before the transition into gametocytes. The fusion of male and female gametocytes gives rise to oocysts. Unsporulated oocysts are shed in the feces and become infective when sporulated after a few days in the environment. Infection in humans may begin after ingestion of uncooked meat containing tissue cysts or food and drinks contaminated with sporulated oocysts from the environment. Inside humans, bradyzoites (from cysts) or sporozoites (from oocysts) infect enterocytes in the small intestine, where differentiate into tachyzoites. Tachyzoite reaches the vascular system and infects other cells (1) – characterizing the acute phase of the disease. Tachyzoites differentiate into bradyzoites, which form tissue cysts and persist for years in the host without causing any clinical manifestation (chronic phase of disease). The most common clinical outcomes correspond to ocular lesions (3), neurological effects (4), or congenital malformations (5). Congenital toxoplasmosis occurs when tachyzoites cross the placenta and infect the fetus. The severity of fetus damage depends on the period of pregnancy.

The history of *T. gondii* and toxoplasmosis discovery could be organized into four stages: the discovery of the etiological agent, the discovery of the parasite in humans, diagnosis, and identification of the definitive host. Currently, the life cycle and the sources of transmission are well known. *T. gondii* is a mandatory intracellular parasite and is taxonomically classified as the domain: Eukaryota; clade: Alveolata; phylum: Apicomplexa; class: Conoidasida;

subclass: Coccidia; order: Eucoccidiorida; family: Sarcocystidae; genus: *Toxoplasma*; species (unique): *Toxoplasma gondii* (NCBI 2021). The worldwide prevalence of toxoplasmosis varies from 10 to 90%, depending on the region due to diversity of environmental and socioeconomic conditions (Dubey 2008; Hill and Dubey 2002; Robert-Gangneux and Dardé 2012). The transmission to humans occurs mainly through the ingestion of tissue cysts from infected meat and contamination of soil, water, or food with oocysts (Cook et al. 2000; Hill and Dubey 2002). Figure 1 shows a schematic overview of the infection in the definite and intermediate hosts, illustrating the sources of transmission and the stages of the parasite's life cycle. This information is detailed in the following sections.

2. INTERMEDIATE HOST INFECTION

2.1. Asexual Reproduction of *Toxoplasma gondii*

The worldwide prevalence of the *T. gondii* could be explained by the parasite's capacity to infect almost all cell types from virtually any warm-blooded animal (Boothroyd 2009; Attias et al. 2020). The intermediate hosts are infected by *T. gondii* stages from different sources (tachyzoites, bradyzoites, and sporozoites), but only the asexual reproduction occurs in their bodies (Frenkel 1973; Attias et al. 2020).

Fecal–oral transmission (ingestion of sporulated oocysts) is the most common pathway through which *T. gondii* spreads from the primary hosts to secondary hosts (Martorelli Di Genova et al. 2019). Only felines can transmit oocysts, and the intermediate hosts may consume the oocysts through contaminated water, soil, or food. After consumption of oocysts, animals may experience an acute infection, and immunocompetent humans might present something like mild to moderate flu symptoms (Johnson and Johnson 2021).

After the ingestion of oocysts, the sporozoites are released under the presence of bile salts and trypsin in the small intestine. Sporozoites invade the host's distal small intestine and after passing enterocytes, convert into tachyzoites in cells of the lamina propria (Speer and Dubey 1998), which then disseminate through the host (Hutchison, Dunachie, and Work 1968; Dubey, Lindsay, and Speer 1998). Even though they do not possess a specialized locomotion organelle, they can move through gliding, flexing, undulating, or rotating, and disseminate in the host, being then the form found in the acute infection (Dubey, Lindsay, and Speer 1998). During *T. gondii* replication, two daughter cells are built within the mother cell (endodyogeny). After multiple division cycles, the tachyzoites egress, leading to the rupture of host cell membrane and promoting its dissemination throughout the host (Sheffield and Melton 1968; Bisio and Soldati-Favre 2019). Tachyzoites and bradyzoites are the only two stages found in the intermediate host once the infection had been established. Under the pressure of the host immune system, tachyzoites convert into bradyzoites (brady = slow in Greek). This late, slowly multiply by endodyogeny inside tissue cysts, and can persist in the host bodies for their whole life (Dubey, Lindsay, and Speer 1998). Bradyzoites have a higher resistance to proteolytic enzymes when compared to tachyzoites (Jacobs, Remington, and Melton 1960).

2.2. Host Cell Adhesion and Invasion

All the infective stages of *T. gondii* possess an apical complex harboring a conoid, two preconoidal rings, and secretory organelles (rhoptries and micronemes) that essentially are responsible for the host cell invasion (Carruthers and Sibley 1997; Dubey, Lindsay, and Speer 1998; Liu et al. 2017). The conoid is a motile structure consisting of 14 spirally wound tubulin fiber polymers. Bellow the conoid is the apical polar ring, which is responsible for organizing the subpellicular microtubules (Nichols and Chiappino 1987; Hu, Ross and Murray, 2002; Tosetti et al. 2020). Rhoptries and micronemes participate directly in the host cell infection because these secretory organelles concentrate essential proteins for cell adhesion and invasion (Hakimi, Olias, and Sibley 2017; Attias et al. 2020).

Morphologically, bradyzoites and tachyzoites are very similar. However, ultrastructure analysis revealed that they differ in aspects such as the protein content and shape of the rhoptries, number of micronemes and dense granules, and amylopectin granules quantity and nucleus location (Speer, Clark, and Dubey 1998; Dubey, Lindsay, and Speer 1998). Bradyzoites have more micronemes and amylopectin granules and less dense granules compared to tachyzoites (Dubey, Lindsay, and Speer 1998). Sporulated oocyst contains two sporocysts that have four sporozoites each. A prominent residual body is left after sporozoites are formed; the residual body is enclosed in a single-unit membrane. Sporozoites are 2 to 8 µm in size with a subterminal nucleus (Dubey, Miller, and Frenkel 1970; Dubey, Lindsay, and Speer 1998).

Various proteins were identified in the infection process of tachyzoite. Micronemal proteins are called MICs and include perforin-like proteins, adhesins, and proteases (Carruthers and Sibley 1997). Rhoptries contains rhoptry proteins (ROPs) and rhoptry neck proteins (RONs) that participate in the host cell invasion and alters the host's response (Boothroyd and Dubremetz 2008; Fox et al. 2016). The dense granule proteins are known as GRAs, which contribute to parasitophorous vacuole (PV) formation (Carruthers and Sibley 1997). Proteins such as ROP18, ROP5, and GRA7 were identified in higher expression levels in virulent *T. gondii* strains and their functional analysis revealed their important roles in the pathogenicity and survival of the parasite in the host (Hakimi, Olias, and Sibley 2017).

The parasite invasion occurs by either phagocytosis or active penetrating through the host cell's membrane. In the second case, the parasite adheres and invades the cell by gliding motility in which cytoskeleton and micronemal proteins participate (Speer et al. 1997; Tardieux and Baum 2016). This movement allows adhesion to the host cell by microneme proteins, designated as MIC. For example, MIC1 or MIC13 adhere to sialic acid residues in the host cell surface (Monteiro, Soares, and de Souza 1998; Kato 2018).

After the adhesion process, the invasion takes place via the moving junction assembly that is formed by the strong interaction of *T. gondii* rhoptry neck proteins (RON2, RON4, RON5, RON8), apical membrane antigen 1 (AMA1), and host proteins (Kato 2018; Attias et al. 2020). The rhoptry protein complex is secreted and inserted in the host cell membrane, RON2 directly binds to the micronemal protein AMA1 at *T. gondii* apical end, and RON4, RON5 and RON8 associate to the host cell's cytoskeleton proteins (see chapter 3; Besteiro et al. 2009).

The moving junction motion towards the interior of the cell results in the PV formation that is an invagination of the host plasmatic membrane that surrounds the protozoa (Dubey, Lindsay, and Speer 1998; Kato 2018). Proteins secreted from rhoptries and dense granules

alter the PV interior and membrane, and allow the tachyzoites' survival and replication (Attias et al. 2020).

2.3. Tissue Cyst Formation

Tachyzoites can differentiate into bradyzoites (forming tissue cysts) after 10 to 14 days post-infection *in vivo* (Dubey 1996). Tissue cyst is a resistant structure that persists in the host for years, establishing the chronic infection. The cyst protects *T. gondii* from the host's immune system and allows the species' perpetuation when ingested by other hosts (Cerutti, Blanchard, and Besteiro 2020).

The conversion of tachyzoite into bradyzoites can be induced *in vitro* by stress generation such as alkaline pH, high temperature, mitochondrial inhibition, and high nitric oxide levels (Dubey, Lindsay, and Speer 1998; Lyons, McLeod, and Roberts 2002). Myb-like transcription factor (BFD1) is an important gene promoter to bradyzoite differentiation, the absence of BFD1 did not allow *T. gondii* differentiation after induction *in vitro* and *in vivo*, with no brain cyst formed in mice (Waldman et al. 2020).

The conversion between tachyzoite and bradyzoite forms is associated with morphological and molecular biological changes, including specific expression of the surface antigens (such as SAG1, SAG2A, SAG2B, LDH1, ENO2 in tachyzoite and BAG1, SAG2C, SAG2D, SAG4, LDH2, ENO1 in bradyzoite) and alterations in the metabolism, for example, the fermentation to lactate has a significant role in bradyzoites energy production compare to tachyzoites (Denton et al. 1996; Lyons, McLeod, and Roberts 2002).

The tissue cyst presents a robust wall constituted by two layers, one outer layer formed by compact filaments and one internal layer formed by loose filaments (Lemgruber et al. 2011). That robust wall has specific antigens like cyst-wall glycoprotein (CST1) that binds to *Dolichos biflorans* lectin (DBA) and a cyst-matrix molecule termed matrix antigen (MAG)1 (Lyons, McLeod, and Roberts 2002). The cyst wall is not permeable to molecules bigger than 40 kDa what protects bradyzoites from the drugs currently used (Lemgruber et al. 2011). The cyst size depends on the stage but ranges from 30 to 70 µm in diameter and most of which are found in muscle cells and neurons (Wanko, Jacobs, and Gavin 1962; Dubey 1996).

3. HUMAN TOXOPLASMOSIS

Until the discovery of *T. gondii*, the coccidia group was considered to have a simple life cycle, infecting only one host, and establishing the infection in the intestinal cells of the hosts. Now, it is known that *T. gondii* is a coccidian parasite not restricted to the intestines of felids since it became adapted to a diversity of animals, and the domestication of the animals, for example, probably led to human infection. The parasite is transmitted by several modes, including the fecal-oral cycle, carnivorous, and transplacental transmission (Dubey 2009; Dubey et al. 2012), as detailed in Figure 1.

The horizontal transmission of *T. gondii* occurs by ingestion of oocysts from the environment and tissue cysts (or tachyzoites) from meat or primary offal (viscera) of different

animals. Vertical transmission occurs when tachyzoites infect the fetus through the placenta (Tenter, Heckerroth, and Weiss 2000).

Most of the immunocompetent people when infected by *T. gondii* do not have symptoms, but tissue cysts could persist in the host and reactivate the disease in immunosuppression states (CDC – National Center for Health Statistics 2018). In the early stage of infection, the parasite invasion and proliferation induces cell destruction, which may provoke fever, lymphadenopathy, and inflammation in organs such as the heart, liver, and skin (Frenkel 1973).

The immunological state of the host is an important aspect of the different symptoms that could be manifested. The immune system is didactically divided into innate and acquired immune systems (Mukhopadhyay, Arranz-Solís, and Saeij 2020). Epithelial barriers, macrophages, granulocytes, dendritic cells, natural killer (NK) cells, and complement system compose the innate immune system. The lymphocytes are the acquired immune system actors, and all of their activities are orchestrated by soluble factors called cytokines (Carrillo 2017). Infection with *T. gondii* leads to the production of interleukin (IL)-12 and interferon-gamma (IFN γ), which induce pro-inflammatory effects and, in immunocompetent hosts, are responsible for the effective elimination of the tachyzoites (Fisch, Clough, and Frickel 2019).

The immune system status is also related to toxoplasmosis reactivation, tissue cysts may rupture and reactivates the infection especially in immunosuppressed hosts (Dubey et al. 2016). Under certain conditions including low levels of nitric oxide, IFN- γ , TNF- α , or IL-12 and lack of T cells, the bradyzoites can revert into tachyzoites and acute symptoms could be observed (Lyons, McLeod, and Roberts 2002).

T. gondii can disseminate in the host because it infects resident leukocytes and dendritic cells, using their migratory characteristic, to transport themselves in the bloodstream (see chapter 9). These infected cells circulate to the draining lymph nodes using the lymphatic vessels, therefore, *T. gondii* hijacks the leukocytes and disseminates to different tissues intracellularly or extracellularly by binding to external surface ligands present in host leukocytes (Da Gama et al. 2004; Randall and Hunter 2011). The protozoa dissemination also occurs through the lymphatic circulation or bloodstream, and depending on the strain and the host state, *T. gondii* is capable of invading organs such as the brain, eye, and placenta that are tissues inaccessible to diverse pathogens, and that is the reason toxoplasmosis can manifest in its severe forms: neurological, ocular or congenital (Harker, Ueno, and Lodoen 2015).

3.1. Neurological Manifestations

The central nervous system is protected by the blood-brain barrier (BBB) that is formed by different types of cells, for instance, endothelial cells, pericytes, vascular smooth muscle cells, astrocytes, microglia, and neurons that regulate the molecules' and pathogens entrance (Sweeney et al. 2018).

However, *T. gondii* overcomes this extremely difficult obstacle formed by BBB, mainly by the migration of infected leukocytes that cross the barrier in a Trojan horse way (Lachenmaier et al. 2011), the large wooden horse with a hollow body filled with Greek warriors that massacred Troy (Cobussen 2014).

Analysis *in vivo* showed that *T. gondii* is observed particularly in the frontal and parietal lobes and around the vessels (Dellacasa-Lindberg, Hitziger, and Barragan 2007). The central

nervous system infection will cause, in most cases, one or multiple brain abscesses that could be manifested as fever, headache, hemiparesis, cranial nerve palsies, ataxia, change in consciousness, stiff neck, photophobia, or seizures (Elsheikha, Marra, and Zhu 2020).

3.2. Ocular Manifestations

That Trojan horse effect is not exclusive to the central nervous system, it could occur in other tissues like the eye which is an immune-privileged tissue that has low immune cell activation and inflammation (Furtado et al. 2012; Greigert, Bittich-Fahmi, and Pfaff 2021). *T. gondii* infected dendritic cells are capable of adhering and transmigrating across human retinal endothelium via intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and activated leukocyte cell adhesion molecule (ALCAM), which allows *T. gondii* to infect the tissues and injure the organ (Furtado et al. 2012).

Ocular manifestations are also observed due to congenital transmission, as retinochoroiditis with symptoms such as eye pain, photophobia, tearing eyes, blurred vision, and progressive loss of vision that may result in total blindness (CDC – National Center for Health Statistics 2018). The ocular symptom can occur late after the initial diagnosis of toxoplasmosis, therefore, an extended clinical follow-up is indicated (Brandão-de-Resende et al. 2020). Recurrences of ocular toxoplasmosis can even occur in immunocompetent hosts, being more severe in elderly, pregnant, or immunodeficient patients with extended lesions. In these episodes, new lesions are produced and can gradually deteriorate the vision ability (Greigert, Bittich-Fahmi, and Pfaff 2021).

3.3. Congenital Malformations

The severity of the damage in the unborn child is higher when the parasite infection occurs early in the pregnancy, mainly in the first trimester. *T. gondii* infection can induce miscarriage, a premature child, and abnormal size of the head. The aspects related to the congenital transmission and the damage in the newborn are not fully understood, but it is known that dysfunctional inflammatory response in the mother, and the underdeveloped immune system from the fetus, are factors associated with the vertical transmission of parasite and severity of the clinical manifestations in the infected newborns (Gómez-Chávez et al. 2020). Sometimes the newborn does not show manifestation at birth, but the ocular toxoplasmosis can happen throughout the baby's life, and the signs of the illness involve vision and hearing loss, mental and psychomotor disability, hematological abnormalities, and seizures (CDC – National Center for Health Statistics 2018).

Importantly, approximately 90% of the women that are *T. gondii* infected during pregnancy do not show symptoms (Hampton 2015), therefore, prenatal care must contain toxoplasmosis evaluation, prevention education, and correct damage management.

4. DEFINITIVE HOST INFECTION

The oocyst is the key stage of all coccids, the first group of important animal parasites ever discovered. Several species of felines, including domestic cats, have an important role in the transmission of *T. gondii* because they are the only host that produces the oocysts of this parasite (Dubey et al. 2020). Millions of oocysts are eliminated in the feces of felines and can survive in the environment for months (Dubey and Frenkel 1972; Dubey and Jones 2008; Dubey et al. 2012). The oocysts, when released in the feces of cats, are not infective, but once in the environment, under aeration, humidity, and warm conditions, they sporulate in 1 to 5 days and become infective (Dubey 1998). The unsporulated oocysts are 10 x 12 µm in diameter and contain a wall that consists of two colorless layers under light microscopy. Polar granules are absent and the sporont almost fills the oocyst. The sporulated oocysts are 11 x 13 µm in diameter and have a resistant double-wall containing in its interior two sporocysts, measuring 2 x 8 µm each. They can remain infectious in moist soil or sand for up to 18 months. The sporulated oocysts then contaminate water, soil and food, and consequently infect intermediate hosts such as birds and mammals, including humans. (Hogan et al. 1960; Dubey, Lindsay, and Speer 1998; Tenter, Heckeroth, and Weiss 2000).

4.1. Sexual Reproduction of *Toxoplasma gondii*

The sexual reproductive stage of the *T. gondii* life cycle is known as gametogony. The formation of gametes begins when cats (and other felines) ingest any *T. gondii* stage (tachyzoites, sporozoites, or bradyzoites) from other infected hosts. When they ingest tissue cyst, its wall is ruptured by proteolytic enzymes of the stomach and small intestine, causing the release of bradyzoites. Bradyzoites can invade any nucleated cell, just like tachyzoites and sporozoites, and enterocytes are the preferential targets in the intestinal tract. Inside the cells of the lamina propria, bradyzoites or sporozoites can differentiate into tachyzoites. After several rounds of division, *T. gondii* tachyzoites can disseminate to extra-intestinal tissues. Some bradyzoites penetrate enterocytes and in 3-15 days, in these cells, initiate the development of asexual forms (schizonts). This stage named schizogony corresponds to the differentiation of the pre-sexual stage that were grouped into five distinct morphological types of schizonts (A to E) (Dubey and Frenkel 1972; Elsheikha, Marra, and Zhu 2020). Type A is the smallest parasite; type B is characterized by a centrally located nucleus; C is elongated with subterminal nuclei; type D is smaller than type C and contain only a few PAS-positive granules; and the type E resembles the subtype D and suffer division with the formation of a residual body (Dubey and Frenkel 1972; Moura, Amendoeira, and Barbosa 2009). The schizonts multiply and originate merozoites that egress and may infect enterocytes again in 2 to 4 rounds, generating an increase in the number of this parasite form. After that, microgametes (male) and macrogametes (female) are formed. The male gamete has two flagella to swim in the intestinal lumen, allowing it to find and penetrate the female gamete creating the diploid zygote. The formation of the oocyst wall begins after the fertilization of the female gamete and the immature oocysts are discharged into the intestinal lumen by the rupture of the enterocyte. *T. gondii* persists in intestinal and extra-intestinal tissue of cats for

at least several months, and possibly for the life of the animal (Dubey 2004; Moura, Amendoeira, and Barbosa 2009).

The period between the infection by the etiologic agent and the appearance of the first detectable forms of the parasite in cats is 10 days and the oocysts are eliminated in the feces for 1 to 2 weeks. During this period, the animal is asymptomatic and does not have antibodies to *T. gondii*, however, it is estimated that only 1% of the population of infected cats eliminate oocysts in the environment (Dubey and Frenkel 1972; Dubey et al. 2012).

The reasons for the establishment of sexual reproduction of *T. gondii* exclusively in the feline intestine are still of high interest. Temporal analysis of the transcriptome of the cat intestine during the first 96 hours post *T. gondii* infection revealed significant changes in transcripts of genes involved in immune response and metabolic pathways (Wang et al. 2018). A recent study showed that sexual differentiation in *T. gondii* could be inhibited by the histone deacetylase HDAC3, in a process mediated by microorchidia (MORC) protein and the Apetala 2 transcription factor (Farhat et al. 2020).

Studies using feline intestinal organoids have shown that the high abundance of linoleic acid in cats, compared with other nonfelid mammals hosts, seems to be a critical factor for the exclusive occurrence of sexual reproduction (Martorelli di Genova et al. 2019; Elsheikha, Marra, and Zhu 2020). *In vitro* tests demonstrated the induction of sexual development when *T. gondii* was cultured in feline intestinal epithelial cells supplemented with linoleic acid. Furthermore, felids are the only mammals that lack delta-6-desaturase activity in their intestines, which is required for linoleic acid metabolism, resulting in systemic excess of linoleic acid. In addition to that, the inhibition of murine delta-6-desaturase and diet supplementation with linoleic acid allowed sexual development in mice. Then, this mechanism of species specificity is the first one defined for a sexual parasite cycle, highlighting how host diet and metabolism shape the coevolution with microbes (Martorelli Di Genova et al. 2019).

Cats usually shed large numbers of oocysts in primary *T. gondii* infection. In uncontrolled environments, it is considered rare to eliminate oocysts after reinfection or to reshedding oocysts in the absence of reinfection. However, some studies showed that this immunity does not persist for all the life of the cat (Dubey, Lappin, and Thulliez 1995; Dubey 1998). A second shedding of oocysts could be induced in cats challenged with *T. gondii* about 6 years after primary infection. In addition, in some cases, short-term reshedding of oocysts has been observed without reinfection of the cat. It is currently not known which factors induce the reshedding of oocysts under natural conditions (Tenter, Heckeroth, and Weiss 2000; Weiss and Dubey 2009).

Furthermore, experimental evidence shows that cats can shed oocysts after the primary infection. In this study, 13 cats were infected with two strains: VEG (genotype III), and TgDoveBr1 (genotype II). In experimental re-infection of cats, the excretion of *T. gondii* oocysts can remain high for years after the first infection, mainly when different strains are used. The environmental contamination seems to be much higher than previously thought because it was detected that 71% of the cats shed oocysts after tertiary infections. Thus, the authors provide evidence that the excretion of *T. gondii* oocysts was higher over the years in experimentally re-infected cats, especially when heterologous strains were used (Zulpo et al. 2018).

4.2. Seroprevalence and Treatment in Cats

The role of felines is of paramount importance in the transmission of the disease. The risk of infection is associated with the number of infected cats as the environment becomes more contaminated. Several studies demonstrate the variety of feline seroprevalence found in Brazil (Table 1).

Table 1. The occurrence of *T. gondii* in felines in Brazil.

Local(City/State)	N# (Prevalence %)	Methodology*	Reference
Jaguapitã/PR	163 (73%)	IIF	Garcia et al. 1999
Santa Maria/RS	180 (21.6%)	IIF	Oliveira et al. 2000
São Paulo and Paraná ^a	191 (19.4%)	IIF	Langoni et al. 2001
Niterói/RJ	41 (29.3%)	ELFA/IHA	Netto et al. 2003
Santa Isabel do Ivaí/PR	58 (84.4%)	MAT	Dubey et al. 2012
São Paulo ^b	237 (35.4%)	MAT	Pena et al. 2005
Araçatuba/SP	400 (25%)	IIF	Bresciani et al. 2006
Porto Alegre/RS	245 (64.8%)	IHA/IIF	Pinto et al. 2009
Lages/SC	300 (14.3%)	IIF	Rosa et al. 2010
Porto Alegre/RS	90 (16.7%)	IHA	Schnell 2011
Curitiba/PR	282 (16.3%)	IIF	Cruz et al. 2011
São Luis/MA	200 (50.5%)	IIF	Braga et al. 2012
Cascavel/PR	171 (28.7%)	IIF	Andrade et al. 2015
Lages/SC	107 (35.5%)	IIF	Moura et al. 2015
Palotina/PR	100 (29%)	IIF	Souza et al. 2017

Total number of samples collected.

* IIF: Indirect Immunofluorescence Assay; ELFA: Enzyme Linked Fluorescent Assay; MAT: Modified Agglutination Test; IHA: Indirect Hemagglutination.

^a Only states are indicated.

^b The authors collected animals from the state of São Paulo.

The diagnosis is based mainly on the detection of antibodies by serological methodologies, but histological and molecular methods, as well as mice bioassays and Sabin-Feldman reaction, were also used to analyze the occurrence (Tavares and Fagundes 2018).

Feline infections are typically subclinical and congenitally infected kittens are the most likely to have clinical signs. The common symptoms of *T. gondii* infection can include fever, ocular inflammation, anorexia, lethargy, abdominal discomfort and neurologic abnormalities (Elmore et al. 2010). Although clinical manifestation is related, there are no pathological clinic occurrences. Thus, it is not possible to diagnose the disease by pathognomonic imaging, and the confirmation of the diagnosis depends on the isolation of the parasite, histopathologic analysis, and positive serodiagnosis (Lappin et al. 1989; Dubey, Lappin, and Thulliez 1995).

For this reason, the treatment is indicated only when the disease is symptomatic. In cases of diarrhea or lymphopenia, it is indicated the administration of clindamycin hydrochloride because it has good intestinal absorption. This treatment leads to clinical improvement within 24 to 48 hours after the beginning of its administration. In the case of ocular signs, the clinical improvement occurs after a week of the treatment. However, the drug may cause local

irritation in the digestive system, with side effects such as anorexia, vomiting and diarrhea when administrated in excess (Bresciani et al. 2014).

The use of glucocorticoids is contraindicated in case of systemic impairment because it can exacerbate the manifestation of the disease, whereas the topical use of this drug in association with clindamycin hydrochloride is indicated in the case of ocular lesion. In addition, experimental models using enrofloxacin, a drug with relatively low side effects, demonstrate what seems to be an important alternative in the treatment of clinical toxoplasmosis manifestation (Dubey and Lappin 2006; Bresciani et al. 2014).

In summary, the biological aspects that make the cats the exclusive host for *T. gondii* sexual reproduction are still not well understood. Subclinical symptoms in cats turn the serological diagnostic important to understand the prevalence of disease and help with the control. The treatment is indicated for the improvement of the cat welfare in case of clinical symptoms. There isn't any efficient treatment impairing the shedding of oocysts, and consequently, it is hard to avoid the contamination of the environment. Furthermore, the diversity of wild felines also complicates the control of parasite transmission.

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Chapter 2

ULTRASTRUCTURE OF TACHYZOITE, SPOROZOITE AND BRADYZOITE FORMS

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ABSTRACT

Among the three infective stages of *Toxoplasma gondii*- tachyzoites, bradyzoites and sporozoites- the ultrastructure of tachyzoites, the stage characteristic of the acute phase of toxoplasmosis, is the best known and was the first to be described. The multiple instruments and techniques involving electron microscopy were, and still are, key tools in reaching the present knowledge on it (Dubremetz and Ferguson 2009). Starting with the first images obtained by Meyer and De Mendonça (Meyer and De Mendonça 1955), a considerable number of information on *T. gondii* ultrastructure was achieved and compiled in a classic review by Dubey et al. (Dubey, Lindsay, and Speer 1998b). The ultrastructure of *T. gondii* developmental stages can be considered a ‘work in progress’ since this early picture has been refined since then, both concerning the ultrastructure and the functional dynamics of this organism.

1. INTRODUCTION

Toxoplasma gondii tachyzoites, bradyzoites and sporozoites are highly polarized elongated cells with the anterior end more acute, in contrast to a blunter rear end, resulting in a shape that has been compared to a teardrop or a banana. All the typical organelles and structures of eukaryotic cells, e.g., nucleus, mitochondrion, endoplasmic reticulum and Golgi complex, are present (Figure 1A). In tachyzoites, the nucleus is centrally positioned and is rather spherical but its upper pole forms a shallow cup that accommodates the cisternae of the

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Golgi complex. As in other cell types, the outer membrane of the nucleus is part of the rough endoplasmic reticulum that spreads throughout the cytoplasm.

However, the apical complex is the defining structure of the phylum Apicomplexa. In *T. gondii* it is composed of the pellicle, cytoskeletal elements, and secretory organelles, namely micronemes and rhoptries. Dense granules are the third class of secretory organelles present in all three stages. *T. gondii* displays other particular organelles, and some are typical of a specific infective stage. These include amylopectin granules are one of the most conspicuous differences between bradyzoites and tachyzoites, where these are rarely seen. The apicoplast, an organelle of symbiotic origin, is present in all three forms and is exclusive of the phylum. Figure 1B is a model illustrating the main cellular organization of a tachyzoite of *T. gondii*.

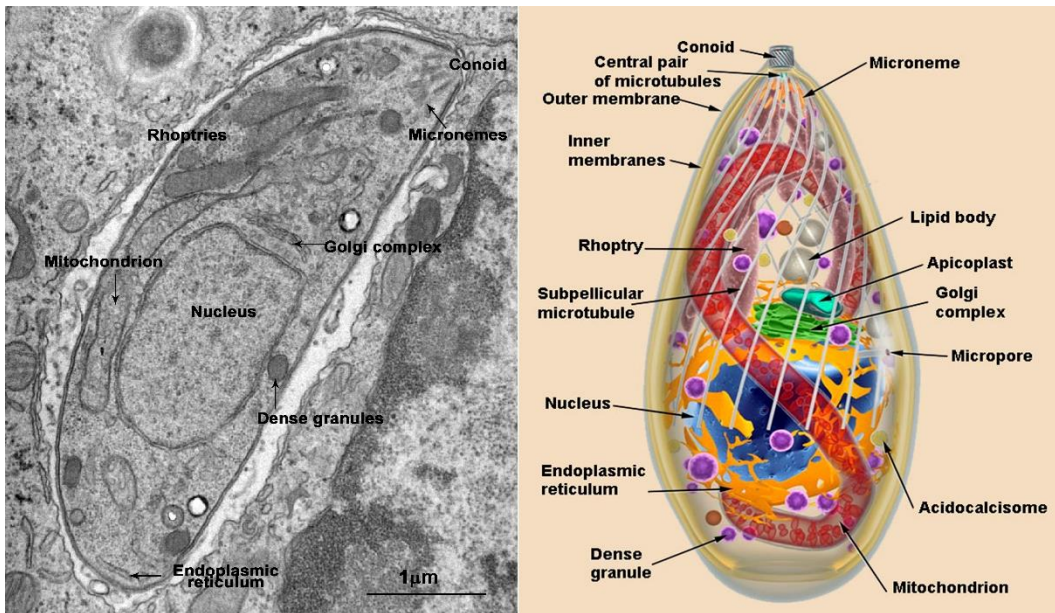


Figure 1. Cell organization of *T. gondii* tachyzoites. A- Transmission electron microscopy of a tachyzoite inside a parasitophorous vacuole (not all organelles were captured in a single section). B- Three-dimensional scheme of a tachyzoite with main structures and organelles represented. (Electron micrograph from Attias M. personal archive; Model modified from Figure 2a published in Attias et al. 2020. *Parasites Vectors* 13, 588).

2. THE PELLICLE

Toxoplasma gondii is surrounded by three layers of membrane, constituting the pellicle. The first and more external layer is the continuous plasma membrane. The two inner layers of the pellicle constitute the inner membrane complex (IMC) and are absent from the apical and basal poles of the parasite, and the area of the micropore - a cytosomal structure present in the mid-portion of the cell body. Below the cytoplasmic face of this complex and in association with it, lies the subpellicular network (SPN), a highly organized network of intermediate filament-like proteins of the articulins family (Mann and Beckers 2001; Lemgruber et al. 2009) (Figure 2A-C; Lemgruber et al. 2009). Freeze fracture of *Toxoplasma*

and *Sarcocystis* revealed particularities in the distribution of intramembranous particles in each membrane of the pellicle (Figure 3A). These membrane sacs are delineated by the sutures between each one and were classified as cap, central, and basal, according to their positioning (Chen et al. 2015). The cap IMC region is a single membrane sac that has a conical shape and surrounds the apical portion of the parasite. Apical annuli, circular 8+1 rosettes of protein particles of unknown function are distributed in this membrane (Dos Santos Pacheco et al. 2020).

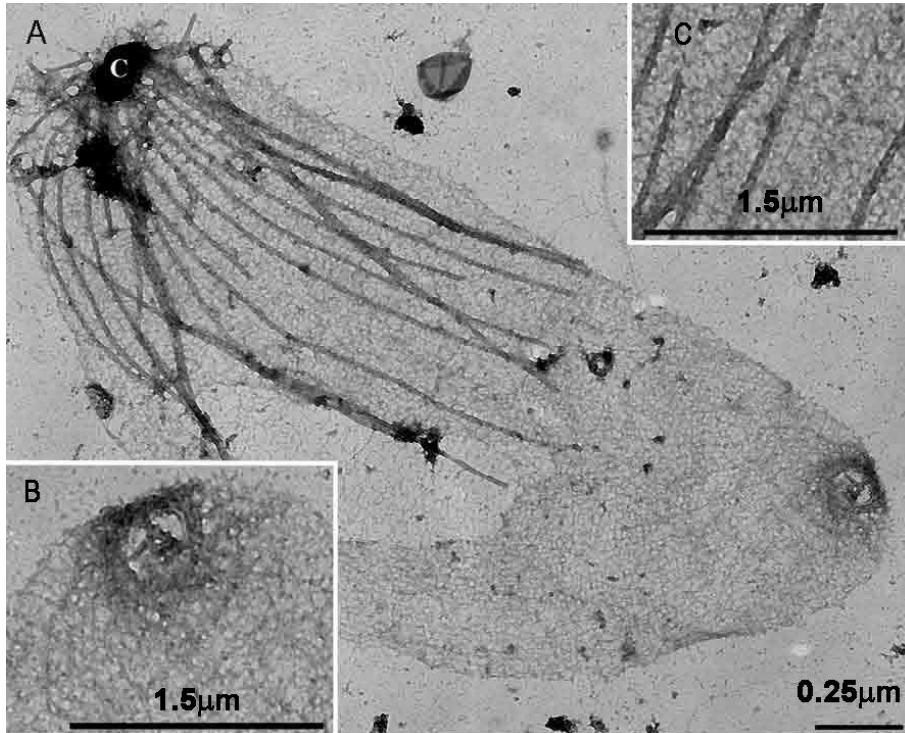


Figure 2. After detergent extraction, the subpellicular microtubules, the conoid (c) became visible. The subpellicular network is present in the full extent of the parasite, ending in an electron-dense circular structure (B), while microtubules extend only to about 2/3 of the parasite length. (C) The microtubules are much thicker than the filaments of the subpellicular network (images published as Figure 1A in Lemgruber et al. 2009. Mem Inst Oswaldo Cruz. 104(5):706-9).

The IMC is made of rectangular flattened vesicles aligned in longitudinal rows and joined in a jig-saw puzzle-like fashion. The membranes of the IMC are characterized by particles in double rows radiating from the apical portion that correspond in number and arrangement to the underlying microtubules (Porchet and Torpier 1977; Cintra and de Souza 1985).

The IMC is critical for the parasite motility, hence, its ability to invade host cells. The outer leaflet of the IMC is associated to an actin-myosin machinery (J. M. Dobrowolski and Sibley 1996). IMC membrane alveoli are absent from apical and bottom extremities of the parasite and around the micropore, a cytostomal opening in the midportion of the cell body (Nichols, Chiappino, and Pavesio 1994) (Figure 1).

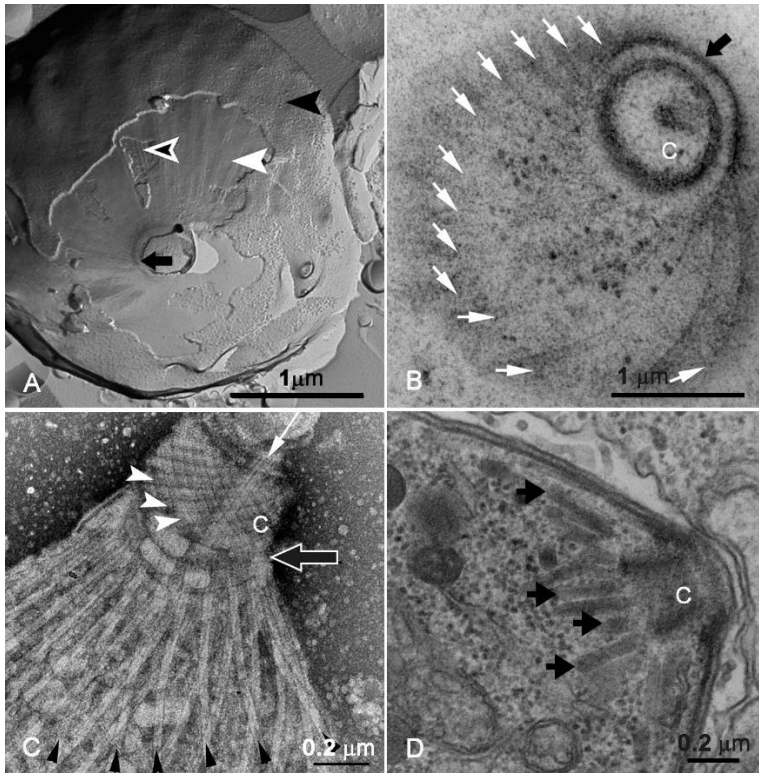


Figure 3. (A) Freeze fracture replica of the anterior portion of a tachyzoite showing the position of the apical ring (arrow), and the three layers of the pellicle indicated by arrowheads of different colors. The plasma membrane (black), and the two inner complex membranes, with an arrangement of intramembranous particles that correspond to the diameter and the distribution of the subpellicular microtubules that run below it (white and black/white arrowheads). (B) Tangential thin section of the anterior portion of a tachyzoite showing 13 of the 22 subpellicular microtubules in a spring-like spiral (white arrows) around the apical ring (black arrow) and the conoid in a cross section inside it (c). Views of the conoid (c) by negative staining (C) and ultrathin section (D). Arrowheads in c point to conoidal fibers in typical inclination. The thin arrow points to the central pair of microtubules and the thick arrow to de apical ring. Black arrows in (D) point to micronemes that crown the base of the conoid. Electron micrographs from Attias M. personal archive.

3. CYTOSKELETON

Toxoplasma gondii tachyzoites, bradyzoites and sporozoites share the most morphological features concerning the cytoskeletal organization (Morrisette and Sibley 2002). Microtubules, microfilaments and intermediate filaments, as well as myosins, are present, but with many particular differences compared to other eukaryotic cells.

3.1. Microtubules and Tubulin Related Structures

Tachyzoites, bradyzoites and sporozoites are elongated and polarized forms of *T. gondii* that share a quite similar organization of the subpellicular microtubules (SPMT), that are 22

in number and radiate from the apical polar ring (APR) (Figure 3B), that is a microtubule-organizing center and end at approximately two-thirds of the length of the parasite (Figure 2A) (Nichols and Chiappino 1987; W. de Souza 1974).

3.2. Conoid

The conoid is part of the cytoskeleton and is settled in the apical portion of the cell body, inside the APR. Being motile, the conoid can move above and below the APR. It is a hollow cylinder mainly constituted by tubulin in a particular spring-like spiral organization (Figure 3 B). Each conoidal fiber extends about halfway across the conoid diameter and about six fibers are counted in the longitudinal cross-section (Hu, Roos, and Murray 2002). Tubulin subunits in the conoid are assembled in a comma shape with 9 subunits, an organization very distinct from the 13 protofilaments of a microtubule (Hu, Roos, and Murray 2002). The conoid is topped by two apical rings and a pair of closely associated microtubules (~400-nm-long) runs inside the conoidal space (Figure 3C) (Morrissette and Sibley 2002). The conoid plus the two conoidal apical rings constitute the ‘conoid complex’ (reviewed in Dos Santos Pacheco et al., 2020).

3.3. Actin and Myosin

Most of the actin present in *T. gondii* is in the monomeric globular form (J. Dobrowolski and Sibley 1997; J. M. Dobrowolski and Sibley 1996) and could only be visualized with the use of jasplakinolide (Shaw and Tilney 1999). *T. gondii* class XIV myosin motors participate in the motility of *T. gondii* and other apicomplexan parasites. This actomyosin based motor is part of complex invasion machinery known as the glideosome that includes all layers of the pellicle, plus the subpellicular network (SPN) and the SPMT (Boucher and Bosch 2015; Fréchal et al. 2017).

3.4 Subpellicular Network (SPN)

A third component of the cytoskeleton is the subpellicular network (SPN), a network of 8-10 nm filaments containing alveolins- artichurin-like proteins- between the IMC and the SPMTs (Mann and Beckers 2001; Lemgruber et al. 2009; Tosetti et al. 2020) (Figure 2A).

4. SECRETORY ORGANELLES

4.1. Micronemes and Rhoptries

Micronemes and rhoptries (Figure 1) are secretory organelles essential for tachyzoites, bradyzoites and sporozoites to invade host cells (Dubois and Soldati-Favre 2019; Carruthers 1999), being part of the apical complex (Figure 4). For this reason, they are addressed

together. Micronemes secrete adhesins required for gliding motility and invasion while rhoptries contents are secreted only upon contact with the membrane of the host cell being essential for the formation of the moving junction and the formation of the parasitophorous vacuole (PV) membrane. Rhoptries also secrete proteins that neutralize cell defense mechanisms subverting its functionality (Besteiro et al. 2009).

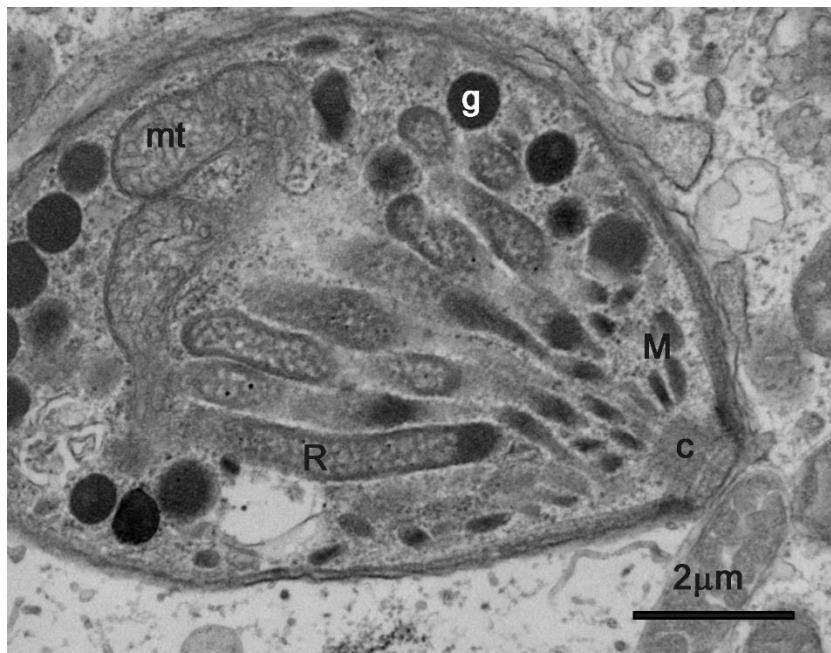


Figure 4. The conoid (c) and secretory organelles; micronemes (M) and rhoptries (R) constitute the apical complex. Dense granules (g) are a third class of secretory organelle. (mt) mitochondrion. Electron micrograph from Attias M. personal archive.

Micronemes are small rod-like organelles abundant in number that are disposed around and below the APR (Figures 3D and 4), while rhoptries are club-shaped organelles smaller in number (10-14 in tachyzoites) that converge to the conoidal space (Paredes-Santos, de Souza, and Attias 2012).

Both rhoptries and micronemes differ in numbers among each stage: Tachyzoites have more rhoptries than sporozoites and bradyzoites, while bradyzoites have far more micronemes than sporozoites and tachyzoites have fewer micronemes than both (Dubey, Lindsay, and Speer 1998b).

Two domains can be identified in rhoptries: the neck and a larger compartment, the bulb (Figure 4). When observed by transmission electron microscopy, in tachyzoites the bulb has a labyrinthine lacunar texture, while in bradyzoites they are more compact and electron-dense while in sporozoites both types are found (Dubey, Lindsay, and Speer 1998b).

Observation of freeze-fracture replicas of rhoptries revealed, both in tachyzoites and bradyzoites, intramembranous particles arranged in distinctive parallel arrays. Tubulovesicular subcompartments and free particles were observed in the rhoptry lumen on freeze-fractured replicas. Several pore-like structures localized in the bulb portion were observed in cryo-fixed, deep-etched samples (Lemgruber et al. 2011).

4.2. Dense Granules

Dense granules are the third type of secretory organelles and are present in all three zoite stages of *T. gondii*. As its nomenclature indicates, these are membrane-limited electron-dense microspheres measuring 200 nm average (Figure 1 and 4). Their number is higher in tachyzoites and sporozoites, while bradyzoites have fewer of them (Dubey, Lindsay, and Speer 1998a). The contents of these granules belong to a family of proteins named GRAs (C Mercier, Cesbron-Delauw, and Sibley 1998; Cesbron-Delauw 1994). Secretion of dense granules occurs constitutively during parasite division cycles inside the PV, contributing both to the enlargement of the PV membrane and the formation of the intravacuolar network that acts both in keeping the parasites linked to each other and the vacuolar membrane (Magno et al. 2005). They are also critical for parasite survival since they modulate metabolic and immunogenic responses of the host (Hakimi, Olias, and Sibley 2017). Dense granules are distributed throughout the cytoplasm but have been recently shown to accumulate in the apical pole of extracellular parasites (Venugopal et al. 2020).

5. OTHER ORGANELLES

5.1. Amylopectin Granules

Amylopectin granules are characteristically seen in bradyzoites (Figure 5) and sporozoites and are very rare in tachyzoites. These granules contain a crystalline storage polysaccharide that stains in red with PAS (Guérardel et al. 2005). They probably represent a reserve of energy to be consumed from one developmental stage (bradyzoite or sporozoite) to another (tachyzoites). In the intestine of cats, type B schizonts, that divide by endodyogeny, result in merozoites containing PAS-positive inclusions, corresponding to amylopectin granules (Speer and Dubey 2005).

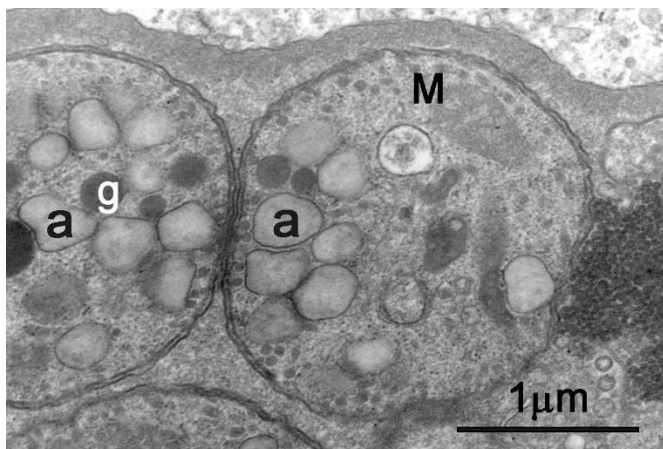


Figure 5. Amylopectin granules (a) in the cytoplasm of two bradyzoites seen in cross-section, dense granules (g) and micronemes (M) peripherally disposed of. Electron micrograph from Attias M. personal archive.

5.2. Lipid Bodies

Rarely seen in tachyzoites, lipid bodies are often seen in sporozoites and absent from bradyzoites (Dubey, Lindsay, and Speer 1998b). This accumulation of lipid bodies in sporozoites, stage resulted from the sexual reproduction in felids intestine, may be related to the absence of Δ -6-desaturase in the cat gut, an enzyme necessary for linoleic acid metabolism (Di Genova et al. 2019).

5.3. Nucleus

The nucleus usually measures about 1 μ m in diameter but is not perfectly spherical, the more apical side as a shallow cup above which the Golgi complex accommodates (Figure 6). The chromatin forms clumps and is peripherally distributed with a central nucleolus. In tachyzoites, the nucleus is centrally located in relation to the longitudinal axis, while in bradyzoites and sporozoites it is positioned in the lower half of the cell body (Dubey, Lindsay, and Speer 1998a). During division by endodyogeny, the nucleus assumes a horseshoe shape. In this stage, each extremity of the nucleus houses the mitotic spindle, here called centrocone, linked to a spindle pole (see chapter 5; Brooks et al. 2011).

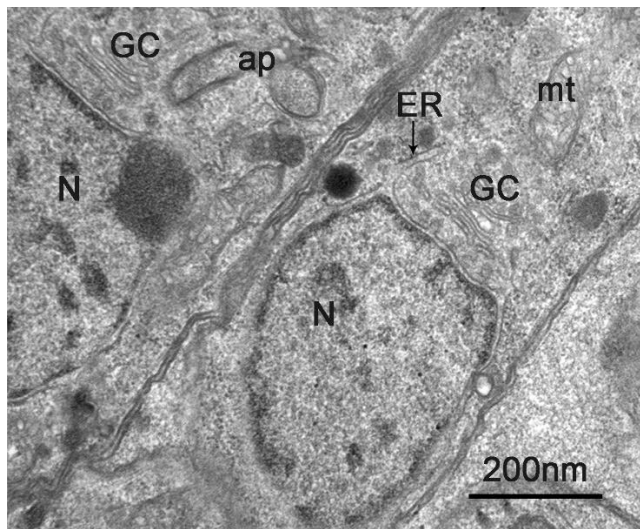


Figure 6. Two tachyzoites in longitudinal section. The nucleus (N) is positioned medially. Above it, the apicoplast (ap), the Golgi complex (GC), and a fragment of the mitochondrion (mt) with the characteristic tubular cristae above it. Electron micrograph is a courtesy of Dr. Erica Martins-Duarte (UFMG) personal archive.

5.4. Endoplasmic Reticulum

The rough endoplasmic reticulum of *T. gondii* spreads in all directions from the outer nuclear membrane (Figures 1 and 6). The lipophilic cationic fluorescent dye DiOC6 (used to

visualize the endoplasmic reticulum) labeled the peripheral region of the protozoan. By transmission electron microscopy, the outer nuclear membrane, profiles of the endoplasmic reticulum, the inner membrane complex of tachyzoites were labeled by KI-OsO₄ technique; suggesting that the inner membrane complex may be a specialized region of the endoplasmic reticulum in *T. gondii* tachyzoites (E. J. T. De Melo and De Souza 1997).

5.5. Golgi Complex

The Golgi complex in *T. gondii* is composed of a single stack of 5 or 6 lamellae situated just above the nucleus (Figure 6). During endodyogeny, the Golgi expands laterally and is cleaved in two in the middle plane, each half designated to a daughter cell (Pelletier et al. 2002; Hartmann et al. 2006).

5.6. Mitochondrion

As observed in other protists, *T. gondii* has a single and long mitochondrion that may assume different shapes and positions depending on the localization of the parasite (Figures 1, 4, 5, 6). Inside the PV, it usually assumes a peripheral (close to the pellicle) “lasso” shape (self-closed), while in the extracellular space it could either assume a “sperm-like” or a “collapsed shape” (Ovcariakova et al. 2017). Previously, reconstruction from either serial sections (E. J. L. Melo, Attias, and De Souza 2000) and, more recently, slice and view series (Paredes-Santos, de Souza, and Attias 2012) have confirmed this diversity of shapes. Furthermore, in dividing tachyzoites, the mitochondrial division is also coordinated (Nishi et al. 2008) and forms two distinct branches that will be included each in one of the two daughter cells (M. Attias, Miranda, and De Souza 2019). The mitochondrial cristae are bulbous with a narrow neck. The mitochondrial matrix is usually clear. In Apicomplexa mitochondrial genomes encode only three proteins (Sheiner, Vaidya, and McFadden 2013; Vaidya and Mather 2009). Nevertheless, genomic sequencing of *Toxoplasma gondii* and *Plasmodium* spp. revealed genes encoding all TCA cycle enzymes (Reviewed in Sheiner et al., 2013), furthermore, the TCA cycle was demonstrated to be fully active in *Toxoplasma* tachyzoites (MacRae et al. 2012).

5.7. Apicoplast

Among the eukaryotic cells, there are a few organelles that are considered as originated from an endosymbiotic process. These include the mitochondria and the chloroplast. They are characterized by the presence of a small genome, and the presence of two membranes. Early electron microscopy studies revealed the presence in *T. gondii* of a not characterized structure enveloped by several membranes. Kilejian (1975) observed an extrachromosomal DNA molecule in *Plasmodium lophurae*. This observation was, later on, extend to *T. gondii* (Borst et al. 1984). This area was subsequently extensively analyzed in *Plasmodium* species (reviews in Waller and McFadden 2005; McFadden 2011). In the case of *T. gondii*, a key work was

reported by Kohler et al. (1997). It is now clear that the structure containing the extrachromosomal DNA, designated as apicoplast in *Plasmodium* by McFadden et al. (1996), is bounded by four membranes. The presence of multiple membranes is in close agreement with the idea that the organelle originated from a secondary endosymbiosis process (Gould, Waller, and McFadden 2008). Now it is clear that the apicoplast originated from the chloroplast of a single-celled red alga that was engulfed by the apicomplexan ancestor (McFadden and Yeh 2017).

From the morphological point of view, the apicoplast can be described as an organelle localized at the anterior region of the protozoan, near the nucleus and the Golgi complex (Figure 6). Electron microscopy analysis revealed the presence of four-unit membranes delimiting the apicoplast. Additional membrane profiles can be occasionally seen, but most probably correspond to part of the endoplasmic reticulum and/or the Golgi complex. The two inner membranes are originated from the ancestral plastid. The third one is derived from the red algae plasma membrane. The fourth one originated from the plasma membrane of the protozoan ancestor during the internalization of the endosymbiont, forming a kind of phagosomal/endosomal membrane. The apicoplast matrix appears as a finely granular structure with some fibrous texture. The granules correspond to the 70S ribosomes. Cytochemical analysis indicates that the apicoplast DNA may appear as a discrete nucleoid concentrated in a small portion of the apicoplast (Striepen et al. 2000). During endodyogeny, the apicoplast elongates to form a dumbbell-shaped organelle with a central constriction that will originate one organelle for each daughter cell (Striepen et al. 2000). It is important to point out that the apicoplast is not photosynthetic.

5.8. Acidocalcisomes

Following its initial description in trypanosomatids (Review in Docampo et al. 2005), acidocalcisomes were also seen in members of the Apicomplexa group, including *T. gondii* (Moreno and Zhong 1996). The organelles appear as spherical electron-dense structures with a diameter varying from 150 to 400 nm and enveloped by a typical unit membrane around 6 nm thick. Usually, tachyzoites present around 10 acidocalcisomes, distributed throughout the cell (Figure 7A and B). They correspond to structures previously designated as volutin or metachromatic granules, or black granules (Review in Moreno et al. 2007). In conventional thin sections, most of the acidocalcisomes display an empty appearance, with a small dense material that stays attached to the inner face of the membrane (Figure 7C). This is probably due to the extraction of the organelle content during the steps of preparation of the parasites for electron microscopy. However, observation of whole cells adhered to the EM grid showed that the whole organelle is electron-dense (Figure 7A). Presently it is known, based on biochemical and electron microscopy studies, that the electron density is due to the presence of a large amount of pyrophosphate, polyphosphate, calcium, magnesium, and other elements, as detected by X-ray microanalysis (Figure 7B). It contains a linear polymer of orthophosphate residues linked by high-energy phosphoanhydride bonds forming the polyphosphates.

The acidocalcisome presents an acidic pH, and is labeled with the weak base acridine orange, and seen by fluorescence microscopy. The acidic pH is mainly due to the presence in the organelle membrane of two proton pumps; a vacuolar H⁺-ATPase and a vacuolar H⁺-

pyrophosphatase (Miranda et al. 2008). In addition, a Ca^{2+} -ATPase is present. The acidocalcisomes were once considered as part of the lysosome-like compartment. The Ca^{2+} content in the acidocalcisome is very high, although most of it is bound to polyphosphate. The high concentration of phosphorous is in the form of inorganic pyrophosphate and polyphosphate. The concentration of Ca^{2+} in the cytoplasm of *T. gondii* is around 100 nM, a value that resembles that found in mammalian cells (Reviewed in Lourido and Moreno 2015). Given a large amount of this cation in the acidocalcisome this organelle has been considered as a special Ca^{2+} storage organelle in addition to others such as the endoplasmic reticulum and mitochondria. Since fluctuations in cytosolic free Ca^{2+} concentrations regulate several key cellular functions, such as organelles secretion, cell motility, host cell invasion, and egress, it is assumed that the acidocalcisome may be involved in the regulation of multiple functions. Recently, it was shown that this protozoan actively incorporates P using a gradient of Na^+ ions to drive P uptake across the plasma membrane via a transmembrane protein designated as TgPIT. This transport mechanism is electrogenic and coupled to a plasma membrane Na^+/K^+ -ATPase (Asady et al. 2020). Ablation of TgPIT partially impairs P importing and the subsequent synthesis of polyphosphates. These parasites present a much larger number of acidocalcisomes, and a reduced volume (Asady et al. 2020).

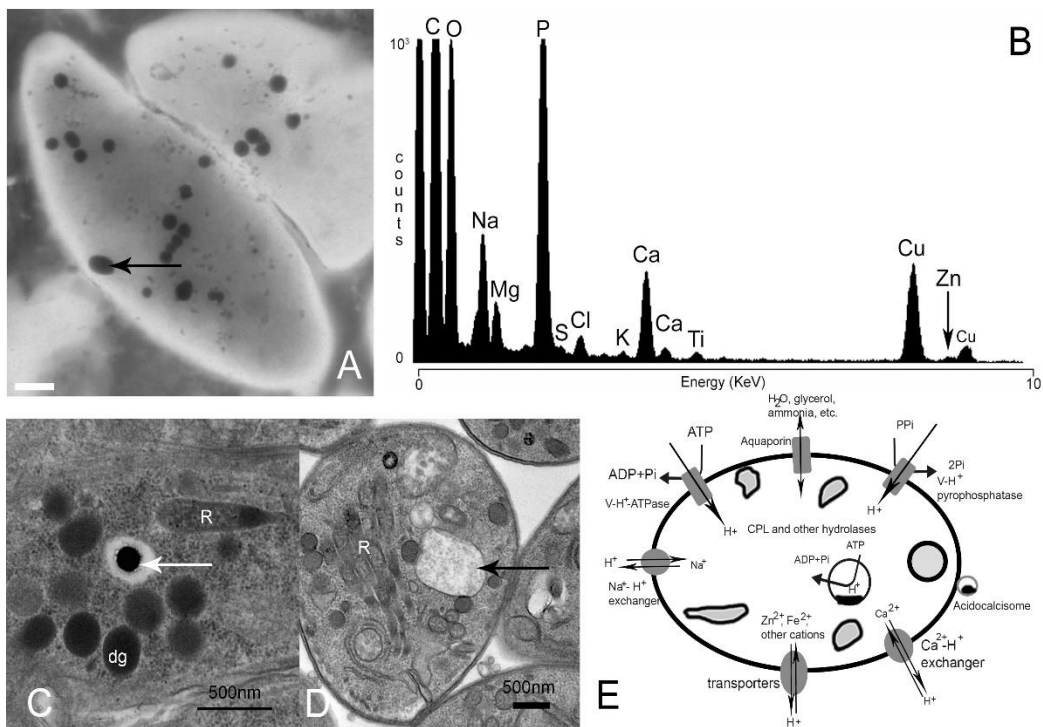


Figure 7. (A) Electron spectroscopic image (ESI) of a whole-cell of *T. gondii*. Electron-dense structures correspond to acidocalcisomes. (B) Corresponding x-ray spectrum obtained from the region pointed in A (arrow = electron-dense organelle). (C) Ultrathin section of *T. gondii*. Acidocalcisome with the typical dense core pointed by arrows. R-rhoptries, dg- dense granules. (D) Transmission electron micrograph of tachyzoites in the extracellular space with the transient TgLACVET. (E) Main pumping activities in association with the acidocalcisome. Images are courtesy of Dr. Kildare Miranda personal archive.

5.9. Large Anterior Cytoplasmic Vacuole of Extracellular Tachyzoite (TgLACVET)

In the extracellular medium, tachyzoites of *T. gondii* present a vacuolar organelle of variable size and shape, displaying a less electron-dense material than that seen in the cytoplasm (Figure 7D). It is localized in the anterior region of the protozoan, just above the nucleus. Sometimes a dense material, similar to that found in acidocalcisomes is present. In others, vesicles of variable diameters are present (Miranda et al. 2010). It closely associates with acidocalcisomes. Immunocytochemical analysis using fluorescence and electron microscopy revealed the presence of a plant-like vacuolar pyrophosphatase, a vacuolar proton ATPase, a cathepsin L-like protease, an aquaporin as well as $\text{Ca}^{2+}/\text{H}^{+}$ and $\text{Na}^{+}/\text{H}^{+}$ exchange activities, stressing the similarity with the plant vacuole (Figure 7E). The vacuole increases in size when tachyzoites are incubated in the presence of HgCl_2 , an inhibitor of aquaporin water channels.

Components of the organelle, as identified using some of the markers mentioned above, are seen in dividing tachyzoites within the PV. Apparently, there is a fragmentation of the vacuole, giving rise to small vesicles localized close to the trans component of the Golgi complex. These components may correspond to the VP1 compartment previously described by Harper et al. (2006). On the other hand, the large vacuole, that we designate as the TgLACVET (Large Anterior Cytoplasmic Vacuole of Extracellular Tachyzoite), may correspond to a large vacuole previously described in tachyzoites invading host cells (Drozdzowicz et al. 2003).

Asady et al. (2020) reported the attainment of a knockout of the gene coding for a membrane protein involved in the transport of phosphate (TgPIT). They observed a set of vesicles budding from the plasma membrane resembling acidocalcisomes. It was suggested that TgPIT is also localized in the membrane of the TgLACVET. Altogether the available information point toward a role of TgLACVET, together with acidocalcisomes, in the control of parasite homeostasis and osmotic stresses.

6. INTRAVACUOLAR STRUCTURES

Once inside the PV, the tachyzoites of *T. gondii* starts secreting the contents of dense granules that will both contribute to the enlargement of the vacuole to accommodate the rosette of daughter cells that will result from division by endodyogeny (see chapter 5) and give rise to a system of membranous tubules: the intravacuolar network (IVN) (Corinne Mercier et al. 2002; Sibley et al. 1995) (Figure 8). The parasites in the PV remain attached to a membranous sac: the residual body. Far from being a waste bag, this structure plays an important role in maintaining the synchronicity of the endodyogeny cycles, metabolic cooperation and triggering of egress (Marcia Attias, Miranda, and De Souza 2019).

The tubules of the IVN connect the parasites to one another and the rosette of tachyzoites to the inner surface of the PV (Figure 8). The average diameter of the tubules ranged from 30-35 nm and in cross-section, an electron-dense dot was seen at the center of the lumen. The tubules could bifurcate along its trajectory (Magno et al. 2005). More recently, using Helium Ion Scanning Electron Microscopy, where samples do not need to be sputtered with a

conductive metal coating, the surface of the tubules had a less smooth appearance, with bumps, that may correspond to branching points. Besides it, very tiny filamentous structures could be observed, as well as the previous description that the tubules do not fuse either with the parasite pellicle and the PV membrane (Wanderley de Souza and Attias 2015).

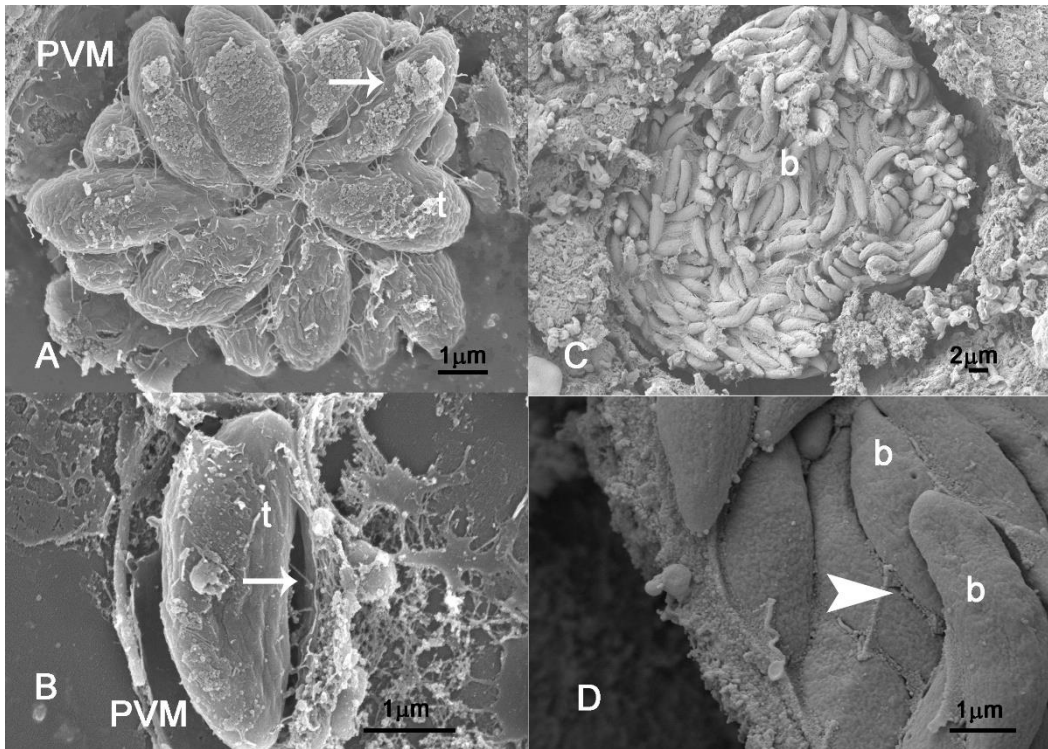


Figure 8. Scanning electron micrographs of PV (A) and (B) and tissue cysts (C), and (D). A rosette of tachyzoites (t) inside a PV (A). The parasites are linked to each other by the posterior end. A network of membranous tubules (arrows) contributes to keeping the rosette arrangement and the attachment to the PV membrane (PVM) (B). Tissue cyst in the brain of a mouse containing hundreds of bradyzoites (b) tightly packed leaving little space for the cystic matrix that is mainly granular (D). Electron micrograph from Attias M. personal archive.

Bradyzoites, on the other hand, remain in tissue cysts (Figure 8) where they pack, leaving little room for the accumulation of a granular matrix that is very distinct from the IVN both in appearance and composition. The cyst itself is surrounded by a corrugated membrane and a thick wall rich in N-acetyl-galactosamine residues, labeled by DBA (*Dolichus biflorus*) lectin.

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Chapter 3

***TOXOPLASMA GONDII* ENTRY MECHANISMS AND ESTABLISHMENT OF THE PARASITOPHOUS VACUOLE**

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ABSTRACT

Toxoplasma gondii is a successful intracellular parasite for developing effective motility mechanisms, invasion, intracellular growth, and egress to infect other cells, avoiding all of these steps being destroyed by the host. Regarding the mechanisms of entrance into the cells, the most studied is one directed by the parasite through a process known as an active invasion. This process depends on gliding motility and comprises the secretion of molecules from the parasite's secretory organelles, such as the micronemes and rhoptries, which will intermediate the invasion. This mediation occurs by the moving junction creation, a transient structure containing proteins secreted by *T. gondii*, and other molecules' participation from the host cell. The junction protects *T. gondii* in the parasitophorous vacuole in the process of assembling, excluding its membrane molecules that would address it to the fusion with the host lysosomal system. If the parasite's interiorization into the cell is active, it could develop normal replication by endodyogeny, forming rosettes throughout the lytic cycle. The parasite may enter the cell by mechanisms triggered by the host cell, such as phagocytosis and distinct endocytic processes, such as macropinocytosis and clathrin-mediated endocytosis, where some participation of the parasite also occurs. The fate of parasites that have been internalized the cell through endocytic processes, such as by phagocytosis, is, in general, the elimination by different intracellular mechanisms. Thus, the infection is not successful. After the parasite entry, the parasitophorous vacuole establishes close to the host cell organelles contributing to its growth. Inside the vacuole, *T. gondii* can secrete molecules that prevent the host elimination mechanisms, such as the occurrence of a classic autophagy pathway or the vacuolar membrane's disruption, thus giving course to

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infection in the cells. Such molecules may also turn the PV membrane-permeable through cytoplasmic molecules.

1. INTRODUCTION

When interacting with its host cells, *Toxoplasma gondii* does not have a highly specific mechanism for recognition and adhesion. This process is interposed by several host cell surface molecules, as surface glycoproteins, as well as by proteins secreted and exposed on the parasite surface, that will facilitate the process of adhesion to different types of host cells thus explaining its capacity to infect most of the cells. Upon contacting the cell, recognizing and adhering to it, the parasite can develop a gliding mechanism that facilitates its locomotion and invasion. It involves interacting with the parasite's proteins associated with the cytoskeleton and interacts with the host cell. In this invasion process provided by gliding, the parasite secretes proteins that mediate its active invasion with the moving junction formation. This transitory structure prevents the formed parasitophorous vacuole (PV) from fusion with the host endolysosomal pathway, favoring the development of the cycle replication of *T. gondii*. The parasite can also internalize the cell through pathways that have the host cell as a protagonist and can influence the fate of the infection, occasionally culminating in eliminating the parasite.

2. HOST CELL RECOGNITION AND ADHESION

The recognition of the host cell that is a crucial step for the *Toxoplasma* entry into the host cell does not appear to be limited to any host membrane subdomain, as these subdomains are not included in the membrane of the recently formed PV (Charron and Sibley 2004). The most discussed hypothesis is that for internalization into the cell, the parasite secretes an arsenal of molecules that will act either as ligands or receptors, favoring its entry into any cell type. Despite this lack of specificity, the beginning of the parasite contact with the cell may include three distinct morphological profiles, which were documented when *T. gondii* interacted with neutrophils, these are: (a) parasite internalization keep up with host cell emission of filopodia, (b) the formation of tubular membrane expansion around the parasite and (c) deepening of the host cell membrane at the entrance region (MacLaren, Attias, and De Souza 2004) (Figure 1).

The early stage of the entry process occurs through *T. gondii* with the host cell by its apical end. Scanning electron microscopy of initial stages of interactions shows the protozoan's adhesion to the host cell surface through different regions of the protozoan. However, successful infection only happens when the anterior end of *T. gondii* is involved in the interaction. Areas of cell adhesion and recognition of surface molecules between the parasite and the host cell occurs by low-affinity bonds with molecules constitutively exposed in the parasite's membrane's outer surface, anchored to it by glycosyl-phosphatidyl-inositol (GPI). These molecules are the SAGs (surface antigens), the SRS (SAG related-sequence), and the SUSAs (non-SAG antigens) (Reviewed by V. Carruthers and Boothroyd 2007). In addition, they recognize receptors in different cells, such as heparan sulfate, proteoglycans,

and laminin (Haas and Plow 1994; Carruthers et al. 2000; Ortega-Barria and Boothroyd 1999).

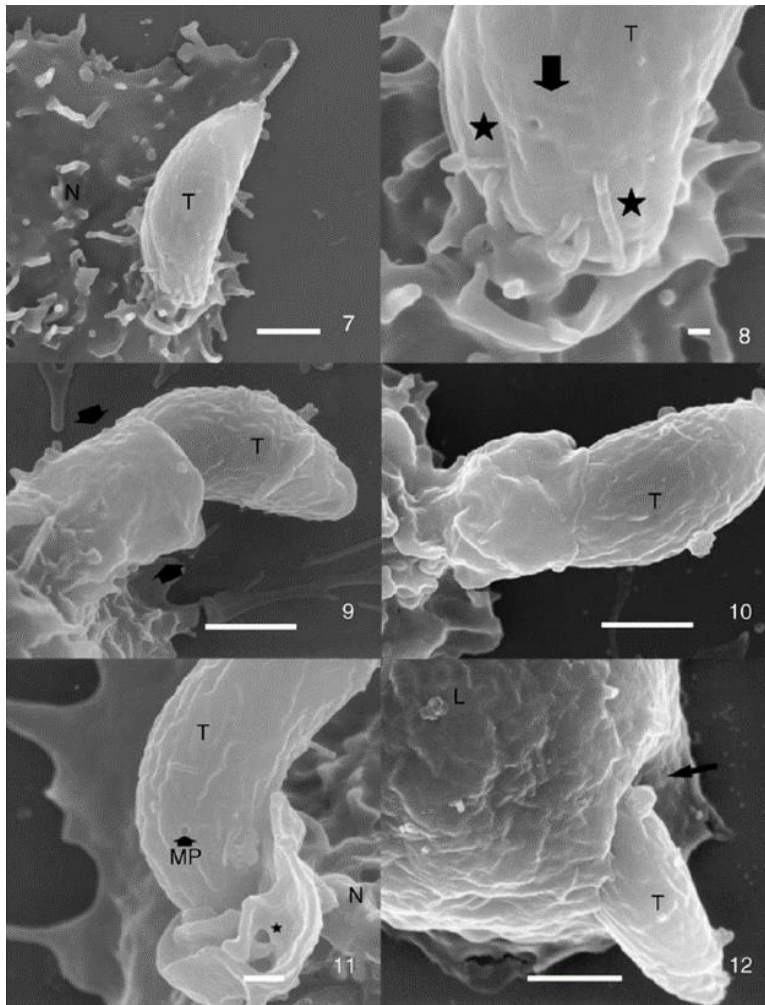


Figure 1. Scanning electron microscopy showing *T. gondii* and the host cell neutrophil. In the parasite's entry mechanism in this cell, the neutrophil (N) extends filopodia around the parasite (stars) (7 and 8), could form a tunnel around the parasite (9, 10, and 11) or still could be seen the formation of a cavity in the host cell's membrane. Bars: 1 mm (7, 9–12) and 0.1 mm (8). (After MacLaren, Attias, and De Souza 2004).

Early studies aimed to understand the parasite process with the cell through its surfaces employed lectins and neoglycoproteins labeled with colloidal gold particles for the ultrastructural localization of carbohydrate residues sugar-binding sites in thin sections tachyzoites of *T. gondii*. However, it was found that the lectins and glycoproteins labeled the rhoptries. Still, not the parasite surface and the data inferred participation of the organelle in the release of molecules that are known today fundamental for the parasite and host cells relation (De Carvalho et al. 1991).

The study of the recognition process of the host cell by the parasite that was done through previously staining the components of the host cell membrane, such as proteins and lipids,

proved that these could be transferred to the parasite membrane and the PV membrane (PVM). In the epithelial Vero cells, the labeled molecules were found at the contact between the parasite and the host cell and then localized on the PV membrane and intravacuolar parasites (Figure 2). The parasites labeled with these membrane components probes before the infection could attach to the host cell surface but lost the fluorescent probes after the invasion (Pacheco-Soares and De Souza 1998). Regarding the components labeled on the macrophage's membrane, protein and lipids were incorporated into the parasite's membrane and the PVM. The authors also observed this phenomenon after treating the macrophages with cytochalasin-D, preventing phagocytic events (Pacheco-Soares and De Souza 2000a).

De Carvalho et al. (1993) also analyzed the possible role of surface components of macrophages in the interaction with tachyzoites of *T. gondii* by evaluating the effect of digestive enzyme treatment during interiorization tachyzoites of *T. gondii*. The surface of untreated or cytochalasin-treated macrophages was altered by treatment with neuraminidase, phospholipase C, trypsin, protease, and hyaluronidase, induced a rise in the infection. Thus, the removal of sialic acid residues from the cell surface (by neuraminidase), phosphate present on the plasma membrane phospholipids (by phospholipase C), and acid mucopolysaccharides (by hyaluronidase) could facilitate the association between the parasite and the cell surface molecules. Furthermore, the treatment of the macrophages with trypsin significantly inhibited parasites' uptake, suggesting that macrophages possess a trypsin-sensitive protein on their surface that would be important in the invasion by *T. gondii*. Macrophages' infection after treatment with cytochalasin D blocked the typical phagocytic process and inhibited the cells' infection by *T. gondii*. This inhibition caused by cytochalasin D could be reversed when the cells were also treated with phospholipase C. At the same time, this profile was not observed in the treatments with neuraminidase and hyaluronidase. It shows that the first effect described on the increase of infection induced by neuraminidase and hyaluronidase would occur by a classic phagocytic process, whereas that obtained using phospholipase resulted from active penetration (De Carvalho et al. 1993).

Thus, sialic acid is an abundant molecule on the cell surface, and it is recognized as an important receptor for host cell invasion by several protozoa, including *T. gondii*. It was verified that when *T. gondii* infects host cells presenting different levels of sialic acid residues (wild-type Chinese hamster ovary (CHO) cells and glycosylation mutants, Vero and LLCMK2 cells), the parasite could also present different levels of interiorization. The infection was to a lower extent in cells that presented few or no surface-exposed sialic acid residues. The same profile was observed if sialic acid residues were removed by neuraminidase treatment, different from those described for macrophages in previous studies. In contrast, adding sialic acid residues to surface-exposed glycoconjugates favored the infection by *T. gondii* (Monteiro, Soares, and Souza 1998). A more recent report presented a novel protein putative TCP-1 chaperonin in *T. gondii* (TgTCP-1) with a specific binding activity to sialic acid and DC and Vero cells in vitro. The binding of TgTCP-1 protein to DC and Vero cells was inhibited by incubation with free sialic acid or by neuraminidase treatment of the cells, similarly, a reduction in the *T. gondii* invasion in Vero cells was detected after pre-incubation of the cells with recombinant TgTCP-1 protein (N. Yang et al. 2020).

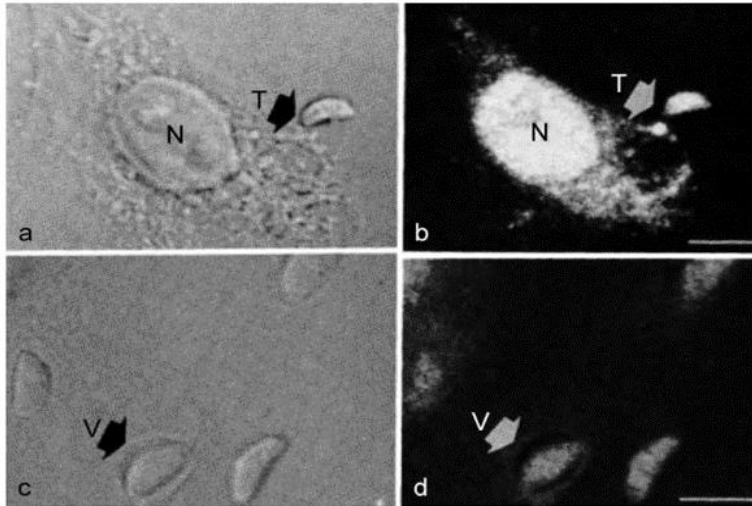


Figure 2. The transfer of molecules from the host cell membrane to the parasite in the interaction process. Vero cells were previously labeled with DTAF labeled membrane lipids and then interacted with *T. gondii* for 5 (A and B) and 30 minutes (C and D). The labeling was transferred from the host cell membrane to the parasite (T) (A and B). After 30 minutes of interaction, the PVM was also labeled, and the surface of parasites appear labeled (V) (C and D). Bars: Figs a and b = 25 μm ; c and d = 10 μm (After Pacheco-Soares and De Souza 1998).

T. gondii can also adhere to a wide variety of host cells through the mediation of micronemal adhesins (MICs), inserted into the parasite's plasma membrane after the apical exocytosis of micronemes. In a recent review, Liu and colleagues (2017) presented the associations already described between these adhesins and many surface molecules in the host cell (Liu et al. 2017). It was highlighted the association of MIC1 and MIC13 to the host surface sialylated oligosaccharides (Fourmaux et al. 1996; Friedrich, Matthews, and Soldati-Favre 2010); the interaction of MIC2 with glycosaminoglycans (V. B. Carruthers, Giddings, and Sibley 1999), and the adhesion molecule 1 (ICAM-1), which is considered essential for transmigration of parasites across host cellular barriers (Barragan, Brossier, and Sibley 2005). In addition to the description of MIC3 as an important adhesin for *T. gondii* invasion, Zhang et al. (2019) have investigated the participation of ROP9 (roptry protein) and the surface protein SAG2 in the invasion process. It was found that the transcription and expression levels of these proteins in invading *T. gondii* were higher compared to free parasites. Furthermore, these proteins specifically bind to both heparin and host cell surface, and the previous incubation of these proteins with the host cells prevented the *T. gondii* invasion (Zhang, Jiang, and Chen 2019).

3. GLIDING MOTILITY

T. gondii and other members of the Apicomplexa do not have a visible locomotion system, like the cilia and flagella, or display an ameboid movement such as other parasites. Thus, *T. gondii* and other apicomplexans rely on a piece of machinery called glideosome for their motility (Opitz and Soldati 2002), a molecular complex composed of an actin-myosin

system (actin filaments, myosin, and associated proteins). Glideosome localizes under the plasma membrane and is responsible by power the parasite gliding motility, allowing it to migrate across biological barriers, and actively invasion into the host cell (Vanderberg 1974; Aikawa et al. 1978; Håkansson et al. 1999) and it is also important to the parasites' egress from infected cells (Blackman and Carruthers 2013), contributing to the spread of the infection.

The parasite gliding on the cell surface helps recognize and interact with its surface molecules, and thus the parasite becomes well adhered to the host cell. However, this constant motility is also essential for the invasion. So, the glideosome machinery, whose activity is needed to maintain the parasites' roaming profile, is confined to a space in the pellicle, between the plasma membrane (PM) of the parasite and the inner membrane complex (IMC) (Opitz and Soldati 2002). The IMC is formed by flattened vesicular sacs connected to a network of an intermediate filament-like protein named alveolins that associates the IMC to the subpellicular microtubules. Thus, the glideosome is firmly attached to the cytoskeleton and can associate with the host cell's extracellular receptors, enabling the locomotion and invasion process. This attachment is provided by MICs that could act as specific binders to the host receptors (Paing and Tolia 2014).

Thus, the gliding results from the backward translocation of these adhesion-receptors complexes, driven by the myosin motors along the parasite's actin filaments, in the space between the IMC and the parasite's PM. In this mechanism, while the parasite glides onto a surface, whether a cell or a glass slide, trails left by the parasite could be visualized due to translocation of proteins through the membrane and then their shedding on the substrate. Thus, the parasite releases surface membrane proteins on the trails such as SAG1 (Dobrowolski and Sibley, 1996), SAG2, SAG3, and p35, and lipids (Håkansson et al. 1999). These trails are easily followed after staining with specific antibodies (L. David Sibley, Håkansson, and Carruthers 1998). It was a process firstly described in *Plasmodium* spp. (Vanderberg 1974).

Furthermore, it could be mediated, for example, by parasite's adhesins proteins with transmembrane domain, represented in *Plasmodium* by proteins from the thrombospondin-related anonymous protein (TRAP) family and in *T. gondii*, the homologous MIC2 (L. David Sibley, Håkansson, and Carruthers 1998). When in contact with the host cell or other substrate, MIC2 secretion occurs at the apical pole of the parasite, most probably in the area localized just below the polar ring from where the sub-pellicular microtubules emerge (Paredes-Santos, de Souza, and Attias 2012) and interact with the substrate surface and the parasite actin-myosin system, similar to the integrins in other models (Meissner, Ferguson, and Frischknecht 2013). It results in the microneme-substrate's translocation complex and forwards gliding on the substrate or into the host cell (Soldati and Meissner 2004) (Figure 3). In addition, recent reports showed that the MIC2 adhesin undergoes proteolytic processing by C-mannosyltransferases that act on specific domains, the adhesin's TSR domains. These domains are responsible to associates the MIC2 cytoplasmic domain, which is connected to the parasite actin-myosin motor, to the extracellular domain bound to host cell molecules, and the C-mannosylation on it promotes the parasite adhesion to host cells and could be a factor associated with the virulence (Albuquerque-Wendt et al. 2020).

The trails deposited by the parasite were also verified during the *T. gondii* sporozoites' interaction with the host. After the invasion, the sporozoites residing in the PV were shown to release a long, membranous tail, named "sporozoite-specific trail," composed of many dense

granule proteins (GRAs). This shedding of trails may represent an important mechanism in the transcellular migration by intravacuolar *T. gondii* sporozoites (Tartarelli et al. 2020).

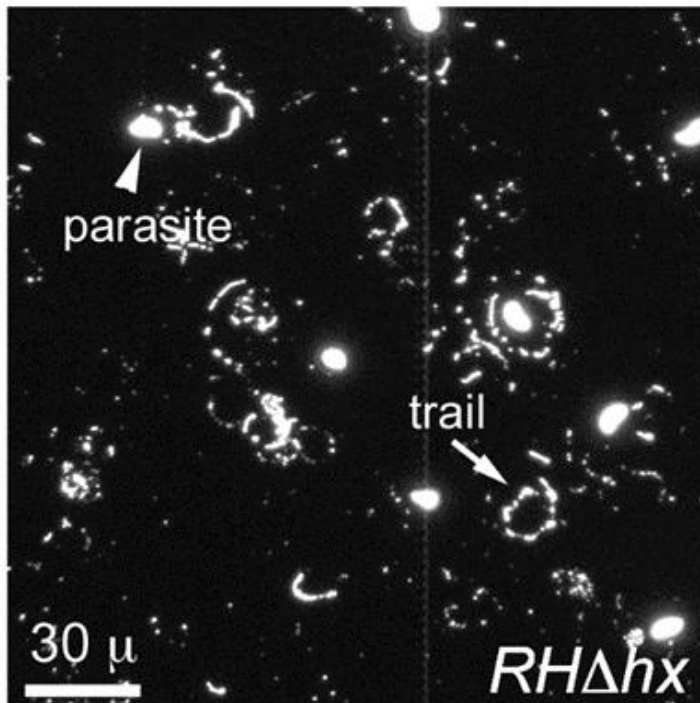


Figure 3. The *T. gondii* gliding with deposition of trails.

In the trail assays of tachyzoites from RH strain (RH Δ hx), the samples were stained with mouse anti-SAG1 antibody and goat anti-mouse Alexa-488 to label the trails (arrow) deposited by the parasites (After Heaslip et al. 2011. PLoS Pathogens 7(9): e1002201. doi: 10.1371/journal.ppat.1002201. Image under the terms of the Creative Commons Attribution License CC BY 4.0).

The translocation of adhesin-substrate complexes is regulated by actin filaments polymerization that serves as a substrate for short single-headed myosin heavy chain myosin A (MYOA). MYOA is static and is connected to the parasite's IMC (L. David Sibley 2003). The MYOA's feature of not being mobile added the relative shortage of actin filaments of the parasite, suggesting that the local polymerization induces the parasite's motility and that the glycolytic enzyme aldolase could participate process (Jewett and Sibley 2003). The cytoplasmic domain of some adhesins, such as MIC2 and AMA-1, is also associated with aldolase, which acts as a connector between the extracellular adhesive domain of the adhesins and the parasite's cytoskeleton, providing the vital link to generate motility and being also crucial to the parasite invasion (L. David Sibley 2003; Boucher and Bosch 2015). Thus, *T. gondii* that does not express aldolase presented defective motility and invasion (Starnes et al. 2009), which may have been associated with incomplete glycolysis that could generate toxicity. It was related that aldolase is dispensable for the gliding in parasites grown in the absence of glucose (Shen and Sibley 2014). Another connector, the glideosome-associated connector (GAC), also associates with the parasite F-actin and translocates along with the parasite during the locomotion and invasion, binding to the tail of the adhesin MIC2 (Jacot et al. 2016).

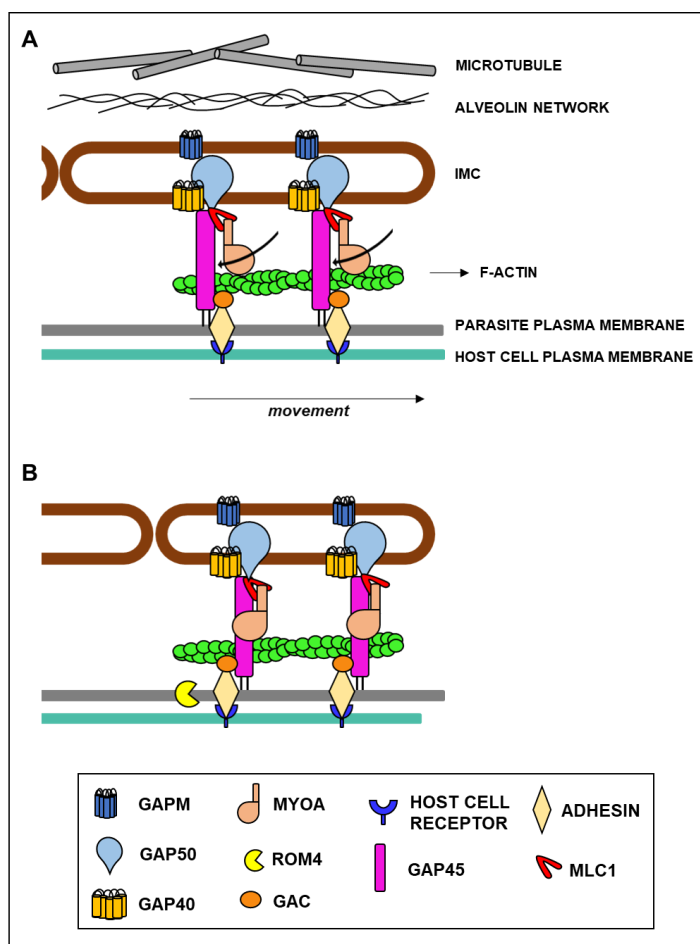


Figure 4. Gliding motility and the glideosome complex. A - A network of intermediate filament-like proteins, termed alveolins, connects the IMC to the subpellicular microtubules. The glideosome complex comprises the MYOA motor and its myosin light chain, MLC1, linked to the IMC proteins GAP40 and GAP50 through GAP45. GAP45 maintains the spacing of the parasite plasma membrane and the IMC. The connector GAC associates F-actin and adhesins. The glideosome is also linked to the cytoskeleton through GAPM proteins, thus enabling traction forces. B - MYOA head domain on the F-actin changes the conformation following ATP hydrolysis drives the parasite's forward movement. Rhomboid-like protease 4 (ROM4) cleaves the transmembrane domain of adhesins to disengage their interaction with host receptors.

Glideosome is also composed of other molecules, such as myosin light chain 1 (MLC1) (Herm-Götz et al. 2002), GAP45, GAP50 (Gaskins et al. 2004; Baum et al. 2006), and GAP40 (Frénal et al. 2010). The head domain of MYOA converts the chemical energy released by hydrolysis of ATP into directed movement along with the parasite F-actin. MLC1 binds to MYOA and collaborates to convert energy into motion (Green et al. 2006; Bosch et al. 2007; Bookwalter et al. 2014). In *T. gondii*, MLC1 brings MYOA to its site of action within the pellicle, anchoring it to the IMC through its association with GAP45 (Frénal et al. 2010). This association ensures the pellicle's integrity during the motility by forming a bridge anchored into the PM and maintains the appropriate spacing between the IMC and the parasite PM (Frénal et al. 2010). To generate traction, MYOA must be firmly fixed to the

cytoskeleton of the *T. gondii* (Tardieux and Baum 2016), possibly through the proteins GAP50 (Gaskins et al. 2004) and GAP40 (Frénal et al. 2010) on the IMC side and on extracellular substrates of the host cell on the other side. The indirect association between IMC and the subpellicular microtubules is provided by proteins associated with glideosome with multiple-membrane spans (GAPMs) present on IMC and contact with alveolins GAP50 (Bullen et al. 2009). GAP40, GAP50, and GAPMs are essential in IMC's biogenesis during intracellular replication (Harding et al. 2016) (Figure 4).

Therefore, the parasite's actin is an essential component of motility and, consequently, the host cells' invasion. In apicomplexan parasites, the actin is kept in low concentration when in polymerized or filamentous form, as F-actin, besides being unstable and maintaining substitutions in residues of amino acids that generally stabilize the interaction between actin monomers in other organisms (Skillman et al. 2011; Vahokoski et al. 2014). Although the evidence of filamentous actin is crucial to the locomotion and invasion process, actin filaments' visualization has been extremely laborious. In *Plasmodium*, F-actin proved to be short (100nm) and heterogeneous when evaluated by electron microscopy and ultracentrifugation (Schüler and Matuschewski 2006), and imaging studies demonstrated the formation of an extensive F-actin cytoskeleton in both gametocytes and ookinetes (Hliscs et al. 2015; Siden-Kiamos, Louis, and Matuschewski 2012). *T. gondii* actin is encoded by one single gene, ACT1, that presents nearly 80% of sequence identity with the actin isoforms in mammals and 93% similarity with ACT1 from the apicomplexan *Plasmodium* (Janice M. Dobrowolski, Niesman, and Sibley 1997). Biochemical studies indicate that most *T. gondii* actin (>97%) is found in the monomeric state (Janice M. Dobrowolski, Niesman, and Sibley 1997). The actin filaments are not found in nonmotile tachyzoites examined by conventional electron microscopy, except under a membrane-permeable actin-polymerizing and filament-stabilizing drug stimulus Jasplakinolide, that caused the polymerization of actin filaments at the anterior end of the tachyzoite in association with the conoid (Shaw and Tilney 1999). However, the expression of actin-chrome bodies allowed the visualization by the first time *T. gondii* F-actin. It revealed some functions for parasite F-actin, which are not directly related to the gliding and invasion process. F-actin structures were found in the parasite cytosol and a network that connects parasites in the PV, allowing an exchange of vesicles. When actin was deleted in the parasite, the network was not formed, and the parasites replicate asynchronously (Periz et al. 2017). Using the same approach, Periz et al. (2019) described that this F-actin network mediates the micronemes recycling from the mother-to-daughter parasites. In this process, the residual body works as a storage and sorting region for these organelles (Periz et al. 2019).

Del Rosario et al. (2019) analyzed the F-actin dynamics during host cell invasion and through super-resolution microscopy. Real-time imaging identified a concentration of F-actin at the top of a pre-invading parasite, an F-actin ring at the junction area during an invasion, and networks of perinuclear and posteriorly localized F-actin. Furthermore, parasites with the dysfunctional actin-myosin system, genetically altered, presented a significant reduction of junctional and perinuclear F-actin, and this modification has also affected the passage of the parasite nucleus through the junction complete the invasion. Thus, this study states that F-actin machinery favors nuclear passage, pushing it through the constriction (Del Rosario et al. 2019).

In the dynamic process of actin regulation, these parasites also have a limited repertoire of actin-binding proteins (ABPs). They do not have the actin-related protein 2/3 complex

(ARP2/3), part of the actin nucleation machinery (Gordon and Sibley 2005). Instead of these, two ABPs proteins — actin-depolymerizing factor (ADF) e profilin — contribute to monomers' high content hijacking free globular actin (Mehta and Sibley 2010; Skillman et al. 2012). Thus, the parasite's actin is maintained as a heterogeneous mixture of sequestered actin monomers and dynamic filaments of varying lengths that can be originated through an unusual polymerization dynamic. Formins (FRMs) act as potent nucleators of actin filaments (Baum et al. 2006). Three different actin nucleators are present in *T. gondii* and direct the dynamic of F-actin filaments. It was described that the formin FRM1, from the conoidal area, directs the F-actin availability that is necessary for the parasite motility, invasion, and egress; FRM2 localizes to the apical juxtanuclear zone of the parasite and is related to the apicoplast inheritance, while FRM3 is localized at the residual body and mediates the intravacuolar cell-cell communication (Tosetti et al. 2019).

According to the current model for the gliding, the force generated for motility and invasion relied exclusively on F-actin polymerized at the parasite's apical tip and translocated within the IMC and PM space parasite (Tosetti et al. 2019). However, new studies suggest the involvement of other mechanisms in the motility of *T. gondii*, such as a secretory-endocytic cycle that produces retrograde membrane flow. This process induces the recycling of receptors, creating attachment sites, regulating the parasite surface during the motility, and generating the retrograde membrane flow (Gras et al. 2019).

3.1. Regulation of Adhesin Secretion

The specific signaling pathways associated with the motility and invasion of *T. gondii* are not still understood. However, in response to the loss of integrity of infected cells, a reduction in potassium levels increases the intracellular calcium levels (Moudy, Manning, and Beckers 2001). This peak in calcium concentration serves as a signal for activating specific kinases and coordination of microneme secretion and, therefore, affects gliding motility, invasion, and egress (Brochet and Billker 2016; Lourido and Moreno 2015). For example, the kinase TgCDPK1 is essential for microneme (Lourido et al. 2010), and TgCDPK3 is important to start the motility because it phosphorylates TgMYOA (Gaji et al. 2015).

Yang et al. (2019) showed that a *T. gondii* guanylate cyclase-ATPase (TgGC) is also critical for calcium signaling. TgGC-deficient parasites were defective in motility, host cell attachment, invasion, and the subsequent host cell egress. Furthermore, TgGC is important for increasing cytosolic calcium concentration and microneme secretion, which is crucial for transducing extracellular pH and potassium levels to activate cytosolic calcium flux (L. Yang et al. 2019).

Other molecules and proteins are having a part in this process, such as cGMP-dependent protein kinase (PKG), that triggers the secretion of micronemes and the egress of the parasite, independently of calcium participation (Lourido, Tang, and David Sibley 2012; Collins et al. 2013; Govindasamy et al. 2016). The phosphatidic acid has also been identified as an important lipid mediator for the microneme secretion and the parasite's natural egress, acting parallel to calcium (Bullen et al. 2016; Bisio et al. 2019). The production of inositol 1,4,5 triphosphate by phosphatidylinositol phospholipase C (PI-PLC), and the competing calcium mobilization that occurs before the secretion of micronemes, are linked to the generation of diacylglycerol (DAG), which could be interconverted into phosphatidic acid (Bullen et al.

2016). The microneme surface has an acylated pleckstrin homology domain-containing protein (APH), crucial to detecting phosphatidic acid. In the parasites in which APH was depleted, the microneme secretion was blocked, affecting the glideosome function (Bullen et al. 2016). A C2 domains-containing protein (DOC2.1), also conserved along with the phylum, mediates micronemes' exocytosis in a calcium-dependent manner (Farrell et al. 2012). Another protein, located in the apical polar ring of tachyzoites, named *T. gondii* RNG2 (TgRNG2), has been shown to affect the secretion of micronemes (Katris et al. 2014).

The small GTPase Rab11A, which participates in the exocytosis in eukaryotic cells, is crucial for the cytoskeleton-driven transport of dense granules in *T. gondii*, also driving the release of their content. Thus, it was described that Rab11A also regulates parasite motility and adhesion to host cells, being the MIC2 secretion affected in parasite mutants. Furthermore, it was also observed an accumulation of Rab11A-positive vesicles and dense granules at the apical pole of extracellular and invading parasites, suggesting that Rab11A-dependent charge delivery regulates secretion during parasite interiorization into host cells (Venugopal et al. 2020).

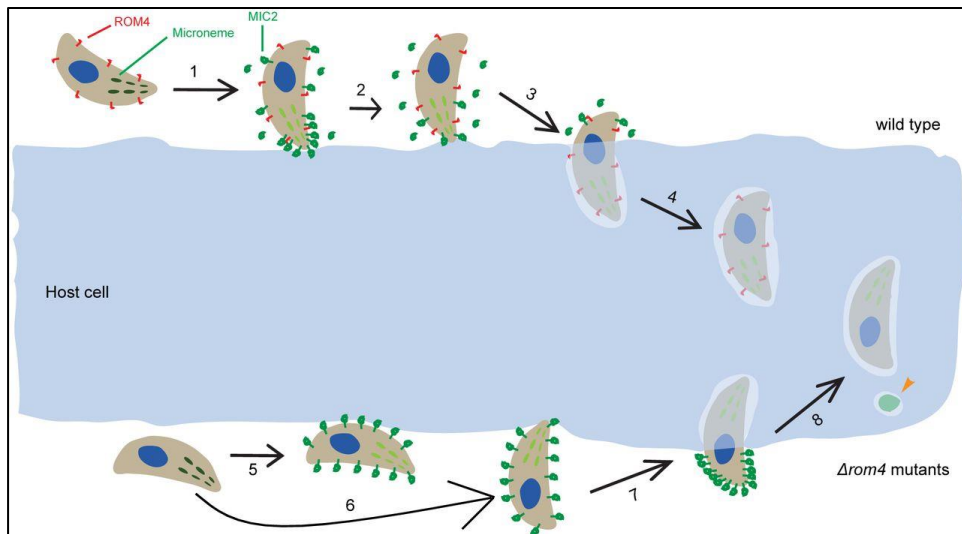


Figure 5. Model for MIC2 processing by TgROM4 during the invasion.

After contact with host cells, parasites discharge their micronemal adhesins (such as MIC2) to the surface (1). Adhesins are translocated to the posterior end by the motor complex (1-3). Normally, in WT parasites, ROM4 cleaves MIC2 to prevent aberrant host cell binding. ROM4-mediated cleavage allows MIC2 to form a temporal apical-to-posterior gradient that helps the parasite reorientate and establish an MJ (1). After the MJ formation, the microneme secretion stops, and surface MIC2 is shed by ROM4 (2 and 3). The parasite internalizes the host cell (3), and no MIC2 is on the surface when it is fully internalized (4). TgROM4-mutant parasites cannot cleave surface MIC2, lose the MIC2 gradient, often bind to host cells laterally (5), and cannot form the MJ, reducing invasion ability. If the mutant parasite orientates correctly and forms an MJ (6), it normally invades the host cell (7). The unprocessed MIC2 that is accumulated on the surface is removed during active penetration (7). After its full entry, capped MIC2 is separated from the parasite (arrowhead) (8) (After Shen et al. 2014. MBio 5 (5). Doi: 10.1128/mBio.01795-14. Image under Creative Commons Attribution 4.0 International license).

The concentration gradient of adhesins secreted by micronemes during the invasion process in a certain region of the parasite's membrane contributes to the reorientation of the parasite on the host cell's surface fundamental step for invasion. In *T. gondii*, a conserved serine protease (serine-protease intramembrane protease-4 (TgROM4) rhomboid-like) cleaves the adhesins (such as MIC2 and AMA-1) in its transmembrane domain, according to the need. This cleavage causes an interruption in the parasite's ligand and receptor interactions in the host cell. However, the deletion of TgROM4 does not affect *T. gondii* survival *in vitro*, even if not compensated by another protease (Rugarabamu et al. 2015; Buguliskis et al. 2010; Shen et al. 2014). The mutant parasite for ROM4 has its gliding process affected because the adhesin-receptor complexes migrate to the posterior pole of the parasite, and its motility becomes stationary, with an increase of the parasite adhesion and affecting the apical orientation, indispensable for its invasion (Rugarabamu et al. 2015; Shen et al. 2014) (Figure 5).

4. ACTIVE INVASION

After host cell recognition and adhesion, together with the constant gliding process, the parasite is prone to invade the cell to develop the infection. Electron microscopy analysis of some apicomplexan parasites along the invasion process (J. F. Dubremetz and Torpier 1978; King 1981; Russell and Sinden 1981) highlighted that the apical pole of the parasite was in contact with the plasma membrane of the host cell and then was noticed a tight transitory junction between the parasite and the membrane of the host cell, which allowed the parasite entry process, possible as a result of the association between the surface ligand capping, parasite motility and microfilaments (Aikawa et al. 1978). This junction was described as a moving junction (MJ) and was observed for the first time using electron microscopy analysis of the merozoites from *Plasmodium knowlesi* invading erythrocytes (Aikawa et al. 1978). As it is known, a considerable amount of the *T. gondii* population actively invades its host cells through MJ formation (Reviewed by Horta et al. 2020).

In the most well-known entry process of *T. gondii* into host cells, it is believed that the parasite itself directs the process, which is crucial to the parasite's fate. The active invasion can influence the composition of the PVM, preventing the fusion with host lysosomes (Morisaki, Heuser, and Sibley 1995). Organelles of the parasite's apical complex – micronemes, rhoptries – participate in the process by exocytosis of their contents (Jean François Dubremetz et al. 1993; Carruthers and Sibley 1997). It is worth noting that these secretory organelles, including the dense granules, discharge their contents sequentially during the interaction with the host cell. The coordinated, sequential release of micronemes, rhoptries, and then dense granules suggests their contents are involved in attachment, PV formation, and modification of the host cell, respectively (L. David Sibley et al. 1999). The secretion by micronemes and rhoptries is modulated by calcium, an important mediator of cell invasion. It was identified that parasites' knockdown of TgFER2, a calcium sensor protein, had the rhoptries secretion and invasion affected, not forming the MJ (Coleman et al. 2018). The parasite cytosolic calcium signaling activates motility, and after the invasion is completed, the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A

catalytic subunit 1 (PKAc1) of *Toxoplasma* participate in the suppression of calcium signaling (Uboldi et al. 2018).

During the invasion, remodeling in the host cell actin cytoskeleton formed a complex composed of F-actin, ARP2/3, and cortactin at the site of formation of MJ (Gonzalez et al. 2009). This remodeling of the cytoskeleton is necessary to anchor the parasite to the host cell and initiate deformation of the host cell membrane during invasion (Gaji, Huynh, and Carruthers 2013).

The host cell participation in the parasite entry has long been considered in several studies. Thus, the previous treatment of the cells with cytochalasin, a drug that interferes with actin filaments, followed by *Toxoplasma* infection, caused changes in the morphology of the cells and considerably inhibited the parasite's internalization (Ryning and Remington 1978; S. R. L. Silva, Meirelles, and De Souza 1982). It is important to highlight that after the treatment, the cells were washed. The medium was replaced by a new one in which the parasites were included, proving that the residual presence of the drug could not interfere with the parasite actin. Another study pointed out that the maintenance of host cell actin cytoskeleton integrity is determinant for the invasion by *T. gondii* and showed that ADP-ribosylation factor-6 (ARF6), a molecule that regulates membrane trafficking and actin cytoskeleton rearrangements at the plasma membrane, is recruited to the PV of *T. gondii* and also presented a role in the parasite invasion (C. V. da Silva et al. 2009). On the other hand, some studies have reported that the invasion could also be dependent on the parasite's actin filaments. The parasite invasion into cytochalasin D-resistant host cells was blocked by cytochalasin D treatment. In contrast, parasite mutants that presented motility and invasion in the presence of cytochalasin were able to invade wild-type host cells in the presence of drugs (J. M. Dobrowolski and Sibley 1996). The conditional genetic excision of actin from *T. gondii* severely compromised motility, invasion, and egress (Andenmatten et al. 2013; Drewry and Sibley 2015), although the parasites can still be maintained in culture for several days, which indicates the possibility of the co-existence of an actin-independent motility mechanism (Egarter et al. 2014; Whitelaw et al. 2017).

The molecular organization of the MJ comprises a set of proteins specifically secreted by the neck of the rhoptries (RON2, RON4, and RON5), creating a complex on the host cell membrane, in which RON2 has a domain that would serve as a receptor for the parasite. The binding of this receptor, RON2, to the protein AMA1, that is secreted by the parasite's micronemes and is anchored to the parasite membrane, would constitute a general mechanism of recognition for any cell type by *T. gondii* (Tonkin et al. 2011; Besteiro, Dubremetz, and Lebrun 2011). Exposed in both sides of the junction, AMA1 and RON2 form a compact complex that maintains the proximity among the parasite and host cell membranes (M. Lamarque et al. 2011; Tyler and Boothroyd 2011). The link between AMA1–RON2 in *T. gondii*-host cell interaction is a strong affinity bond required to support the invasion process's strength and tension (Tonkin et al. 2011). When AMA1 is actively linked to RON2 in the active invasion, AMA1 becomes less susceptible to cleavage by rhomboid proteases, which allows the two proteins to keep the connection between the cells intact throughout the invasion (Krishnamurthy et al. 2016).

RON2 is linked to AMA1 by a small ectodomain, and the other domain of RON2 is located on the cytosolic surface of the host cell membrane, as part of a complex with the proteins RON4, RON5, and RON8 (M. Lamarque et al. 2011; Besteiro et al. 2009; Straub et al. 2009) (Figure 6). This complex delineates the MJ in *T. gondii* and recruits cytosolic

proteins from the host cell such as ALG2-interacting protein X (ALIX; also known as programmed cell death 6-interacting protein), tumor susceptibility gene 101 protein (TSG101), CBL-interacting protein of 85 kDa (CIN85; also known as SH3 domain-containing kinase-binding protein 1) and CD2-associated protein (CD2AP), comprising a set of adapter proteins known to connect the membrane proteins to the underlying cytoskeleton (Guérin, Corrales, et al. 2017). Also, toxofilin secretion by *T. gondii* along the invasion causes disassembly of the actin network at the entrance site of the parasite, allowing the RON complex to be associated with a newly formed F-actin ring structure in the MJ site (Gonzalez et al. 2009; Delorme-Walker et al. 2012). Besides actin interaction, the host microtubules have also been localized at the parasites' entry site that early invades the host cell, specifically on the MJ. The host microtubule disruption significantly reduced the parasite invasion early after stimulating parasite invasion but not at later time points and did not affect parasite contact, attachment, motility, or penetration (Sweeney et al. 2010). It was also reported that the host cell' β -tubulin is a binder of RON4 in the invasion process (Takemae et al. 2013). However, other host cell components are crucial for invasion. Just as heparan sulfate was described above as important for parasite adhesion to host cells, it also appears to have a role in invasion. A binding assay with CHO mutant cells indicated that heparan sulfate is required for binding to RON4. RON4 recombinant protein binds directly to heparin, and sulfation on glucosamine of heparan sulfate is important for RON4 binding. It was also showed that RON4 interacts with glypican (Takemae et al. 2018).

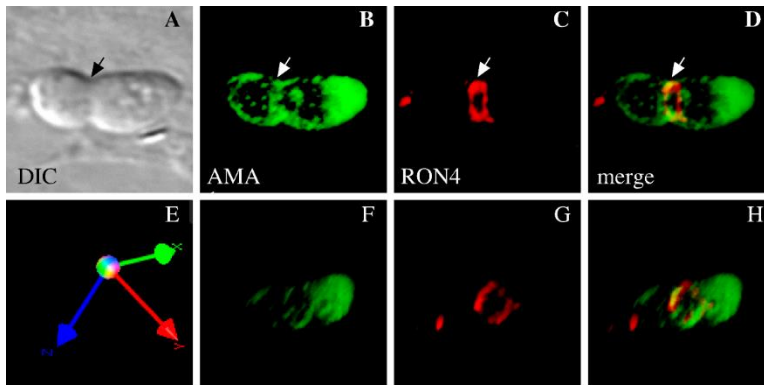


Figure 6. AMA1 and RON4 at the MJ during *T. gondii* invasion.

The parasites were allowed to invade after the synchronization process. The samples were then processed with rabbit antisera raised to recombinant RON4 and Texas red-conjugated goat-anti-rabbit antibody and an antibody specific for the cytoplasmic tail of TgAMA1 (CL22). (A–D) The parasite has approximately 40% of it internalized in the host cell. The arrows show the MJ. (E) Axis of rotation for the reconstruction and (F–H) the new view of the images present in (B–D) are shown (After Alexander et al. 2005. PLoS Pathogens 1 (2): e17. Doi: 10.1371/journal.ppat.0010017. Image under the terms of the Creative Commons Attribution License CC BY 4.0).

The AMA1–RONs complex could provide a physical link between the parasite's cortical cytoskeleton and the host cell, acting as a stable structure in which the parasite applies traction during the invasion. Genetic approaches in *T. gondii* have validated the contribution of the complex AMA1–RONs to the invasion (Beck et al. 2014; M. H. Lamarque et al. 2014; Straub et al. 2011; Mital et al. 2005); however, the parasite's motility was not substantially affected. Other studies suggest that AMA1 has a function in signal transduction. The

connection between the ectodomain of AMA1 and RON2 induces the dephosphorylation in the AMA1 cytosolic tail, increasing invasion efficiency (Krishnamurthy et al. 2016). Although some components of the MJ have been depicted as essential for its assembly, mutant parasite for RON4 showed a modest decrease in the invasion capacity; however, in these mutant parasites, the expression of the endogenous N-terminal fragment of RON5 was affected, highlighting that RON4 stabilizes the other members of the MJ (Wang et al. 2017).

Recently a new protein secreted by rhoptries, known as RON4L1, was identified and showed to be part of the MJ complex in *Toxoplasma*. During the invasion, RON4L1 is associated with MJ and targeted to the host membrane's cytosolic face. Parasites RON4 L1 knock-out could lead mice to deathless efficiency than the normal parasites, although the protein is not essential for invasion in vitro. The co-existence of independent MJ complexes in tachyzoite of *Toxoplasma* is possible because the RON4L1 protein and its traffic to the MJ are not affected without RON2 RON4, and RON5 (Guérin, El Hajj, et al. 2017).

After the invasion, the newly formed PV detaches from the plasma membrane through an unknown mechanism. The inhibition of dynamin by Dynasore, which blocks the GTPase activity of dynamin, suggests a role of the host cell dynamin in the PV pinch (Caldas, Attias, and De Souza 2009). It was recently showed that *Toxoplasma* parasite twisting motion mechanically produces a host cell membrane rupture to conclude the invasion in a safe vacuole (Pavlou et al. 2018). This step requires further analysis.

5. OTHER MECHANISMS FOR *T. GONDII* INTERNALIZATION

Although the main *Toxoplasma* invasion mechanism described nowadays is the active invasion, commanded mainly by the parasite (described in a topic above), early studies about the entry process of *T. gondii* emphasized the contribution of the host cells in the parasite entry process and also discussed the intracellular fate of *Toxoplasma* in macrophages, HeLa cell and fibroblasts (Jones, Yeh, and Hirsch 1972). The entry mechanism into these cells was described as phagocytosis, with pseudopods extended by the cells around the parasites in a phagocytic vacuole. Another interesting feature observed in the *Toxoplasma* infection in macrophages was these cells' ability to control the parasite growth concerning the HeLa and fibroblasts, which are not professionals in phagocytosis (Jones, Yeh, and Hirsch 1972). Another study of the same group investigated the parasite fate after the entry into these cells, and morphologic evidence highlighted that living parasites altered the phagocytic vacuolar membrane in macrophages, fibroblasts, and HeLa cells, thus preventing the fusion of the PV with lysosomes; what is known today as a result of an active invasion process. However, when in contact with the cells or those added in a viable state but later acquired an appearance of destruction inside the vacuoles, the dead parasites were located in vacuoles fused to lysosomes (Jones and Hirsch 1972).

Other studies considering the ultrastructural analysis corroborated the findings of Jones and colleagues. Meirelles and De Souza (1983) and Meirelles and De Souza (1985) described some features of the invasion and survival of *T. gondii* in macrophages and fibroblast cell lines. Silva et al. (1982) reported that *T. gondii* could infect by different mechanisms, generating distinct PV profiles: one type presenting a small space between the parasite membrane and PVM, and a second type of the vacuole seemed to be very large and contained

tubular structures inside (S. R. L. Silva, Meirelles, and De Souza 1982). In the macrophages that were simultaneously infected with epimastigotes of *Trypanosoma cruzi* and tachyzoites of *T. gondii*, it was verified, as expected, the fusion of the macrophage's lysosomes with vacuoles containing *T. cruzi*. However, lysosomes were not fused with vacuoles containing *T. gondii* (Meirelles and De Souza 1983). It was then verified the survival of *T. gondii* when in interaction with chicken macrophages *in vitro*, while the infection by other parasites such as *Leishmania* and *T. cruzi* were cleared (Meirelles and De Souza 1985).

One of these types of entry mechanisms described is the phagocytosis, in which the internalization of *T. gondii* does not involve the secretion of proteins that are stored in secretory organelles and also does not require specific orientation of the parasites in contact with the host cell, as observed in the active penetration process. In this mechanism, the parasites are internalized by cells as macrophages in a large vacuole, involving host cell changes (Morisaki, Heuser, and Sibley 1995). Phagocytosis occurs by gradual engulfment of the parasite, culminating in the fusion of its spacious phagosome created with the host cell endosomes/lysosomes acidification, destroying intravacuolar parasites (Morisaki, Heuser, and Sibley 1995; D G Mordue and Sibley 1997). It is possible to resume and highlight some features of the active invasion that differs from phagocytosis, based on the PVM composition, on the fate of the parasite after entrance into the cell, and in the host cell's morphology that holds the parasite. The parasite is apically oriented on the active invasion and invaginates the host cell plasma membrane simultaneously with apical organelles. The host cell cytoskeleton suffers almost no change during the parasite invasion, generating a vacuole positioned close to the parasite, presenting a neutral pH, and avoiding fusion with endosomes and lysosomes because of its membrane composition. Still, the active invasion is a fast process in which the PV is formed in 15-30 s after the invasion (L. David Sibley 1995).

However, a more recent report showed an invasion mechanism triggered by the *T. gondii*, representing a link between endocytosis and active invasion pathways. Thus, when interacting with phagocytic professional cell macrophages, the parasite *T. gondii* from an avirulent strain could enter into the cell by the classic process of phagocytosis and actively invading from within the phagosomal compartment to form a PV. This phagosome to vacuole invasion pathway (PTVI) could represent a link between the parasite's endocytic and penetrative routes to enter the host cell. This pathway was described as a more efficient pathway to an invasion of cells like macrophages by avirulent strains, as the infected macrophages acquire a hypermotility and could be spread by the organism, favoring the immune stimulation, controlling the acute infection e collaborating to the establishment of the chronic infection (Zhao et al. 2014). More recent work has reported another way for *T. gondii* invasion into the macrophages through microscopic video analysis, named pseudopod-assisted invasion. In this mechanism, *T. gondii* is engulfed by a tube-like structure on peritoneal macrophage phagosomes and then escapes from the phagosomes. After the escape, the parasites re-invade macrophages through intercellular junctions between their apical and host cell membranes (Jin et al. 2020).

As a successful parasite, *Toxoplasma* seems to have other entry mechanisms into the host cells. It was recently reported that endocytic processes are involved in the internalization of *T. gondii* (Portes et al. 2020). This study used cells of the fibroblastic lineage (HFF1) and mice peritoneal macrophages previously treated with inhibitors of distinct endocytic pathways. The inhibitors for clathrin-mediated endocytosis inhibited the internalization of tachyzoites. Also, the treatments that interfere with macropinocytosis increased parasite attachment to the host

cell surface significantly blocked parasite internalization. It was confirmed after specific labeling for macropinocytosis (Rab5 effector rabankyrin 5 and Pak1) associated with parasite-containing cytoplasmic vacuoles. These results indicate that *T. gondii* in mammalian cells can take place by clathrin-mediated endocytosis and macropinocytosis. As the macropinocytosis inhibitors have inhibited the internalization of *Toxoplasma* in a large proportion and as it is already known other possible mechanisms to parasite's entry, such as active invasion, it cannot be ruled out that the drug treatments also interfere with steps of other entry processes. The authors discussed the possibility that active penetration and macropinocytosis are complementary mechanisms.

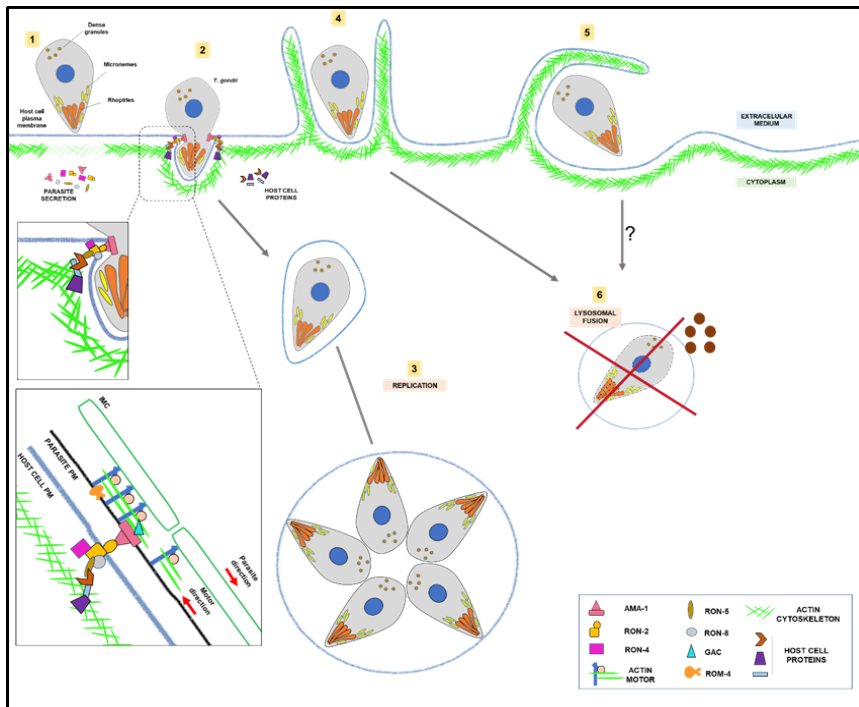


Figure 7. Schematic representation of the entry mechanisms of *Toxoplasma gondii*.

Active invasion with moving junction formation during host cell invasion (1) Rhoptry secretion. The parasite redirects its position with the apical complex near the host cell PM. As a result, the actin filaments' organization is looser, as induced by secretion of parasite profilin, the rhoptry proteins are secreted into the host cell cytoplasm. (2) Moving junction formation. The parasite and host cell proteins' secretion of proteins create the molecular junction between the parasite and host cell PM. The Rhoptry proteins, RON2, RON4, RON5, and RON8, form a complex with host cell proteins of the ESCRT family, which associates the parasite to the host cell actin filaments the extracellular domain of RON2 to AMA1 that is on the parasite PM. In the parasite's cytoplasm, the cytosolic domain of AMA1 connects to the parasite actin-myosin motor by the glideosome-associated connector (GAC) and the rhomboid protease ROM4 uncouple the complex allowing the parasite movement. IMC, inner membrane complex. (3) Once invading the host cell by active invasion, the parasite can replicate in a safe PV free of fusion with lysosomes and degradation. There are also mechanisms triggered by the host cell that mediates parasite entry, such as (4) phagocytosis or (5) macropinocytosis, with the participation of actin filaments from the host cell and emission of pseudopods or macropinosomes, respectively. (6) When the entry is not active/directed by the secretion of proteins by the parasite, the PV could become susceptible to fusion with lysosomes and may cause the parasites' degradation and death, as described when the entry occurred phagocytosis.

Further studies are necessary to clarify some points such as the possible fate of parasites after any entry mechanism is adopted (Portes et al. 2020). The *T. gondii* entry into the host cell through macropinocytosis was described in a study in which myosin motors' contribution to the parasite invasion was investigated. Parasites MYOA-deficient could enter the host cells by a mechanism similar to macropinocytosis. After the entry and junction formation, the host cell formed protrusions with actin mobilization to surround these deficient parasites, then residing inside a typical vacuole. In some cases, the host cell membrane expansions failed to cover the parasite. This study also highlighted the host cell membrane dynamics participating in the *Toxoplasma* invasion process (Bichet et al. 2016). Figure 7 summarizes the main mechanisms of *T. gondii* entry into the host cells studied up to now, including the main pathway of active invasion, phagocytosis, and macropinocytosis.

6. ESTABLISHMENT OF THE PARASITOPHOUS VACUOLE

After successfully entering the host cell, *T. gondii* resides within a PV, in which the membrane creates a safe niche for survival and replication of the parasite. Thus, the vacuole is a target for recognition by the host immune defense mechanisms, and there are some important processes involved in the host responses and evasion mechanisms of the parasite that occurs at or around the vacuole and deserve to be highlighted.

When an Apicomplexa parasite actively invades the host cells, some proteins are secreted by the parasite's organelles. There is an invagination of the host cell membrane, culminating in the formation of PV within a few seconds (Morisaki, Heuser, and Sibley 1995). The PV is largely host-derived, evidenced by its lipid composition and capacitance measurements of patch-clamped host cells, indicating that more than 85% of the PVM comprises the host cell membrane constituents (Suss-Toby, Zimmerberg, and Ward 1996). It was also evident in a study that shows through the insertion of a fluorescent probe into the host cell membrane, and it was internalized with the PVM after the invasion process (Dana G. Mordue et al. 1999). The surface of host cells was labeled before tachyzoites invasion. After binding the parasite to the host cell, the labeled lipids and proteins were transferred to the protozoan surface and subsequently found in the PV (Pacheco-Soares and De Souza 1998; 2000a). The nature of components exposed on the parasite surface when it enters into contact with the host cell surface also influences the surface components internalized to be part of the PV membrane. Thus, labeling the macrophage surface with cationized ferritin particles followed by *T. gondii* infection showed that the region of parasite-macrophage interaction presented a considerable concentration of anionic sites. Then it also could be internalized with the parasites and associate with the PVM. No labeling was observed on the PV when the parasites were previously fixed or opsonized (L. De Carvalho and De Souza 1990) (Figure 8).

Still, concerning the transfer of molecules between the host cell surface and the parasites, collaborating to the PV membrane formation, a study employed lectins labeled to follow the fate of lectin-binding sites on the host cell surface. It was found that few lectin-binding sites were internalized together with the tachyzoites to compose the PV membrane. However, these sites were observed in small vesicles close to the PV. Furthermore, intramembranous particles, identified as integral membrane proteins, were seen in the membrane lining the PV. At the same time, saponin-cholesterol complexes were not observed in the PV membrane but

were found only in the parasite inner membrane complex. (Pacheco-Soares and De Souza 2000b) (Figure 9).

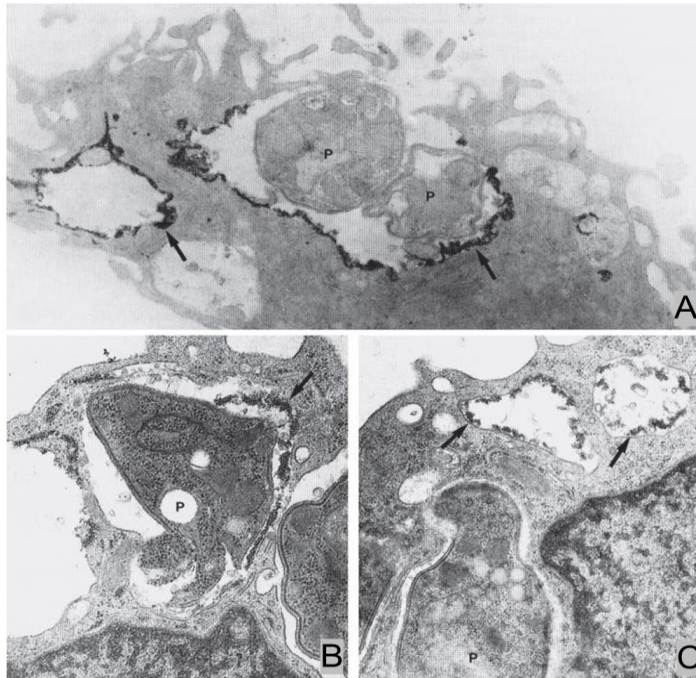


Figure 8. Localization of anionic sites, detected using cationized ferritin particles, during the interaction of control (A and B) and antibody-coated (C) *T. gondii* tachyzoites with macrophages. Initially, there is a concentration of the anionic sites at the site of parasite internalization. These sites are incorporated into the membrane lining the internalized parasites (B) but are excluded when interacting with opsonized tachyzoites (C). Labeled cytoplasmic vacuoles were also found (arrows). A- x 12,000; B - x 22,000; C - x 15,000; P = parasite; (After Carvalho and De Souza 1990).

Some studies were performed in the past years to understand the formation of the PVM by detecting some enzymes that compose the macrophage plasma membrane. The cytochemical detection shows that the enzyme Na⁺-K⁺-ATPase is present on the host cell plasma membrane and in the PV, but no labeling of the PVM was observed for the Mg²⁺-ATPase enzyme (L. Carvalho and De Souza 1989) (Figure 10). Still, although it has been previously reported that *T. gondii* does not induce a respiratory burst during interaction with macrophages (Seth E. Anderson and Remington 1974; Murray and Cohn 1979; 1980), biochemical studies had shown that when *T. gondii* coated with IgG interacts with macrophages, a respiratory burst occurs (S. E. Anderson, Bautista, and Remington 1976). De Carvalho and De Souza reported that it would be due to the Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme activity that generates reactive oxygen species (ROS) mediate cellular defense against pathogens. In this study, the enzyme was cytochemically detected in the PV containing *T. gondii* opsonized by specific IgG antibodies but not control parasites vacuoles. In this case, the enzyme is probably internalized and becomes part of the vacuole membrane, being active during the initial attachment of the parasite and inside the PV (L. Carvalho and De Souza 1989). A recent study showed the importance of this enzyme activity in naive macrophages to clear type III *T. gondii* (Matta et

al. 2018). Other enzymes were identified as participants in the interaction between the parasite and the host cells as detected after incubation of the parasite and the host cells with general or specific inhibitors of tyrosine kinases. These treatments induced the formation of filopodium-like surface projections in the macrophages, increasing the tachyzoites' attachment to the cell surface. Specific labeling with anti-phosphotyrosine, phosphothreonine, and phosphoserine antibodies show phosphoproteins in macrophages interacting with tachyzoites and around the PV. These observations suggest that protein phosphorylation is important to the *T. gondii*-host cell interaction process (Ferreira, De Carvalho, and De Souza 2003).

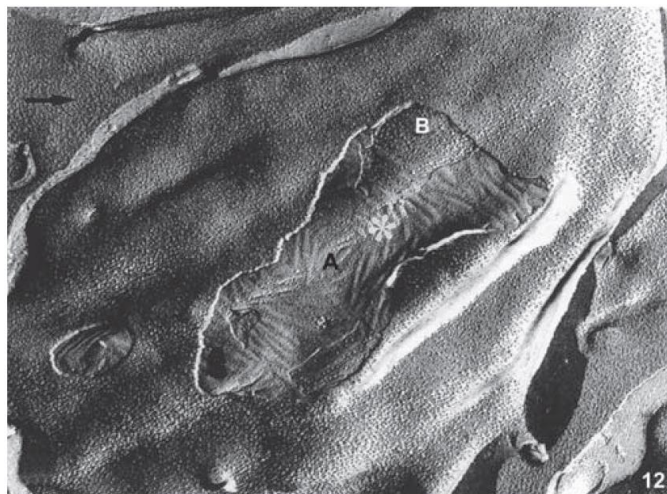


Figure 9. Freeze-fracture replica of cells infected with *T. gondii* and fixed in the presence of digitonin. Membrane particles are seen on the fracture faces of the membrane lining the parasitophorous vacuole (PV) (arrow). However, this membrane and the parasite plasma membrane do not present a cholesterol-digitonin complex, which is abundant in the protozoan inner membrane complex (asterisk); x 12,000; (After Pacheco-Soares and De Souza 2000b).

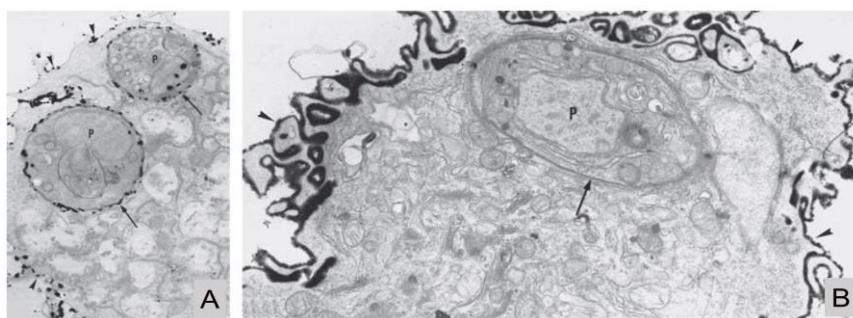


Figure 10. Cytochemical localization of Na^+/K^+ -ATPase (A) and Mg^{2+} -ATPase (B) in macrophages infected with *T. gondii*. For the first enzyme, labeling of the host cell plasma membrane (arrowheads) as well as of the parasitophorous vacuole (PV) (arrow in A) is evident. On the other hand, no labeling of the PV membrane was observed for Mg^{2+} -ATPase (arrow in B) P = parasite; x 12,000; (After Carvalho and De Souza 1989).

As described before, during the invasion, the secretion of proteins from the rhoptries and micronemes generates the MJ and creates the nonfusogenic vacuole, avoiding the lysosomal acidification and parasite clearance. The rhoptry secretion is also important, besides the invasion, to the PV formation. After the formation of the MJ, there is the secretion of the rhoptry content as evacuoles into the host cells, and these evacuoles contain ROP1 and ROP2 that can fuse with and modify the PVM (Håkansson, Charron, and Sibley 2001; Reviewed in (Clough and Frickel 2017).

The parasite's access to the nutrients from the host cytosol is allowed by PVM, which was modified by the parasite to act as a sieve to the diffusion of small molecules (up to 1300 Daltons) (Schwab, Beckers, and Joiner 1994). It is possible because of the presence of the proteins from dense granules GRA17 and GRA23 (Gold et al. 2015). However, because of the complexity of the transport of proteins across the PVM, some mediator proteins participate. In the case of transport of GRA16 and GRA24 across the PVM into the cytosol, the secreted protein Myr1, that is before cleaved by TgAsp5, was found to be essential to the process of protein export to the host compartments (Hsiao et al. 2013; Coffey et al. 2015; Hammoudi et al. 2015; Curt-Varesano et al. 2016). It was recently reported that several MYR1-interacting proteins, GRA44, GRA45, and MYR4, are essentials for the translocation of the *Toxoplasma* effector protein GRA16 and the upregulation of human genes c-Myc and cyclin E1 in infected cells (Cygan et al. 2020).

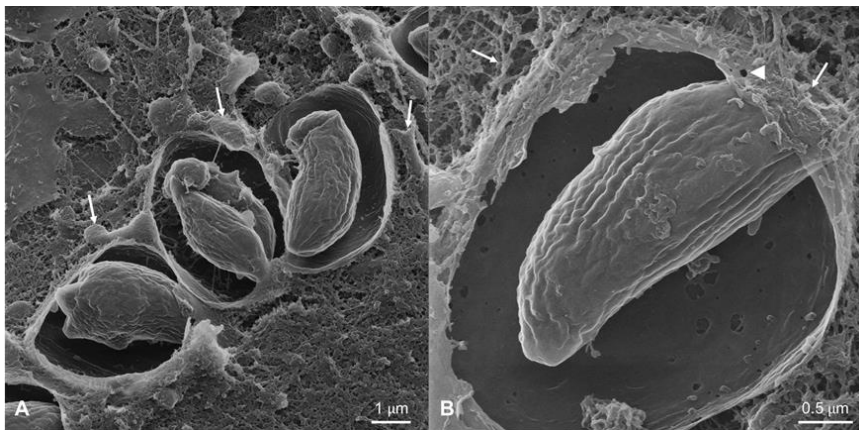


Figure 11. Helium Ion Microscopy of the relationship of the PV and the host cell.

(A) Parasitophorous vacuoles of *Toxoplasma gondii*. Organelles (probably mitochondria) are seen around them (m). (B) The PV membrane can be seen from the cytoplasmic side. The appearance of the pores (arrowhead) is similar to those observed in the inner surface. Host cell endoplasmic reticulum profiles could be seen around the vacuole (arrow) (After De Souza and Attias 2015).

Some host organelles are strategically organized around the PV. Thus, the parasite recruit microtubule-organizing center (MTOC), host cell mitochondria, and endoplasmic reticulum (ER) to its PVM after the invasion (Jones and Hirsch 1972; Edesio Jose Tenorio de Melo, de Carvalho, and de Souza 1992; Sinai, Webster, and Joiner 1997; Laliberté and Carruthers 2008). It is suggested that fusion between the ER and the PV occurs because some projections from the ER towards the vacuole are formed and the glucose-6-phosphatase, an enzyme marker of the ER, was seen in the ER profiles and within PV. The increase in the size of the PV, necessary during the parasite replication, could be allowed by this ER interaction (Edesio

J.T. Melo and De Souza 1997) (Figures 11 and 12). The host ER-PVM association is described to make the possible antigen-cross presentation in dendritic cells infected by *T. gondii* (Goldszmid et al. 2009). The connection between the PVM and the organelles appears to be strong, as, after the subcellular fractionating approach, the association was maintained (Sinai, Webster, and Joiner 1997). The host intermediate filaments and microtubules are disposed around the PVM and are crucial to the PV location near the nucleus and the PV shape maintenance (Halonen and Weidner 1994; E. J.T. Melo, Carvalho, and De Souza 2001). Also, the MTOC is repositioned from the host nuclear membrane to the PVM (Coppens et al. 2006). Lysosomes and endocytic vesicles are also near the PVM and enable the parasite's cholesterol uptake (Coppens et al. 2006; Coppens, Sinai, and Joiner 2000).

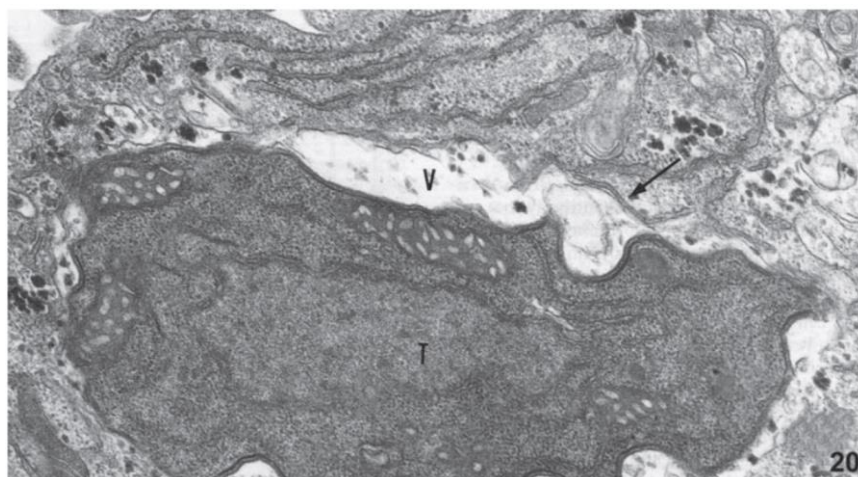


Figure 12. Transmission electron microscopy image of a thin section showing a tachyzoite (T) of *T. gondii* within a parasitophorous vacuole (V). Profiles of the host cell endoplasmic reticulum (arrow) are associated with the membrane lining the vacuole; x 22,000; (After Edesio J.T. Melo and De Souza 1997).

A tubulovesicular network composed of nanotubules connects these membranes between the PVM and the *T. gondii*'s PM. This intravacuolar network (IVN) is essential to parasite development, increasing the area of molecular exchanges between the parasite and the host cell (Mercier et al. 2002). Magno et al. 2005 showed that these nanotubules of the IVN present 30–35 nm thick and serve as a support to the tachyzoites inside the PV, providing a connection between the parasites and their disposition in the form of a rosette after the replication by endodyogeny (Magno et al. 2005). De Souza & Attias, in 2015, by super-resolution electron microscopy, described a thickness of $28,6 \pm 5$ nm for the nanotubules and identified new structures on the membrane surface of the parasite that seems to be involved with secretion mechanisms in intracellular parasites, while not seen in extracellular parasites. The nanotubules of the IVN (Figure 13), which are secreted by the parasite at the beginning of the invasion, are connected by small adhesive bonds. It was noticed filamentous material interconnecting the IVN tubules to each other, the parasite, and the vacuolar membrane's inner surface (De Souza and Attias 2015).

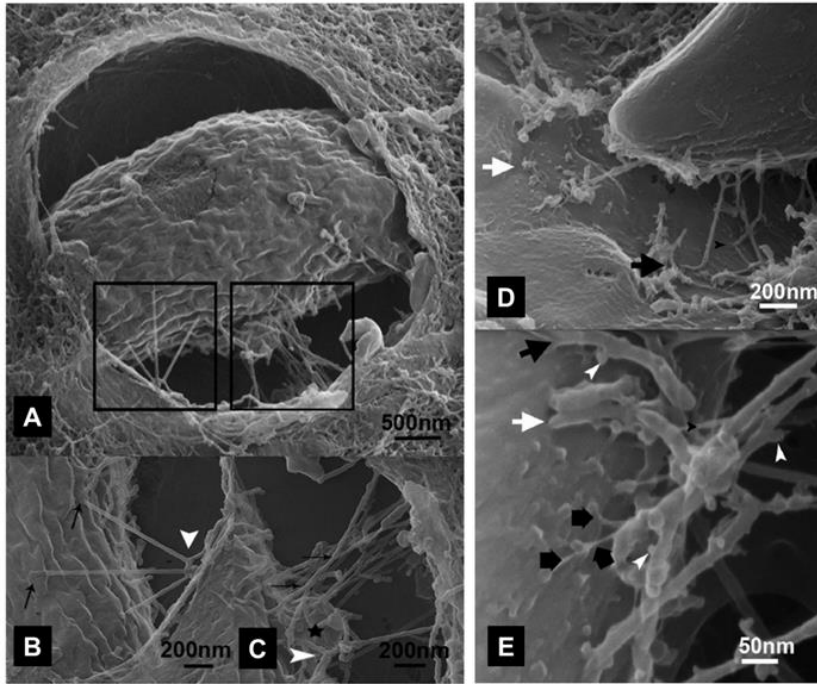


Figure 13. Helium Ion Microscope (HIM) images of the *T. gondii* PV.

(A) After parasite invasion, there is the secretion of dense granules creating the IVN (squares). (B) and (C) Zoom of the areas in the squares. (B) Three straight tubules apposed to the parasite surface and anchored to a shorter one in the PVM (C) It was identified bifurcating tubules (arrowhead) and crossing tubules (thin black arrows) composing the IVN. (D) The IVN tubules are also link the parasite and the PVM through tiny projections (black arrowhead). The surface of the PVM is rough (thick white arrow); (E) High magnification images of IVN allowed to see areas that may correspond to the point of origin of a new branch of the tubule (white arrowheads); closed tubule touching the surface of the PVM (thick white arrow), tubules that appears continuous with the PVM (thick black arrow) and tiny filamentous structures that link the IVN tubule to the PVM inner surface (small black arrowheads) (After De Souza and Attias, 2015).

The GRAs proteins are associated with the intravacuolar network and give stability and conformation (Mercier et al. 2005). Tachyzoites secrete the GRA proteins into the PV space after its formation (Jean François Dubremetz et al. 1993; Leriche and Dubremetz 1990). These proteins are secreted as soluble proteins that are then associated with the IVN or to the PVM (Mercier et al. 2002). There is also the secretion of multi-lamellar vesicles from a posterior invagination of the parasite into the PV lumen to compose an intravacuolar network (L.D. Sibley et al. 1995; Mercier et al. 2002). It was recently described that these tubules contain F-actin that appear to collaborate with the organizing of the IVN in the formation of the residual body and for the transit of vesicles between tachyzoites in the vacuole (Periz et al. 2017; Tosetti et al. 2019). The IVN is also involved in the host's lipid salvage (Caffaro and Boothroyd 2011; Nolan, Romano, and Coppens 2017) and host cytosolic proteins (Dou et al. 2014). Deletion of GRA2, a component of these tubules, caused a decrease in host cytosolic proteins' ingestion (Dou et al. 2014). Recent studies also reveal that IVN-associated GRA proteins regulate the formation and maturation of the cyst matrix and cyst wall structures (Guevara et al. 2019).

6.1. Host Autophagy Controls the *Toxoplasma* Growth

Toxoplasma has developed several mechanisms for survival and growth inside the host cell, and these strategies are associated with the isolated compartment provided by the P.V. In the absence of the host immune mechanisms, *Toxoplasma* escapes from the endolysosomal host system; however, some host's pathways can eliminate the parasite's safe niche, such as the autophagy. Despite being a catabolic process fired to recycle cell content and damage organelles, the host autophagy machinery could help control the infection by *T. gondii* (Besteiro 2019). In the classical autophagy process, the machinery involved includes Atg7 (E1-like), Atg3 (E2-like), and the Atg12-Atg5-Atg16L1 (E3-like) complex, and the Atg8 proteins such LC3 to the autophagosome isolation membrane (Mizushima, Ohsumi, and Yoshimori 2002). The membrane-bound LC3 associates with the parasite through adaptor proteins such as NDP52 and p62 that bind to ubiquitinated proteins on the pathogen (Randow 2011; Gomes and Dikic 2014), and then autophagosome membrane delineate and fuses with lysosomes to degradation.

This pathway is dependent on TNF- α and is activated by the binding of CD40 on macrophages surface into the CD40 ligand (CD40L or CD154) on activated CD4 T cells, controlling the *Toxoplasma* infection on the brain (Reichmann et al. 1999; Andrade et al. 2006) and reducing the parasite replication in the peripheral tissues during the acute phase (Subauste and Wessendarp 2006). Therefore, the autophagy is activated after the CD40 binding, which recruits TRAF6, inducing the autocrine production of TNF α (Mukundan et al. 2005; Subauste, Andrade, and Wessendarp 2007). There is a synergism between the TRAF-6 signaling, which occurs via ULK1, and TNF α signaling via Beclin-1, triggering autophagy. The parasite could block this pathway through the activation of the host EGFR. Thus, on the invasion process, the micronemes' secretion of MIC3 and MIC6 induces the EGFR activation, causing the host Akt's activation by PI3K-dependent phosphorylation. The Akt, when activated, prevents the LC3 and autophagic membranes recruitment to PVM (Muniz-Feliciano et al. 2013). In mammalian cells, the Akt phosphorylation results in the mTORC1 activation, which negatively regulates autophagosome formation, inhibiting autophagy (Kim et al. 2011). During the invasion and M.J. formation, the parasite inhibits activating the stimulators of autophagy, PKR, and eIF2 α , through the FAK-Src pathway (Portillo et al. 2017).

6.2. Non-Classical Mechanisms of Parasite Control and Autophagy Proteins

Besides controlling the *Toxoplasma* infection by the classical autophagy pathway, autophagy proteins can be involved in processes activated through intracellular infections but are unrelated to classical autophagy. One of these mechanisms is the LC3-associated phagocytosis (LAP), which refers to the recruitment of LC3 and other autophagy proteins to generate a single-membrane phagosome that carries on pathogens or dead cells that have been phagocytosed by the host cell (Sanjuan et al. 2007). The proteins involved include ATG3, ATG5, ATG7, ATG12, ATG16L, Beclin 1, and PI3KC3; other proteins such as ULK1 are not participating in this process (Martinez et al. 2015). LAP is a result of a faster fusion with lysosomes and plays a protective role against pathogens.

The autophagy proteins are also important inductors of the parasite clearance in murine cells through recruitment of the host Immunity-related GTPases (IRGs) and guanylate-binding proteins (GBPs) to the PVM, culminating in its disruption, which could result in the parasite clearance independently of the formation of the autophagosome (Reviewed in (Clough and Frickel 2017). Thus, although the host cells are provided with mechanisms for controlling the parasite infection by *T. gondii*, it has developed efficient mechanisms to deal with these host defenses. These mechanisms triggered by the parasite are based on its virulence factors, usually proteins found in organelles such as rhoptries and dense granules, the ROPs, and GRAs, respectively. Many ROPs have been studied and described as virulence factors that blocked the host mechanisms carried out to destroy the parasites present in the P.V. (Saeij et al. 2007; 2006; Taylor et al. 2006; Behnke et al. 2012). These pathways will be covered in more detail in chapter 7: "The balance between host cell-autonomous immunity and *Toxoplasma gondii* evasion strategies."

CONCLUSION

Toxoplasma has an efficient invasion system that guarantees its entry and prevents phagocytosis by the host cell and its effector mechanisms. After entry, already inside the PV, *T. gondii* can secrete molecules that are its factors associated with a virulence that prevent the success of host elimination mechanisms such as the induction of a classic autophagy pathway or the disruption of the vacuolar membrane. These processes could be different according to the cell or host type, and the responses triggered by the parasite are generally strain-specific and associated with its virulence. Considering that the mechanisms described here refer to the most studied *Toxoplasma* strains, we cannot fail to emphasize the need to investigate these pathways and mechanisms using strains with different profiles. In short, these pathways are part of a balance between the host defense and the parasite's ability to replicate, remaining in its PV to be protected and obtaining nutrients necessary for its growth, always evading host responses and spreading the infection in the organism.

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Chapter 4

CYSTOGENESIS PROCESS IN *TOXOPLASMA GONDII*

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ABSTRACT

Toxoplasma gondii is an intracellular protozoan parasite. A key event for the longtime persistence of the parasite in a host is its ability to convert from tachyzoites, the fast-dividing form, into bradyzoites, the slow-dividing form, under the pressure of host immune response. *T. gondii* cysts are formed mainly in the Central Nervous System (CNS) during the transition from acute to the chronic phase of infection. Bradyzoites secrete glycoproteins to form the cyst wall and the intracystic matrix, markedly modifying the parasitophorous vacuole. In this chapter, we review the main ultrastructural features of the cyst and the process of cystogenesis, as well as the strains and mechanisms capable to favor or induce cyst formation *in vitro* and *in vivo*.

1. INTRODUCTION

Toxoplasma gondii is a protozoan parasite adapted to intracellular life that infects all nucleated cells from warm-blooded animals. In the acute stage of infection, this parasite actively invades host cells forming a parasitophorous vacuole (PV), where the tachyzoite forms replicate by endodyogeny (see chapter 5; Sheffield and Melton 1968; Dubey, Lindsay, and Speer 1998). After several rounds of replication, tachyzoites egress from host cells, causing cell rupture. Multiple courses of the lytic cycle in adjacent cells cause tissue destruction and pathology (Blader et al. 2015). In the acute stage of infection, interferon- γ -

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mediated immune response triggers the conversion of tachyzoites to bradyzoites, a slow-dividing form/stage, which characterizes the chronic phase of toxoplasmosis. Bradyzoite resides in a modified PV called tissue cyst (Sullivan and Jeffers 2012) (Figure1), which preferably localizes inside cells of the central nervous system and skeletal muscle. As these are located at immune-shielded tissues, *T. gondii* remains protected from the action of the immune response mechanisms of the host (Weiss and Kim 2000).

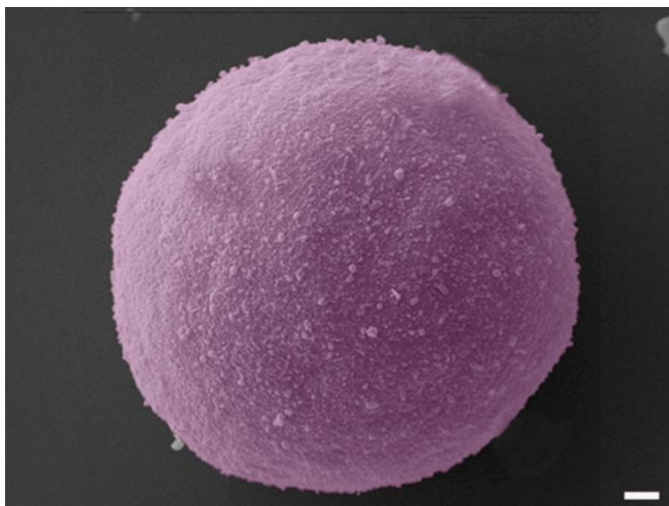


Figure 1. Scanning electron micrograph of a cyst of the Me49 strain isolated from infected mice. Cyst wall presents an irregular and rough surface. Scale bar = 1 μ M. Electron micrograph from Lemgruber L & Vommaro RC personal archive.

The process of cystogenesis, which comprises the conversion of tachyzoites (that reside in a PV) to bradyzoites and the conversion of PVs to cysts, is an extraordinary event in *T. gondii* biology. Cyst formation permits parasite persistence throughout the lifespan of intermediate hosts, allowing a ‘silent’ parasite transmission among intermediate hosts, through carnivorousism (Dubey et al. 2008).

The bradyzoites are morphologically different from the fast-replicating tachyzoites in several aspects: accumulation of amylopectin granules, repositioning of the nucleus to the posterior region, higher compaction of rhoptries and a higher amount of micronemes (see chapter 2). Bradyzoite molecular markers include surface and cytoplasmic antigens of the parasite. Bradyzoite antigens can be detected as early as 24 hours after induction of the differentiation process, indicating a possible compromise with differentiation right after the parasite enters the host cell (Weiss and Kim 2000; Paredes-Santos et al. 2013). Bradyzoites alternate between G0/G1 latency state. The entry or not in the S phase and the permanence in G1 are decisive for the conversion. For the conversion to occur, tachyzoites start replicating in a longer cycle, culminating in a genetic commitment leading to the expression of antigens characteristic of bradyzoites followed by complete conversion. Some proteins of the SAG (surface antigen) family are expressed in both stages, however, other proteins are expressed only in the bradyzoite stage, such as SAG2C, SAG2D, SAG4A, SAG5A, SRS9 and BSR4. These proteins may be involved in the persistence of cystic forms (Jung, Lee, and Grigg 2004; Kim and Boothroyd 2005).

Bradyzoites in the early stages of conversion present increased expression of heat shock proteins. One of the main markers of bradyzoites, the BAG1 protein is an HSP class 30 chaperone (Bohne et al. 1995) and parasites knockout for BAG1 originate fewer cysts (Bohne and Roos 1997; Bohne et al. 1998; Zhang et al. 1999). HSPs 90, 70 and 27 present in bradyzoites are associated with the development of *T. gondii* cysts (Toursel et al. 2000; Weiss et al. 1998). MAP kinases and cyclic nucleotides, including cyclic AMP and cyclic GMP are also important molecules, which act responding to stressful situations and have their expression increased during conversion (Eaton, Weiss, and Kim 2006; Lacey et al. 2007).

Bradyzoites do not present an active electron transport chain or the Krebs cycle, obtaining energy mainly through the glycolytic pathway and fermentation. Bradyzoites accumulate amylopectin granules in their cytoplasm and this form of glucose storage is metabolized to lactate, the main energy supply of this stage of *T. gondii* (Coppin et al. 2003; 2005; Denton et al. 1996; Guérardel et al. 2005). The regulation of the glycolytic pathway is differentiated in bradyzoites because it depends on the pyruvate kinase and lactate dehydrogenase to metabolize amylopectin. Lactate dehydrogenase has specific stage isoforms and lactate dehydrogenase 2 (LDH2) is the specific isotype expressed in bradyzoites. LDH2 expression is essential for the formation of cysts (Al-Anouti et al. 2004; Yang and Parmley 1997). The enolase enzyme ENO1, involved in energy metabolism appears as a typical enzyme in bradyzoites, located in the cytoplasm (Dzierszinski et al. 2001; Ferguson and Parmley 2002).

2. CYSTOGENESIS *IN VITRO*

In vitro systems - the study of the conversion of tachyzoite to bradyzoite and the encystment process was first established in the 1960s (Jacobs, Remington, and Melton 1960; Matsubayashi and Akao 1963). The use of antibodies that recognize specific antigens and protein expression techniques coupled to GFP (green fluorescence protein) was important to follow the steps of the differentiation. In this way, several protocols for induction of *in vitro* encystation have been developed (Bohne, Heesemann, and Gross 1993; Weiss et al. 1995; Dzierszinski et al. 2004; Ferguson 2004). A common feature in all of them is the promotion of a stressful environment for the host cell and the parasite leading to conversion.

The procedures used to obtain cysts in culture include deprivation of essential nutrients, as arginine or pyrimidine (Fox, Gigley, and Bzik 2004; Tomavo and Boothroyd 1995); alterations in culture conditions, as the increase of temperature (43°C), acidic (6.6 - 6.8) or basic pH (8.0 - 8.2) and incubation with sodium arsenate (Lane et al. 1996; Soete, Camus, and Dubrametz 1994; Weiss et al. 1995). The use of sodium nitroprusside as an exogenous nitric oxide donor, the addition of oligomycin, an inhibitor of mitochondrial ATP synthase function or antimycin-A, an electron transport inhibitor in the respiratory chain, were also well exploited to obtain tissue cysts (Bohne, Heesemann, and Gross 1994; Tomavo and Boothroyd 1995). Other researchers achieved the formation of cysts using tunicamycin, preventing the first stage of N-glycosylation and causing the endoplasmic reticulum stress or the herbicide fluoridone inhibitor of the abscisic acid, which controls the intracellular level of calcium (Nagamune et al. 2008; Narasimhan et al. 2008). The use of IFN- γ and pro-inflammatory cytokines are efficient mechanisms for inducing the formation of cysts in infected

macrophages, but it is not a good system for fibroblasts (Bohne, Heesemann, and Gross 1993; Soete, Camus, and Dubrametz 1994). Therefore, the type of host cell seems to play a decisive role in the process. The energy metabolism of host cells can make them more or less permissive to the formation of cysts (Weilhammer et al. 2012). Cysts are found preferentially in differentiated cells, which do not divide and have a long life, as are cells from the nervous and the skeletal muscular systems. Several studies have shown that differentiated myotubes prevent the growth of the tachyzoite stage and favor the conversion and the permanence of bradyzoites (Swierzy and Lüder 2015).

An effective spontaneous cystogenesis system was achieved in skeletal muscle cells infected with bradyzoites and incubated for 96 hours without any artificial inducers (Ferreira-da-Silva et al. 2009; Guimarães, de Carvalho, and Santos Barbosa 2008). Strains that have a slower replication rate, such as Me49 (type II) and Prugnnaud (Pru) (type II) are more cystogenic, making their use more advisable for *in vitro* cyst formation than fast replicating strains, such as RH (type I). This ability to differentiate in tissue cysts is frequently lost with the prolonged passage in culture, with the parasites acquiring a faster rate of replication. However, with the isolation of strains of *T. gondii*, which spontaneously form true cysts in cell cultures, it became easier to investigate this process.

The atypical EGS strain was isolated from human amniotic fluid of a patient with toxoplasmosis in Brazil and corresponds to an atypical strain (Vidigal et al. 2002; Ferreira et al. 2004). EGS proved to be highly virulent and, at the same time, cystogenic for mice (Brandão et al. 2009). These characteristics were confirmed *in vitro*, as this strain formed thick-walled cysts, in the absence of any stress condition in different cell lineages (Figure 2). Hence, this kind of strain constitute useful tools for examining the differentiation process of *T. gondii* (Paredes-Santos et al. 2016; 2013).

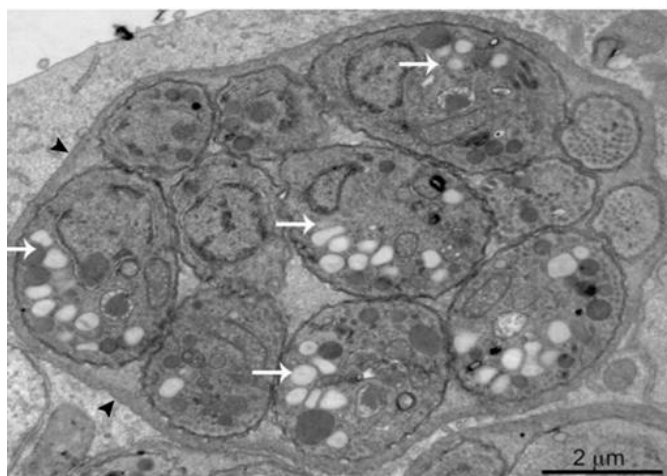


Figure 2. Transmission electron micrograph (TEM) of a tissue cyst of the EGS strain. Inside the cyst, several bradyzoites presenting amylopectin granules (white arrows) can be observed. The matrix is electron-dense; the cyst wall is uniform, compact and electron-dense (arrowheads). Electron micrograph from Paredes-Santos TC & Vommaro RC personal archive.

3. MECHANISMS THAT INDUCE CYSTOGENESIS

After the establishment of either artificial or spontaneous cystogenesis induction protocols and the identification of biochemical and molecular mechanisms that allow the monitoring of cystogenesis, some advances about the mechanisms that lead to this conversion were elucidated. Through studies of the action of IFN- γ , which controls the conversion of tachyzoites to bradyzoites, it was possible to observe that bradyzoites have a different cell cycle and that the genetic impairment of the conversion occurs between phases S and M. An essential condition for conversion is a lower rate of replication of tachyzoites. Nitric oxide may play an important role, delaying the cell cycle, triggering the conversion, and controlling the overgrowth of parasites (Bohne, Heesemann, and Gross 1994).

Transcription factors have been characterized as having a role in regulating stage-specific genes involved in cystogenesis. Completely converted bradyzoites, enter a G0 quiescence state, and therefore it is common to find odd numbers of parasites in a cyst, resulting from an asynchronous division process (Radke et al. 2003).

The control of translation of stage-specific genes is related to a quick and effective response to stressful situations and involves the phosphorylation of a translation initiation factor TgIF2 α , which has a homolog in humans- the eukaryotic initiation factor-2 (eIF2). The phosphorylation of this factor inhibits the translation of globular proteins and promotes the translation of proteins related to stress survival. All agents used to induce cystogenesis *in vitro* induce phosphorylation of TgIF2 α (Sullivan et al. 2004). This factor is phosphorylated by specific kinases activated under stress related to nutrient deprivation, endoplasmic reticulum stress and thermal shock (Narasimhan et al. 2008).

Transcriptional control has also been shown to be essential for the conversion of tachyzoites to bradyzoites. *Toxoplasma* epigenetic machinery is able to modify histones by acetylation and methylation, which is conserved in eukaryotic cells that pass through differentiation, as well as modifications in the nucleosomes (Dalmaso et al. 2009; Saksouk et al. 2005). Transcriptional control occurs by increasing the expression of transcriptional regulatory proteins such as the Apicomplexan AP2 proteins (ApiAP2- transcription factors that contain the plant-related DNA binding domain Apetala2), which prevents the transcription of bradyzoite genes during the tachyzoite phase (Radke et al. 2013; White and Radke 2014). AP2 proteins act as regulators of bradyzoite genes binding to the promoter regions of bradyzoite markers as BAG1 and B-NTPase (Nucleoside Triphosphate Hydrolases). It has been shown the transcription of 15 AP2 mRNAs fluctuating during *T. gondii* cell cycle and that are upregulated during bradyzoite conversion. The AP2 proteins AP2XI-4 and AP2IV-3 have been described as bradyzoite activators, as deletion of AP2XI-4 led to the downregulation of bradyzoite markers and impairment of cyst formation. Otherwise, AP2IX-9, AP2IX-4, and AP2IV-4 have been characterized as bradyzoite repressors (Radke et al. 2013; 2018; Walker et al. 2013; Hong, Radke, and White 2017; Huang et al. 2017).

4. THE CYST WALL AND MATRIX

T. gondii is auxotrophic for several nutrients, including cholesterol, purines, and tryptophan, which must be obtained from the host cell. Although the PV provides a perfect environment for parasite proliferation and protection from host immune responses, it is also a barrier for nutrient harvesting by the parasite. Tachyzoites can obtain and digest host-derived proteins (Dou et al. 2014), and the PVM is permeable to molecules of up to 19 kDa (Schwab, Beckers, and Joiner 1994; de Souza and Attias 2015). The dense granule proteins GRA 17 and GRA 23 are secreted by the tachyzoites and mediate the exchange of small molecules between *T. gondii* and the host cell cytoplasm, forming pores in the PVM (Gold et al. 2015).

T. gondii cysts may contain from two to several thousand individual bradyzoites. The viability of parasites within the cysts is possible due to the permeability of the cyst wall to nutrients that can pass from the host cell cytoplasm into the cyst. On the other hand, the cyst wall prevents the interaction of intracellular parasites with the components of the immune response. It has been shown the cyst wall is permeable to negatively charged molecules and those up to 10 kDa. GRA proteins may participate in the transport of small molecules across the cyst membrane and cyst wall (Ferguson and Hutchison 1987; Guimarães et al. 2007; Lemgruber et al. 2011). The actual permeability properties of this barrier still need to be further investigated.

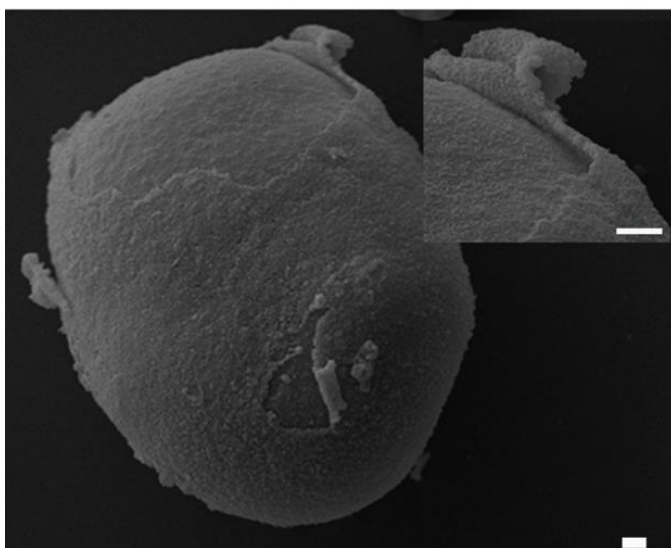


Figure 3. SEM of a cyst of Me49 strain isolated from infected mice. Two layers of the cyst wall can be seen in detail in the inset. Scale Bar = 1 μ M (Micrograph from Lemgruber L & Vommaro RC personal archive).

Analysis by scanning electron microscopy (SEM) showed the cyst wall presents an irregular and rough surface (Figures 1 and 3). The cyst is delimited by a cyst wall that represents a remnant of the PV membrane (PVM), which becomes convoluted, and the accumulation of a large amount of fibrillar and electron-dense material in the internal face of the PVM (Figure 4A). In addition, the cyst wall presents small vesicles and tubules immersed in the fibrillar network (Figure 4B) (Tomita et al. 2013; Lemgruber et al. 2011).

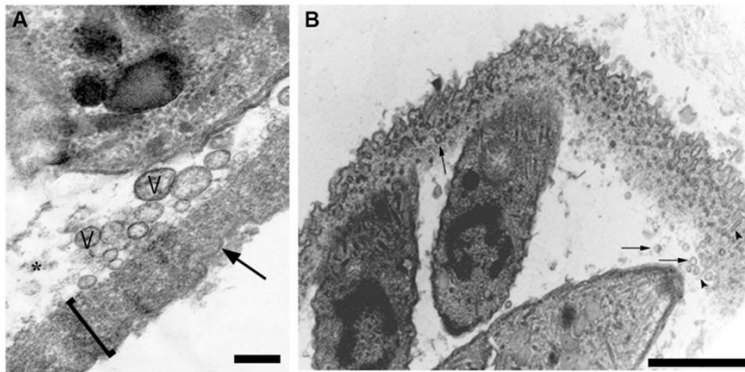


Figure 4. TEM of a cyst of Me49 strain isolated from infected mice. A- A large amount of fibrillar and electron-dense material can be seen accumulated underneath a convoluted membrane (arrow), forming a thick wall (bracket). The cyst matrix is filled with an amorphous granular material (*) and vesicles of different sizes (V). Scale bar= 0,1 μ M. B- Ultrathin section of an osmium-imidazole-buffered fixed cyst showing a great number of vesicles (arrows) and tubules (arrowheads) near and within the cyst wall. Scale bar = 1 μ M (Micrographs from Lemgruber L & Vommario RC personal archive).

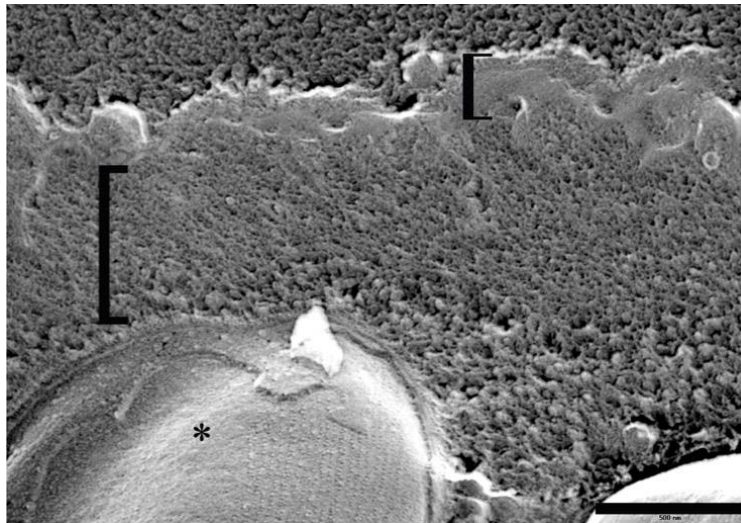


Figure 5. Quick Freeze - Freeze Fracture - Deep Etch - Rotatory Replica of a cyst of the Me49 strain isolated from infected mice. The cyst wall components form two distinct layers: one more compact (smaller bracket), facing the outer part of the cyst; and another which is looser (larger bracket), with a sponge-like aspect, near the cyst matrix. Bradyzoite (*). Scale bar = 0,5 μ M. (Figure 3A in Lemgruber et al. 2011. Cellular Microbiology, Volume: 13, Issue: 12, Pages: 1920-1932.).

T. gondii cyst wall is formed by two layers, as shown by SEM (Figure 3) and metal replicas of freeze-fracture with deep etch (Figure 5) (Lemgruber et al. 2011). An outermost thin layer, composed of compact material and an innermost, thicker layer showing a sponge-like appearance that faces the cyst matrix and is composed by loosely arranged filaments. The dense granule proteins, GRAs, are known to form the intravacuolar network (IVN) in the tachyzoite PV and the same occurs in the tissue cysts. The convoluted cyst membrane and the filamentous components of the cyst wall are recognized by antibodies against GRA1, GRA3 and GRA7. TEM of isolated cysts fixed with the osmium-imidazole technique revealed the

presence of straight tubular structures within the cyst wall. These tubules had a smooth surface and a mean diameter of 65 nm. Several vesicles with a mean diameter of 67 nm also appear within the cyst wall (Figure 4B).

The cyst wall is rich in proteins highly glycosylated that are labeled by the lectins - DBA (*Dolichus biflorus* agglutinin), which recognizes residues of N-acetyl-galactosamine and WGA (Wheat germ agglutinin), which recognizes residues of N-acetyl-glucosamine (Derouin et al. 1981; Sethi et al. 1977). In addition to glycosylation, another post-translational modification, sumoylation, was found in cystic wall-forming proteins. It is responsible for the correct addressing of proteins during cystogenesis (Braun et al. 2009).

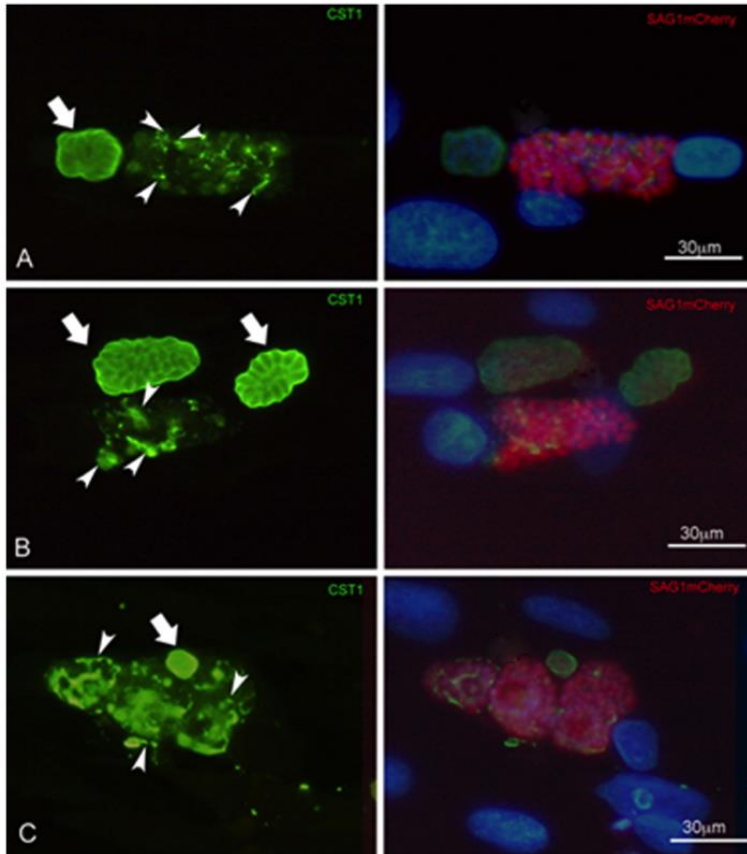


Figure 6. Immunofluorescence of cyst wall deposition during conversion of tachyzoites of the EGS strain expressing SAG1-mCherry proteins. HFF cells were infected for 96h. The initial appearance of cyst wall elements was detected with SALMON-E anti-CST1 antibody. A-C Parasites in CST1positive vacuoles (green - arrows) did not express the SAG1-mCherry protein (red). The vacuoles that contained SAG1 positive parasites had spots of wall protein deposition (arrowhead). DAPI - blue. (Images from Paredes-Santos TC & Vommaro RC personal archive).

Remarkably, it is possible to follow the deposition of cyst wall components before the full conversion of tachyzoites to bradyzoites (Figure 6). Bradyzoite protein markers are expressed at different steps throughout the process of conversion. The bradyzoite proteins BAG1 and CST1 (cyst wall protein) were found early during *in vitro* differentiation (Paredes-Santos et al. 2013). On the other hand, the proteins LDH2 (lactate dehydrogenase) and

surface proteins as SRS9 (SAG1-related sequences) appear to be expressed later following *in vitro* induction (Yang and Parmley 1997; Kim and Boothroyd 2005).

CST1 is the major protein in the cyst wall. It is a highly glycosylated protein of 250 kDa, rich in mucins in the N-terminal region that present a high affinity for the DBA lectin. CST1 is in the deposition region of the cyst wall, just below the membrane and has a structural role in the support of the wall. Parasites knockout for CST1 or that do not express the mucin-rich region, are able to encyst *in vivo* but are fragile and break during cyst isolation (Tomita et al. 2013; Zhang and Smith 1995; Zhang et al. 2001). The complementation of the phenotype leads to the restoration of the stability of the wall, demonstrating that the region of glycosylation is important for its integrity. The glycosylation is essential for addressing proteins to the wall. The inhibition of an initial precursor of the parasitic o-glycosylation pathway makes the persistence of infection *in vivo* reduced and the recognition by DBA is lost (Caffaro et al. 2013). Another cyst wall protein, CST2 was shown to be essential for the virulence and the establishment of the chronic phase of *Toxoplasma* infection, as the cyst burden in mice infected with CST2-KO was undetectable (Tu et al. 2019).

Cyst matrix is filled by a diffuse material between bradyzoites. It is formed by the intracystic network (ICN), an intricate network of tubules similar in diameter to those of the intravacuolar network, but shorter, denser, and more compacted and vesicles of different sizes (Figures 7 and 8). Images of transmission electron microscopy (TEM) suggest an intense secretory activity of bradyzoites. The cyst matrix of cysts from animal tissue or cell culture presents a large number of vesicles (Figures 8 and 9). These vesicles were observed budding from the cell body and the posterior pole of bradyzoites forming the tubule-vesicular network of the ICN (Lemgruber et al. 2011).

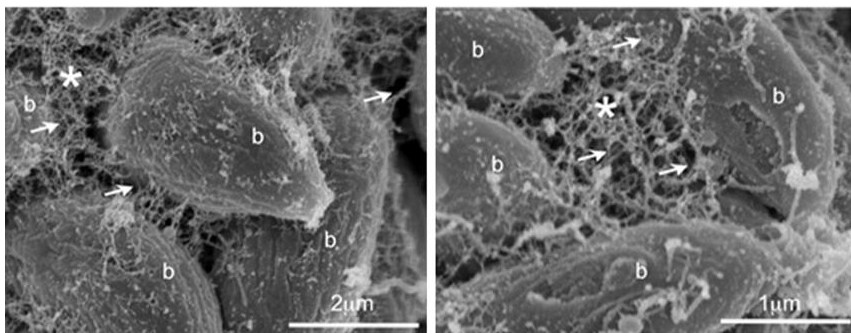


Figure 7. Scanning electron microscopy of cysts of the EGS strain obtained in LLC-MK2 cells after removal of the host plasma membrane and cyst membrane with dry cleavage methodology. The cystic matrix (*) is composed of short tubules (white arrows) that interconnect the parasites (white arrows). (Micrographs from Paredes-Santos & Vommaro RC personal archive)

MAG-1 is a cystic matrix protein that is only present in the matrix and is expressed in low levels in tachyzoites. Dense granule proteins as GRA1, GRA2, GRA3, GRA4, GRA5, GRA6, GRA7 and GRA8 were detected in the cystic matrix (GUIONAUD et al. 2010; Parmley et al. 1994), as well as NTPases and serine protease inhibitors, which remain in the PV matrix (Torpier et al. 1993; Ferguson 2004).

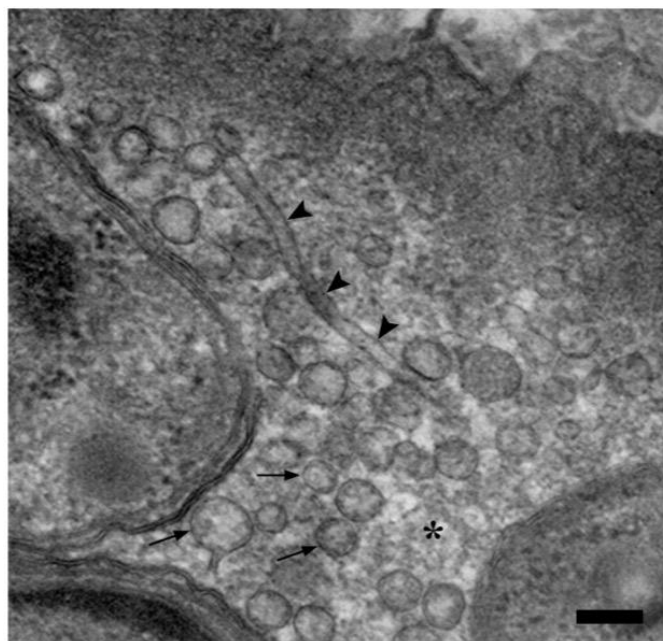


Figure 8. TEM of a cyst of Me49 strain isolated from infected mice. Fibrillar and amorphous granular material fills the space (*) between bradyzoite, as well as large number of vesicles of different sizes (arrows), larger in diameter than the intracystic network elements (arrowheads). Scale bar = 0,1μM (Micrograph from Lemgruber L & Vommaro RC personal archive).

CST1 protein appeared exclusively on the cyst wall while GRA1, GRA2, GRA3, GRA5 and GRA6 appeared both in the cyst matrix and wall (Ferguson 2004; Lane et al. 1996; Lecordier et al. 1999; Tu et al. 2019). Knockouts for GRA4 and GRA6 proteins formed fewer cysts *in vivo*, demonstrating the importance of dense granule secretion for the formation and maintenance of the cystic wall (Fox et al. 2011). The protease inhibitor TgPI1 is secreted by the dense granules and knockout parasites for this protein form a greater number of cysts *in vitro* and *in vivo* (Pszenny et al. 2012).

In addition to the dense granules proteins, present in the matrix and cyst wall, a study comparing the transcriptome of tachyzoites and bradyzoites from cell culture and mice brain cysts, identified two bradyzoite-specific proteins: one with a MAR-like adhesive domain (Microneme Adhesive Domains)- the MCP4 (Microneme Adhesive Repeat domain-containing protein 4), and the pseudo-kinase BPK-1. Both proteins appear in the cystic wall (Buchholz et al. 2011). BPK-1 is essential for resistance to the active pepsin enzyme, present in the low pH of gastric juice, which is the first barrier faced by the parasite after the oral ingestion of tissue cysts (Buchholz, Bowyer, and Boothroyd 2013).

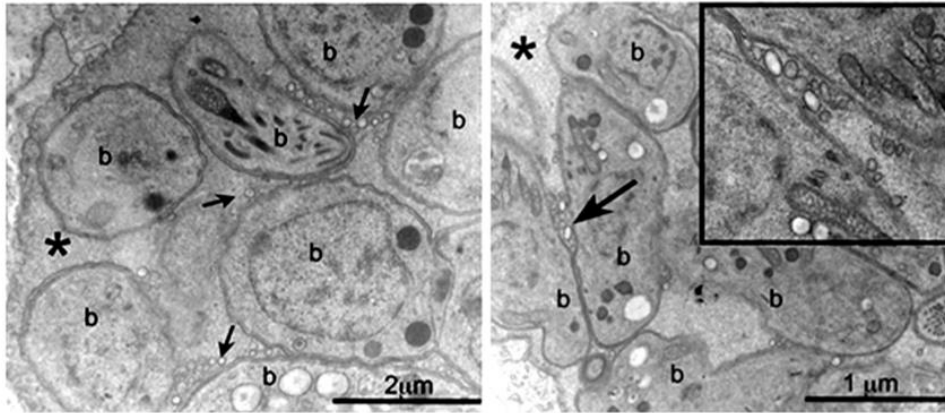


Figure 9. TEM of an immature cyst of the EGS strain showing cystic matrix (*) filled with several vesicles (black arrows). Inset shows the vesicles among parasites. (Micrographs from Paredes-Santos & Vommaro RC personal archive).

5. MODIFICATIONS OF THE HOST CELLS DURING CYSTOGENESIS

In the context of tachyzoite infection host cell organelles suffer a dramatic repositioning, redirecting host cell nutrients to the PV (Coppens and Romano 2018). As soon as the tachyzoite invades a host cell, the non-fusogenic PV is located or migrates to the perinuclear region (Wang, Weiss, and Orlofsky 2010), and the centrosomes are pulled to the vicinity of the PV (Walker et al. 2008), leading to a global reorganization and recruitment of host cell structures, including microtubules, mitochondria, the endoplasmic reticulum, the Golgi complex, and lysosomes, around the PV (Melo and Souza 1997; Magno et al. 2005). Mitochondrial profiles and ER tubules surround the PV and are become in close contact with the PVM. The association of mitochondria with the PVM seems to vary between parasite strains. Tachyzoites also divert host cell lysosomal content to the PV by vesicular transport dependent on host cell microtubules, which are inserted into the PV and governed by the secreted *Toxoplasma* protein GRA7 (Coppens and Romano 2018). Besides, the parasite scavenges sphingolipids from the host Golgi complex, by sequestering Golgi-derived Rab vesicles (Romano et al. 2013).

Comparatively to tachyzoites, less is known about the interaction of *T. gondii* cysts with the host cell cytoplasm, and the host cell architecture during and after cyst formation. While a proportion of parasites within cysts remain in the G0 stage (White, Radke, and Radke 2014), the cyst grows with time, because some bradyzoites continue to divide at a slow rate (Watts et al. 2015) a demonstration that cysts are metabolically active and are expected to scavenging nutrients from the host cell. Survival within the strengthened cyst wall, although guarantees long-term infection persistence (Ferguson and Hutchison 1987), represents a great obstacle to the diversion of host resources to the cyst. Cysts possess a size-selective permeability to molecules of up to 10 kDa (Lemgruber et al. 2011). Anionic markers, which adhere to the cyst wall membrane can also reach the cyst lumen (Guimarães et al. 2007).

Experiments with the EGS strain, which form cysts spontaneously in human foreskin fibroblasts (HFF) culture and mice, were carried out to analyze the modifications suffered by

host cells during the encystation process. In cells infected with bradyzoites, host cell microtubules and intermediate filaments formed a 'cage' around cysts. The host cell microfilaments were not seen in the cyst periphery. The presence of cysts did not disrupt the organization of host cell microfilaments, or stress fibers organization. Likewise, the cortical pattern of actin filaments did not change during cyst formation. In contrast, the intermediate filament network reorganized around the cysts. These modifications of the cytoskeleton are similar for both the PV and cysts (Halonen and Weidner 1994; Halonen, Weiss, and Chiu 1998).

The distribution of microtubules in host cells on cyst formation is similar to that observed previously for the tachyzoite infection. In *in vitro* infection with bradyzoite, a cage of microtubules surrounded the cysts. The treatment with the microtubule stabilizer taxol did not disrupt the cage of microtubules around cysts (authors' non-published data) and the number of cysts increased by 50% in taxol-treated cells, compared with untreated samples, which suggests the stabilization of a microtubule cage favors bradyzoite development and cyst formation (Paredes-Santos et al. 2018). Host cell microtubules and intermediate filaments, but not the actin cytoskeleton, reorganize under bradyzoite infection in cells containing cysts of *T. gondii*.

Infection with the cystogenic strain EGS also showed that organelles such as ER lamellae accumulate juxtaposed the cyst and appeared interdigitated with cyst wall folds. On the other hand, the Golgi complex did not appear associated with or near the cyst or on the nuclear periphery. In the same system using the EGS strain infection, the host cell mitochondria did not appear adhered to the cyst wall but were concentrated around cysts. Lysosomes observed with anti-LAMP-1 accumulate around the cyst wall, while uninfected cells had lysosomes distributed throughout the cytoplasm and surrounding the nucleus (Paredes-Santos et al. 2018).

CONCLUSION

Understanding the cystogenesis process, induction factors and the characterization of the transcriptional regulatory proteins are crucial to identify new potential targets for toxoplasmosis chemotherapy.

Many issues, such as the acquisition of nutrients by bradyzoites and the passage or transport of molecules through the cyst wall, the secretion of proteins that form the cyst wall and their transport and assembly to the wall, as well as the participation of GRA proteins in these processes still require investigation and are essential to elucidate the key steps of cyst formation.

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Chapter 5

***TOXOPLASMA GONDII* CELL DIVISION: BACK TO THE SOURCE**

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ABSTRACT

Cell division is fundamental to survival. It allows species to prevail, and its regulatory mechanisms underlie a plethora of fundamental biological processes ranging from evolution to disease. For apicomplexan parasites, cell division also constitutes a mechanism of pathogenesis. New cells emerge, invasion competent, from the infected host cell, ready to invade neighboring healthy cells.

The asexual generation of progeny entails the multiplication and segregation of genetic material from a mother cell and its packaging into a variable number of daughter cells. How different cell types regulate the temporal and physical coordination of these processes, and their output, varies widely in nature.

In this chapter, we will describe how *Toxoplasma gondii*, the so-called “model” apicomplexan organizes cell division, as an example of the remarkable adaptability and divergence of cell division in this phylum. We will describe the asexual cell division mechanism used by one of the clinically relevant life forms of the parasite; the tachyzoite. This process, known as endodyogeny, has been described in exquisite structural detail, and more recently, new insight into the regulatory mechanisms has emerged. This chapter will focus on the factors triggering the onset of cell division and on the structural aspects underlying the sequential and hierarchical building of a new *T. gondii* cell.

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1. INTRODUCTION

Mammalian cell division cycle is divided in four distinct stages. Nuclear DNA synthesis and replication occur upon entry into S-phase. Concomitant with the entry into the S-phase, the centrosome, the main microtubule organizing center (MTOC) of the cell, duplicates and begins moving towards opposing poles of the cell. G2 serves as a “checking” stage whereby the fidelity of DNA duplication is monitored and DNA repair pathways are activated if needed. Only if everything checks out mitosis, or M-phase, follows. During mitosis, the nuclear envelope disaggregates, duplicated chromosomes condense and by ways of contacting the mitotic spindle, organized by the centrosome, move to opposing poles of the cell. With a few exceptions, cytokinesis, defined as the physical separation of two new cells, follows immediately. Following this, the cell enters G1 or interphase, the longest stage of the cell cycle and a stage of preparedness for the next round of division. Organelles are duplicated during this stage.

Toxoplasma gondii, as most of the parasites in the phylum Apicomplexa, infects a variety of host cells and can scale the number of progeny accordingly, to suit different niches. Different modes of cell division are associated with distinct life forms, and transitions between hosts and cell types. *T. gondii* can exist in most warm-blooded intermediate hosts as either a fast-dividing tachyzoite or a slow-dividing bradyzoite. In felid species, the definitive host, bradyzoites can differentiate into merozoites, which can further differentiate into gametes. The latter have the potential of fusing, thereby initiating the sexual replicating track. This chapter will focus on the mode of division used by tachyzoites and bradyzoites, known as endodyogeny (Sheffield and Melton 1968). Note, however, that *T. gondii* enteric stages multiply by other mechanisms, known as endopolygeny and schizogony (Ferguson 2009). Key to this ability is a highly flexible cell and division cycle, which will be discussed in detail below. Nonetheless, this chapter will focus on the best studied mode of division, describing its structural and molecular underpinnings, endodyogeny.

Endodyogeny occurs by very distinct mechanisms to those found in mammalian cells. First, the cell cycle in *T. gondii* is composed of only three distinguishable stages; G1/interphase, S/M-phase and budding. In cell culture, one full cycle takes roughly six hours to complete. DNA replication begins with S-phase entry. This stage is temporally marked by the duplication of the centrosome and the concomitant elongation of the Golgi apparatus. The start of the M-phase follows. Though these stages can be morphologically distinguished, they don't occur temporally separated and hierarchically, as they do in mammalian cells. In fact, S phase and mitosis partially overlap, with no in between G2 “checking” stage (Radke et al. 2001; Khan et al. 2002).

Nuclear mitosis occurs in the presence of a virtually intact nuclear envelope, and with little appreciable chromatin condensation. This mechanism of nuclear division is known as closed or semi-closed mitosis. Nuclear segregation is driven by the mitotic spindle, nucleated by cytosolic centrosomes positioned adjacent to each other. Spindle microtubules contact kinetochores through a semi-open region of the nuclear envelope, exhibiting pores that give way to microtubules of the spindle. As centrosomes gradually separate from each other, dragging along their associated genetic material, mitosis progresses.

Daughter cells are not formed by equal segregation of the content driven by forces at opposing poles, and fission of the mother cell cytosol (i.e., binary fission), but rather

assemble *de novo* within the mother. Nuclear and organellar segregation occur progressively and concomitantly with the growth and development of internal daughter cells. Two new cells emerge from the original mother cell, acquiring in the process its plasma membrane. This process, known as budding, encompasses *de novo* assembly of many of the cellular components of a tachyzoite, and major disassembly of the mother cell, as well as, segregation of duplicated organelles.

2. STRUCTURAL INSIGHT INTO ENDODYOGENY

2.1. Nuclear Events – S Phase and Mitosis

Direct visualization of chromosomes by classical techniques is impaired by a lack of appreciable chromatin condensation in mitosis. Hence, early electron microscopy studies of the asexually dividing *T. gondii* tachyzoites failed to identify *bona fide* stages of mitosis or to decipher *T. gondii*'s karyotype. Early efforts to reconstruct the parasite's karyotype by pulsed-field gel electrophoresis and assignment of linkage groups by hybridization identified 9 distinct bands with 10 physical linkage groups of sizes ranging from 2 Mb to over 6 Mb (Sibley and Boothroyd 1992). More recently, genome sequencing and assembly established that *T. gondii* has a 60-70 MB haploid genome, distributed in 14 chromosomes, coding for over 7500 genes (Kissinger et al. 2003). In time, the genomes of over 60 strains of *T. gondii* have been sequenced, and minimal variations in genome size and number of coding genes among strains, identified. Subsequently, third generation sequencing and re-assembly of the *T. gondii* genome, together with 3D mapping of genomic contacts by Hi-c, showed that the initial karyotype overestimated one chromosome; *T. gondii*'s genome is distributed in 13 nuclear chromosomes (Xia et al. 2020; Berna et al. 2020; Bunnik et al. 2019). In addition to the nuclear genome, *T. gondii* also bears two DNA-containing organelles: the apicoplast and the mitochondrion. The replication of the Apicoplast nucleoid and its segregation is coupled to the nuclear cycle (Martins-Duarte et al. 2021). The timing and mechanism of mitochondrial DNA replication remain poorly understood.

The start of the S-phase is marked by the duplication of the centrosome, the microtubule organizing center of nuclear mitosis in *T. gondii* (Figure 1). S-phase is the shortest phase of the cell cycle, taking up roughly half-hour to complete (Radke et al. 2001). Nuclear DNA duplication is functionally based on core DNA replication machinery conserved in other eukaryotes (White and Suvorova 2018). Mini-chromosome maintenance complex (MCM) DNA helicase, DNA Polymerase cofactors such as PCNA, topoisomerase and functionally specialized DNA polymerases have been identified in the genome of *T. gondii* (Naumov et al. 2017; White and Suvorova 2018; Guerini, Behnke, and White 2005). Interestingly, DNA synthesis detectably progresses only up to amplifying the DNA content to 1.8 times before the onset of cytokinesis. The last stretch of DNA synthesis is concomitant to the emergence of daughter cells (Radke et al. 2001).

In addition, specialized proteins have been shown to play a role in DNA synthesis in this parasite. A protein named TgECR1 was identified by functional complementation of a temperature-sensitive mutant that uncontrollably replicated chromosomes at the restrictive temperature. TgECR1 displays a dynamic localization, appearing first adjacent to the nucleus

in the early S-phase and in the nucleus in the late S-phase. Following nuclear division, TgECR1 is downregulated (Naumov et al. 2017). Though the exact function of ECR1 remains to be deciphered, it was shown to form a stable complex with the cyclin-dependent kinase-related kinase 5 (TgCrk5) and to interact with the ubiquitin-mediated protein degradation machinery and the minichromosome maintenance complex. These interactions are likely essential for ECR1's regulatory role of chromatin replication.

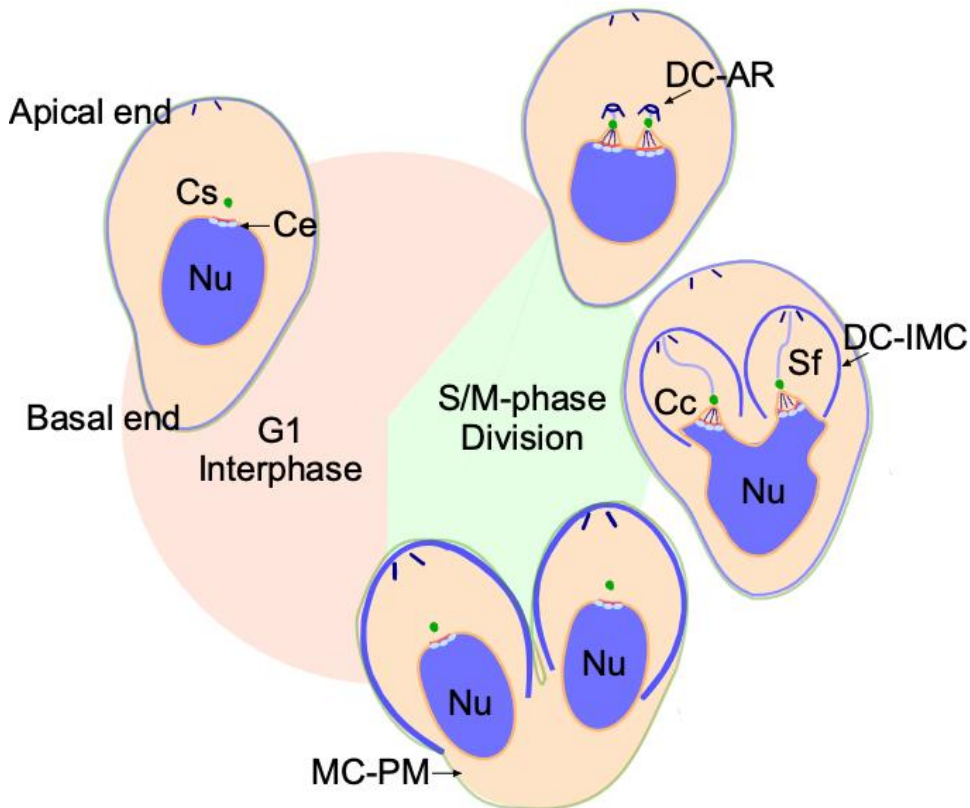


Figure 1. Endodyogeny in *Toxoplasma gondii*. Schematic representation of the major events occurring during the asexual replication of *T. gondii*. The cell cycle is divided in three stages; G1 or interphase – the longest of the 6 hour cell cycle–, S-phase and M-phase. S-phase entry is marked by the duplication of the centrosome (Ce), and the marked protrusion of the centrocone (Cc). Daughter cell formation (DC) is triggered shortly after S-phase entry. It initiates with the polymerization of a fiber composed of homologs of the Striated fiber assemblin proteins (Sf-fiber). The Sf-fiber positions the daughter cell's apical ring (DC-AR). Shortly after, other cytoskeletal components, such as components of the inner membrane complex (DC-IMC) begin assembling hierarchically. The centromeric (Ce) region of chromosomes persists adjacent to the centrosome throughout the cell cycle. The physical connections of the chromosomes to the centrosome, and of the centrosome to the daughter cell ensure the coordination of nuclear mitosis and cytokinesis. Finally, before the emergence of two new cells completes, these inherit the mother cell's plasma membrane (MC-PM). Note that the segregation and biosynthesis routes of organelles are not represented for simplicity. The Golgi and the apicoplast duplicate at the onset of S-phase, and are segregated by association with the centrosome. The mitochondrion enters new cells immediately before cytokinesis. Specialized secretory organelles such as the rhoptries and dense granules are synthesized *de novo*, while micronemes are a mixture of inherited and *de novo* synthesized.

A long-standing question in the field has been, how do Apicomplexa equally distribute the nuclear content between two daughter cells, in the context of a shared cytosol, without condensing the DNA or breaking down the nuclear envelope? Approaching how duplicated DNA segregates in *T. gondii* requires an understanding of its peculiar nuclear anatomy.

The first observations of dividing *Toxoplasma gondii* tachyzoites by transmission electron microscopy noted “an electron-dense body of the nucleus,” which the authors named “the E-body” (E- Endodyogeny). Because of its high electron density, this structure was proposed to contain DNA. The authors also noted that the E-body changed morphology during division, and hence proposed this structure to be “the instigating factor of endodyogeny” (van der Zypen and Piekarski 1968). This structure was later observed in other cyst-forming coccidian apicomplexans, such as *Eimeria* (Dubremetz 1971; Kelley and Hammond 1972), and later renamed with its currently used denomination: “the centrocone.” Albeit an extranuclear structure, devoid of chromatin, the centrocone is a structure intimately related to nuclear division. It is a conical elaboration outlined by the outwards folding of the nuclear envelope. The position of the centrocone is intimately related to that of the centrosome (Figure 1). The centrocone morphology varies along the cell cycle. During interphase, the position of the centrocone is noticeable as the structure persists throughout the cell cycle. However, at the start of mitosis, when centrosomes have not appreciably separated yet, the centrocone noticeably protrudes from the nucleus. It then duplicates and segregates along with the centrosome to two distinct, albeit adjacent, sites of the nucleus.

More recently, a protein carrying multiple membrane occupation and recognition nexus (MORN) motifs was shown to localize to ring structures at the apical and posterior end of the parasites, and to the centrocone (Gubbels et al. 2006). Overexpression of TgMORN1 resulted in severe defects in nuclear segregation and daughter cell formation. However, null mutants of the protein progress normally through mitosis, thereby suggesting TgMORN1 is not required for centrocone function (Lorestani et al. 2010). Nonetheless, TgMORN1 serves as the only molecular marker available to identify the centrocone structure by Immunofluorescence assays, and has allowed the detailed study of the morphological changes suffered by the structure during the cell cycle.

Live imaging of TgMORN1-YFP expressing parasites confirmed the centrocone dynamics observed earlier by TEM, and have additionally allowed *live* observation of the temporal and spatial correlation of the centrocone morphological changes with other simultaneous events of cell division; namely centrosome duplication, chromosome segregation and daughter cell formation. In this respect, it should be noted that all major stages of canonical mitosis (metaphase, anaphase and telophase) can be clearly distinguished and identified in *T. gondii*.

Microtubules of the mitotic spindle are housed within the centrocone during mitosis and penetrate the nuclear envelope through pores, contacting the kinetochores. A homolog of the microtubule plus end-binding protein EB1, which in other systems stabilizes the mitotic spindle microtubules, was identified in *T. gondii*. TgEB1 localizes to the nucleus outside of division, but quickly re-localizes to the centrocone region upon entry into the M phase (Chun Ti Chen et al. 2015). TgEB1 has been shown to play an important role in regulating the proper progression of mitosis. Null parasites of the protein exhibit a delay in replication, whereby straying kinetochores can be observed during segregation (Chun Ti Chen et al. 2015).

The nucleus is highly structured into defined territories. Distinct chromatin domains are permanently maintained in specific regions of the nucleus. Centromeres are typically a single location on a chromosome where the kinetochore protein complex assembles during mitosis. The kinetochore is the point of attachment for microtubules of the mitotic spindle. Centromeres are marked by the presence of a variant histone H3, known as CenH3 or CenPA. Immunofluorescence assays revealed that in *T. gondii* CenH3 and its associated DNA cluster at the periphery of the nucleus at a location intimately related to the position of the centrosome and the centrocone (Brooks et al. 2011) (Figure 1 and 2). Normally, upon entry into interphase/G1 centromeres of most cell types dissociate from the mitotic spindle and redistribute throughout the nucleus; in *T. gondii* this is not the case. Following mitosis, centromeres remain clustered at the periphery of the nucleus, at a location intimately associated with the position of the centrocone and the centrosome (Figure 2).

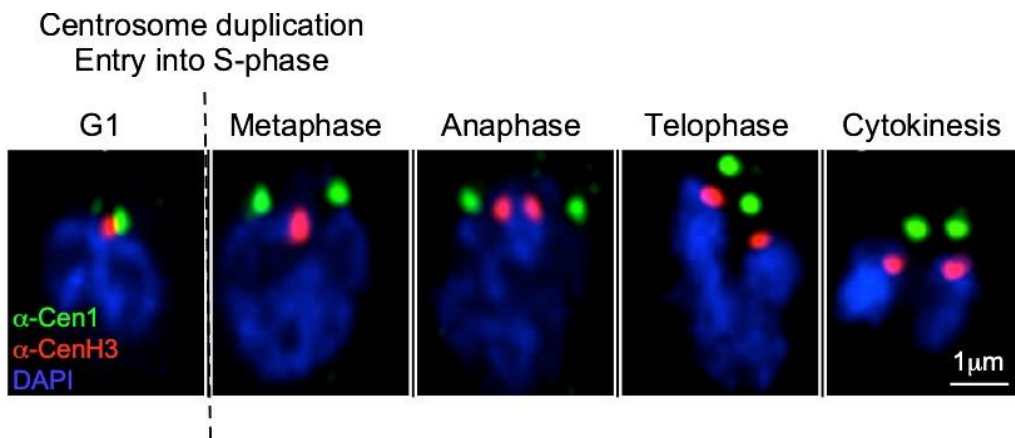


Figure 2. Nuclear Mitosis in Endodyogeny of *Toxoplasma gondii*. Early electron microscopy studies suggested that nuclear mitosis in *T. gondii* followed a similar progression scheme as that observed in metazoan, based on the morphology of the mitotic spindle. However, due to the lack of chromatin condensation into individual chromosomes, the stages of mitosis at the chromosomal level were not deciphered until markers of different substructures became available. Mitosis has been more recently visualized by Immuno-fluorescence assays. This series of images show pictures of nuclei of parasites at distinct stages of the cell cycle (as indicated). The DNA is marked by DAPI. The centrosome is labeled with anti-centrin1 antibody (green). The chromosomes' centromeres are labeled with anti-CenH3 antibody (red) (Adapted from: Brooks et al. 2011). Note that centromeres remain clustered and in close physical proximity to the centrosome at all stages.

The position of clustered centromeres is mirrored by kinetochore proteins, which also persist in their location throughout the cell cycle. Components of the kinetochore, Ndc80 and Nuf2 have been characterized in *T. gondii*. Both TgNdc80 and TgNuf2 display a clustered localization apposed to the position of the centrocone (Farrell and Gubbels 2014). Generation of a TgNuf2 conditional knock-down strain revealed its essentiality for chromosome segregation. However, in the absence of TgNuf2, centromeres remained clustered. Thereby suggesting that microtubule binding is not required for the centromeres' interaction with the centrocone and centrosome. Consistently, microtubules of the mitotic spindle are not present in interphasic parasites (Maria E Francia et al. 2020). Instead, it has been proposed that centromere clustering could be maintained by specialized components of the nuclear envelope, namely, peripherally associated proteins of the nuclear pore complex (Maria E

Francia et al. 2020). The molecular components bridging the interaction between nuclear envelope components involved in centromere clustering, the centrocone and the centrosome, in interphase have not been identified. Note however that the kinetochore components are indeed required for centromere-centrosome connection during division; in the absence of mitotic spindle binding to kinetochore components, chromosomes are not segregated, and nuclei “fall off” the cell, straying far from the centrosome.

The compartmentalization of the nucleus in *T. gondii* has also been evidenced by the localization of the heterochromatin-binding protein TgChromo1 (Gissot et al. 2012). This protein, specifically binds to heterochromatin, flanking the centromeres in physical proximity of the centrocone and marking the telomeres. Fluorescence in Situ Hybridization (FISH) of the DNA binding regions of TgChromo1 revealed that telomeric DNA sequences are also clustered in the nuclear periphery (Gissot et al. 2012). More recently, with the advent of Hi-c, multiple additional nuclear territories have been defined in several Apicomplexa. These have been shown to be characteristic of each life stage, and to be related to transcriptionally active and repressed regions of the genome (Bunnik et al. 2019). The link between this compartmentalization, and its importance for the proper progression of nuclear mitosis is still to be deciphered. However, it has been proposed by multiple authors that compartmentalization could be a mechanism to safeguard genome and epigenome integrity throughout the unusual nuclear mitoses of Apicomplexan parasites.

2.2. Daughter Cell Assembly

New daughter cells are assembled in a stepwise and highly ordered process that is temporally and spatially guided by cytoskeletal self-organization. 22 subpellicular microtubules shape each new cell. These are organized by a microtubule organizing center, located at the cell apex, known as the apical polar ring (APR). The name reflects its ring-shaped morphology (Katris et al. 2014). In coccidian species of the apicomplexan phylum, such as *T. gondii*, the APR surrounds the conoid, a hollow barrel-shaped motile structure composed of 14 atypical comma-shape tubulin fibers, composed of 9 protofilaments (K. Hu, Roos, and Murray 2002). The conoid protrudes from the cell tip in response to calcium signaling, especially during host cell invasion in extracellular parasites. When not protruding, the conoid rests between the APR and two apical pre-conoidal rings (PCRs). At the very apex, a single cone-shaped membrane plate called the apical cap is found. Several proteins have been reported to localize at the apical cap including nine apical cap proteins (called AC1 to AC9) (A. L. Chen et al. 2015, 2017). TgCentrin2 labels a peripheral ring of six annuli at the lower boundary of the apical plate (K. Hu et al. 2006; Leung et al. 2019).

The positioning of the daughter cell APR, the apical cap and the conoid are determined very early during cell division. Two new apical complexes are formed *de novo* in the mother cell's cytosol, at a position intimately related to that of the centrosome. The position of daughter cell formation is defined by a structure named “the SFA fiber”: a self-organizing polar fiber (M. E. Francia et al. 2012) (Figure 1). SFAs, or striated fiber assemblins, are the major components of the striated microtubule-associated fibers (SMAFs) in the flagellar basal apparatus of flagellated green algae (Lechtreck, Rostmann, and Grunow 2002). In *T. gondii*, the SFA fiber emanates from and is tethered to the centrosome (M. E. Francia et al. 2012). Two proteins, TgSFA2 and TgSFA3, have been identified as essential components of the

SFA fiber in *T. gondii*. These protein components of the fiber accumulate at the centrosome following its duplication in S-phase. As S-phase and mitosis progress, the fiber actively grows extending out towards the mother cell's cytosol. At this end, components of the daughter cells' apical complexes begin assembling, early in mitosis. This connection inevitably positions the founding structures of the daughter cell cortex adjacent to the nucleus. As described above, at all times the chromosomes are tethered to the centrocone and centrosome. Hence, chromosomes and daughter cells are indirectly physically linked through their mutual connections to the centrosome. Genetic ablation of either one of the proteins identified in the SFA fiber renders parasites completely unable to form daughter cells. Note, however, that nuclear mitosis progresses normally in these mutants.

The MTOC used by *T. gondii* to organize the cortical cytoskeleton (i.e., the APR) has been proposed to have evolved from a complex structure that in evolutionarily related free-living and parasitic alveolate algae, such as *Chromera velia*, *Colpodella vorax* and *Perkinsus marinus*, includes a basal body that serves to organize apical motile flagella (O'Shaughnessy et al. 2020; de Leon et al. 2013; M. E. Francia et al. 2012; Maria E. Francia and Striepen 2014; Dos Santos Pacheco et al. 2020). This hypothesis has been separately proposed by different authors in light of their specific findings. For instance, *T. gondii* possesses two SAS-6 homologs; TgSAS6 and TgSAS6L. SAS-6 is a widely conserved protein among eukaryotes that determines the nine-fold symmetry of the microtubule-based barrels (centrioles) that make up the centrosome and basal body in all systems where this protein has been characterized. While TgSAS6 is found at the centrosome, TgSAS6L is found at the APR (de Leon et al. 2013). Likewise, the early connection of the SFA fiber to the APR of the daughter cells during division exemplifies the repurposing of a protein function. SFAs anchor the basal body through a system of fibers known as the rootlet in evolutionarily related organisms (M. E. Francia et al. 2012).

The inner membrane complex (IMC) is a specialized membranous system localized beneath the parasite's plasma membrane. It is composed of flattened vesicles made of three fused membrane plates. The IMC is lined by an additional filamentous membrane known as the subpellicular network (SPN) (Mann and Beckers 2001). The SPN is composed of filament-like proteins known as alveolins, or IMCs, which interweave the cortical microtubules. This intricate membrane system provides structure, shape and stability to the parasite. It also plays a pivotal role in motility as this is where the force generating complex required for *T. gondii*'s gliding motility is located (Heintzelman 2003). The integrity of this complex is required for the organization of subpellicular microtubules, the APR and conoid assembly (Tosetti et al. 2020).

Dozens of IMC proteins that span the entire length of the parasite have been identified in *T. gondii* (Anderson-White et al. 2011; A. L. Chen et al. 2015; Torres et al. 2021). Of these, IMC15 and IMC32 are some of the earliest detectable proteins of the IMC to be laid out during daughter cell formation (Anderson-White et al. 2011; Torres et al. 2021). Around the same time, SAS6-L, a marker of the APR, RNG2 a marker of the conoid (Katris et al. 2014), and proteins of the apical cap AC9 and AC10, (Tosetti et al. 2020) assemble to begin forming a new cell.

Elegant experiments using photoactivation of different IMC proteins, demonstrated that the formation of the daughter IMC occurs exclusively *de novo*, and it is followed by the recycling of maternal IMC. The latter occurs following the emergence of daughter cells from the mother (Ouologuem and Roos 2014). The dynamic of expression and assembly of IMC

components onto the daughter cell scaffold has been documented by the study of fluorescent protein fusions along the cell cycle (Anderson-White et al. 2011). Overall, it has been shown that different IMC proteins are assembled hierarchically and sequentially, and that many of them are essential for daughter cell formation (Harding et al. 2016). The temporal control of their expression underlies this dynamic (Ouologuem and Roos 2014). In addition, sub-compartments within the IMC have been identified whereby specific IMC sub-compartment proteins (ISPs) localize. ISP1 localizes to the apical cap portion of the IMC. This protein is one of the earliest detectable components of the IMC observable during daughter cell assembly (Beck et al. 2010). ISP2 and ISP4 localize to a central compartment of the IMC (Fung et al. 2012; Beck et al. 2010) while ISP3 localizes to both the central and basal regions of the IMC. The specific localization of all three ISPs is required to maintain these domains separately, whereby disruption of ISP1's localization results in the re-localization of ISP2 and ISP3 to the apical cap. The specific distribution of ISPs is dependent upon N-terminal myristoylation and palmitoylation. It has been proposed that ISP1 serves a sort of "gate-keeping" function determining the hierarchical targeting of proteins to specific regions of the IMC. Finally, a class of proteins named ISC, localize specifically to the transverse and longitudinal sutures between the membrane sacs of the IMC. ISCs have been shown to be important for the maintenance of parasite morphology (A. L. Chen et al. 2017; Lentini et al. 2015; Anderson-White et al. 2011; Mann and Beckers 2001; Harding and Meissner 2014; Tosetti et al. 2020; Porchet and Torpier 1977; N. S. Morrisette, Murray, and Roos 1997).

Following assembly of the apical MTOC, the cortical cytoskeleton grows towards the basal end of the parasite by the addition of tubulin monomers to the subpellicular microtubules (N. Morrisette and Gubbels 2020; Gubbels et al. 2006). However, before the assembly of daughter cell microtubules and the IMC, and before the mother cell's cytoskeleton disassembles, the daughter cell basal complex, marked by the appearance of TgMorn1 rings, starts forming (Nishi et al. 2008; K. Hu et al. 2006; K. Hu 2008). A number of proteins, other than TgMorn1, have been identified as members of the *T. gondii* basal complex. These include: TgHAD2a, TgDLC, TgCentrin2, TgIMC5, TgIMC8, TgIMC9, TgIMC13, TgIMC15, Tg14-3-3, TgMSC1a, TgMyoC, and TgMyoJ (Anderson-White et al. 2011; K. Hu 2008; Lorestani et al. 2010; Engelberg et al. 2016).

The basal complex has been proposed to be the force generating complex that resolves septation at the end of daughter cell formation. However, it has been shown that septation does not complete during daughter cell formation. Parasites growing within the same vacuole remain physically connected by an extensive filamentous network composed of F-actin (Periz et al. 2017, 2019). This connection was shown to underlie the coordinated progress through successive rounds of replication amongst parasites within the same host cell, residing in the same parasitophorous vacuole. Only upon egress from the host cell, does septation complete. Nonetheless, TgCentrin2 has been put forward as an eventual mediator of septation. TgCentrin2 could potentially generate contractile force in response to changes in local calcium concentrations (shown to occur during egress from the host cell) via its EF-hand domain (K. Hu 2008). TgMyoJ a non-canonical myosin, which colocalizes with TgCentrin2 has also been shown to be important for constriction. In the absence of TgMyoJ, TgCentrin2 does not localize to the basal end, and constriction fails (Fréchal et al. 2017). Finally, TgMorn1 has been shown to play a role in septation, as mutants expressing non-functional versions of this protein result in cojoined multi-headed parasites that fail to separate from each other, even when extracellular (Lorestani et al. 2010).

In addition to organizing the parasite's cortical cytoskeleton, the highly polarized anterior end of the parasite houses the secretory organelles known as rhoptries, micronemes, and the dense granules (Paredes-Santos, de Souza, and Attias 2012). These specialized organelles participate in multiple processes essential for *T. gondii*'s parasitic lifestyle, including attachment to the host cell to license invasion, gliding motility and egress.

The rhoptries are membrane bound organelles, bundled at the very tip of the cell. Rhoptries exhibit a characteristic club-shaped morphology, whereby a bulb-shaped bottom and a thinner anterior section, known as the rhoptry neck, can be distinguished. Rhoptries secrete effector proteins needed for invasion and host-cell modification upon entry, which are essential for the modulation of the host cell defense response (Boothroyd and Dubremetz 2008). Hence, these organelles play pivotal roles in the parasitic lifestyle of *T. gondii*. During daughter cell formation, rhoptries are formed *de novo*. The process of rhoptry biosynthesis entails first the formation of a globular vesicle next to the Golgi apparatus, named the pre-ROP compartment. This is followed by a maturation stage, and the anchoring of the mature organelles to the apical tip of the forming daughter cells. Disruption of several proteins involved in vesicular trafficking block rhoptry biogenesis or cause the mistargeting of rhoptry resident proteins to other organelles. Some examples include the dynamin-related protein B (DrpB) (Breinich et al. 2009), the sortilin-like receptor TgSORTLR (Sloves et al. 2012), clathrin heavy chain 1 (CHC1) (Pieperhoff et al. 2013) and the RabGTPases Rab5A and Rab5C (Kremer et al. 2013). A clathrin adaptor complex protein, named TgAP1, was shown to regulate rhoptry biogenesis by activating rhoptry protein exit from the Trans Golgi Network (TGN). TgAP1 also participates in rhoptry post-Golgi maturation (Venugopal et al. 2017).

Finally, rhoptry tethering to the apical end of the parasite has been shown to be dependent on the proper palmitoylation of a protein named Armadillo Repeats-Only (TgARO) (Frénal et al. 2013; Mueller et al. 2013). How rhoptries successfully reach the apical end of the growing daughter cell has been studied in great detail. Disruption of actin polymerization interferes with rhoptry positioning. Equally, functional disruption of Myosin F, a member of the alveolate specific Myosin XXII class, interferes with this process. TgARO interacts with myosin F (MyoF) mechanistically linking rhoptry positioning and an actomyosin-based positioning mechanism (Mueller et al. 2013, 2016; Jacot, Daher, and Soldati-Favre 2013). The tethering palmitoylation of TgARO is mediated by the Palmitoyl Acyl Transferase TgDHHC7 (Beck et al. 2013; Frénal et al. 2013). TgARO also plays a role in holding rhoptries in bundles (Mueller et al. 2016).

Micronemes are apically located rod-shaped secretory vesicles loaded with a plethora of effectors required for invasion, egress and motility. These include adhesin complexes, perforins, and proteases. Subpopulations of micronemes with distinct morphology and protein content have been described in *T. gondii*. Micronemes are formed *de novo* during the late stages of daughter cell formation, in each separate daughter cell. In addition, micronemes of the mother are recycled back into the daughters from the residual body – a plasma membrane bound residue of the original mother cell. The residual body acts as a sorting hub for recycled material. In this way, each daughter obtains roughly half of the mother's micronemes and *de novo* forms the other 50%. The network of F-actin bundles which interconnects individual parasites within the same parasitophorous vacuole plays a pivotal in the redistribution and recycling of microneme vesicles (Periz et al. 2019). *De novo* synthesis of micronemes, is based on similar principles governing the biosynthesis of rhoptries, whereby the endosomal

trafficking system is heavily involved in the task (Dubois and Soldati-Favre 2019). However, two distinct populations of micronemes, engaged in distinct trafficking routes, have been identified (Juliette Morlon-Guyot et al. 2015, 2018). Differential sorting of microneme and rhoptry proteins to their resident compartments is affected by the disruption of clathrin adaptor TgAP1. Disruption of the $\mu 1$ subunit results in the mis-sorting of microneme proteins at the level of the TGN (Venugopal et al. 2017). Likewise, conditional depletion of TgSORTLR leads to a severe defect in rhoptry and microneme biogenesis without apparently affecting other organelles (Sloves et al. 2012; Dubois and Soldati-Favre 2019).

Dense granules are vesicles of tremendous importance for the parasite's survival within the host cell. These secretory organelles are loaded with a variety of host-cell and parasitophorous vacuole modifying factors with functions that include induction of gene expression changes in the host cell, among others (Bougdour et al. 2013; Bougdour, Tardieux, and Hakimi 2014). Dense granules are synthesized at the Golgi, and then transported to their sites of content release (Coppens et al. 1999). Trafficking of dense granules requires MyoF for transport along actin filaments (Heaslip, Nelson, and Warshaw 2016).

Time-lapse microscopy of fluorescent markers targeted to various subcellular structures described the timing of organelle partitioning (Nishi et al. 2008). The elongation of the Golgi apparatus, together with centrosome duplication, are amongst the first observable morphological changes at the onset of the S-phase. As the daughter cell cytoskeletal scaffold grows out from the apical end, the first organelle to be encapsulated is the divided Golgi. Further extension of the cytoskeletal scaffold results in the partitioning of the apicoplast, followed by the nucleus and the endoplasmic reticulum. The mitochondria is the last organelle to be segregated.

The apicoplast is a non-photosynthetic chloroplast-like organelle, with essential functions in metabolism and survival. The apicoplast bears its own 35kb circular genome. DNA duplication of the apicoplast is coupled to that of the nuclear DNA, whereby apicoplast nucleoid division and segregation initiate at the beginning of S-phase and conclude during mitosis (Martins-Duarte et al. 2021). The segregation of the apicoplast into daughter cells occurs by its physical association to the centrosome (Striepen et al. 2000). In particular, it has been shown that myosin F, an essential motor participating also of centrosomes positioning, is involved in apicoplast inheritance; depletion of MyoF results in parasites losing the apicoplast (Jacot, Daher, and Soldati-Favre 2013). Finally, a dynamin-related protein named DrpA assembles multimeric spirals that upon GTP hydrolysis mediate the fission of the apicoplast before cytokinesis (van Dooren et al. 2009).

T. gondii possesses a single mitochondrion whose localization and morphology along the cell cycle are dynamic (Melo, Attias, and De Souza 2000; Ovcariakova et al. 2017). Little is known about the mechanism of mitochondrial genome duplication and maintenance, and its coordination with cell cycle progression. In fact, the mitochondrial genome sequence itself is still in the works (Namasivayam et al. 2020; Berna et al. 2020). Mitochondrial duplication starts at the early stages of daughter formation, concomitant with IMC formation. The mitochondrion branches at multiple locations, however, it does not enter the developing daughters up until the very end of the division cycle (Nishi et al. 2008; Ovcariakova et al. 2017; Melatti et al. 2019). A homolog of the Fis1p protein of yeast, Fis1, was shown to interact with an outer mitochondrial membrane protein named LMF1. Fis1 homologs play a role in recruiting fission mediating dynamins in other systems. Disruption of LMF1 in *T. gondii* severely impacts mitochondrial morphology, and impairs proper organellar segregation

(Jacobs, Charvat, and Arrizabalaga 2020). Three dynamin-related proteins: DrpA, DrpB, and DrpC can be identified in the genome of *T. gondii*. DrpA plays a role in apicoplast fission and DrpB is involved in rhoptry biogenesis, as outlined above. It was shown that DrpC is important for mitochondrial biogenesis, and proposed that it perhaps mediated fission of the organelle; depletion or dominant-negative expression of TgDrpC results in interconnected mitochondria (Melatti et al. 2019). However, it was recently shown that DrpC appears to be involved in vesicle trafficking and endocytosis, and it was proposed that it might not mediate mitochondrial fission directly (Heredero-Bermejo et al. 2019).

3. REGULATION OF CELL CYCLE PROGRESSION

3.1. Coordination by Physical Tethers

Chromosomal centromeres are constantly tethered to the centrosome (M. E. Francia et al. 2012; Farrell and Gubbels 2014). This connection is seemingly mediated by components of the nuclear envelope specifically localized at the centrocone. A similar physical association with the centrosome has been described for the apicoplast and the Golgi on the cytosol [56, 115, 116]. Finally, an SF-fiber tethers the centrosome to the daughter bud MTOC and the associated invasion machinery (rhoptries and micronemes) providing a robust mechanism for the assembly of a complete invasion-competent new parasite cell. Hence, it is clear that the centrosome plays a pivotal role in coordinating chromosomes, Golgi and apicoplast segregation, with the assembly of daughter cells. This coordination has been shown to be lost in different mutant parasite lines. For example, mutants of the SFA proteins, TgSFA2 and TgSFA3, which form the SF-fiber, carry out mitosis normally, but do not assemble daughter cells. Likewise, mutants of kinetochore components carry out normal daughter cell assembly while failing to pack their nuclear content into them (Farrell and Gubbels, 2014). Proper centrosome duplication and migration are also important for proper progression of cell division, as mutants failing to duplicate or separate centrosomes progress with nuclear mitosis, but assemble a single daughter (Chen and Gubbels 2013; Suvorova et al. 2015; Semenovskaya et al. 2020). A number of proteins resident to the centrosome, including TgCep250 and TgCep530, have been shown to physically link nuclear mitosis and daughter cell budding. Depletion of either protein does not affect mitosis progression, but results in parasites forming without nuclei (Courjol and Gissot 2018; Chen and Gubbels 2019). Additionally, a recently characterized zinc-finger containing protein, named TgZFP2, was shown to accumulate at the centrosome upon daughter cell formation. Though its exact function is not understood, ablation of TgZFP2 leads to a futile cell division cycle whereby the connection between the daughter cells and the nucleus is lost (Semenovskaya et al. 2020). Collectively, these data have supported a model put forward a number of years ago, proposing that nuclear mitosis and daughter cell assembly are not co-regulated, but rather occur concomitantly and are temporally coordinated only by their mutual tethers to the same organizing center. It has been proposed that this physical mechanism of regulation ascribes parasites with the flexibility to regulate their output whilst using other modes of division such as endopolygony and schizogony. In these modes of division nuclear mitosis and daughter cell formation occur temporally separated. This, however, has not been formally shown.

More recently, the flexibility of cell division regulation in *T. gondii* has been further elucidated by the demonstration of a functional compartmentalization of the centrosome (J. Morlon-Guyot et al. 2017; Ferguson and Dubremetz 2013; Suvorova et al. 2015; C T Chen and Gubbels 2015). The centrosome in *T. gondii* has been shown to be divided into two distinct protein “clouds” whereby the centrioles likely reside within the cytosolic cloud, or “outer core”, and a second domain, named the “inner core”, is physically adjacent to the nucleus. The outer core contains proteins such as TgSAS6 – the protein that ascribes centrioles with their characteristic geometry, TgCentrin1- a *bona fide* luminal marker of centrioles-, and TgCep250. The latter also co-localizes with TgCep250L1 at the inner core. TgCep250 is a large protein containing multiple coiled-coiled domains – a common feature of centrosomal proteins. Its homologs in mammalian cells play a role in maintaining the centrioles together before the onset of cell division. In higher eukaryotes, the Never in mitosis (NIMA)-kinase Nek1 is responsible for the phosphorylation of Cep250 during the early stages of mitosis, which leads to centrosome separation. In *T. gondii* a Nek1 ortholog shows a dynamic localization, associating with the centrosome only upon its duplication. A mutant of TgNek1 fails to separate the centrosome suggesting a mechanism of centrosome separation akin to the one observed in metazoan operates in this parasite. Consistent with its role in providing a platform for daughter assembly, unseparated centrosomes in a TgNek1 mutant, assemble a single daughter (Chen and Gubbels 2013). In *T. gondii*, the function of TgCep250 was explored. The ablation of TgCep250 caused centrosomal cores to stray from each other, suggesting this protein may play a role in maintaining the physical proximity between the cores. However, it was shown that TgCep250 is not a substrate of TgNek1 (Chen and Gubbels 2019). The function of TgCep250L1 at the inner core remains to be deciphered.

3.2. Global Regulators

Aurora kinases are serine/threonine protein kinases with prominent roles in regulating key events in cell division including chromatin condensation, centrosome and spindle function, and cytokinesis. Three aurora kinase-coding genes have been identified in *T. gondii*. TgArk2 localizes at the mitotic spindle, and its disruption does not cause any delay in cell cycle progression or aberrant cell division. In contrast, knock-down of TgArk3 results in pronounced defects in parasite division and a major growth deficiency. Though the mechanistic link between TgArk3’s disruption and cell division has not been untangled, it could be linked to its localization at the outer core of the centrosome and the budding daughter cells’ cytoskeleton where it most likely exerts its function (Berry et al. 2016).

In higher Eukaryotes, the chromosomal passenger complex (CPC) promotes chromosome bi-orientation by detaching incorrect kinetochore-microtubule attachments, playing an essential role in controlling the fidelity of DNA segregation. The CPC is formed by Aurora kinase B (ARKB), Inner centromere protein INCENP, Borealin and Survivin. In *T. gondii* TgArk1 is an ARKB homolog. In addition, two INCENP homologs INCENP1 and INCENP2, have been reported (Suvorova et al. 2015; Berry et al. 2018). However, no Survivin nor Borealin homologs could be identified in the genome. It was shown that the proteins of the putative *T. gondii* CPC (TgArk1, INCENP1 and INCENP2) dynamically vary their localizations along the cell cycle, whereby the interaction between TgArk1 and both TgINCENPs, is essential for TgArk1’s translocation to the nucleus. Genetic ablation of the

CPC components showed that TgINCENP1 appears to be dispensable, while TgArk1 and TgINCENP2 are required for cell division (Berry et al. 2018). It was proposed that TgArk1 could be involved in catalyzing the timely opening of the nuclear envelope during spindle assembly, enabling its interactions with the chromosomes' kinetochores (Berry et al. 2018).

Mitogen-activated protein kinases (MAPKs) are a conserved family of protein kinases that regulate signal transduction, proliferation, and development throughout eukaryotes. The genome of *T. gondii* encodes for three MAP-related kinases: MAPKL1, MAPK2 and ERK7. Both MAPKL1 and MAPK2 have been implicated in the regulation of centrosome duplication. A temperature sensitive mutant of MAPKL1 over-duplicates the centrosome at restrictive temperature, leading to the assembly of an aberrant number of daughter cells (Suvorova et al. 2015). TgMAPKL1's substrates, however, remain unidentified. On the other hand, parasites lacking MAPK2 fail to duplicate their centrosomes, do not complete DNA replication and do not initiate daughter cell budding. However, MAPK2 mutant parasites continue to grow and replicate their Golgi apparatus, mitochondria, and apicoplasts (Hu et al. 2020). The remaining MAP-related kinase, Erk7, was shown to regulate the synthesis of the conoid (O'Shaughnessy et al. 2020; Back et al. 2020). Conditional null parasites for this protein exhibit a striking phenotype whereby conoid assembly is completely abrogated.

The coordinated and timely onset of successive cell cycle stages is largely controlled by Cyclin-dependent kinases (CDKs) in mammalian cells. The cyclic expression and ubiquitin-dependent degradation of cyclins control the timely onset of the CDK/cyclin complex activity (Ventura and Giordano 2019). It is well established that the ubiquitination machinery and the degradation of proteins via the proteasome-dependent degradation pathway play pivotal roles in regulating the progression of the cell cycle in metazoan. The abundance of cyclins, for example, is controlled in this way. Waves of ubiquitination enrichment are particularly associated with cell cycle progression in *T. gondii*. Changes in this particular protein post-translational modification, the ubiquitinome, are particularly associated with the cell cycle, in comparison with other PTMs (Silmon De Monerri et al. 2015).

A number of Cdk-related kinases (Crks; TPK2, TgCrk1, TgCrk2, TgCrk4, TgCrk5 and TgCrk6) and in some cases their partner cyclins, have been identified and characterized in *T. gondii* (Khan et al. 2002; Alvarez and Suvorova 2017; Naumov et al. 2017). TPK2 is a functional homolog of the CDC2 cyclin-dependent kinases, able to interact with mammalian cyclins A, B1, D3 and E, and to phosphorylate human Histone H1. Dominant-negative mutant of TPK2 arrest in S-phase.

TgCrk2 partners with a P-type cyclin. Its activity was shown to be essential for transitioning out of G1, while its depletion results in a G1 arrest. TgCrk6 and TgCrk4 are required for progression through S-phase and mitosis, participating of spindle assembly and centrosome duplication, respectively. Their cyclin partners have not been identified, nor have their substrates been deciphered. TgCrk1 and its partner TgCycL, were shown to be essential for daughter bud assembly (Alvarez and Suvorova 2017). Finally, TgCrk5 was shown to localize at the centrocone, and to interact with ECR1 (a factor involved in DNA duplication, see above), which suggests it may play a role in S-phase and mitosis (Naumov et al. 2017).

Progression of the cell cycle in *T. gondii* is also encompassed by stage-specific transcriptome changes (Behnke et al. 2010). The coordinated progression of cell cycle subtranscriptomes was elucidated using synchronized parasite populations. Recent findings using single cell RNA-sequencing of asynchronously growing *T. gondii* are in agreement with these earlier findings. Single cell transcriptome analyses allow parasites grouping in their

respective cell cycle stage by identifying their underlying transcriptomic programs (Xue et al. 2020). The regulation of these transcriptional waves is mediated by an extensive lineage of transcription factors called ApiAP2s, for Apicomplexan Apetala 2, whose homologs were first characterized in plants (Jeninga, Quinn, and Petter 2019; Hakimi and Deitsch 2007). The function of most ApiAP2s in *T. gondii* is unknown. However, many of *T. gondii*'s ApiAP2's have been shown to play a role in cell cycle progression.

AP2XII-2 expression peaks during S and M-phase. Consistently, loss of AP2XII-2 results in slowed tachyzoite growth due to a delay in S-phase progression (Srivastava, White, and Sullivan 2020). TgAP2IX-5, controls cell cycle events downstream of centrosome duplication. TgAP2IX-5 binds to the promoter of hundreds of genes and controls the activation of the budding-specific cell cycle expression program (Khelifa et al. 2021).

The mutation of the nuclear factor Cactin arrests parasites in G1 (Szatanek et al. 2012). Transcriptional analysis in this context determined that several ApiAP2s were upregulated. These include: TgAP2X-7, TgAP2VIIa-6, TgAP2XII-4, and TgAP2VIII-4, indicating that these ApiAP2s could suppress cell cycle progression to S-phase.

Noteworthy, the transcriptional regulation of cell cycle progression intersects with the regulation of gene expression underlying differentiation and virulence. For example, TgAP2XI-5 is expressed throughout the cell cycle. However, it binds the palindromic GCTAGC motif specifically during S and M-phase, upregulating the expression of several genes, including a number of virulence factors (Lesage et al. 2018; Walker et al. 2013). The interplay between transcription factors promoting the expression of cell division required genes, and inhibiting differentiation pathways, determines the parasite's commitment with either one of the pathways. Evidence suggests that commitment to differentiation of tachyzoites into bradyzoites occurs during S and M-phase (White, Radke, and Radke 2014). In this context, AP2XII-2 has been shown to interact with AP2IX-4. The latter was shown to interact with microrchidia (MORC) (Srivastava, White, and Sullivan 2020). MORC is a transcriptional repressor complex composed of multiple effectors controlling both differentiation and cell division (Farhat et al. 2020). Conversely, AP2IX-5 acts as a transcriptional repressor promoting the inhibition of the differentiation pathway to ensure the continuation of asexual replication (Khelifa et al. 2021).

CONCLUSION

Toxoplasma gondii tachyzoites divide by a fascinatingly intricate modular division scheme called endodyogeny, representing the smallest of scales of internal daughter assembly amongst the modes of division found in the Apicomplexa phylum. Whilst the onset of cellular events that determines entry into a specific stage of the cell cycle is controlled by global soluble regulators, coordination of the multiple synchronously occurring events in *T. gondii*'s cell division are seemingly orchestrated only by reciprocal physical connections to the centrosome. Whereas the onset of particular stages of the cell cycle seems to be regulated by orthologs of metazoan regulators, such as the Crks, Arks, and MAPKs, *bona fide* checkpoints do not seem to operate as concurrently occurring nuclear mitosis and daughter cell formation can progress uncoupled when physical tethers are interrupted by exogenous manipulation.

This particular mode of regulation has been proposed to underlie the flexibility of choice in cell division mechanism used in different life stages or under certain conditions by these parasites. Tremendous progress has been made in understanding the underlying molecular players governing these phenomena in tachyzoites, both based on individual efforts and higher throughput analyses both in *T. gondii* and in related organisms (Gubbels et al. 2008, 2020). The ready access to whole-genome sequencing, and the plethora of tools to rapidly generate mutants of essential and non-essential proteins using CRISPR/Cas9 genome-editing technology (Shen et al. 2014), and conditional knock-down strategies (Sheiner et al. 2011; Brown, Long, and Sibley 2018), has precipitated the characterization of multiple structural and regulatory factors. However, the underlying molecular mechanisms regulating cell division in life stages other than the tachyzoite remain virtually unexplored. The molecular basis of the alternative modes of cell division used by *T. gondii* bradyzoites, schizonts, merozoites and gametes remain obscure. The identification of master regulators of differentiation (Farhat et al. 2020; Waldman et al. 2020) and the recent technological breakthroughs allowing *in vitro* access to sexual stages (Di Genova et al. 2019) will be pivotal in unlocking access to these stages. Genome wide CRISPR screens, which have already served in identifying key virulence factors in tachyzoites (Sidik et al. 2016; Young et al. 2019), can now profit from these technologies to seize the opportunity to identify the core subset of genes required for common principles of all cell division modes, as well as the specific principles governing each individual mode of division used by this parasite.

Overall a deep understanding of the mechanisms of cell division brings us one step closer to devising effective strategies to prevent the clinical manifestations of acute toxoplasmosis. Expanding our breadth of knowledge on the molecular basis of proliferation to other life stages of the parasite will allow us to interfere with admixing and genetic recombination in sexual stages, as well as horizontal transmission by infected tissues and by oocyst shedding cats.

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Chapter 6

EPIGENETIC REGULATION AFFECTS ESSENTIAL MECHANISMS IN *TOXOPLASMA GONDII*

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ABSTRACT

How chromatin structure changes, allowing genetic information get accessed by different proteins is a key question to understand gene expression in all eukaryotes. In the *Toxoplasma* genome, an extensive repertoire of chromatin remodelers and histone modifiers is present, representing essential pieces to understand epigenetic regulation in this parasite. In addition to unique transcriptional factors, this epigenetic machinery represents a complex regulatory mechanism that must be further explored. Epigenetic regulation is represented essentially by three pillars: histone post-translation modification (PTMs), non-coding RNAs and DNA methylation. Mapping of all the histone PTMs in *Toxoplasma* revealed shared and unique modifications, including at least ten different types of modifications. The numerous potential combinations of PTMs are called the “histone code” and can trigger or inactivate several essential processes by either interfering with DNA accessibility or by recruiting chromatin remodeling complexes. Among the identified modifications, histone acetylation was remarkably abundant. Reversible acetylation/deacetylation has been explored as drug targets due to their role in gene expression control. So far, the available data reveals that *T. gondii* possesses a complex and robust histone code and understanding the code and the enzymes responsible for the modifications could reveal new key elements to elucidate essential processes and identify promising new targets to more efficient treatments.

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Keywords: histones, epigenetic regulation, HDAC inhibitor

1. INTRODUCTION

The alternation between different hosts, different developmental forms, and cell cycle control are major events in *Toxoplasma gondii*'s life cycle that require refined control of gene expression (Radke et al. 2005). As soon as the parasite enters the host cell, several factors related to the parasite's multiplication need to be expressed. The expression of these genes is silenced as soon as the parasite evasion occurs, initiating the expression of another set of genes related to the gliding movement for the parasite to find a new host cell. Soon, the parasite must enter this new cell, and for that, invasion-related proteins must be available. The egress and invasion of the parasite from the host cell happens in seconds, and the change in gene expression needs to be tightly regulated (Behnke et al. 2010). The factors regulating gene expression are directly linked to how genetic information is stored, accessed, and read. Cell division in *T. gondii* happens through an endodyogeny process, where two daughter cells are formed inside the mother cell. During this process, the parasite never loses the nuclear envelope. Also, the characteristic chromosomes formed during mitosis are not observed, with neither 30 nm fiber formation nor higher-order chromatin condensation level (Striepen et al. 2007). Importantly, *Toxoplasma* transcriptional control is achieved by 68 members of a single family of transcription factors similar to those found in plants, called AP2 factors (De Silva et al. 2008; Dixon et al. 2010; Behnke et al. 2010).

Due to the paucity of specific transcription factors, epigenetic regulation is an essential regulatory mechanism in this parasite, including several factors related to chromatin organization, such as chromatin remodeling and histones modifiers. Interestingly, these chromatin remodeling factors found in *T. gondii* are divergent from those found in other eukaryotes.

Histones are small, essential proteins with a globular domain, which fold the DNA into nucleosomes, the fundamental unit of chromatin. The nucleosomes are formed by 147 base pairs (bp) of DNA around an octamer of histones, composed of two copies of each histone (H2A, H2B, H3 and H4) (Khorasanizadeh 2004; Allis and Jenuwein 2016; Kim 2018; Peterson and Laniel 2004). Each nucleosome is separated by about 10 to 60 bp of DNA involved by the histone linker H1, although so far there is no available data confirming the existence of histone H1 in *T. gondii*. In addition to this globular domain, histones have N- or C-terminal tails that are exposed outside of the nucleosome's core.

Thus, histones have a central role in epigenetic regulation. Core histones, which will form nucleosomes, are classified into two groups, canonical and variant histones. Canonical histones have their transcription coupled with DNA replication, whereas, in variant histones, transcription occurs independently of replication. The presence of histone variants can alter the properties of nucleosomes granting them specialized functions (Kamakaka and Biggins 2005).

S. cerevisiae : ~~~MAPKSTTKTSKKGKPATSKGEKSTKAIAKTAKTAAKEEASKSYSRELIIEGLALRKERGGSRPALKKFIK--ENYPVGSASNFDLYFNNAIKKGVEAGDEQEPG--PA : 110
H. sapiens : MSETVPAPAASA----APEKPLAGKKA-----KKPKAANAASKKKPA[PSVSBELIVQAASSSSKERGGVSLAAKLKALAAGYDVEKNNSRIKLGIKSL----VSKGTILVQTGTGAS : 105
L. major : ~~~~~~
T. brucei : ~~~~~~
T. cruzi : ~~~~~~

* * * * *

20 40 60 80 100

S. cerevisiae : GAVKLAKKSPSEVKK-----EVEVSPKPKCAATTSVSATAKSAASTLAFKKRVKKSPPTVA[KASSESTLYTEFELIKSMPOIN : 192
H. sapiens : GSFKLNKRASSVTETPGASKVATKTKATGASKKKKATGASKSVKTPKKAK---KCALP---RUSCKNPKKETVVEKKVAKSPA-----KAAVKKAAKA : 197
L. major : ~~~~~~
T. brucei : ~~~~~~
T. cruzi : ~~~~~~

* * * *

120 140 160 180 200 220

S. cerevisiae : DKGSGSELVKKVKKDFESSKLKTSNFDFLENSAIKKCVENGEMIQPKGPSGIINKKKVKVLST : 258
H. sapiens : ~~~~~~FVTRPKTAAP---KK-----AAPKKK~ : 215
L. major : ~~~~~~ZVRKAVKVVKAVKTAKK---SSK-----KSSAKK~ : 105
T. brucei : ~~~~~~ZAKKA~ : 76
T. cruzi : ~~~~~~ZAKKAPKKAUKKA-----PKKK~ : 80

v
kk

Figure 1. Sequence alignment of histone H1 or histone H1-like. Amino acid sequence alignment for the histone H1 from *Saccharomyces cerevisiae* (ID number: GFP66013); *Homo sapiens* (ID Number: AAN06699.1), *Leishmania major* (ID number: LmCAA11592.1); *Trypanosoma brucei* (ID number: TbCAB76182.1), and *Trypanosoma cruzi* (ID number: TcPB181318.1). Histone H1 from humans has one classical globular domain marked by the box in the second line which is similar with *S. cerevisiae* globular domain. *S. cerevisiae* (first line) has two globular domains. Both are marked with boxes. Protozoan parasites such as *Trypanosomatids* do not show the globular domain. They present a unique H1, named H1-like since it corresponds only to the C-terminal domain of the human H1. Conserved and identical amino acids are marked in grayscale (conserved in gray, identical in black).

The histones in *T. gondii* comprise the four canonical histones H3, H4, H2A, all in single gene copy, and two isoforms of histone H2B, H2Ba, and H2Bb, where the latter is detectable only in sexual stages. In addition, *T. gondii* possesses five histones variants: CenH3 with conserved function as centromeric histone (Dalmasso, Sullivan, and Angel 2011), H3.3 that differ from H3 only by four amino acids (Sullivan Jr 2003), H2A.X, H2A.Z and H2B.Z. Some studies have already shown that H2A.Z and H2B.Z are located together at transcription initiation sites, while H2A.X dimerizes with H2Ba and are enriched in repressed genes. In addition, H2A.X appears to be associated with DNA repair (Bogado et al. 2014).

So far, the linker histone (histone H1), essential for the formation of higher-order chromatin structure, has not been described or even identified in *T. gondii* or any other apicomplexan parasites. The histone H1 family is evolutionarily less conserved and much more variable in size, structure, and sequence than the core histones, making it difficult to be identified bioinformatically (Kasinsky et al. 2001) (Figure 1).

2. THE “HISTONE CODE”

Histones have long been considered only for their structural function, but nowadays, they are active and dynamic components concerning all the DNA-dependent processes (Strahl and Allis 2000; Allis and Jenuwein 2016). Post-translational modifications (PTM) in histones are critical components in the gene expression regulation in all eukaryotes, including *Toxoplasma gondii*, affecting the structure of the nucleosomes.

Due to their intrinsically disordered structure, and because they protrude out of the nucleosome, it is known that the histone tails can interact with other proteins, and they are the target of several PTMs (Post-translational modifications). Currently, around 20 types of PTMs in histones have been described in eukaryotes, including acetylation, phosphorylation, methylation, ubiquitination, among others. The same is observed in *Toxoplasma* histones, where several unique and conserved PTMs have also been identified (Nardelli et al. 2013).

Changes in chromatin architecture can make the DNA more accessible (euchromatin) or less accessible (heterochromatin) by the transcription machinery acting as a barrier for it or for other proteins (Croken, Nardelli, and Kim 2012). Besides transcription, histone PTMs also affect other DNA-dependent processes such as DNA replication, repair, and recombination, either by altering their interactions with DNA or recruiting other remodeling proteins (Bannister and Kouzarides 2011).

The various modifications can be understood as a language or the “histone code,” first proposed by Strahl and Allis (2000). The authors proposed that each modification can act alone, together or sequentially, resulting in the activation or blocking of a particular biological process. For each modified residue, there are writers, erasers, and readers. Writers are the enzymes that add modifications, such as histone acetyltransferases (HATs) and histones methyltransferases, while erasers remove such modifications, such as histone deacetylases (HDACs) and lysine demethylases (KDM). The proteins called readers have specific domains that enables them to recognize these modifications; for example, proteins that recognize acetylation contain a domain called bromodomain; methylation, chromodomain; or phosphorylation 14-3-3 domains (Allis and Jenuwein 2016; Chen et al. 2017).

3. POST-TRANSLATIONAL MODIFICATIONS IN *TOXOPLASMA*

In *T. gondii*, the histone code was mapped in 2013, identifying 249 peptides with 108 different modifications. Among them, conserved PTMs have been found, especially in histones H3 and H4 N-terminal tails. However, some unique and specific modifications were also identified, with unknown functions. Some PTMs that indicate gene activation, such as H3K9ac, H3K14ac, H3R17me, H4K8ac, H4K12ac, and H4K16ac, were found abundantly in *T. gondii*. Classic gene suppression markers, such as H3K9me, H3K36me, and H3K79me, are also present. Other PTMs were also identified, such as lysine succinylation, propionylation, formylation, and crotonylation, indicating a specific and unique histone code for *T. gondii* (Nardelli et al. 2013).

Among the different PTMs, histone acetylation is one of the most studied and may have several functions in gene expression regulation, especially related to transcription activation or silencing (Khorasanizadeh 2004; Bannister and Kouzarides 2011; Allis and Jenuwein 2016). Acetylation favors gene expression since it neutralizes the positive charges of lysines, decreasing the interaction between histones and DNA, making it less condensed and facilitating access to transcription activators. Deacetylation of histones causes the opposite effect, making chromatin more condensed and repressing gene expression (Khorasanizadeh 2004; Kim 2018; Chen et al. 2017). The acetylation/deacetylation balance is made by histone acetyltransferases (HATs) and histone deacetylases (HDACs), named as such for being first described in histones. However, it is important to note that both HATs and HDACs can act on non-histone proteins.

HATs catalyze the reversible acetylation reaction, using acetyl-CoA as a cofactor to add an acetyl group to the lysines present mainly in the N-terminal portion of histones (Bannister and Kouzarides 2011). There are two types of HATs named HAT-A and HAT-B. Type A HATs are nuclear and more heterogeneous than type B, and can be classified into three families: GNAT, MYST and PCAF (p300 / CBP association factor) (Marmorstein and Zhou 2014; Harris et al. 2019). According to their location, these protein families are responsible for the acetylation of histones in the nucleosomes (Bannister and Kouzarides 2011). Type B HATs are cytoplasmic and are involved, mainly in the acetylation of newly synthesized histones, specifically in histone H4 lysines 5 and 12, an important pattern for depositing new histones in the newly synthesized DNA (Parthun, et al, 2007).

Toxoplasma has members of both GNAT and MYST families of histone acetyltransferases, but not p300 /CBP, since this family is present only in metazoans (Dixon et al. 2010). From the GNAT family, two proteins were identified: GCN5A and B, which have different acetylation targets (Bhatti et al. 2006). GCN5 proteins act in multi enzymatic complexes called SAGA (Spt-Ada-Gcn5 Acetyltransferase), which is relatively conserved among eukaryotes (Grant et al. 1997). On the other hand, unlike other organisms, the GCN5 of *T. gondii* have unique N-terminal extensions, which probably can result in particular mechanisms of action (Harris et al. 2019).

GCN5A and B are located in the parasite nucleus. These two proteins have a bromodomain downstream of the catalytic domain (Naguleswaran et al. 2010), responsible for recognizing acetylated lysines. GCN5A is not essential for parasite survival and is responsible for the acetylation of histone H3 lysine 18 (H3K18) and is important to alkaline stress response and tachyzoite-bradyzoite differentiation. GCN5B is responsible for acetylating multiple lysines on the tail of histone H3. Its gene knockout was unsuccessful which may indicate that this is an essential

protein for *Toxoplasma* survival (Vanagas et al. 2012). By co-immunoprecipitation assay, it was observed that GCN5B is located mainly in the gene bodies, indicating a function in transcription extension. The authors also identified other partners of the protein complex, which is quite divergent and has new and specific components in Apicomplexa (Harris et al. 2019).

Two proteins represent the MYST family, also called A and B, which have CHD (chromodomain) and zinc finger domains. MYST-A is more expressed in tachyzoites, and its deletion and overexpression were not viable, suggesting that acetylation through this enzyme is essential for the parasite's survival. MYST-A has two isoforms, one longer than the other, both present in the nucleus and cytoplasm of the parasite (Bhatti et al. 2006; Smith et al. 2005; Vanagas et al. 2012). TgMYST-B seems to target histone H4 and it is involved in the recruitment of serine/threonine kinases, important proteins for progression in the cell cycle and DNA repair (Vonlaufen et al. 2010; Kim 2018).

T. gondii has 7 histone deacetylases divided into four distinct classes, according to the domain, cofactor and sequence similarity to yeast proteins (Seto and Yoshida 2014). Class I HDACs includes TgHDAC2 and TgHDAC3 that have a classic HDAC domain, require Zn^{2+} as a cofactor, they are homologous to yeast Rpd3, and mainly nuclear. Class II, TgHDAC1 and TgHDAC5, can shuttle between nucleus and the cytoplasm, they are homologous to yeast HdaI, they have a classic HDAC domain, but they can still have other domains, such as inositol-phosphate kinase. In class III are the sirtuins, TgSir2A and TgSir2B, which have domain and homology to the yeast protein Sir2. TgSir2A and TgSir2B are considered non-classic because they use NAD^{+} as a cofactor for their activity. Finally, class IV, represented only by TgHDAC4, has mixed characteristics between classes I and II.

The classic HDACs (I, II, and IV) have a catalytic domain with eight central parallel β -sheets and 16 α -helices forming the main structure, which is similar to the arginase domain (Finnin et al. 1999). These proteins can be redundant, which means that in the absence (by knockout or knockdown) of some classic HDAC, another HDAC can compensate and perform the target proteins deacetylation (Seto and Yoshida 2014).

Little is known about the HDACs of *T. gondii*, only that they have some conservation with mammalian proteins. Most of them have large insertions of amino acids inside the HDAC domains, making them unique. A genetic screen using the CRISPR/ Cas9 strategy suggests that classic HDACs from *T. gondii* would be essential, while class III HDACs would be dispensable for the parasite's survival (Sidik et al. 2016). The only HDAC characterized is TgHDAC3, which has about 60% similarity to human HDAC1 and is located in the parasite nucleus. This enzyme is related to cell cycle control and differentiation from tachyzoites to bradyzoites. Importantly, it was verified, by chromatin immunoprecipitation analyzes, that TgHDAC3 is present in the co-repressor complex, which is found in the promoter regions of specific bradyzoite genes that are silenced in the tachyzoite forms. In contrast, these tachyzoites have TgGCN5-A acetyltransferase located in the promoter region of transcriptionally active genes (Figure 2A and B) (Saksouk et al. 2005). By inhibiting TgHDAC3, the authors observed an increase in histone H4 acetylation, mainly in the promoter regions just before the transcription start sites. Among the most hyperacetylated genes were those exclusively expressed in bradyzoites and sporozoites, indicating that these are the main target genes for TgHDAC3. In addition, in type II strains, an imbalance of bradyzoite-specific surface glycoproteins was also observed. Thus, inhibiting TgHDAC3 favors differentiation for bradyzoites by increasing the histones acetylation in these genes (Bougdoor et al. 2009).

TgHDAC2, together with TgHDAC3, is a class I HDAC, and according to the *Toxoplasma* genome database (ToxoDB.org), it is cell cycle-regulated. Microarray data indicates an increase in expression during the S phase of the cell cycle suggesting a function during DNA replication.

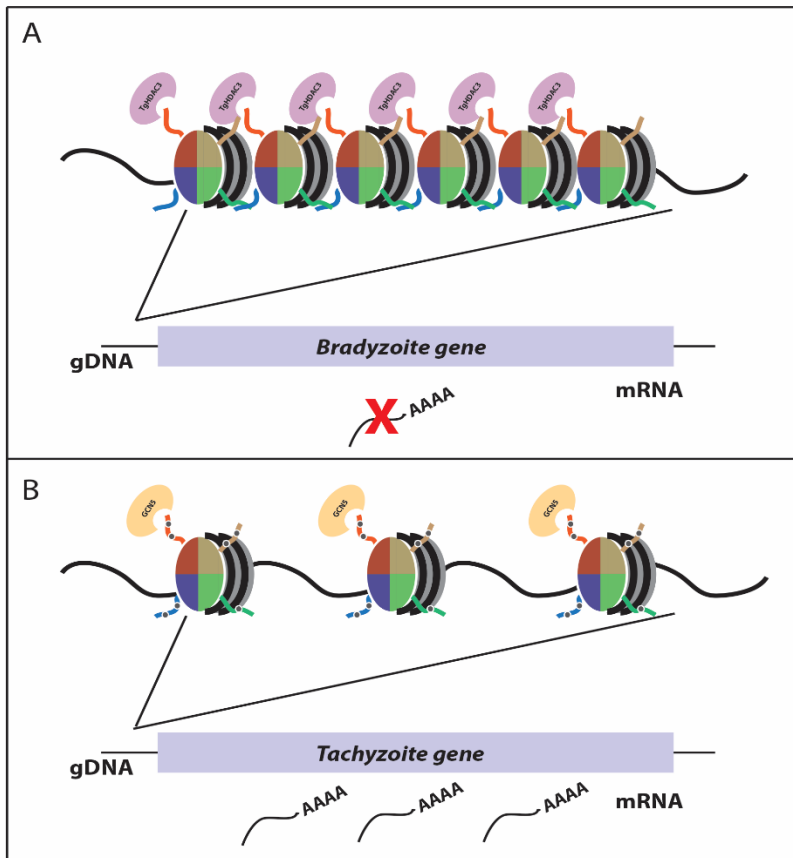


Figure 2. Schematic design of the effect of acetylation on *Toxoplasma gondii*. (A) Histone deacetylase TgHDAC3 is enriched in the promoter regions of silenced genes, such as bradyzoite genes. TgHDAC3 affects the local chromatin architecture by removing acetylation, which re-establishes the strong DNA-histone interaction preventing access to proteins such as the transcriptional machinery. (B) Actively transcribed genes are enriched in GCN5 acetyltransferases, which transfer an acetyl group to the lysines of histones, loosening the chromatin structure, allowing access to the transcriptional machinery.

In addition to the most well-known function in gene silencing, class I HDACs are known to act directly on the replication fork, removing specific acetylation for DNA replication progression. The specific acetylation of H4K16, which is enriched in zones of onset of DNA replication, is removed by class I HDACs to restore chromatin after replication, as this acetylation prevents DNA from compacting. In mammals, the absence of HDACs 1 and 2 leads to hyperacetylation of H4K16 in nascent chromatin, causing a reduction in the speed of the replication fork and activation of the response to replication stress (Bhaskara et al. 2013). So, it is possible to speculate that TgHDAC2 might play a similar role in *T. gondii*. Our group

also observed that TgHDAC2 plays an important role in virulence, directly impacting host cell invasion (manuscript in preparation).

TgHDAC1 is expressed constitutively throughout the life and cell cycle of *T. gondii*. Besides the HDAC domain, TgHDAC1 has an Inositol polyphosphate kinase domain, with unknown function. Like TgHDAC1, TgHDAC5 also has an auxiliary domain, and it is constantly expressed during the cell cycle. Finally, TgHDAC4, a class IV HDAC, has a classic HDAC domain at the C-terminal region. Little is known about class IV HDACs in all eukaryotes since only HDAC11 from mammalian was so far included in this class and characterized.

Regarding the non-classical HDACs, TgSir2A and TgSir2B have a slight increase in expression in the G1 phase of the cell cycle and they are more expressed in bradyzoites and oocysts, indicating a function in these developmental forms (ToxoDB.org). So far, there is no information about the function of these enzymes.

Histone methylation is another well-known PTM in all eukaryotes and occurs in both lysines and arginines. Unlike other PTMs, such as acetylation, histone methylation does not change the amino acid's charge (Michalak et al. 2019). Histone methylation can have several different functions, depending on the methylated residue, but some are conserved, including activation or inhibition of transcription and DNA repair (Michalak et al. 2019). Arginine methyltransferases (PRMTs) and lysine methyltransferases (KMT) are the proteins responsible for adding this modification. PRMTs are considered conserved across species, while KMTs are more divergent (Sautel et al. 2007).

T. gondii genome encodes five PRMTs. PRMT5 was included in class II, and the others belong to class I. PRMT2 is a non-canonical methyltransferase and has little homology to human PRMTs (Yakubu et al. 2017).

PRMT1 from *T. gondii* is found mainly in the cytoplasm and it is not essential for the parasite's survival (Bissati et al. 2016). In the absence of this protein, parasites showed morphological changes during cell division and a modest decrease in virulence in mice. Bisset and collaborators (2016) also observed an increase in histone H3 monomethylation, indicating that this could be one of the targets of PRMT1.

PRMT4, on the other hand, is in the nucleus of the parasite and it is essential for the survival of the parasite (Saksouk et al. 2005). Furthermore, it is mostly associated with activation of transcription, and its inhibition led to differentiation into bradyzoites (Yakubu et al. 2017). PRMT5 is a class II methyltransferase found in the cytoplasm of tachyzoites and in the nucleus of bradyzoites, suggesting a distinctive function in those forms. Like its mammalian ortholog, this protein is associated with gene repression, as it is responsible for the arginine methylation in histone H4, a classic silencing marker (Liu et al. 2019).

Among the most well-known KMTs in *T. gondii* is TgSet8. It was believed that this protein was exclusive to metazoans. Although similar in structure and phylogeny, TgSet8 can transfer up to three methyl groups in H4K20, contributing to gene silencing, unlike its counterparts that only perform monomethylation (Sautel et al. 2007).

T. gondii has a large number of demethylases. There were seven proteins with a JmjC domain (Jumonji) and two JARID-like H3K4 and JMJD1-like H3K9 demethylases and two orthologous to JMJD6 family of proteins known for demethylating H3R2 and H4R3 (Bougdoor et al. 2010).

Another modification identified is ubiquitylation. Ubiquitylation is a key post-translational modification in lysines, where two or more ubiquitins can be incorporated

(polyubiquitination) or just one (monoubiquitination). This modification plays a role in essential cellular processes such as signaling, protein-protein interaction, transport, and directing proteins for degradation via the proteasome. This protein turnover is essential for the cell cycle's progression as it degrades poorly folded proteins, maintaining cell integrity (Monerri et al. 2015). Ubiquitylation occurs through the glycine residue present in the C-terminal portion of ubiquitin, which is covalently bound to the side chain on the target protein's lysine. The removal of the modification is mediated by deubiquitylating enzymes, which contain several families of proteins. In *Toxoplasma*, some candidate proteins were found that possibly participate in the ubiquitination/proteasome pathway (Ponts et al. 2008). Among the deubiquitination enzymes found, one of them is orthologous to the mammalian UCHL3, the first to be characterized in *Toxoplasma* (Frickel et al. 2007). Another characterized deubiquitinase is TgOTUD3A, cytoplasmic and associated with the cell cycle (Dhara and Sinai 2016).

In 2015, the ubiquitin proteome was mapped in *Toxoplasma*, where more than 500 ubiquitinated proteins were identified, with different functions and locations, such as cytoskeleton proteins and the inner membrane complex. Enrichment of this modification was observed during the cell cycle, suggesting that this modification is important for its progression (Monerri et al. 2015). In histones, ubiquitination in H2A.X is associated with DNA damage (Pan et al. 2011; Monerri et al. 2015). Lysine 24 of histone H3 appears to be an important regulatory site since the presence of different PTMs such as acetylation, methylation, and ubiquitination has been detected in this residue. This modification was also identified in another H3 residue and also in H2B.Z, showing that this modification is present in the chromatin of *T. gondii* and may have conserved functions.

It has also been seen that this modification is important for importing proteins into the apicoplast, and the enzyme that conjugates ubiquitin located in the organelle is essential for the transport of proteins into it and is essential for the parasite (Agrawal et al. 2013).

Phosphorylation is a vital modification whose balance is made by the enzyme's kinases and phosphatases. Phosphorylation is the most common post-translational modification and plays essential roles in innumerable cellular processes. The *Toxoplasma* cell cycle appears to be regulated by phosphorylation events.

In histones, phosphorylations are mainly associated with mitosis and DNA repair (Allis and Jenuwein 2016; Nardelli et al. 2013). In *Toxoplasma*, phosphorylations have been identified in serine 10 of histone H3, a classic marker of mitosis (Sautel et al. 2007). Other marks identified were H4S1 and H3T107; the latter's function still not characterized yet (Nardelli et al. 2013). In mammals, during DNA damage, an increase in phosphorylation was detected in the SQ motif of H2A.X and H2A1. In *Toxoplasma*, this motif is present in both histones, but it is only complete in H2A.X. Induced oxidative stress increases the phosphorylation in H2A.X and the transcription of both histones H2A.X and H2A1 (Dalmaso et al. 2009). After treatment with resveratrol, a drug with an unclear mechanism of action but appears to induce DNA damage in *T. gondii*, this modification was also observed. The same treatment also led to deacetylation in histones H3 and H4 (in lysine 16). H4K16ac is usually a marker associated with active transcription, DNA replication, and DNA repair by homologous recombination (Contreras et al. 2021).

Although the *T. gondii* phosphoproteome has already identified phosphorylated residues in histones, the phosphatases and kinases involved in this process have not yet been fully characterized. *T. gondii* has at least one histone phosphatase, PP1, and two histone kinases

with Snf1 domain (Dixon et al. 2010). PP1 is localized in both nuclear and cytoplasmic, but its function is still unclear (Daher et al. 2007). Experiments with phosphatase inhibitors have shown that PP1 is involved in cell invasion, and it is essential for parasite survival (Delorme et al. 2002). Among cyclin-dependent kinases, TPK2 has been shown to phosphorylate histones *in vitro* and appears to play an essential role in life cycle regulation (Khan et al. 2002).

4. THE THREE PILLARS OF EPIGENETIC REGULATION

Epigenetic regulation involves chemical or structural chromatin modifications, causing alteration in gene expression without changing the primary DNA sequence. This form of regulation comprises two other mechanisms in addition to PTMs: DNA methylation and non-coding RNAs (ncRNAs) (Allis and Jenuwein 2016).

DNA methylation and its relationship to gene expression were proposed many decades ago, and its importance in the development of several organisms has already been reported. In addition, it is involved in several vital functions, such as genomic imprinting and inactivation of the X chromosome (Jin, Li, and Robertson 2011; Chen et al. 2017). DNA methylation consists of the action of cytosine-5 DNA methyltransferase (DNMT), which covalently adds a methyl group (CH₃) to the carbon 5 in the cytosine pyrimidine ring in a CpG (cytosine-phosphate-guanine) dinucleotide. When it occurs in promoter regions, this process usually silences gene transcription since it recruits proteins that bind to DNA and chromatin remodelers, restricting access to the transcription machinery. On the other hand, methylation on gene bodies is usually associated with active transcription (Chen et al. 2017; Hamm and Costa 2011; Gissot and Kim 2008).

Initially, researchers were skeptical regarding the presence of DNA methylation in *Toxoplasma* since no methylated cytosine had ever been identified by mass spectrometry. It was only in 2017, when Wei and collaborators identified two DNMT counterparts (TgDNMTa and TgDNMTb) responsible for DNA methylation in the parasite. Using a more sensitive technique (MethylC-seq), they verified the presence of DNA methylation sites in tachyzoite and bradyzoite forms. In bradyzoite forms, both enzymes have higher levels of transcription and activity, and consequently, more DNA methylated sites were observed. These modifications are mainly involved with metabolism and resistance to the host's immune system. Methylated non-CpG sites were also identified, in CHG and CHH sites (where H can be any nucleotide, except G), related to energy metabolism (Choi, Keyes, and Horrocks 2006; Wei et al. 2017).

The second mechanism involves different classes of ncRNAs (ncRNAs- non-coding RNA), consisting of RNAs that are not translated into proteins. These RNAs are fundamental pieces in regulating the chromatin structure and gene expression at different levels, from transcription to translation (Moazed 2009; Reinhart and Bartel 2002). They can recruit chromatin remodeling enzymes or assemble ribonucleoprotein complexes that act on chromatin or histone modifications (Chen et al. 2017; Hamm and Costa 2011).

Among the different classes of non-coding RNAs, lncRNAs (long non-coding RNA) have several functions in gene regulation, including chromatin structure remodeling through histone-modifying enzymes, mRNA stabilization, splicing, and others. Small RNAs have

similar functions, mostly silencing gene expression through the RNA interference pathway. However, small interfering RNAs (siRNAs) and lncRNAs can act together through RNA scaffolds, affecting chromatin structure leading to gene silencing (Holoch and Moazed 2015).

The RNA interference pathway begins with the processing of a long double-stranded RNA by the enzyme RNase-II Dicer. The siRNAs generated guide Argonaute proteins (AGO) to form the RISC complex (RNA-induced silencing complex) that finally recognize and cleave the mRNA of interest (Hannon 2002; Meister and Tuschl 2004; Ullu, Tschudi, and Chakraborty 2004). This mechanism regulates gene expression by degrading messenger RNAs, repressing translation, modifying the chromatin structure, and protecting the genome's integrity against invasive nucleic acids, such as viruses and transposons (Braun et al. 2010).

In *Toxoplasma*, a search in the genome database identified a Dicer protein, an AGO family member, and an RdRp (RNA-dependent RNA polymerase). Based on the structural characteristics of the identified proteins, it is suggested that the *Toxoplasma* RNAi pathway is phylogenetically and functionally related to plants and fungi machineries (Atayde, Tschudi, and Ullu 2011; Ullu, Tschudi, and Chakraborty 2004).

In 2006, the only argonaut protein of *T. gondii* was characterized. TgAgo is a small protein mainly located in the cytoplasm, with a role in gene silencing. It contains the conserved PIWI domain in addition to a PAZ domain (Riyahi et al. 2006). This protein is methylated in the N-terminal region resulting in the recruitment of a Tudor SN (TSN) nuclease, which more potently silences genes silencing (Musiyenki et al. 2012).

There are three classes of small RNAs: (a) siRNAs are double-stranded and guide the degradation of mRNAs by base pairing, via the classic interference RNA pathway; (b) miRNAs (microRNAs), that seem to inhibit translation by binding in the 3' UTR region of mRNAs and (c) the small heterochromatic RNAs that are similar to siRNAs that are present in heterochromatin chromosomal regions (Braun et al. 2010).

It has already been seen that the most abundant classes of RNAs in *Toxoplasma* are miRNAs and siRNAs, which are molecules derived from repetitive sequences and similar to those of plants. Braun et al. (2010) identified that siRNAs are related to proteins necessary for gene silencing, including TgHDAC3 deacetylase, suggesting that these RNAs might be related to heterochromatin formation. It was also observed that miRNAs sediment with polyribosomes, indicating that they have a translational regulation role. The presence of TgAGO was related to both classes, reaffirming the silencing function of these non-coding RNAs (Atayde, Tschudi, and Ullu 2011; Ullu, Tschudi, and Chakraborty 2004; Braun et al. 2010).

Toxoplasma interference RNA was proved functional after being used to study genes in the glycolytic pathway in 2006 (Ananvoranich et al. 2006). It was also used to suppress adenosine kinase, which had significantly reduced mRNA levels (Yu et al. 2008). Another study efficiently inhibited the expression of the DHFR-TS gene, causing the parasite's death in vitro and in vivo, increasing the survival of mice (Azimi-resketi et al. 2020).

5. HDAC INHIBITORS

Despite the existence of antiparasitic drugs for the treatment of toxoplasmosis, these are only effective against the tachyzoite stage of the parasite, not showing activity during the

latent stage. So far, the therapy is not well tolerated by some patients, particularly those under immunosuppressive therapy or with AIDS, with severe side effects. Constant failure in the treatment of congenital toxoplasmosis by conventional therapies has been described, in addition to reports of the appearance of drug resistance (Konstantinovic et al. 2019; Dunay et al. 2018).

In this regard, HDACs show essential roles in parasite pathogenesis, controlling essential parasite pathways, regulating gene expression and cell cycle progression, become a key target for generating novel therapies that can overcome the mentioned issues (Fioravanti et al. 2020; Vanagas et al. 2012).

Histone deacetylase (HDAC) inhibitors, known as “epidrugs” (HDACi), were first developed for cancer treatment, and due to their potential for drug targets, they are now being also investigated for parasitic diseases (Fioravanti et al. 2020; Vanagas et al. 2012; Bose, Dai, and Grant 2014).

Parasites are similar to tumors since both undergo intense metabolic activity without the host's control. Besides, like tumors, parasites hide and escape from the host immune system. Among the common factors, the search for HDAC inhibitors as antiparasitic agents started after discovering the cyclic tetrapeptide apicidin, with a broad-spectrum antiparasitic activity. Advances have been made in several protozoan parasites, including *Toxoplasma gondii*, *Plasmodium falciparum*, *Schistosoma mansoni*, *Leishmania spp*, *Trypanosoma cruzi* (Andrews, Haque, and Jones 2012; Fioravanti et al. 2020).

The sensitivity to some HDAC inhibitors has been shown in *T. gondii*. However, efforts are needed to identify aspects of the HDACs that diverge between human and *T. gondii*, their molecular importance in parasite virulence/maintenance, catalysis, and regulation mechanism, as these form the basis for novel and highly specific antiparasitic drugs (Fioravanti et al. 2020).

HDACis exhibit potent anti-toxoplasma effects *in vitro* and *in vivo*, as a promising and improved therapy against *Toxoplasma gondii* (Fioravanti et al. 2020). So far, the HDACi approved by FDA are SAHA, Romidepsin, Belinostat, and Panobinostat, showing promising results for parasitic diseases such as schistosomiasis, leishmaniasis and malaria (Table 1). Since novel HDACis exhibit potent anti-toxoplasma effects *in vitro* and *in vivo*, this could lead to a new and improved therapy against *Toxoplasma gondii* (Fioravanti et al. 2020).

Table 1. FDA-approved HDACi for parasitic diseases

HDACi	Use for cancer treatment	Effect in inhibing parasites
SAHA	Cutaneous T-cell lymphoma (CTCL)	<i>P. falciparum</i> , <i>P. vivax</i> , <i>T. gondii</i>
Romidepsin	CTCL	<i>Plasmodium Knowlesi</i>
Panobinostat	Multiple mieloma e CTLC	<i>Plasmodium Knowlesi</i>
Belinostat	Peripheral T-cell lymphoma	<i>Plasmodium Knowlesi</i>

Relevant HDACis with antiparasitic activity are included in several classes, such as Cyclic tetrapeptide HDACi (eg. Apicidin and its derivatives), Short- Chain fatty Acid HDACi (Valproic acid, sodium butyrate and its derivatives), Hydroxamate HDACi (eg. TSA and

SAHA) and Class III HDACi (eg. sirtinol, surfacton, splitomicin and hyperforin) (Bose, Dai, and Grant 2014; Andrews, Haque, and Jones 2012).

HDACis target histones and non-histone proteins, acting through a broad range of interactive mechanisms, determining cell fate by either direct or indirect mechanisms. HDACis are pleiotropic factors that act through a wide variety of distinct but associated mechanisms. These mechanisms by which HDACi cause lethality include proteolysis inhibition, reversion of miRNA silencing, upregulation of apoptosis proteins, activation of the death receptor pathway, induction of the endogenous CDK inhibitor, ROS generation and induction of DNA damage, disruption of heat shock protein (Hsp 90) via acetylation, inhibition of DNA repair, interference with the S-phase checkpoint, disruption of the homologous and non-homologous end junction, interference with the proteasome function and disruption of mitotic spindle assembly (Bose, Dai, and Grant 2014).

The first HDAC inhibitor for parasites was discovered in a fungus, called Apicidin, a metabolite discovered in 1996. Apicidin presented strong activity in vitro against *P. falciparum*, *T. gondii*, and other parasites. Apicidin is included in the cyclic tetrapeptide class and generates hyper-acetylation of histones in *P. falciparum*, inhibiting deacetylation by PfHDAC1. This compound also causes substantial modifications to the transcriptional cascade of the parasite's cycle. On the other hand, this compound's lack of specificity for protozoan HDACs against the mammalian ones prevents its clinical use. Besides, the low bioavailability of apicidin is another barrier for it to become clinically suitable (Darkin-Rattray et al. 1996; Andrews, Haque, and Jones 2012).

Structural similarities between HDACs lead to the disability of apicidin to selectively inhibit *P. falciparum* and *T. brucei* compared to mammalian cells. To address this problem, apicidin analogs with indole modifications and quinolone replacements have been tested against *P. falciparum* parasites. Some quinolone derivatives enhanced specificity for *P. falciparum* vs. mammalian cells. Ever since, a range of synthetic analogs has been verified for *P. falciparum*, *Toxoplasma gondii*, *Trypanosoma* and *Leishmania* species (Andrews, Haque, and Jones 2012).

Another cyclic tetrapeptide with HDACi activity is the FR235222, recently shown to have potent activity against *T. gondii* tachyzoites. This compound caused hyper-acetylation of *T. gondii* histone H4. The absence of the TgHDAC3 gene in mutant strains of the parasite conferred resistance to the compound. Data from chromatin immunoprecipitation and microarray experiments showed the effects of FR235222 -mediated -hyper-acetylation on H4 over all 14 chromosomes of the parasite. The FR235222 treatment also induced differentiation of the tachyzoite stage parasites into the bradyzoite stage (Maubon et al. 2010; Boudour et al. 2009).

Short-chain fatty acid-type HDAC inhibitors include valproic acid, sodium butyrate, and its derivatives. The HDACis included in this class have weak inhibitory activity against the mammalian HDACs. However, these compounds have other targets than the HDAC enzymes, making it difficult to distinguish which effect is due to epigenetic modifications. Some of these compounds, like valproic acid, proved to be clinically efficient. Valproic Acid has been used as an antiepileptic drug and mood stabilizer and has been tested as an HDAC inhibitor. Valproic Acid and sodium butyrate have poor activity in vitro against *T. gondii* tachyzoites, *P. falciparum* and *S. mansoni*. Also, the compound presents low specificity for the parasites compared to human cells (Andrews, Haque, and Jones 2012).

The Hydroxamate-based HDAC inhibitors include TSA, and synthetic compounds, like the suberoylanilide hydroxamic acid (SAHA), approved in 2006 by the FDA for clinical trials for T-cell lymphoma. The hydroxamates are the most studied antiparasitic HDACi. Several studies have investigated the effect of these classes of HDACi on *Plasmodium* and *Schistosoma* growth. TSA is a potent inhibitor of in vitro growth of *P. falciparum*; however, it is not FDA approved, considering that it is lethal to human cells as it is to the parasite, being unsuitable for clinical trials. In contrast to TSA, SAHA has weaker activity against *P. falciparum* in vitro but improved specificity against the parasite. SAHA also presented ex vivo growth inhibition activity of *P. falciparum* and *P. vivax* strains obtained from infected people. Hydroxamates such as TSA, SAHA, SBHA, and scriptaid have been tested for activity against *T. gondii* parasites. All of those showed similar lethality levels against the tachyzoite, whereas SBHA presented lower levels than those. As for *P. falciparum*, TSA is less selective than the other three compounds. Curiously, low levels dosage of these compounds presented a stimulatory effect on parasite proliferation and survival in the tachyzoite stage and a suppressive effect on its infectivity (Fioravanti et al. 2020; Andrews, Haque, and Jones 2012).

Various Class III HDACi (Sir 2-inhibitors) have been tested for their lethality against *P. falciparum* and *Leishmania*, including sirtinol, surfaction, splitomicin and hyperforin. Overall, this class of HDACi presents poor parasite inhibition. Other compounds included in this class are nicotinamide and nicotinic acid which are both products in the Sir2 catalysis reaction. The first acts as an inhibitor of both acetylated peptides and NAD⁺. It was observed that in vitro administration of nicotinamide conferred a lagging growth to the parasite, whereas nicotinic acid presented no effect. So far, similar inhibitory activity levels were achieved for humans and overall SIRT protein from the parasites, indicating poor specificity (Andrews, Haque, and Jones 2012).

A series of synthetic aminophenyl hydroxamates and amino benzyl hydroxamates were screened for their activity against *T. gondii*, presenting high efficiency as anti-parasitic activity (Loeuillet et al. 2019).

In this manner, as the epigenetics play an important role in the parasitic cycle, the impact of the development of novel HDAC inhibitors in *Toxoplasma* is expected to increase, possibly improving the treatment of toxoplasmosis with lower toxicity and less side effects than the existing treatments, being an alternative especially to immunosuppressive patients (Lim and Othman 2014; Fioravanti et al. 2020).

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Chapter 7

THE BALANCE BETWEEN HOST CELL-AUTONOMOUS IMMUNITY AND *TOXOPLASMA GONDII* EVASION STRATEGIES

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ABSTRACT

Toxoplasma gondii, the causative agent of toxoplasmosis, is an obligate intracellular parasite with global distribution. The isolated strains of this parasite have a population structure with clonal and genetic diverse lineages reflecting their location and capacity to infect different warm-blooded vertebrate hosts resulting in distinct virulences. *T. gondii* actively penetrates virtually any nucleated cell and establishes an intracellular replicative niche named the parasitophorous vacuole. During host cell invasion, the parasite evades many microbicidal systems of the host cell. Some of these evasion mechanisms are based on virulence factors secreted from specialized secretory organelles, which, depending on the strain virulence and the host, are important for circumventing the host cell-autonomous immunity. *T. gondii* alters host cell signaling, evades the production of reactive oxygen species and nitric oxide, IRG and GBPs (GTPases that compose the microbicidal defense system in rodents) and autophagy. These microbicidal systems and the molecular mechanisms that regulate their evasion will be presented.

1. INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite that infects virtually any nucleated cell of warm-blooded animals, including humans (Dubey 2010). Classical genetic studies with *T. gondii* strains isolated from North America and Europe revealed that this parasite

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comprises three clonal lineages; type I, virulent strains; type II and type III vary between intermediate and avirulent strains (Darde et al. 1992; Howe and Silbey 1995). However, in South America, *T. gondii* is genetically diverse and classified as recombinant strains (Khan et al. 2007). *T. gondii* belongs to the *Apicomplexa* phylum presenting at the apical end an apical complex containing the conoid, which is part of the cytoskeleton machinery, and two specialized secretory organelles: micronemes and rhoptries (Morrisette and Sibley 2002). *T. gondii* actively penetrates the host cells (Morisaki et al. 1995) in a tightly regulated process requiring a coordinated sequential content release from three secretory organelles: micronemes, rhoptries and dense granules (Carruthers and Sibley 1997). Initially, micronemes secrete proteins with adhesive domains that bind to the host cell surface (Carruthers et al. 1999). Next, rhoptries secrete from its neck portion RON proteins that form anchor points in the plasma membrane of the host cell for the formation of the moving junction (Alexander et al. 2005; Besteiro et al. 2011; Shen and Sibley 2012). Later, rhoptry bulbous proteins (ROPs), with many described as virulence factors, are secreted. Finally, dense granules secrete the last wave of several proteins (GRA) with distinct destinations such as the lumen of the parasitophorous vacuole (PV), the intravacuolar network, the PV membrane (PVM) (Cesbron-Delauw et al. 2008) and the host cell nucleus (Hakimi et al. 2017). Although the PV is formed mainly by the host cell plasma membrane, many intramembranous proteins are excluded from the PVM, necessary to avoid the fusion of the PV with lysosomes and parasite degradation (Jones and Hirsch 1972; Mordue et al. 1999).

T. gondii infection induces a strong immune response in the host mediated by interferon-gamma (IFN- γ) that acts in the acute and chronic phase of the infection (Suzuki et al. 1988; Suzuki et al. 1989). IFN- γ regulates one of the most potent immune responses in the host, controlling the expression of hundreds of genes involved in innate and adaptive immunity (MacMicking 2012; Schneider et al. 2014). Some of these genes compose cell-autonomous immunity, such as the ones that form protein complexes as nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) (Nathan et al. 1983), inducible nitric oxide synthase (iNOS) (Stuehr et al. 1991; Alley et al. 1995), p21-47 k Da immunity-related GTPases (IRGs) (Gilly and Wall 1992) and p65-73 k Da guanylate binding proteins (GBPs) (Cheng et al. 1983; Cheng et al. 1985) and classical autophagy (Matsuzawa et al. 2012) (Table 1).

Microbicidal activation of macrophages induces higher expression of NADPH oxidase that produces reactive oxygen species (ROS) (Nathan et al. 1983). It has been shown that virulent *T. gondii* type I strains are controlled by ROS produced by macrophages from activated and stimulated peritoneal mice cavities (Murray et al. 1979), and avirulent type III strain of *T. gondii* is also controlled by ROS production of naïve macrophages (Matta et al. 2018; Kochanowsky et al. 2021) (Table 1). iNOS synthesizes nitric oxide (NO) (Stuehr et al. 2001), a microbicide molecule that controls *T. gondii* replication *in vitro* (Adams et al. 1990) and *in vivo* (Scharton-Kersten et al. 1997) (Table 1). Classical autophagy (refer in this chapter as autophagy) is a cellular process evolutionarily conserved in eukaryotes, induced by cell stress (mainly starvation) and focused on the catabolic and recycling process involving protein and organelle degradation by lysosomes (Zhang 2015; Subramani and Malhotra 2013). Autophagy is directly involved in the control of a variety of pathogens (Deretic and Levine 2018). This process is performed and regulated by more than 30 ATG gene products, including ATG8 (microtubule-associated protein 1 Light Chain 3 - LC3). However, subsets of these proteins are also involved in different cellular processes known as “autophagy-related processes” (Subramani and Malhotra 2013), also known as “noncanonic autophagy”. IRGs

and GBPs are a subfamily of proteins induced by IFN- γ and accumulate at the PVM, causing PV vesiculation and deformation, exposing and killing susceptible *T. gondii* strains (Hunter and Sibley 2012; Hakimi et al. 2017) (Table 1). Before the decoration of IRGs and GBPs to the PVM this structure needs to be loaded with LC3, a central autophagic protein (Subauste 2019; Besteiro 2019). However, upstream and degradative steps of autophagy are not involved in this process. Thus, this is a classic example of “autophagy-related process”. Nonetheless, autophagy has also been implicated in the immunity against *T. gondii* (Subauste 2019) (Table 1). It has been shown that the ligation of CD152 to CD40 expressed by host cells infected with *T. gondii* triggers the host autophagy machinery that kills *T. gondii* with a clear involvement of initiators and terminal ATG proteins and lysosomes (Besteiro 2019; Subauste 2019).

However, during the coevolutionary process with the host, many evasion strategies to subvert host innate and adaptive immunity were selected in *T. gondii* (Hakimi et al. 2017), such as host signaling modulation, evasion from ROS (Wilson et al. 1980), inhibition of NO production (Seabra et al. 2002), phosphorylation and inactivation of IRGs and GBPs (Hunter and Sibley, 2012; Hakimi et al. 2017) and evasion from autophagy (Besteiro, 2019; Subauste, 2019) (Table 2). The strategies described above used by the host to control *T. gondii* (Table 1) and the main evasion mechanisms adopted by the parasite to subvert the host cell-autonomous immunity (Table 2) will be discussed in the following topics.

Table 1. Host innate immune response against *Toxoplasma gondii*

Host innate immunity	Signaling pathway	Microbicidal mechanism against <i>T. gondii</i>	Reference
IFN- γ - JAK-STAT	STAT1	NADPH oxidase upregulation and ROS synthesis	Murray et al. (1979), Murray and Cohn (1980)
IFN- γ - JAK-STAT	STAT1	iNOS upregulation and NO synthesis	Adams et al. (1990), Hayashi et al. (1996), Scharton-Kersten et al. (1997), MacMicking (2012), Schneider et al. (2014), Khan et al. (1997)
IFN- γ - JAK-STAT	STAT1	IRGs upregulation	Martens et al. (2005), Ling et al. (2006), Hunn et al. (2008), Zhao et al. (2009a), Khaminets et al. (2010)
IFN- γ - JAK-STAT	STAT1	GBPs upregulation	Degrandi et al. (2007), Yamamoto et al. (2012), Selleck et al. (2013), Kravets et al. (2016), Matta et al. (2018)
CD40	JNK/CaM KK β /AMP K/ULK1	CD40-mediated autophagy	Reichmann et al. (2000), Andrade et al. (2006), Portillo et al. (2010), Liu et al. (2016), Besteiro (2019), Subauste (2019)

AMPK, AMP-activated protein kinase; CaMKK β , calcium/calmodulin-dependent kinase kinase β ; GBP, guanylate binding proteins; IFN- γ , interferon-gamma; iNOS, inducible nitric oxide synthase; IRG, immunity-related GTPases; JAK, Janus kinase; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; NO, nitric oxide; ROS, reactive oxygen species; STAT, signal transducer and activator of transcription; ULK1, Unc-51 like autophagy activating kinase

2. HOST-*TOXOPLASMA GONDII* COADAPTATION: AN ARSENAL OF SURVIVAL STRATEGIES

2.1. Host Signaling Pathways and Regulation of Host Gene Expression by *Toxoplasma gondii*

At the cellular level, sensing of external signals is detected by cell receptors and transmitted via signaling pathways that integrate through other signaling cascades, forming an intricate communication network that changes the cell phenotype. The Janus kinase (JAK) signal transducer and activator of transcription (STAT) signaling pathway is one of the most well studied signaling cascades, responding for more than fifty molecules such as hormones and cytokines (Shuai et al. 1992; Müller et al. 1993; Morris et al. 2018).

Interferons (IFNs) are important cytokines involved in the stimulation of cell-autonomous immunity against *T. gondii* (Cerávolo et al. 1999). After *T. gondii* detection by the immune system IFN- γ is produced (Suzuki et al. 1988), binds to its cell surface receptor and activates the JAK-STAT signaling pathway, regulating the expression of hundreds of genes involved in immunity against this parasite (MacMicking 2012; Schneider et al. 2014). STAT1 upregulates the expression of NADPH oxidase and iNOS, enzymes that catalysis the synthesis of ROS and NO, respectively, important microbicide molecules that control *T. gondii* replication (Adams et al. 1990; Schariton-Kersten et al. 1997) (Table 1). STAT1 also triggers the expression of IRGs and GBPs, effector molecules that are involved in PVM damage and consequent *T. gondii* control (Hakimi et al. 2017) (Table 1).

Although the STAT family members and other important transcription factors develop a crucial role in the immune response against *T. gondii* by regulating the cell-autonomous immunity and cytotoxicity necessary for parasite control, *T. gondii* evades from host innate immunity through virulence factors secreted during host cell invasion that regulates host gene transcription, and consequently modulates host cell activation. During host cell invasion, *T. gondii* secretes from dense granules the *T. gondii* inhibitor of STAT1-dependent transcription (TgIST), a virulence factor that translocates to the host nucleus and recruits the Mi-2 nucleosome remodeling and deacetylase (Mi-2/NuRD) complex (Table 2). This complex binds to STAT1 promoters, resulting in chromatin alteration, signal blockage and consequent repression of IFN- γ gene expression (Gay et al. 2016; Olias et al. 2016). The STAT1 translocation to the host nucleus occurs normally, however, the Mi-2/NuRD complex silences this transcription factor by its sequestration (Gay et al. 2016; Olias et al. 2016). Furthermore, this evasion mechanism does not depend on *T. gondii* virulence, as all parasite clonal lineages inhibit STAT1 transcriptional activities and block IFN- γ response (Rosowski and Saeij 2012).

The reprogramming of host cell transcriptional machinery by *T. gondii* is not restricted to STAT1, but also occurs in STAT3 and STAT6, two important host transcription factors involved in anti-inflammatory Th2 response polarization (Saeij et al. 2007; Butcher et al. 2011) (Table 2) that is differently modulated according to the *T. gondii* strain (Jensen et al. 2011; Butcher et al. 2011). *T. gondii* type I and type III have a highly active allele of ROP16 (ROP16_{I/III}), which is not shared by *T. gondii* type II, caused by a single polymorphic residue substitution in this kinase domain (Yamamoto et al. 2009). Thus, the phosphorylation of STAT3 and STAT6 mediated by ROP16 occurs differently, depending on the parasite strain. The secretion of ROP16_{I/III} results in a sustained STAT3 and STAT6 phosphorylation and

consequent polarization of host response in the Th2 profile (Jensen et al. 2011; Butcher et al. 2011), as well as downregulating IL-12 production, limiting the protective Th1 inflammatory response (Saeij et al. 2007). Furthermore, ROP16 triggers arginase-1 expression in the host in a STAT6 dependent manner that is also recognized as an important evasion mechanism to *T. gondii* (Butcher et al. 2011) (Table 2).

Table 2. *Toxoplasma gondii* effectors and their specific host target involved in subversion of host immunity

<i>Toxoplasma gondii</i> effector	Host target	Effect elicited in the host	Reference
TgIST _{I/II/III} *	STAT1 blockage	Repression of IFN- γ gene expression	Gay et al. (2016); Olias et al. (2016), Rosowski and Saeij (2012)
ROP16 _{I/III}	STAT3 phosphorylation	Downregulation of IL-12 production; induction of Th2 profile	Jensen et al. (2011), Butcher et al. (2011)
ROP16 _{I/III}	STAT6 phosphorylation	Arginase 1 induction	Butcher et al. (2011)
GRA7 _{II} /GRA14 _{II} /GRA15 _{II}	NF-kB Activation	IL-12 production <i>in vitro</i>	Rosowski et al. (2011), Ihara et al. (2020)
ROP18 _I	p65 phosphorylation	Suppression of proinflammatory cytokine expression	Du et al. (2014)
Catalase/SOD	ROS	ROS suppression	Murray and Cohn (1979), Murray et al. (1980), DaMatta et al. (2000), Guillermo and DaMatta (2004)
PS	TGF- β 1/Smad 2/Smad 3	NF-kB blockage; iNOS degradation/blockage; NO inhibition	Seabra et al. (2002), Dobbin et al. (2002), Lüder et al. (2003), Seabra et al. (2004b), Padrão Jda et al. (2014), Cabral et al. (2018), Damasceno et al. (2021)
ROP5 _I -ROP17 _I -ROP18 _I -GRA7 _I complex	IRGs proteins	IRG system suppression	Fentress et al. (2010), Fleckenstein et al. (2012), Hermanns et al. (2016)
ROP18 _I	Irgb6 and Irga6 phosphorylation	Blockage of Irgb6 recruitment to PV; Blockage of Irga6 activity	Fentress et al. (2010), Fleckenstein et al. (2012)
ROP5 _I	Prevention of Irga6 oligomerization	Blockage of Irga6 activity	Fleckenstein et al. (2012)
GRA7 _I -ROP18 _I	Irga6 phosphorylation	Blockage of Irga6 activity	Hermanns et al. (2016)
ROP5 _I -ROP18 _I	Gbp1	Blockage of Gbp1 recruitment to PV	Selleck et al. (2013)
ROP54 _I	Gbp2	Blockage of Gbp2 recruitment to PV	Kim et al. (2016)
MIC3 _{I/II} /MIC6 _{I/II}	EGFR activation	Blockage of LC3 autophagy pathway	Muniz-Feliciano et al. (2013)

* Type I, II or III strains. EGFR, epidermal growth factor receptor; NF-kB, nuclear factor kappa B; PS, phosphatidylserine; ROS, reactive oxygen species; SOD, superoxide dismutase; TGF- β 1, transforming growth factor- β 1; TgIST, *T. gondii* inhibitor of STAT1-dependent transcription

The modulation of host gene transcription by *T. gondii* virulence factors is not restricted to the STAT family members, and the role of ROPs and GRAs in nuclear factor kappa B (NF- κ B) modulation has been reported (Du et al. 2014). The NF- κ B family of transcription factors is composed by five members in mammals: p65 (RelA), RelB, c-Rel, p105/p50 (NF- κ B1) and p100/52 (NF- κ B2) (Nabel and Verma 1993). In resting cells, NF- κ B is associated with I κ B and stays in an inactive form in the cytoplasm. After stimuli recognition by specific cell receptors of growth hormone, cytokines, or pathogens molecules, such as bacteria lipopolysaccharide (LPS), NF- κ B is activated by phosphorylation of two serine residues in the N-terminal region of the I κ B molecules, leading to polyubiquitination of I κ B and consequent proteasome degradation (Ghosh and Karin 2002). After I κ B degradation, NF- κ B is released and translocate to the cell nucleus, controlling the transcription of target genes involved in inflammation, apoptosis, cytokines and immunoregulation (Ahn and Aggarwal 2005). The major role of NF- κ B in immunity against *T. gondii* infection has been described (Caamaño et al. 1999; Caamaño et al. 2000; Mason et al. 2004). RelB-deficient C57BL/6 mice challenged with *T. gondii* type I (RH) strain succumb 10-15 days after infection, caused by a low level of IFN- γ production and a reduced NK cell activity compared with WT mice (Caamaño et al. 1999). Furthermore, NF- κ B2 CBA/CaJ knockout (KO) mice infected with *T. gondii* type II (ME49) strain have a reduced capacity to respond to IFN- γ and enhanced expression of the pro-apoptotic Fas molecule by CD4⁺ and CD8⁺ T cells, causing increased levels of apoptosis and consequent deficiency in the long-term immunity against *T. gondii* (Caamaño et al. 2000). Taken together, these results show the main role of NF- κ B in immunity against *T. gondii* in the chronic phase of infection (Caamaño et al. 2000). However, the modulation of the NF- κ B pathway by *T. gondii* depends on the strain (Butcher et al. 2001; Shapira et al. 2002; Molestina et al. 2003), and the role of parasite virulence factors from different genetic backgrounds in this modulation has been reported (Rosowski et al. 2011; Du et al. 2014) (Table 2). Secretion of GRA15 by *T. gondii* type II strain is responsible for a high level of NF- κ B activation and nuclear translocation (Rosowski et al. 2011). Furthermore, GRA15 from *T. gondii* type I and type III strains do not activate nuclear translocation of NF- κ B (Rosowski et al. 2011) (Table 2). It was showed that ROP18 from *T. gondii* type I strain phosphorylates p65 (RelA) targeting this protein for degradation in a ubiquitin-dependent way, inducing termination of NF- κ B pathway and consequent suppression of proinflammatory cytokine expression, favoring parasite survival in murine and human immune system (Du et al. 2014). Recent reports describe the partial involvement of GRA7 and GRA14 and the essential role of GRA15 from the *T. gondii* type II strain in the activation of NF- κ B (Ihara et al. 2020) (Table 2). Furthermore, deficiency in GRA7, GRA14, and GRA15 resulted in the downregulation of the transcription of genes regulated by NF- κ B (Ihara et al. 2020) (Table 2).

2.2. Reactive Oxygen and Nitrogen Species Partially Control *Toxoplasma gondii*

ROS production by phagocytes, known as “oxidative burst”, is recognized as one of the first microbicidal mechanisms of activated leukocytes to be understood (Dupré-Crochet et al. 2013; Thomas 2017). NADPH oxidase is a protein complex in the plasma membrane and

phagocytic vacuole responsible for ROS production. NADPH oxidase (Nox) is composed of different isoforms (Nox1, Nox2, Nox3, Nox4, Nox5, and the dual oxidases Duox1 and Duox2) with distinct functions. NADPH oxidase produces O_2^- that dismutates into H_2O_2 that is converted, enzymatically or not, into $HO^\bullet$ and $HOCl$, all those radicals are microbicidal to most microorganisms and classified as ROS (Dupré-Crochet et al. 2013).

With the discovery of the “oxidative burst” as part of the cell-autonomous immunity of leukocytes, model protozoans started to be tested. The killing of *T. gondii* by ROS produced by distinct host cells was demonstrated (Table 1). At this time, known microbicidal agents (NO, IRGs, GBPs) and processes (autophagy) were not described yet in activated macrophages. This multilayer of microbicidal mechanisms as we know it today complicates the study of how *T. gondii* is controlled by distinct host cells from the plethora of intermediate hosts of this parasite. However, ROS was implicated in the killing of *T. gondii* by impairing ROS production with the use of ROS scavengers and KO of essential genes that form NADPH oxidase in host cells (Table 1).

Macrophages from mice peritoneal cavities that receive different inoculums killed *T. gondii* with distinct microbicidal capacity efficiency, which was associated with higher ROS production (Murray et al. 1979) (Table 1). This was further explored using mice peritoneal macrophages with the same distinct stimulus treated with condition medium from spleen cells stimulated with *T. gondii* antigen, BCG or Con A. The present lymphokines in the spleen cells condition medium (mainly IFN- γ) further induced H_2O_2 production of macrophages that were associated with increased killing of *T. gondii* (Murray and Cohn 1980). Although scavengers of ROS could restore *T. gondii* growth in activated macrophages, some contradictory results were found using macrophages activated with lymphokine. The addition of catalase and superoxide dismutase (SOD) (both are enzymatic ROS scavengers) before and after *T. gondii* challenge did not reduce significantly the microbicidal capacity of activated macrophages, clearly indicating an oxygen-independent microbicidal mechanism against this parasite (Sibley et al. 1985).

IFN- γ activated dendritic cells are also able to control *T. gondii* growth by ROS production not involving NO production. This was demonstrated by using scavengers of ROS that could partially or entirely restore replication of intracellular *T. gondii* in DCs activated by IFN- γ (Aline et al. 2002). Using *in vivo* infections of mice, transfer of infected macrophages to mice and flow cytometry analysis, it was shown that ROS is important for *T. gondii* control *in vivo*. However, the parasite can also down-modulate the production of ROS after infection. It was concluded that the balance between parasite killing and inhibition of ROS production by infected macrophages is important to keep the host alive controlling the parasite, as well as to maintain the parasite in the host (Shrestha et al. 2006).

ROS was also implicated in *T. gondii* control by macrophages after ATP treatment (Correia et al. 2010). Extracellular nucleotide (as ATP) is present in inflamed tissues after being released by necrotic and apoptotic cells, being part of the tissue response and hemostasis feedback (Coutinho-Silva and Savio 2021). Not surprisingly, extracellular ATP is involved in the control of this parasite. Naïve macrophages infected with *T. gondii* treated with ATP presented fewer parasites, higher ROS production, no NO production and lysosomal fusion with the PV containing parasites (Correia et al. 2010). The participation of ATP in *T. gondii* control has been related to human toxoplasmosis and with the inflammasome (Jamieson et al. 2010; Coutinho-Silva and Savio, 2021) and experimentally

tested in mice. Using KO mice for P2X7 receptor (recognize ATP) it was found that *T. gondii* infection caused higher mice death, liver damage and cyst numbers, lower inflammatory infiltrate, proinflammatory response and ROS production (Moreira-Souza et al. 2019). Thus, there is a clear proinflammatory response caused by the release of nucleotide by the lytic cycle that is necessary for the immune response and control of the parasite.

It has been demonstrated that *T. gondii*-mediated ROS production induces the secretion of macrophage migration inhibitory factor (MIF) important for the control of the parasite (Kim et al. 2017). MIF^{-/-} macrophages were susceptible to *T. gondii* growth *in vitro*. In addition, Nox4^{-/-} macrophages, but not Nox2^{-/-}, did not control parasite growth with concomitant impairment of MIF expression. Finally, mice deficient in Nox4 were susceptible to *T. gondii* type I strain (RH) and presented more brain cysts when infected with type II strain (ME49). Thus, it seems that *T. gondii* infection induces ROS production by Nox-4, leading to the increase of MIF expression and controls the parasite (Kim et al. 2017).

ROS production by naïve macrophages has also been implicated in the direct control of avirulent *T. gondii* type III strain (CTG) (Matta et al. 2018). Naïve macrophages cleared this avirulent parasite strain by ROS production. However, the levels are not very high, as detected by zymosan particles used as control. This was further demonstrated by the partial reversibility of *T. gondii* control when Nox1 and Nox2 were KO from macrophages, showing that NADPH oxidase is the possible origin of the ROS produced, which is essential for parasite control. KO mice for Nox1 and Nox2 were also more susceptible than wild-type (WT) mice for the infection with this strain. Pyroptosis, a type of cell death linked to inflammasome activation and production of ROS, was not implicated in the process, but GPBs were (Matta et al. 2018).

Recent studies confirmed the role of ROS in *T. gondii* type III strain control (Kochanowsky et al. 2021). Human foreskin fibroblasts (HFF) infected with ROP16 KO parasites from *T. gondii* type III strain (CEP, aka CTG) exhibits and increase in ROS accumulation, but not NO or Gbp5, showing that ROP16 from type III parasites develops a crucial role in ROS suppression. Interestingly, the major role of ROP16 from type III parasites in ROS suppression seems to be dependent on the host cell type. In IC-21 cells, a mouse macrophage-like cell line, ROP16 KO CEP strain exhibits ROS accumulation, when compared to WT parasites. In contrast, the THP1, human monocyte-like cell line, infected with ROP16 KO CEP do not exhibits ROS accumulation, indicating that the mechanism of ROS suppression may vary between host and cells types. As previously discussed, ROP16 activates STAT6 that is crucial for parasite growth and survival (Butcher et al. 2011). Deletion of STAT6 from both HFF and murine ear tip fibroblasts infected with *T. gondii* CEP strain cause and accumulation of ROS, resulting in defect in parasite growth and survival, showing the major role of STAT6 in parasite fitness and ROS for the control of type III *T. gondii* (Kochanowsky et al. 2021).

Although ROS is involved in the control of *T. gondii* growth, as soon as ROS was examined as a microbicidal agent against this parasite, evasion mechanisms were described. In a cell-free system, *T. gondii* can resist 100 fold more ROS than *Trypanosoma cruzi*, and this was correlated to the catalase (Murray and Cohn 1979) and SOD (Murray et al. 1980) content the parasite expresses. The antioxidative system of the *T. gondii* was further examined and catalase and SOD activity was similar between strains showing that this system is not relevant to explain virulence diversity (Sibley et al. 1986). Catalase KO *T. gondii* was created, showing that this enzyme is important for resistance to ROS and mice virulence

(Ding et al. 2004). Probably because of this robust antioxidative system, this parasite infects mice peritoneal macrophages without inducing a respiratory burst at the contact sites of the cells plasma membranes (Wilson et al. 1980) as also demonstrated for chicken macrophages derived from blood monocytes (DaMatta et al. 2000) and the HD11 chicken macrophage-like cell line (Guillermo and DaMatta 2004) (Table 2). However, as described above, ROS is involved in the direct control of *T. gondii* and may be relevant as a signaling molecule for immune response as demonstrated for MIF production (Kim et al. 2017).

Recently, it was found that the *T. gondii* initiation factor 2 kinase-B (TgIF2H-B) gene is importante for the parasite to sense oxidative stress, summing up on how the parasite respond to ROS production (Augusto et al. 2021). The *T. gondii* type II strain (ME49) KO for TgIF2H-B could replicate faster in HFF suggesting this gene may regulate replication of the parasite. Induction of oxidative stress resulted in lower levels of IF2 phosphorylation, indicating that TgIF2H-B sense ROS. KO parasites presented higher ROS and less catalase in response to the oxidative stress. These KO were also able to better survive in LPS activated J774.1 macrophage-like cell line activated with LPS. The KO parasites demonstrated less capacity to convert to tissue cysts and became more virulent in mice. Taken together this gene sense oxidative stress, caused by the host microbicidal response, slowing the replicative capacity of the parasite and converting to bradyzoites for better stress adaptation (Augusto et al. 2021).

We have also demonstrated that a new dinuclear iron (III) compound control *T. gondii* development after infection of host cells (Portes et al. 2015). *T. gondii* type I strain (RH) infecting LLC-MK2, an epithelial-like cell line, could be controlled by the compound presenting an IC₅₀ of 3.6 µM at 48 h of treatment. Treated parasites presented morphological and ultrastructural changes, signs of apoptosis-like cell death, autophagy, and conversion to bradyzoites. Treated cells presented higher intracellular oxidative stress and parasites treated with the compound showed less catalase and SOD activity. Apparently the compound reduces the oxidative capacity of the parasite against ROS (Portes et al. 2015).

Nitric oxide (NO) synthases (NOS) is an enzyme that uses arginine as substrate, producing NO and citrulline. NOS presents different isoforms, with inducible NOS (iNOS) being induced by proinflammatory signals, especially IFN-γ, in several cell types (MacMicking et al. 1997). Microbicidal activation of macrophages induces the expression of iNOS and subsequent NO production, being then one of the principal mechanism responsible for the high microbicidal activity of these cells. NO production was actually discovered in macrophages treated with bacterial LPS (Stuehr and Marletta 1985) and was related to its cytotoxicity capacity against cancer cells (Hibbs et al. 1987). NO was later related against intracellular protozoans within the cell-autonomous immunity of macrophages (De Groote and Fang 1995).

In an *in vitro* setup, NO produced by activated peritoneal mice macrophages control *T. gondii* as demonstrated in different situations after infection with type I strain (RH) (Adams et al. 1990; Nibbering et al. 1991; Langermans et al. 1992), with type I strain (RH) persisting with a slight reduction in these cells, and type II (ME49), III (VEG) and recombinant (P-Br) strains being completely controlled (Damasceno-Sá et al. 2021) (Table 1). LPS activated HD11 chicken macrophage (Guillermo and DaMatta, 2004) and ostriches macrophages derived from blood monocytes activated with LPS and chicken IFN-γ (Miranda et al. 2016) also control *T. gondii* type I strain (RH) but does not eliminate it. Mice studies using

aminoguanidine (Hayashi et al. 1996) or mice KO for iNOS (Scharton-Kersten et al. 1997; Khan et al. 1997) (Table 1) showed that NO is important for the chronic phase, but not for the acute phase of infection. Exacerbated NO production after oral infection is a key agent inducing tissue necrosis that kills the host (Liesenfeld et al. 1999; Khan et al. 1997). Therefore, the immune response should be balanced to avoid excessive NO production, but some NO production is apparently necessary to control the parasite. This is further supported by the fact that NO is necessary for the conversion of tachyzoites into bradyzoites (Bohne et al. 1994). Furthermore, NO is important for the reduction of the brain cysts of *T. gondii* as seen in KO mice for iNOS (Sa et al. 2018).

While studying if NO production by chicken macrophages derived from blood monocytes was involved in *T. cruzi* and *T. gondii* growth restriction, we ended up using mice peritoneal macrophages activated with IFN- γ and LPS as a positive control for NO production. After infection of these control cells with *T. gondii*, we noticed that NO production was reduced in comparison to noninfected activated macrophages (DaMatta et al. 2000, second paragraph of the “Result and discussion” section). It was described at the time that *T. gondii* could evade lysosomal fusion (Jones and Hirsch 1972), and the oxidative burst (Wilson et al. 1980). However, it was quite established that NO controls the parasite. Thus, NO production inhibition of macrophages after the parasite infection was first interpreted by us as an awkward result. Nevertheless, this inhibition was further characterized using activated mice macrophages (peritoneal and blood monocyte-derived) and we showed that *T. gondii* infection could clearly inhibit NO production with higher inhibition when a higher parasite/macrophage ratio was used (Seabra et al. 2002). By varying the parasite/macrophage ratio, we could demonstrate that the NO production inhibition was not due to macrophage destruction, as low ratios did induce the inhibition without altering the number of macrophages by lysis. Indeed, NO inhibition was only detected if the parasite was alive. In addition, infected peritoneal macrophages were iNOS negative (Seabra et al. 2002). The down-modulation of iNOS and inhibition of NO production by *T. gondii* infection were further demonstrated at the same period by other groups (Dobbin et al. 2002; Lüder et al. 2003) (Table 2).

The NO production inhibition was further studied in the two available chicken macrophage-like cell lines MQ-NCSU and HD11 (Guillermo and DaMatta 2004). Both cell lines after LPS activation and *T. gondii* infection produced less NO. LPS activation of HD11 could control *T. gondii* growth by NO production as NG-methyl-L-arginine revert the killing capacity. ROS was not produced at the contact site of parasite and HD11 (Guillermo and DaMatta 2004) as demonstrated for mice macrophages (Wilson et al. 1980) and chicken macrophages derived from blood (DaMatta et al. 2000). In addition, iNOS was revealed in HD11 by detecting its NADPH diaphorase activity as there is no chicken iNOS antibody available. It was found that infection reduces this activity, suggesting that the infection also diminishes the expression of chicken iNOS (Guillermo and DaMatta 2004). Furthermore, we adapted a methodology to culture ostrich macrophages derived from blood monocytes and activated these cells with chicken IFN- γ and LPS leading to high NO production (Miranda et al. 2016). *T. gondii* could grow in unactivated ostrich macrophages, but activation controlled the RH *T. gondii* strain. However, the parasites were not eliminated and could persist in these activated cells (Miranda et al. 2016). Collectively, *T. gondii* can apparently inhibit NO

production of macrophages from mammals and avian hosts showing that the evasion mechanism that avoids NO is probably present in most of its hosts.

To further understand the mechanism involved in NO production inhibition, we first analyzed if *T. gondii* infection could alter the activity of arginine decarboxylase and ornithine decarboxylase in infected mice peritoneal macrophages (Seabra et al. 2004a). These enzymes are part of the polyamine synthesis that uses arginine, the same substrate iNOS uses to produce NO. The infection could induce a higher synthesis of polyamine as it is important for cell growth, consuming the iNOS substrate explaining lower NO production. However, we could not detect any modulation of these enzymes after parasite infection (Seabra et al. 2004a). The parasite grew less in macrophages treated with methyl ornithine (ornithine decarboxylase inhibitor), indicating that polyamines are needed for parasite growth. In addition, tachyzoites of *T. gondii* also show a high affinity for putrescine. Taken together, it seems that the amount of polyamines in macrophages is sufficient for *T. gondii* growth and that the inhibition of NO production does not involve depletion of the iNOS substrate (Seabra et al. 2004a). We further probed into the inhibition of NO production and verified if the transforming growth factor- β 1 (TGF- β 1) was involved. TGF- β 1 is a macrophage modulator that reduces NO production (Ding et al. 1990). We also examined if TGF- β 1 was being released in infected macrophages by phosphatidylserine (PS) exposure of tachyzoites, as this phenomenon was demonstrated for *Leishmania amazonensis* (de Freitas Balanco et al. 2001), and is involved in many different cell interaction situations, known as the apoptotic mimicry concept (Wanderley et al. 2020). We showed that tachyzoites of *T. gondii* expose PS and that the blocking of PS does revert the inhibition of NO production of activated macrophages (Seabra et al. 2004b). The use of an anti-TGF- β 1 neutralizing antibody could also revert the inhibition of NO production and the reduction in iNOS expression. The involvement of this factor was further demonstrated in infected macrophages by the higher expression of Smad 2 and Smad 3, intracellular mediators of TGF- β 1 signaling. In addition, NF- κ B was also removed from the nucleus of activated infected macrophages in a TGF- β 1 dependent manner. Taken together, PS exposed by *T. gondii* induces the release of TGF- β 1 after infection of activated macrophages, reducing iNOS expression, leading to lower NO production and also removing NF- κ B from the macrophage nucleus (Seabra et al. 2004b) (Table 2). We further probed in the exposure of PS by *T. gondii* by isolating the two PS subpopulations of this parasite. The PS⁺ subpopulation exposes PS on the outer membrane, while the PS⁻ subpopulation does not. The analysis of the vacuoles formed after the invasion of macrophages by these subpopulations reveals that the PS⁺ tachyzoites are localized in narrow vacuoles, indicating active penetration. In contrast, the tachyzoites of the PS⁻ subpopulation are retained in large vacuoles, indicative of phagocytosis (Morisaki et al. 1995; Dos Santos et al. 2011). The PS⁺ subpopulation of *T. gondii* is the only one capable of actively penetrating non-phagocytic host cells and inhibiting NO production after infection of activated macrophages. However, *in vivo* infection with the total population (PS^{+/-}) promotes longer survival in mice than infection with isolated subpopulations only. Infection with PS⁺ tachyzoites triggers high parasitemia identified in tissue samples from animals. In contrast, PS⁻ tachyzoites induce an exacerbated inflammatory process. In both cases, the viability of the mice is compromised. It was suggested that both subpopulations are important for maintaining a balance between inflammation and parasite growth (Dos Santos et al. 2011) (Table 2).

We then determined what pathway was being activated for iNOS degradation after *T. gondii* infection of macrophages. By using pharmacological inhibitors we demonstrated in J774-A1 mice macrophage-like cell line that the iNOS was not being degraded by the calpain or the lysosomal pathway, but by the proteasome pathway (Padrão Jda et al. 2014). We also examined if virulence factors secreted by rhoptries and dense granules were involved in the inhibition of NO production (Cabral et al. 2018). The ROP5-17-18 complex, secreted by rhoptries, is involved in the evasion mechanism of IRGs and TgIST, secreted by dense granules, recruits a repressive complex of STAT1 promoters that blocks IFN- γ transcription (Hakimi et al. 2017). In addition, KO parasites for ASP5 and MYR1 (proteins important for the exportation of secreted parasite proteins to the host cells) were also used. None of the deletion mutants of *T. gondii* could revert the inhibition of NO production after parasite infection. However, iNOS degradation varies between the two different mice macrophage-like cell lines (J774-A1 and RAW 264.7) used in this study. As demonstrated before, parasite infection inhibited NO production in both cell lines, but iNOS degradation was detected only in J774-A1, not in RAW 264.7 macrophages. Thus, *T. gondii* does not employ the described virulence factors to inhibit NO production of activated macrophages and the mechanism used to inhibit NO production (iNOS degradation or not) may vary depending on the macrophage lineage (Cabral et al. 2018) (Table 2).

Other host cells do produce NO after activation with IFN- γ or LPS and IFN- γ (MacMicking et al. 1997) and *T. gondii* infection reduces this production. In mice microglia, *T. gondii* infection could inhibit NO production (Rozenfeld et al. 2005). This inhibition correlated to lower iNOS expression in infected cells and was related to TGF- β 1 as described above for peritoneal macrophages (Seabra et al. 2004b). In addition the NO inhibition cause by the parasite infection allowed neurite growth when neurons were cocultured with infected microglia. The finding was related to neuropreservation during CNS infection by *T. gondii* (Rozenfeld et al. 2005). IEC-6 and rat epithelial-like cell line can also produce NO after IFN- γ activation and *T. gondii* infection cause NO production reduction (not published). These results suggest that the capacity to inhibit NO production may be extended to several cell types.

To further examine the capacity of *T. gondii* to inhibit NO production, we study the infection of activated macrophages by four strains of this parasite (Damasceno-Sá et al. 2021). We used strains of different genetics and virulence to mice: RH, type I, is the most virulent; ME49, type II; VEG, type III; and P-Br, recombinant, have lower virulence. All strains were capable of inhibiting NO production after 24 h of infection of peritoneal mice macrophages. However, the inhibition rate was different, with RH capable of inhibiting more (66% to uninfected control) followed by VEG (54%), P-Br (36%) and ME49 (33%). This changed after 48h of infection as macrophages that interacted with ME49 and P-Br produced more NO, and only VEG and RH could inhibit NO production at 31% and 19% compared to uninfected control. Only RH could persist in activated macrophages, the others were eliminated, with VEG being the most resistant of the three strains. iNOS expression followed a similar pattern, being degraded by all strains after 2h of infection, but only the RH strain sustained the low iNOS expression. All strains could induce TGF- β 1 signaling, as seen by the nuclear translocation of phosphorylated Smad-2, at the beginning of the infection, but only the RH strain maintained this signaling during the infection period analyzed. We finally examined the decoration by LC3 to the PVM after infection, and again, only the RH strain

avoided this docking (Damasceno-Sá et al. 2021). Collectively, the evasion from the NO microbicidal system may be a general evasive mechanism of all *T. gondii* strains, and only the virulent RH was capable of avoiding LC3 loading to the PVM, which suggests that this capacity may be relevant for the virulence of this strain in mice (Table 2).

NO is one of the earliest and most widespread signaling molecules in living organisms (Colasanti et al. 2010). It has also been found in many distinct organisms as a part of the cell-autonomous immunity of macrophages (De Groote and Fang 1995). NO production as a microbicidal system may be present in vertebrates since their origin (Colasanti et al. 2010). This molecule not only functions as a microbicidal agent directly killing parasites, but also modulates the immune response by suppressing spleen cells, which may be necessary for host survival (Candolfi et al. 1994). Therefore, the selective pressure to cope with this system not only to evade direct killing, but also to promote host survival, may elucidate why all strains of *T. gondii* develop an evasion system against NO production explaining why all strains, tested so far, against different host cells that produce NO, can evade this microbicidal system.

2.3. Interferon-Inducible GTPases and the Major Role of Parasite Secreted Virulence Factors

The IFN family of cytokines drives one of the most potent and complex host defense network by stimulating hundreds of IFN-stimulated genes (ISG) (MacMicking 2012; Schneider et al. 2014) and among them are the IRGs (Gilly and Wall, 1992), GBPs (Cheng et al. 1983; Cheng et al. 1985), 72-82 kDa Myxoma resistance proteins, and 200-285 kDa very large inducible GTPases (MacMicking 2004; Kim et al. 2012). We will concentrate on IRGs and GBPs as part of the cell-autonomous immunity of host cells important for the control of *T. gondii*.

Similar to IRGs, GBPs are expressed at the basal level in cells and organs (Mostafavi et al. 2016), but are potentially induced in response to IFN- γ and IFN- α/β (Cheng et al. 1983; Decker et al. 1989). Thus, following activation by proinflammatory cytokines, both IRGs and GBPs may control bacteria, protozoa and viruses growth (Taylor et al. 2004; MacMicking 2012; Martens et al. 2005; Gao et al. 2011). The main role of IRGs (Martens et al. 2005; Ling et al. 2006; Hunn et al. 2008; Zhao et al. 2009a; Khaminets et al. 2010) and GBPs (Degrandi et al. 2007; Yamamoto et al. 2012; Kravets et al. 2012; Degrandi et al. 2013; Selleck et al. 2013; Johnston et al. 2016; Kravets et al. 2016; Matta et al. 2018) against *T. gondii* has been extensively reported (Table 1).

The IRGs Irgm2 (GTPI), Irgm3 (IGTP), Irgd (IRG-47), Irga6 (IIGP1), and Irgb6 (TGTP) accumulates at the PV of astrocytes, activated with IFN- γ , after 2h of infection with *T. gondii* type II strain (ME49); this recruitment is GTP-dependent and parasite must be alive (Martens et al. 2005). IRGs induce PV vesiculation, deformation, consequent disruption of the PV and parasite killing. Astrocytes deficient in the examined IRGs were less efficient in *T. gondii* killing, showing that these proteins control parasites (Martens et al. 2005). Results from Ling et al. (2006) revealed the connection between IRGs and autophagic or autophagic-related processes in the control of *T. gondii* type II strain (PTG) *in vivo* and *in vitro* with lysosomal fusion to the PV. WT mice and peritoneal macrophages activated with IFN- γ are able to rapidly eliminate *T. gondii*, differently from Irgm3 KO mice and macrophages, indicating the

crucial role of Irgm3 in parasite control (Ling et al. 2006). After 4h of infection, a higher number of parasites colocalize with lysosome-associated membrane protein 1 (LAMP-1) in WT cells than in Irgm3 KO cells. Texas red-labeled lysosomes colocalized with parasites in WT Irgm3 cells. Inhibition of lysosomes with bafilomycin A1 and autophagy with PI3K inhibitors lead to reduced *T. gondii* elimination involving the autophagy pathway in the killing process. *T. gondii* elimination also involved PVM indentation, vesiculation and disruption, followed by plasma membrane stripping and autophagosome formation around the parasite (Ling et al. 2006).

The concept of regulatory and effector IRGs acting synergistically to mediate an anti-*T. gondii* activity was introduced by Hunn et al. (2008). This led to the GMS and GKS protein motif IRGs classification. The motif difference is the presence of methionine (M-GMS) or lysine (K-GKS) in the catalytic G-domain at the N-terminal region of the protein. Substitution of the lysine (K) residue by methionine (M) changes the biological function: GMS regulates and GKS effects (Hunn et al. 2008). The IRGs are in an active state in the GTP-bound form and are inactivated by their GTPase activity that converts GTP to GDP. By using L929 fibroblasts transiently transfected with expression constructs for distinct IRGs it was found that the GMS proteins family (Irgm1, Irgm2 and Irgm3) regulates the GKS proteins by maintaining GDP bound to the GKS proteins. When GMS proteins are not present GKS proteins bind to GTP and precipitate as aggregates in the cytoplasm. In WT cells, IFN- γ induces the expression of both families of proteins with GMS interacting with GKS proteins at the endoplasmic reticulum maintaining their inactivity by GDP binding. Upon infection with *T. gondii* type II strain (ME49), GKS diffuse to the PVM, binds to GTP, accumulates at the PVM inducing its vesiculation and disruption, releasing the parasite into the cytosol that ends up dead (Hunn et al. 2008). The role of IRGs in *T. gondii* control was further deepened (Zhao et al. 2009a). Fluorescent Irga6 accumulated on the PVM of *T. gondii* type II strain (ME49) in mouse embryonic fibroblasts (MEFs) activated with IFN- γ . Time-lapse photography identified accumulation of Irga6 on PVM, parasite rounding up followed by disruption of PVM and permeabilization of *T. gondii* itself, indicating parasite death within 20-40 minutes after rupture. Infection of MEFs with a dominant negative mutant form of Irga6 exhibits a strong reduction in colocalization of Irga6 with PVM and parasite death supporting the main role of Irga6 in parasite control. Autophagy was not detected in parasite clearance, although some rare cells presented a few LC3-positive vacuoles, but PV containing *T. gondii* did not fuse with lysosomes. After PVM rupture, the exposed *T. gondii* ME49 strain and the host cell died by necrosis (Zhao et al. 2009a). IRGs control of *T. gondii* was further examined (Khaminets et al. 2010). In MEFs infected with *T. gondii* type II strain (ME49) the PVM loading with IRGs occurs extremely rapid, a few PVs were loaded after 2.5 min of infection. IRG loading was in the following hierarchy order: Irgb6 > Irgb10 > Irga6 > Irgm2 > Irgd. Despite both Irga6 and Irgb6 load to the PVM, Irgb6 was found to load in a much higher proportion. Furthermore, the autophagic regulator ATG5 is crucial to the correct loading of all GKS tested onto PVM, since atg5-deficient MEFs have a mislocalization and aggregation of Irga6, Irgb6 and Irgd (Khaminets et al. 2010).

As previously discussed, IFN- γ is a crucial proinflammatory cytokine that stimulates innate immune cells, culminating in the expression of a robust expression of effectors molecules such as GBPs (Shenoy et al. 2007). After stimulation by proinflammatory cytokines and TLR ligands of the murine macrophage-like cell line, ANA-1, mRNA transcripts were analyzed and 10 GBPs were extensively characterized (Degrandi et al. 2007).

Studies in C57BL/6 mice infected with *T. gondii* ME49 strain revealed an increase in mRNA expression patterns of Gbp1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 post-infection. Gbp1, 2, 3, and 5 shows as a vesicle-like structure in the cytoplasm of MEFs after IFN- γ stimulation. Furthermore, for the first time, it was observed an accumulation of Gbp1, 2, 3, 9 around the PV in MEFs, GBP6 and 7 around the PV of mice RAW264.7 macrophages, after IFN- γ stimulation of these cells. The accumulation around the PV of GBPs strongly suggests an anti-*T. gondii* activity similar to IRGs (Degrandi et al. 2007). Using gene targeting methods, the major role of GBPs in *T. gondii* control was reported *in vivo* and *in vitro* (Yamamoto et al. 2012). The mice chromosome 3 (Chr³) contains a cluster of six Gbp genes (Gbp1, Gbp2, Gbp3, Gbp5, Gbp7, Gbp2ps) that is involved in *T. gondii* control. Mice deficient in Gbp2ps alone or deficient in both Gbp2ps and Gbp5 infected with *T. gondii* ME49 strain shown similar survival rates as WT mice, showing that these GBPs are not involved in *T. gondii* control. However, deletion of Gbp^{chr3} causes an enhanced susceptibility of mice to *T. gondii* infection, increasing parasite burden in spleens and lymph nodes compared to WT mice. Furthermore, deletion of Gbp^{chr3} does not alter concentrations of IL-12 and IFN- γ production measured in mice sera, exhibiting similar levels as WT mice, suggesting that mice susceptibility to *T. gondii* infection is not due to deficiency in production of these proinflammatory cytokines. Susceptibility to *T. gondii* infection was observed *in vitro*, since the infection rate was higher in mice peritoneal macrophages deficient in Gbp^{chr3} (Yamamoto et al. 2012). Previous studies also revealed that GBPs interact and colocalize with Irgb6 and *T. gondii* (Virreira Winter et al. 2011), and similar results were also confirmed in WT macrophages. In addition, Irgb6 recruitment to PV was impaired in Gbp^{chr3} deficient macrophages as well as the reduction in the number of Irgb6-positive parasites interrelating GBPs with this IRG for *T. gondii* control (Yamamoto et al. 2012).

The role of Gbp1 recruitment to PVM as necessary for the control of *T. gondii* avirulent type III strain (CTG) has also been reported (Selleck et al. 2013). As previously discussed, IRGs loading to the PVM depends on ATG5 (Khaminets et al. 2010). ATG5 is also involved in the correct localization of Gbp1 in the PV of IFN- γ activated MEFs (Selleck et al. 2013). ATG5 deficient MEFs exhibit an accumulation of Gbp1 as large aggregates that colocalize with p62 and ubiquitin. After infection with CTG parasite, Gbp1 and Irgb6 were not recruited to PVM of ATG5 deficient macrophages preventing clearance of parasites by IFN- γ activation. Indeed, Gbp1 deficient macrophages from C57BL/6 mice are not able to clear susceptible parasites, and Gbp1 deficient mice are more susceptible to *T. gondii* type I strain in relation to WT mice (Selleck et al. 2013). It has been demonstrated that after IFN- γ stimulation Gbp2 is expressed in different primary cells of mice and that this protein is involved in *T. gondii* clearance *in vivo* and *in vitro* (Degrandi et al. 2013). Mice deficient in Gbp2 and infected with *T. gondii* ME49 display a higher amount of cysts in the brain and higher mortality than in WT mice, suggesting the major role of Gbp2 in parasite control *in vivo* (Degrandi et al. 2013). Furthermore, it was found that Gbp2 accumulates at the PVM 10 min after infection and that deficient macrophages, astrocytes and MEFs display a higher number of parasites than WT cells. In addition, Gbp2 accumulation depends on its c-terminal domain as seen by truncated mutants. Overall it can be concluded that Gbp2 is involved in the control of *T. gondii* ME49 replication both *in vivo* and *in vitro* (Degrandi et al. 2013).

Elegant studies using multiparameter fluorescence image spectroscopy and live cell microscopy revealed the crucial molecular interactions between different murine GBPs and deepened the multimerization understanding of Gbp2 crucial for *T. gondii* killing (Kravets et

al. 2016). The nucleotide binding and membrane anchoring are crucial for multimerization of mouse Gbp2, which displays structural transitions between monomeric, dimeric and oligomeric states in vesicle-like structures (Kravets et al. 2016). MEFs containing Gbp2 deficient in isoprenylation and GTPase activity fail to control *T. gondii* ME49 strain replication, caused by a failure in Gbp2 multimerization (Kravets et al. 2016). Furthermore, Gbp2 forms supramolecular complexes that decorate the PVM by interacting directly with Gbp1 and Gbp3. Gbp5 and Gbp6 are also recruited to PVM, although do not interact directly with Gbp2. These Gbps attack directly the parasite membrane after rupturing the PVM, being the first work to infer how these proteins kill the released parasite in the cytosol (Kravets et al. 2016). Interesting findings revealed that Gbp5 restricts *T. gondii* type III strain (CTG) of in naïve macrophages, independent of IFN- γ activation (Matta et al. 2018). CTG parasite clearance is mediated by enhanced NADPH oxidase activity as noted above, but also by Gbp5 induction in the infected cell. Indeed, NADPH oxidase KO mice display enhanced susceptibility to *T. gondii* infection when compared to WT mice. Furthermore, *in vitro* susceptibility to *T. gondii* infection was also observed in Gbp5 KO macrophages establishing that naïve macrophages can control avirulent type III parasites by a dual microbicidal system (Matta et al. 2018).

It was recently showed that Gbp7 is also involved in the killing of *T. gondii* ME49 strain in mice and MEF and macrophages (Steffens et al. 2020). KO mice were generated for this GBP that was highly susceptible for the parasite infection dying at the acute phase of the infection similar to mice KO for IFN- γ receptor. Parasites could also grow in MEF and macrophages activated with IFN- γ from Gbp7 KO mice. It was also demonstrated that Gbp7 localizes to the PVM of the parasite and colocalizes with Gbp2, better describing how these GBPs attach to the PV and end up direct killing the parasite after ligation to the plasma membrane of *T. gondii* (Steffens et al. 2020).

Recent studies revealed the major role of Irgm2 in the recruitment of effectors IRGs and GBPs proteins in *T. gondii* killing (Pradipta et al. 2021). Irgm2 deficient macrophages or dendritic cells (both bone marrow derived) from C57BL/6 KO mice and MEFs display a reduction in the capacity to control replication of *T. gondii* type II strain (Pru), mainly caused by a reduction in Irgb6 and Gbp1 recruitment to PVM. Furthermore, Irgm2 recruitment to parasite PVM seems to be independent of various ATG proteins, such as ATG3, ATG7, ATG9 or ATG14 and also of IRGs and GBPs proteins, such as Irgb6 or Gbp1 as deficient cells lacking these proteins exhibits a normal recruitment of Irgm2 to *T. gondii* PVM. Thus, Irgm2 is crucial to control the parasite by recruiting Irgb6 and Gbp1 to parasite PVM (Pradipta et al. 2021).

The aforementioned studies highlight the role of IRGs and GBPs as crucial components of the cell-autonomous immunity against *T. gondii*, but they only restrict the growth of less virulent *T. gondii* strain. Virulent strains evade IRGs and GBPs mainly through a series of polymorphic rhoptyr kinases and pseudokinases that are differently expressed in clonal genotypes of *T. gondii* (Hunter and Sibley, 2012; Hakimi et al. 2017). Seminal studies using genetic mapping of the F1 progeny clones obtained through genetic crosses in cats between *T. gondii* clonal strains with different virulences resulted in the recognition of target locus that resulted in genes responsible for the distinct virulence phenotypes (Hunter and Sibley 2012; Behnke et al. 2016). The secretory protein encoding a serine-threonine kinase, ROP18, was found, followed by a polymorphic pseudokinase, ROP5, and another relevant kinase, ROP17.

These ROPs form a complex that in virulent strains result in the most effective combined ROPs polymorphism against IRGs (Hakimi et al. 2017) (Table 2).

Virulent *T. gondii* type I strains have virulence factors (mainly ROPs) that alter the traffic and assembly of the IRG proteins onto the PV, allowing the persistence of virulent parasites in *in vivo* primed macrophages (Zhao et al. 2009b). PV from virulent *T. gondii* type I strain (RH) are decorated with Irga6, but not with Irgb6, while avirulent *T. gondii* type III strain (CTG) are readily decorated by both proteins, indicating that evasion from IRGs by type I parasite is not mediated by the exclusion of the whole IRGs components, but from specific IRGs proteins. Furthermore, transgenic expression of ROP18 from type I virulent parasite in the avirulent CTG parasite did not confer resistance to IRGs in these macrophages, suggesting that another polymorphic kinase could be acting synergistically with ROP18 to avoid parasite elimination by IRGs (Zhao et al. 2009b). Further work revealed the main role of ROP18 in parasite expansion in mice (Fentress et al. 2010). *T. gondii* type I strain (GT-1) exhibits a rapid expansion in the peritoneal cavity of mice, while *T. gondii* type III strain (CTG) exhibits only a background level of expansion, and transgenic CTG strain expressing active type I ROP18 behave similarly to GT-1. Type I ROP18 is also important for parasite survival *in vitro* as cultured inflammatory monocytes clear CTG parasites but do not kill CTG parasites that have the active type I ROP18. Virulent GT-1 ROP18 phosphorylates Irgb6 on a crucial conserved GTPase motif avoiding its loading onto the PV allowing the persistence of the parasite in inflammatory macrophages explaining its survival success (Fentress et al. 2010).

The involvement of the pseudokinase ROP5 in IRGs evasion was also reported (Fleckenstein et al. 2012). *In vitro* studies revealed that ROP5 directly interacts with Irga6 and is also required for the correct phosphorylation of IRG target proteins by ROP18. Furthermore, ROP5 also directly inhibits Irga6 activity by preventing its oligomerization. ROP5 works as a co-factor for ROP18 enhancing its capacity to phosphorylate Irga6 and impairing the microbicidal function of IRGs (Fleckenstein et al. 2012). Further analysis identified that ROP17 is a kinase that acts synergistically with ROP18 to phosphorylate IRG preventing its loading to the PVM preserving this niche harboring *T. gondii* type I strain (RH) (Etheridge et al. 2014). ROP17 and ROP5 are secreted in vacuoles to the external face of the PVM, forming a native protein complex that contributes to IRG evasion. ROP17 KO parasites are significantly more susceptible to IFN- γ activated macrophages compared to WT parasites. This susceptibility was markedly higher in ROP17/ROP18 double KO parasites, indicating that both virulence factors contribute to evasion from the IRG system. Similar to ROP18, ROP17 phosphorylates the conserved motif of IRGs proteins. This demonstrates the complexity of the mechanism used by *T. gondii* type I strains to evade IRGs in activated host cells (Etheridge et al. 2014).

GRA7 was also identified as an important virulence factor that protects *T. gondii* type I strain (RH) from IRG loading to the PVM (Hermanns et al. 2016). GRA7 associates with PVM and directly binds to ROP5, forming an additional component to the ROP5/ROP17/ROP18 complex, although does not contribute to the formation of the complex itself. GRA7 is required for Irga6 phosphorylation by ROP18, as GRA7 KO parasites display a reduction in Irga6 phosphorylation and consequent inability to prevent IRG accumulation in MEFs activated by IFN- γ . All that indicates that GRA7 contributes to Irga6 phosphorylation by the ROPs complex (Hermanns et al. 2016).

Recent advances in the *T. gondii* proteome field lead to the identification of GRA60, a novel virulence factor well conserved in *Neospora* and *Hammondia* that is involved in

subversion of host IRG system (Nyonda et al. 2021). *In vivo* analysis revealed that C57BL/6 mice infected with GRA60 KO *T. gondii* type I strain (RH) display a higher survival rate when compared to C57BL/6 mice infected with WT parasites. Furthermore, C57BL/6 mice also exhibit a reduction in cysts burden in the chronic phase of infection when infected with GRA60 KO parasites from type II strain (ME49), when compared to mice infected with WT parasites. Thus, GRA60 is a crucial virulence factor for *T. gondii* in both acute and chronic phase of infection. GRA60 also develops a major role in *T. gondii* survival by inhibiting parasite loading with IRG proteins. IRGs proteins cooperates and hierarchically accumulates on PVM of susceptible *T. gondii* strains leading to PV rupture and consequent parasite death (Hunn et al. 2008; Khaminets et al. 2010). GRA60 is described as a new component of virulence factors complex that interacts with ROP18 as found by co-immunoprecipitation assay. In association with ROP18, GRA60 neutralize both Irga6 and Irgb10 protecting virulent *T. gondii* from host IRG system machinery by a molecular mechanism that is still being clarified (Nyonda et al. 2021).

As for IRGs, GBPs are also targeted by serine-threonine kinases from *T. gondii* type I strains, contributing to parasite survival. The kinase activity of ROP18 of *T. gondii* type I strain (GT-1) is crucial to prevent Gbp1 recruitment to vacuoles in macrophages derived from bone marrow, as ROP18 KO parasites fail to prevent Gbp1 accumulation on the PVM (Selleck et al. 2013). Furthermore, ROP5 KO parasites also fail to control the accumulation of Gbp1 in the parasite PV, showing that both ROP18 and ROP5 are necessary to prevent Gbp1 accumulation onto PV (Selleck et al. 2013). *T. gondii* type III strain (CTG) exhibits a markedly higher number of PV loaded by Gbp1, confirming that only type I strains of *T. gondii* are able to prevent the immunity promoted by Gbp1 (Selleck et al. 2013).

The pseudokinase ROP54 was identified as a virulence factor preventing parasites clearance by Gbp2 (Kim et al. 2016). ROP54 is secreted to the host cell cytoplasm during invasion and loads to the PVM. ROP54 do not bind to the ROP5/ROP17/ROP18 complex, suggesting that ROP54 acts independently. Furthermore, knocking out ROP54 from *T. gondii* type I strain (RH) impacts growth and virulence in both *in vitro* and *in vivo* assays. ROP54 KO *T. gondii* type II strain (Pru) displayed normal growth *in vitro*, but had decreased parasite virulence *in vivo*. Knocking out this pseudokinase causes an enhanced Gbp2 loading onto the PVM (Kim et al. 2016).

Altogether, the studies presented above clearly show that virulence of *T. gondii* strains has to do with the capacity to avoid microbicidal systems based on parasite proteins that are injected into the host cells during infection. These evasion mechanisms (Table 2) highlight our comprehension of the major role of cell-autonomous immunity response mediated by IRGs and GBPs against the intracellular parasite *T. gondii*, and the major parasite virulence factors that are responsible for its evasion, guaranteeing the parasite survival.

2.4. Autophagy and the Major Role of Epidermal Growth Factor Receptor in Blockage of Autophagy-Mediated *Toxoplasma gondii* Degradation

Macroautophagy is one of the most studied types of autophagy, and relies on the formation of a double membrane, the autophagosome (Zhang 2015; Subramani and Malhotra 2013), whereby portions of cytosol are encircled by this structure and, subsequently, fuse with

lysosomes forming the autolysosome leading to the degradation of the cargo (Zhang, 2015; Subramani and Malhotra 2013). Autophagy is a highly regulated process dependent on the molecular machinery of autophagy related proteins (ATG) (Subramani and Malhotra 2013). However, members of the ATG machinery can also participate in many biological processes independent of autophagosome formation and lysosomes degradation, such as cell survival and apoptosis (Yousefi et al. 2006), cell signaling (Pankiv et al. 2010), protein secretion (Duran et al. 2010) and others processes. In those cases, the process is known as “non-canonical autophagy pathway” or “autophagy-related process” (Subramani and Malhotra 2013; Galluzzi et al. 2017). The participation of ATG proteins in autophagy is critical to the whole autophagic process (Tsukada and Ohsumi 1993; Mizushima et al. 2010) that is negatively regulated by the target of rapamycin (TOR) (Díaz-Troya et al. 2008) and positively regulated by AMP-activated protein kinase (AMPK) (Mack et al. 2012). After activation of autophagy by an external signal, AMPK phosphorylates ULK1 (Unc-51 like autophagy activating kinase), an upstream kinase that induces autophagy (Itakura and Mizushima 2010) through the formation of the ULK1 complex in membranes expressing ATG9 (Papinski et al. 2014). ULK1 recruits Beclin1 and phosphatidylinositol 3-kinase (PI3K), forming a complex in the autophagosome membrane (Itakura and Mizushima 2010). The recruitment of this complex to the forming autophagosome membrane causes the production of phosphatidylinositol 3-phosphate by PI3K, acting as a scaffold protein for remodeling ATG proteins recruited by the Beclin1-PI3K complex (Liang et al. 1999; Nascimbeni et al. 2017). The ATG proteins recruited to the forming autophagosome consists of two ubiquitin-like conjugation systems, ATG12-ATG5-ATG16 complex and LC3. The conjugation of ATG12 to ATG5 is mediated by ATG7 and ATG10, and after ATG12-ATG5 conjugation, ATG16 non-covalently binds to ATG12-ATG5, forming the ATG12-ATG5-ATG16 complex (Mizushima et al. 1998; Hwang et al. 2012). In the second ubiquitin-like conjugation system, E1 activation enzyme ATG7 activates ATG8 or LC3 and is further transferred to ATG3 where phosphatidylethanolamine binds to ATG8, forming the LC3-II and is incorporated into the autophagosome membrane (Biering et al. 2017). Finally, the fusion of lysosomes to autophagosomes occurs, resulting in cargo degradation (Klionsky and Emr 2000; Yoshimori 2004; Galluzzi et al. 2017).

Autophagy has been described as a cellular mechanism employed by the host immunity that captures and degrades invading pathogens, including *T. gondii* (Deretic et al. 2013; Shibutani et al. 2015). We will concentrate on the work that showed that *T. gondii* clearance by the host immune response can be initiated by CD40-mediated autophagy (Besteiro 2019; Subauste 2019). *T. gondii* infection may induce host immune system mediating parasite clearance through CD40 activation (Reichmann et al. 2000; Andrade et al. 2006; Portillo et al. 2010; Van Grol et al. 2013; Ogolla et al. 2013; Liu et al. 2016) (Table 1). CD40 is an important member from TNF receptor family and is expressed in many cell types such as nonhematopoietic cells and antigen presenting cells, developing a broad variety of immune responses (Grewal and Flavell 1998). CD4 T cells produce IFN- γ and express at its cell surface CD154, also known as the CD40 ligand - CD40L) inducing potent activation of macrophages enhancing their microbicidal status against *T. gondii* (Subauste 2019). Classical reports from Subauste groups demonstrated that CD40-CD40L is critical to IL-12/IFN- γ production from immune cells mediating the response against *T. gondii* (Subauste 2019). Patients with hyper IgM (HIGM) syndrome, a disease caused by a deficiency in CD40L

expression, exhibit defect in humoral and T cell-mediated immunity, leading to an increase in the susceptibility of these patients to *T. gondii* infection (Subauste, 2019).

Upregulation of CD40 expression in accessory cells was observed in local sites of infection and lymphoid tissues after *T. gondii* infection in C57BL/6 mice (Reichmann et al. 2000). CD40 KO mice infected with *T. gondii* produce a lower level of IL-12 and a similar level of IFN- γ when compared to WT mice, causing the death of the mice by toxoplasmic encephalitis. Although the IFN- γ production is similar in both CD40 KO and WT mice, the lower production in IL-12 by KO mice can explain the enhanced susceptibility to *T. gondii*. It was reported that C57BL/6 macrophages infected with *T. gondii* and stimulated with IFN- γ or CD40L alone are unable to control parasite replication. However, when synergistically stimulated with IFN- γ and CD40L, macrophages were able to reduce the number of the parasite. That way, CD40 is essential to control parasite replication when combined with proinflammatory cytokines, resulting in macrophage activation and the consequent production of effector molecules that play a crucial role in *T. gondii* control (Reichmann et al. 2000).

The relation of CD40 anti-*T. gondii* activity and host autophagic machinery was reported (Andrade et al. 2006). CD40 stimulation of unprimed macrophages from humans and mice infected with *T. gondii* type I strain (RH) caused fusion with endosomes and lysosomes. These findings were confirmed by colocalization of classical markers of phagolysosomal fusion such as LAMP-1, CD63 and cathepsin D with parasite-containing vacuoles. Furthermore, colocalization of parasite-containing vacuoles with LAMP-1 and consequent lysosomal fusion in mouse peritoneal macrophages is triggered by activated CD4⁺ T cells, but not by resting CD4⁺ T cells. This result was confirmed by incubation of CD4⁺ T cells with antibodies against CD154, and consequent fusion inhibition of parasite-containing vacuoles with lysosomes. Genetic approaches also confirmed the crucial role of autophagy as an anti-*T. gondii* mechanism in RAW 264.7 cells deficient in PI3K, a crucial regulator of autophagosome formation. PI3K knockdown inhibits the parasite-containing vacuoles fusion with lysosomes induced by CD40 stimulation in RAW 264.7 cells. The confirmation of autophagy mediating the vacuole containing parasites fusion with lysosomes came with targeting LC3, a specific marker of autophagosomes. Transfection of CD40 activated RAW 264.7 cells with a fluorescent LC3 revealed colocalization of LC3 around PV. However, in unstimulated CD40 RAW 264.7 cells LC3 does not colocalize with PV, confirming the crucial role of CD40 in triggering host autophagy machinery to control *T. gondii* infection (Andrade et al. 2006).

The role of CD40 induction of the host autophagy machinery was reported in microglia cells (Portillo et al. 2010). CD40 stimulation induces recruitment of LC3 around *T. gondii*, caused by upregulation of the autophagy protein Beclin 1. The upregulation of Beclin 1 was dependent on the downregulation of p21 levels, a molecule known to induce Beclin 1 degradation. Furthermore, silencing of Beclin 1 abolish the CD-40 induced activity against *T. gondii* in microglia. *In vivo* analysis also showed an enhanced susceptibility of Beclin 1 KO mice infected with *T. gondii* that display a reduced survival rate and higher parasite loads in the brain and eye, when compared to control mice (Portillo et al. 2010). Similar results were obtained with nonhematopoietic cells (human microvascular brain endothelial cells, human umbilical vein endothelial cells, human and mouse retinal pigment epithelial cells, mouse endothelial-like cell line) treated with CD40 (Van Grol et al. 2013). The activation of these cells with CD40 induced *T. gondii* killing, with involvement of Beclin 1, ATG7 and LC3 loading onto the PVM and fusion with lysosomes. These results demonstrate that autophagy

is being activated by CD40 in nonhematopoietic cells, allowing the parasite control (Van Grol et al. 2013).

Finally, CD40 also triggers Protein kinase R (PKR) activation, a third signaling pathway that regulates the host autophagy machinery activation against *T. gondii* (Ogolla et al. 2013). PKR is a serine-threonine kinase that is ubiquitously expressed and localizes in the cytoplasm of mammals cells (Clemens 1997). Activation of PKR results in phosphorylation and activation of the eukaryotic initiation factor 2 (eIF-2 α), a well characterized pathway that develops an anti-viral protective immunity by inhibiting viral protein translation (Nakayama et al. 2010). The main role of PKR/eIF-2 α mediating an anti-*T. gondii* activity has also been reported (Ogolla et al. 2013). Stimulation of CD40 in microglia cells induces recruitment of the adaptor proteins TRAF6 and TRAF2, mediating PKR phosphorylation that in turn phosphorylates and activates eIF-2 α (Ogolla et al. 2013). This signaling cascade induces autophagy activation, with LC3 accumulation in the vacuole containing *T. gondii* and consequent fusion with the lysosomes, mediating parasite killing (Ogolla et al. 2013). *In vivo* analysis also confirmed the main role of CD40 induction in host immunity against *T. gondii*. PKR KO mice are more susceptible to ocular and cerebral infection with *T. gondii* when compared to WT mice, exhibiting a significant increase in focal inflammation in the brain and eyes, and a general increased mortality confirming the crucial role of PKR in host immunity by triggering autophagy (Ogolla et al. 2013).

A report described the signaling pathway leading to autophagy triggered by CD40 activation in macrophages (Liu et al. 2016). The identification of two major upstream regulators, calcium/calmodulin-dependent kinase kinase β (CaMKK β) and TNF- α , revealed how CD40 triggers autophagy in *T. gondii* infected RAW 264.7 cells. CD40 activation triggers the phosphorylation of AMPK by CaMKK β , and consequent phosphorylation of ULK1 by AMPK inducing autophagy. ULK1 knockdown inhibited the increase in the number of autophagosomes and autolysosomes after CD40 stimulation with CD154, confirming the major role of ULK1 in autophagy. Furthermore, ULK1 also phosphorylates and inhibits mTORC1, a classical inhibitor of CD40 (Gwinn et al. 2006). CD40 also stimulates autophagy through autocrine TNF- α production with consequent JAK phosphorylation that in turn phosphorylates Bcl-2 that detach from Beclin 1. Incubation of RAW 264.7 cells with the JAK inhibitor SP600125 reduced JAK phosphorylation after incubation with CD154 and consequently impaired the increase in the number of LC3+ structures similar to autophagosomes and autolysosomes. Furthermore, neutralization of TNF- α with anti-TNF- α antibody also impaired the JAK/STAT pathway in RAW 264.7 cells stimulated with CD154. These results confirm the major role of JAK/STAT and TNF- α in autophagy triggering after CD40 ligation (Liu et al. 2016). Activation of CD40 in RAW 264.7 cells reduced the percentage of infected cells and the number of parasites per 100 macrophages, caused by accumulation and encasement of LC3 in *T. gondii* and accessory proteins from the autophagy machinery such as ATG5, ATG7, Beclin 1 and lysosomal enzymes (Andrade et al. 2006; Portillo et al. 2010). Taken together, these results show that JNK, CaMKK β , AMPK, and ULK1 are essential molecules for CD40 induction of autophagy to kill *T. gondii* by the increase of LC3 expression and consequent fusion of vacuoles containing parasites with the lysosomal system (Liu et al. 2016).

Subversion of host immunity is crucial for *T. gondii* survival and evasion from host autophagy induced by CD40 has been reported (Muniz-Feliciano et al. 2013; Corcino et al. 2019) (Table 2). It was revealed that *T. gondii* type I (RH) and type II (ME49) strains block

the autophagy pathway in the host through activation of epidermal growth factor receptor (EGFR) in endothelial cells, retinal pigment epithelial cells and microglia (Muniz-Feliciano et al. 2013). EGFR activation in endothelial cells is mediated by MIC proteins of the parasite that contain EGF domains, such as MIC3 and MIC6, promoting EGFR phosphorylation and activation. After EGFR activation by MIC3 and MIC6, EGFR activates PI3K that in turn activates AKT, preventing LC3 accumulation and parasite degradation through autophagy. This result was confirmed by blocking AKT activation with inhibitor IV culminating in LC3 accumulation around *T. gondii*, lysosome fusion to the PV and consequent parasite killing through autophagy, confirming the role of EGFR activation in evasion of the host autophagy-mediated parasite killing (Muniz-Feliciano et al. 2013). Recent advances also revealed the role of EGFR in the *T. gondii* infection of the brain and retina (Corcino et al. 2019). Mice expressing a dominant negative EGFR (no functional signaling) on endothelial cells present an overall lower parasite load including the brain and retina. The endothelial cells of these transgenic mice after infection presented accumulation of LC3 to the PV and parasite killing dependent on ULK1 and lysosomal enzymes. The use of the 3-MA, autophagy inhibitor, in these mice resulted in lower CNS infection. These results demonstrate the importance of EGFR evasion for *T. gondii* survival (Corcino et al. 2019). To evade the CD40 autophagy pathway, *T. gondii* triggers different signaling cascades during host cell infection culminating in parasite evasion from autophagy proteins targets and consequent degradation through lysosomal enzymes (Muniz-Feliciano et al. 2013; Corcino et al. 2019).

As described, *T. gondii* can evade the mentioned microbicidal systems by employing oxidative enzymes like catalase and SOD (ROS), possibly PS exposure (NO), a variety of proteins from the secretory organelles as ROPs and GRAs (IRGs, GPBs) and subversion of the signaling pathway halting autophagy triggered by CD40 (Table 2). This multilayer of microbicidal systems varies between host cells and tissue specificities and also between the distinct host organisms that *T. gondii* infects. This exuberant diverse capacity, with so many evasion mechanisms, is possibly explained by the selective pressure that this parasite has been faced during its life history in distinct hosts. The study of these evasion mechanisms allows the description of some of these microbicidal systems, exemplifying how important it is to study this fascinating parasite.

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Chapter 8

PURINERGIC SIGNALING IN THE HOST RESISTANCE TO *TOXOPLASMA GONDII* INFECTION

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ABSTRACT

Purinergic signaling was first characterized as the one mechanisms responsible for neurotransmission in non-noradrenergic, non-cholinergic nerves. Since then, two families of purinergic receptors were cloned in most tissue and organs: the P1 family (receptors for adenosine) and the P2 family (receptors for purine or pyrimidine nucleotides). Purinergic receptor activation is involved in intracellular signaling cascades that trigger cellular events such as calcium, sodium, and potassium currents, transcription factor activation, induction of motility, cell growth, cell death, production of inflammatory mediators, and microbicidal activity. The contribution of purinergic signaling to the parasite-host interaction was investigated during infection by pathogens such as bacteria, viruses, and protozoa. In the last 10 years, the activation of purinergic signaling has been proposed as an essential component of the immune response to infection by *Toxoplasma gondii* in both cellular and systemic contexts of the infection. In this chapter, we discuss the participation of P2 receptors in the control of *Toxoplasma gondii* infection in hematopoietic and non-hematopoietic cells (*in vitro*), in human toxoplasmosis, and in murine models of experimental toxoplasmosis (*in vivo*).

1. INTRODUCTION

Purinergic signaling was proposed by Geoffrey Burnstock, who observed cellular responses to neurotransmitters such as adenosine triphosphate (ATP) in non-noradrenergic,

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non-cholinergic nerves (Burnstock et al., 1997). At this point, extracellular nucleotides were thought to be co-stimulatory molecules secreted in synapses that participated in cell membrane polarization and signal transmission through central nerve cells (Burnstock 2012). Additional functions for extracellular nucleotides were discovered subsequently, and soon after, specific receptors (named ‘purinergic receptors’) were described as mediators of cell responses (Knight 2009).

According to the activating ligand, and after cloning by molecular structure, purinergic receptors are sensitive to a range of extracellular nucleotides and nucleosides and were initially classified as P1 and P2 receptors. P1 receptors are activated by adenosine molecules, while P2 receptors are divided into two subfamilies (P2Y and P2X) according to their molecular structure, functions, and intracellular cascade. The P2Y receptors are metabotropic receptors activated by purine or pyrimidine molecules such as uridine triphosphate, diphosphate, or monophosphate (UTP, UDP, and UMP, respectively). By contrast, P2X receptors are ionotropic transmembrane receptors that respond to a range of ATP concentrations (Ralevic and Burnstock 1998).

1.1. P2 Receptors Contribute to Immune Responses and Host Protection

P2 receptors are highly expressed in immune system cells and are involved in modulating many functions such as the initiation of inflammation, fibrosis, angiogenesis, and resolution of inflammation (Cekic and Linden 2016). The most intensively investigated P2 receptors in the context of intracellular infection are P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2X₄, and P2X₇ receptors, all of which promote inflammation and mediate pathogen killing by immune system cells (Idzko, Ferrari, and Eltzschig 2014; Pacheco et al., 2018).

The P2Y₂, P2Y₄, and P2Y₆ receptors are metabotropic receptors (mainly Gq protein-coupled) involved in various cellular events. During activation of P2Y receptors, the transport of the subunit contained in protein Gq occurs, activating phospholipase C, then hydrolyzing phosphatidylinositol-4,5-bisphosphate, forming inositol 1,4,5-triphosphate (IP₃) and the diacylglycerol lipid. The binding of IP₃ to receptors in the endoplasmic reticulum causes the release of calcium (Ca²⁺) from this organelle (Abbracchio and Burnstock 1998; Martelli et al., 2005; Hortua Triana et al., 2018). P2Y receptors might mediate all effects of Ca²⁺ signaling as a second messenger to allow the participation of pyrimidinergic receptors in diverse cellular functions, including growth, differentiation, death, and migration (Zhang and Hu 2020). The contribution of P2Y receptors in the immune response against microorganisms was first reported in *Leishmania amazonensis* infection, where UTP promoted the generation of reactive oxygen species (ROS) and other inflammatory mediators such as caspase-1 and interleukin one-beta (IL-1 β) (Marques-da-Silva et al., 2018; Thorstenberg et al., 2020).

The P2X₇ receptor is the only member of the P2X subfamily that presents a longer intracellular C-terminal in its protein structure (Kopp et al., 2019). The extended P2X₇ C-terminal is associated with various domains that allow the P2X₇ receptor to participate in several intracellular cascades (Miranda Costa-Junior, Sarmiento Vieira, and Coutinho-Silva 2011). Depending on cell type, the activation of the P2X₇ receptor by ATP in low concentrations (micromolar) opens a divalent cation-selective channel. In contrast, the activation with high ATP concentration (millimolar) opens a pore permeable to molecules up to 900 kDa (Volonte et al., 2012). P2X₇ receptor activation induces Ca²⁺ influx, activation of

NADPH oxidase inducing ROS, potassium efflux, phospholipase D activation that contributes to phagolysosomal fusion, nitric oxide production, apoptosis, and gene expression of pro-inflammatory cytokines (IL-1 β , interleukin 6 [IL-6], interleukin 12 [IL-12], and tumor necrosis factor alpha [TNF- α]). Also, the activation of P2X7 is involved in the activation of the NLRP3 inflammasome, a multiprotein complex composed of an NLR receptor 3 receptor, ASC adaptor, and the caspase-1 enzyme that processes immature IL-1 β into its mature form to be secreted (Wiley et al., 2011; Savio et al., 2018). The P2X7 receptor participates in immune responses to *Mycobacterium tuberculosis*, *Chlamydia trachomatis*, *Porphyromonas gingivalis*, Dengue virus, and *Leishmania amazonensis*, among microorganisms, promoting innate cellular immunity that combats the pathogens' strategies to avoid host defenses (Miller et al., 2011; Coutinho-Silva and Savio 2021).

1.2. The Contribution of P2Y Receptors in the Cellular Host Defense Against *T. gondii*

Ca²⁺ signaling plays an essential role in regulating cellular functions in *T. gondii*. During infection, *T. gondii* needs Ca²⁺ mobilization to initiate invasion into the host cell, establish the parasitophorous vacuole, and manage its egress from the host cell. During the active invasion, activation of the parasite's Ca²⁺ pathways occurs inducing the secretion of micronemes from the apical pole of the parasite (Carruthers, Giddings, and Sibley 1999). After the invasion, there is the formation of a non-fusogenic parasitophorous vacuole that inhibits the fusion of the endocytic and exocytic pathways of the host cell, thereby escaping from lysosome degradation. The interference with this signaling compromises the main event involved in the successful intracellular parasite life cycle (Billker, Lourido, and Sibley 2009).

Some studies demonstrated that treatment with Ca²⁺ ionophore in monolayers of infected human foreskin fibroblasts leads to the release of intracellular Ca²⁺ stocks and extracellular Ca²⁺ uptake, leading to increased permeability of the host cell membrane. The increase in Ca²⁺ mobilization leads to interference with the parasite's motility, inducing its rapid exit from the host cell (Black, Arrizabalaga, and Boothroyd 2000; Caldas, de Souza, and Attias 2007).

Moreira-Souza et al., (2015) found that, during infection with *T. gondii* in murine peritoneal macrophages, activation of P2Y receptors by UTP and UDP nucleotides controlled the infection in murine macrophages (Figure 1). This effect was mediated by an increase of intracellular Ca²⁺ concentration released from storage in the host cell. Ca²⁺ modulation is mediated by the activation of P2Y₂, P2Y₄, and P2Y₆ receptors and induces the parasite to leave the host cell early after infection (Moreira-Souza et al., 2015).

During each stage of the lytic cycle, parasites use Ca²⁺ signaling to mediate invasion, vacuole formation, and egress from the host cell. The imbalance of Ca²⁺ concentration affects the most important cellular processes of the parasite, its motility, and changes the secretion of rhoptries and micronemes that participate in the development of the parasite active host cell invasion (Black, Arrizabalaga, and Boothroyd 2000; Caldas, de Souza, and Attias 2010; Lourido and Moreno 2015). After P2Y₂, P2Y₄ and, P2Y₆ receptor activation parasites lose the ability to infect another host cell actively and form non-fusogenic parasitophorous vacuoles captured and eliminated by phage-lysosomal fusion in the endosomal pathway.

After activation of P2Y₂, P2Y₄, and P2Y₆ receptors, parasites that left the host cell have a lower capacity to perform active infection (Moreira-Souza et al., 2015). Because Ca²⁺ signalization is responsible for regulating gliding, microneme secretion, invasion, and motility, the *T. gondii* parasites could not invade a new host. In other words, activation of P2Y receptors by nucleotides favored phagocytosis and destruction of the parasite by the endosomal route, including lysosome fusion with parasitophorous vacuoles (Figure 1. A). This interference in the parasite cycle mediated by the activation of P2Y receptors prevented the *T. gondii* replicative cycle's development without causing significant changes in the host cell.

2. THE P2X7 RECEPTOR CONTRIBUTION DURING *T. GONDII* INFECTION

2.1. The Contribution of P2X7 Receptor in the Cellular Immune Response Against *T. gondii*

The first discovery regarding the microbicidal action of purinergic signaling in the context of *Toxoplasma gondii* infection appeared in 2010. It was shown that the activation of the P2X7 receptor, through extracellular ATP (eATP) treatment in infected murine macrophages, was killed parasites in parasitophorous vacuoles, inhibiting the modulation of the host cell by *Toxoplasma* (Figure 1. A) (Corrêa et al., 2010). One of the escape mechanisms used by *T. gondii* is the prevention of lysosome fusion through the expression of molecules on the surface of the parasitophorous vacuole (PV), including lysosome-associated membrane proteins, mannose receptors 6-PO₄, transferrin receptors, and other molecules that make up the primary phagosome, abolishing lysosomal fusion to the PV that is consequently not acidified (Sibley 2003). By contrast, activation of the P2X7 receptor, as reviewed by Miller (2011), increases the concentration of intracellular Ca²⁺ by inducing the activation of a protein known to be a member of the Ras homologous A family, which activates phospholipase D. This protein participates in lysosomal fusion by a pathway not controlled by the parasite. This intracellular pathway, activated by the P2X7 receptor, induces lysosomal fusion to the PV.

Another parasite evasion mechanism is mediated by the expression of antioxidant molecules in the cytosol (peroxiredoxin [Prx] 1 and 2, catalase, superoxide dismutase [SOD] 1) or in the mitochondria (Prx3, SOD2, and 3) and are essential for the protection against the production of ROS from the host (Kwok et al., 2004). NADPH oxidase is an enzyme complex that has part of its complex associated with the cell membrane (gp91^{phox} and gp22^{phox}), and the other part is in the cell cytosol (gp40^{phox}, gp67^{phox}, and gp47^{phox}). When the portion present in the cytosol is activated, it attaches to the membrane portion. It induces superoxide production by transferring electrons from NADPH across the membrane, coupling it to an oxygen molecule, forming a superoxide anion (McCann and Roulston 2013). With the increase in intracellular Ca²⁺, induced by the activation of the P2X7 receptor, there is also the activation of the p38 protein, which is a protein kinase activated by the mitogen. This protein induces the activation of the intracellular subunits of NADPH oxidase (Miller et al., 2011) coupled to the subunit present in the host cell membrane, inducing the production of ROS in

infected cells (Figure 1. A). The findings of this study paved the way for the understanding of the effects triggered by the P2X7 receptor for parasitic control.

Moreira-Souza et al., (2017) analyzed the effect of the P2X7 receptor on the control of *T. gondii* and observed intracellular cascades activated by the receptor. These pathways interfered with the growth of the parasite within murine macrophages. The critical element was the induction of ROS production by NADPH oxidase and mitochondria, 40 min after treatment with eATP induced the activation of the P2X7 receptor in mice bone-marrow-derived macrophages with consequent death of the parasite. Another pathway involved in the P2X7 response is the activation of the NLRP3 inflammasome. Inflammasomes are multiprotein platforms that are important for the cleavage and consequent secretion of pro-inflammatory cytokines such as IL-1 β and IL-18. Inflammasome activation requires two signals; the first is related to recognizing pathogen-associated molecular patterns, followed by the second signal that is damage-associated molecular patterns such as leaking nucleotides and HMGB1 (Schroder and Tschoopp 2010). The primary protein that composes the NLRP3 inflammasome is an intracellular sensor of the family of protein receptors with a nucleotide oligomerization domain (NLRs). The most studied are the inflammasome NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3). The inflammasome NLRP3 is composed of the protein NLRP3, adapter protein (apoptosis-associated speck-like protein-ASC, containing a caspase activation and recruitment domains-CARD). Furthermore, inflammatory caspase 1, which cleaves pro-IL-1 β in its mature form, can leave the cell, activate its receptor (IL-1r), and kill the parasite with the help of mitochondrial ROS (Figure 1A).

The P2X7 receptor gene is highly polymorphic. It was found that a series of single nucleotide polymorphisms (SNPs) can alter the function of the increased receptor, generating greater susceptibility of individuals who have these mutations to diseases such as the increased risk of bone fracture risk in postmenopausal females and infections of intracellular pathogens such as *Mycobacterium tuberculosis* and *Chlamydia* (Fuller et al., 2009; Savio et al., 2018). In the context of *T. gondii* infection in mouse cells, the absence of the P2X7 receptor allows the parasites to replicate more often than cells that have the receptor; the presence of the receptor after parasite death induces apoptosis in the macrophages (Lees et al., 2010) (Figure 1A). In human macrophages, the loss of P2X7 receptor function is associated with homozygosity 1513 A>C (Glu-496 to Ala), that is, with the modification of the nucleotide adenine by a cytokine in the two pairs of genes at position 1513, causing an increase in the parasitic load when compared to individuals who have adenine in the same position (Lees et al., 2010). This finding suggests that it is necessary to have the P2X7 receptor functionally active in mouse and human cells to control *T. gondii*.

Jamieson et al., (2010) analyzed children with congenital toxoplasmosis from the North American National Collaborative Chicago-based Congenital Toxoplasmosis Study cohort and families from Campos dos Goytacazes in the North Fluminense region of Rio de Janeiro in Brazil. They found that the altered function of the P2X7 receptor by SNPs increases the susceptibility to congenital toxoplasmosis. Children with such loss-of-function mutations had more clinical signs associated with retinochoroiditis caused by infection by *T. gondii*. This finding is an example of the deterministic role of the P2X7 receptor in resistance or susceptibility to congenital toxoplasmosis acquired after birth.

We have discussed the importance of the P2X7 receptor for the control of *T. gondii* in macrophages. The presence of the receptor has also been observed in non-immune cells such as epithelial cells (Cesaro et al., 2010; Souza et al., 2012). Intestinal epithelium cells are the

first with which *T. gondii* comes into contact in the host. Cells of the CMT-93 lineage (mouse colon epithelial cells) infected with *Toxoplasma* produce TNF- α , IL-6, and CC motif chemokine ligand 5 (CCL5). CCL5 induces the early recruitment of dendritic cells. The authors found that treatment with 50 μ M of A-740003 (pharmacological inhibitor of the P2X7 receptor) is associated with reducing all these cytokines (Huang et al., 2017). These inflammatory mediators participate in parasite infection control in the intestinal epithelium (Figure 1B).

Studies of human epithelial cells are usually done with cell lines such as FHs 74 Int, derived from the human fetal small intestine epithelium. The expression of the P2X7 receptor in these cells contributes to the activation of the canonical inflammasome NLRP3 and secretion of IL-1 β , inhibiting the proliferation of *T. gondii* (Quan et al., 2018). Understanding the inflammatory factors triggered by the parasite in human intestinal cells reveals a pathway to understanding events during the establishment of infection by the parasite and characterizes the inflammatory mediators that can contribute to host defense as well as in controlling the replication of the parasite and suppressing the parasite spread into the host (Figure 1B).

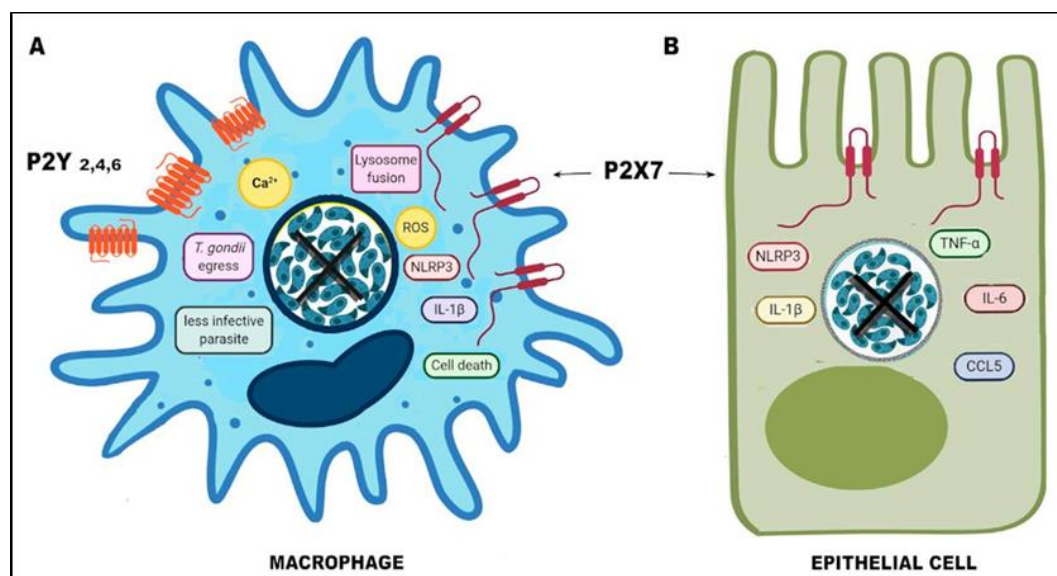


Figure 1. Effect of activation of purinergic signaling on *T. gondii* infection in vitro. In murine macrophages, the activation of the P2Y receptor after *Toxoplasma* infection increases intracellular Ca^{2+} concentrations, inducing the parasite egress. This mechanism reduces the parasite's ability to actively infect the host cell and create a non-fusogenic parasitophorous vacuole degraded by the endosomal pathway. Regarding the P2X7 receptor, after infection in murine macrophages, there is an increase in ROS production, culminating in the death of the parasite by activation of the NLRP3 inflammasome, secretion of IL-1 β , and lysosomal fusion, inducing cell death (A). In intestinal epithelial cells, the P2X7 receptor increases parasite load by producing inflammatory mediators such as TNF- α , IL-6, and CCL5 and activation of the NLRP3 inflammasome with the consequent secretion of IL-1 β (B). Figure created by BioRender.com

In murine macrophages, the activation of the P2Y receptor after *Toxoplasma* infection increases intracellular Ca^{2+} concentrations, inducing the parasite egress. This mechanism reduces the parasite's ability to actively infect the host cell and create a non-fusogenic parasitophorous vacuole degraded by the endosomal pathway. Regarding the P2X7 receptor,

after infection in murine macrophages, there is an increase in ROS production, culminating in the death of the parasite by activation of the NLRP3 inflammasome, secretion of IL-1 β , and lysosomal fusion, inducing cell death (A). In intestinal epithelial cells, the P2X7 receptor increases parasite load by producing inflammatory mediators such as TNF- α , IL-6, and CCL5 and activation of the NLRP3 inflammasome with the consequent secretion of IL-1 β (B). Figure created by BioRender.com.

2.2. The Effect of P2X7 Receptor on the Systemic Immune Response during Experimental Toxoplasmosis

In vivo studies are extremely important for understanding the role of the P2X7 receptor in the body that are associated with local and systemic effects, depending on the period of infection. In the acute phase of infection, where tachyzoites infect intestinal epithelial cells during the first week of infection, the presence of the P2X7 receptor increases the secretion of pro-inflammatory cytokines such as IL-1 β , IL-12, IL-6, TNF- α , and interferon-gamma (IFN- γ) in the serum of infected mice (Corrêa et al., 2017). These mediators are essential to control parasite burden in the peritoneal cavity, and they prevent tissue damage in the spleen and liver, both shock organs in acute toxoplasmosis (Corrêa et al., 2017). In intestines of animals collected by autopsy after oral infection by *T. gondii*, as seen in CMT-93 cells, there was an increase in the production of CCL5, increasing the recruitment of dendritic cells (Huang et al., 2017). Dendritic cells (DC) are innate immune system cells classified as professional antigen-presenting cells. They recognize *T. gondii* through Toll-like receptors present in the cell membrane or cytoplasm after parasite proteins are processed and directed to the DC membrane coupled to the main histocompatibility complex communicates between the innate and the adaptive immune responses. In the context of *Toxoplasma* infection, activation of CD4⁺ T lymphocytes was observed as an essential producer of IFN- γ , a critical cytokine in the control of *T. gondii* that mediates a series of cellular events that limit the replication of the parasite, in addition to destabilizing the PV (Yarovinsky 2014).

After parasite dissemination starts in the first week post-infection, *T. gondii* can establish a chronic phase (beginning 20 days after infection). This phase is characterized by cysts containing parasites present in mouse tissues, e.g., the central nervous system. During the chronic phase of infection (30 days post infection), the P2X7 receptor contributes to pro-inflammatory cytokine production (IL-1 β , IL-12, IL-6) in the mouse spleen (Moreira-Souza et al., 2019). In this phase, the presence of the receptor contributes to the migration of inflammatory cells to the brain. These cells are responsible for the production of IL-1 β , IL-12, and ROS, which act as microbicides that control the proliferation of the parasite, evidenced by the decrease in the number of *T. gondii* cysts in the brain (Moreira-Souza et al., 2019).

In other words, the activation of the P2X7 receptor activates and induces intracellular cascades in host cells and also participates in the acute and chronic phases of the infection. These systemic and local responses increase inflammation as an essential mechanism for controlling proliferation and disseminating *T. gondii*.

CONCLUSION

Purinergic signaling is among the most crucial host components of the immune response to *T. gondii* infection. Various purinergic receptors are modulated during infection and participate in immune responses generated by professional immune cells and epithelial cells. This well-conserved family of receptors participates in the initial moments of parasite-host recognition, recruiting immune cells, increasing host defenses, and shaping the adaptative immune responses that develop weeks after the initial infection. Thus, purinergic signaling can be seen as a new avenue for future pharmacological investigations to manage toxoplasmosis.

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Chapter 9

**METASTATIC-LIKE PHENOTYPE
OF MONOCYTES/MACROPHAGES INDUCED
BY *TOXOPLASMA GONDII* INFECTION:
MMPS, UPA/UPAR AND CD147 INVOLVEMENT
AT EARLY INFECTION AND DISSEMINATION**

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ABSTRACT

In this chapter, we will approach several known mechanisms used by *Toxoplasma gondii* to disseminate throughout the host. Three major mechanisms are discussed: i) paracellular gliding movement; ii) leukocytes hitchhiking parasites and iii) Trojan horse dissemination. We show herein accumulated evidence of the involvement of the host's metalloproteinases of extracellular matrix and serine proteases from the uPA/uPAR pathway, gathered by our group in the last decades. We also show data of in situ expression of matrix metalloproteinases and extracellular matrix metalloproteinase inducer, CD147, at the site of entry and early dissemination of *T. gondii* on acute experimental murine oral infection. The presence of circulating zymogen and active forms of MMP-2 and MMP-9 after oral infection of C57BL/6 and C57BL/6 IFN γ ^{-/-} with *T. gondii* was analyzed. Both forms of MMP-9 and MMP-2 were significantly increased in the serum after 7 days of infection. However, only MMP-9 was detected at the site of entry in infected mice, the ileum, while expression of MMP-2 was faintly detected in the absence of IFN γ . *T. gondii* infection induced the expression of MMP-9 at the lungs during its early dissemination. Expression of CD147 at the ileum and lungs was also increased upon infection. Interestingly, we also detected CD147 at the brain of infected

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mice, at the proximity of parasite foci. To our knowledge, this is the first demonstration of MMP-9 and CD147 expression during in vivo oral infection with *T. gondii* at sites of early dissemination of the parasite and even at the brain. These host molecules are known to be involved in the metastasis of several types of cancer and are used as biomarkers of poor diagnosis of cancer progression. We suggest that *T. gondii* infection-prone infected cells modify their pericellular space to facilitate parasite dissemination throughout the host, taking advantage of cells from the immune system to reach the brain and retina, crossing crucial hosts' barriers.

1. INTRODUCTION

One-third of the world's population is estimated to be infected with *Toxoplasma gondii* (Remington et al. 1995; Pappas et al. 2009). The intracellular parasite has a complex biological cycle, with accidental and definitive hosts, represented by the Felidae family. Sexual reproduction only takes place at the definitive host. (Dubey and Beatie, 1988).

T. gondii infection occurs via oral ingestion of felid oocysts or tissue cysts, with sporozoites or bradyzoites release at the gut. Parasites cross the intestinal epithelial barrier by three major mechanisms: i) by gliding movement driving paracellular migration (Barragan and Sibley 2005; Jones et al. 2012); ii) by a recently proposed mechanism through hitchhiker leukocytes in an integrin independent fashion (Drewry and Sibley 2019), and by iii) by leukocyte facilitated entry in a mechanism known as Trojan horse (DaGama et al. 2004; Lambert and Barragan 2010; Seipel et al. 2010; Schuindt et al. 2012; Brasil et al. 2017).

Parasite reaches lamina propria, where resident macrophages and recent arrived blood monocytes are infected by *T. gondii* and used to reach deeper tissues (Brasil et al. 2017; Drewry and Sibley 2019). Dendritic cells and NK cells from lamina propria might also be infected and contribute to the spread of the infection (Lambert et al. 2006).

1.1. Dissemination of *T. gondii* Throughout the Host

Evasion of the host's immune response is a feature well known among protozoan pathogens, but *Toxoplasma gondii* has developed several and diverse mechanisms regarding careful control of the host's immune response. Most parasites that break the intestinal barrier are eliminated by neutrophils and macrophages at the site of entry. However, some bradyzoites and tachyzoites use immune cells such as macrophages, dendritic cells and neutrophils to disseminate throughout the host, reaching the blood-brain-barrier and blood-retina-barrier, remaining inside quiescent tissue cysts.

T. gondii dissemination via lymphatics and hematogenic pathways inside leukocytes is the main form of parasite spread and vast information on this subject is available (Unno et al. 2008; Lambert et al. 2006; Brasil et al. 2018; Drewry and Sibley 2019). After oral infection *T. gondii* is found inside mouse CD11c⁺ DCs at lamina propria, Peyer's patches and mesenteric lymph nodes, while in the blood parasite is found inside CD11b⁺ monocytes (Courret et al. 2006). Infected monocytes are observed in the extravascular space at the brain after 7 days of infection (Courret et al. 2006). *In vitro* infection of murine macrophages leads to a transient down regulation of integrins beta₂, alpha_v and alpha₄ chains followed by the adhesion to

laminin, fibronectin and collagen IV (Da Gama et al. 2004). Infection of human macrophage lineage THP1 shows downregulation of co-stimulatory molecules CD86, CD83, CD40 and CD1a, suggesting an impairment of infected monocyte/macrophages to stimulate a proper immune response (Seipel et al. 2009).

Dissemination is contained by inflammatory macrophages producing ROS and NO, that function as microbicide molecules. Nevertheless, when peritoneal and blood-derived murine macrophages are activated by IFN γ and lipopolysaccharide (LPS), *T. gondii* infection results in a significant decrease of NO production (Seabra et al. 2002; Lüder et al. 2003; Shrestha et al. 2006). Pre-coating *T. gondii* with annexin V can revert NO inhibition, in a mechanism known as apoptotic mimicry. Annexin V binds to phosphatidylserine (PS) and contributes to an anti-inflammatory profile of these macrophages (Seabra et al. 2004). *T. gondii* PS⁺ tachyzoites but not PS⁻ are the ones responsible for the NO inhibition in vitro and PS⁺ parasites in vivo infection result in increased parasite burden at the peritoneal cavity. On the other hand, PS⁻ parasite infection leads to low parasite burden with strong inflammatory response, suggesting a regulatory role of PS by *T. gondii* subpopulations at the entry site (Seabra et al. 2004).

Infection of DCs leads to an ameboid phenotype with a rapid migration profile in 2D and 3D collagen matrix in an integrin-independent way (Ólafsson et al. 2018). The signaling axis identified in the promotion of this phenotype upon infection involves the GABAergic receptor and L-VDCC (L-type voltage-dependent calcium channel – Ca_v1.3). Treatment with GABA inhibitor decreases parasite load in the brain, spleen and mesenteric lymph nodes (Bhandage and Barragan 2019). However, the precise mechanism used by infected DCs to traverse the epithelial and endothelial barriers is not demonstrated.

On the other hand, it has been recently described that infected monocytes carry parasites through interstitial spaces, involving the parasite molecule ROP17. This mechanism of dissemination is also dependent on the host's Rho/ROCK signaling pathway, and independent of integrin expression, and it has been referred to as hitchhiking (Drewry et al. 2019). At the present, the specific targets of ROP17 inside host cells were not identified.

Interestingly, Rho kinases are essential to MMP expression and stress fibers orientation during interstitial migration, dependent on extracellular matrix density (Foolen et al. 2014). ROCK 1 and ROCK2 siRNA usage decrease the levels of MMP-2 (Gong et al. 2019), suggesting an intersection of Rho/ROCK signaling pathway and MMPs expression/activation.

1.2. Metastatic Like Phenotype of *T. gondii* Infected Leukocytes

After oral contamination with oocysts or tissue cysts from *Toxoplasma gondii*, enterocytes can be infected by bradyzoites that are then converted to tachyzoite forms at the ileum (Dubey 1998). Adjacent cells and intraepithelial leukocytes can also be infected by tachyzoites, and from there disseminate to other tissues by blood and lymphatic circulation (Liesenfeld 2002). At submucosal tissues, the parasite encounters resident dendritic cells (DCs) and macrophages that might facilitate dissemination and crossing blood-brain and blood-retina barriers (Barragan and Sibley 2003; Da Gama et al. 2004; Courret et al. 2006; Dunay et al. 2008). However, the molecular bases of this facilitated transport are not yet well defined.

Adhesive contacts between immune cells are dynamic and transient. They require high flexibility and mobility to develop several functions including immunological surveillance and antigen recognition. Leukocyte migration involves interactions cascades where molecules from different families promote cleavage of nectin, occludin, PECAM and S-cadherins (Bazzoni and Dejana 2004). Extracellular matrix metalloproteinases (MMPs) are known to play a key role during the migration and transmigration process, favoring the immune cell influx, regulating the inflammatory process and extracellular matrix remodeling (Elkington et al. 2005).

MMPs are part of the wide endopeptidase zinc-dependent family, that are involved in several physiological and pathological mechanisms, such as embryonic development and in response to infections (Sternlicht 2001). With exception of MMP-7 and -26, MMPs molecules present 3 structural domains: an N-term prodomain, a catalytic internal domain-containing cysteine and a hemopexin C-term domain (Bode et al. 1999; Visse and Nagase 2003). MT-MMPs are extracellular matrix metalloproteinases inserted at cell surface membrane by a transmembrane domain or by GPI anchors (Elkington et al. 2005).

MMPs are regulated in multiple steps and gene transcription is induced in inflammatory cells by TNF α or IL-12, among other cytokines (Unemori et al. 1991). Additional steps of MMPs control occur at membrane level by favoring cleavage of zymogens in active enzymes. Among the enablers of those interactions is the extracellular matrix metalloproteinase inducer also known as EMMPRIN, basigin, or CD147 (Zhu et al. 2014; Grass and Toole 2015; Muramatsu 2016). Besides its role in protein-protein oligomerization, CD147 seems to be essential to MT1-MMP and MMP-9 synthesis, since its knockdown in monocytes by siRNA inhibits the expression of these molecules in response to *Chlamydia pneumoniae* (Schmidt et al. 2006). Several invasive tumor types are known to show increased expression of MMP-2, MMP-9 and CD147, and the systemic presence of those molecules is related to a bad disease prognostic (Aoki et al. 2019; Dana et al. 2020).

For the past decades, we have been working with this set of molecules involved in the migration of altered metastatic cells, to determine its usefulness in the migration of *T. gondii* infected leukocytes.

Initial studies showed a decrease of invasion index over 3D Matrigel when infected murine macrophages were treated with MMP I inhibitor (Seipel et al. 2010). In the same report, we showed for the first time that macrophage infection by *T. gondii* results in MMP-9 secretion of both activated and unactivated forms. On the other hand, CD44 expression is drastically diminished upon infection while expression of membrane type-1 matrix metalloproteinase (MT1-MMP), disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) are increased (Seipel et al. 2010).

We were able to demonstrate that CD44 as well TIMP-1 and uPAR (urokinase-type plasminogen activator receptor) co-immunoprecipitate MMP-9 in active and zymogen forms from the supernatant of infected murine macrophages (Schuindt et al. 2012). CD44 functions as a docking molecule for MT1-MMP and MMP-9 and are secreted as a multimeric complex in several types of cancer (Redondo-Muñoz et al. 2008; Abdelazeem et al. 2015). Pericellular proteolysis is initiated by uPA/uPAR pathway and PAI-1 is the inhibitor of this pathway (Gontero et al. 2004; Finlayson 2015; Mahmood et al., 2018). The addition of PAI to infected cells drastically decreased the secretion of the CD44/MMP-9/TIMP-1/uPAR complex (Schuindt et al. 2012). Taken together, these results strongly suggest that *T. gondii* infection triggers a metastatic program on the host's cell, contributing to parasite dissemination

throughout the host. Nonetheless, *in vivo* models of *T. gondii* oral infection show contrasting results, regarding the involvement of MMP-9 or MMP-2 in the immune pathogenic outcome in murine toxoplasmosis (Muñoz et al. 2009; Cavalcanti et al. 2011).

In this chapter, we show the results of investigative work about the expression of MMP-2, MMP-9 and CD147 at the early dissemination sites of infection during the acute phase of experimental murine toxoplasmosis. To our knowledge, this is the first demonstration of the involvement of CD147 in *T. gondii* infection although its' molecular role is yet to be determined.

2. MATERIALS AND METHODS

2.1. Animals and Parasites

Swiss mice, C57BL/6 and C57BL/6 IFN γ ^{-/-} were bred and maintained at UENF's Central Animal Facility Thereza Kipnis. *T. gondii* ME49 strain (ATCC#50840) brain tissue cysts were obtained from swiss mice previously (1-3 months) inoculated intraperitoneally with 5 cysts, similar to Dunay et al. 2008. Briefly, brains from sacrificed animals were removed and homogenized in PBS and cysts counted after centrifugation (Sigma Chemical Co.). The experimental protocol was approved by UENF's Animal Experimentation Ethics Committee (292/2015).

2.2. *In vivo* Infection and Tissue Removal

Experimental groups C57BL/6 and C57BL/6 IFN γ ^{-/-} mice were infected with 50 tissue cysts by gavage (n=9 per group). Mock infection was produced by saline gavage of the same volume containing the tissue cysts (n=9 mice per group). Animals were observed daily for survival curves. Five animals were sacrificed after 7 days of infection in CO₂ camera and ilea, lungs and other organs removed and put in freeze medium Tissue Tek™ O.C.T.™ (Sigma). Sera were also collected, clarified, and aliquots were kept at -20°C.

2.3. Immunofluorescence Microscopy

Frozen sections of ileum or lungs (8µm thickness) were rehydrated with PBS BSA 3%; gelatin 0.1%; Tween 20 0.05% and azide 0.05% (buffer A), for 50 min at room temperature and then stained with goat anti-mouse MMP-9 (C-20; 1:200), MMP-2 (C-19; 1:200) or CD147 (T-18; 1:250) (Santa Cruz Inc.). After 2h of incubation, slides were washed twice by immersion in buffer A. Secondary antibodies coupled to biotin (swine anti-goat Caltag Laboratories) were incubated for 1h (1:1000), slides washed twice with PBS by immersion and incubated with streptavidin-PECy5 (e-Bioscience; 1:2.000) for 1h hour. For *T. gondii* localization, slides were pre-stained with rabbit polyclonal *anti-T. gondii* (1:1500) followed by anti-rabbit FITC (e-Bioscience; 1:2.000), following the same steps described above. After washing, slides were mounted in Prolong Gold with DAPI (Sigma Chemicals Co.) and

analyzed with a Zeiss AxioPlan coupled to an AxioVision™ 4.6. system. Ten fields per slide were analyzed.

2.4. *In situ* Zymography

In situ zymography was performed according to Yan and Blomme (2003). Briefly, slides were coated with 0.1% of FITC-gelatin [0.1 mg/ml, w/v and 0.1% (w/v); respectively] were dissolved in water in Tris-HCl pH 7.5 buffer containing 200 mM NaCl, 10 mM CaCl₂ and 2.5% Triton X-100. Slides with frozen tissue sections (8 µm thickness) were put in contact with the FITC coated slides in a moist box for 48h at 37°C in the dark. Gelatin-FITC slides were then analyzed using a Zeiss AxioPlan coupled to an AxioVision™ 4.6 system.

2.5. Immunoprecipitation and Zymography

Immunoprecipitation of clarified serum was performed as described in Schuindt et al. 2011. Protein G agarose (Calbiochem) was incubated with 2 µg of anti-MMP-9 (C-20) [Santa Cruz Biotech] for 18 h at 4°C and mixed with the sera for an additional 3 h incubation. Pellets containing immune complexes were washed with Low RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100). For zymography, the pellet was suspended in sample buffer without β-mercaptoethanol and subjected to SDS-PAGE using 8% polyacrylamide gels containing 0.1% gelatin. After electrophoresis, gels were rinsed with Tris-HCl pH7.5 buffer containing 2.5% Triton X-100 at 37°C. After 48 h of incubation with Tris-HCl pH 7.5 buffer containing 200 mM NaCl, 10 mM CaCl₂ and 2.5% Triton X-100 at 37°C; gels were stained with 0.25% of Coomassie blue and areas of gelatinolytic activity were visualized as clear bands.

2.6. Densitometric Assays

Gels were digitalized using a Canon EOS Rebel XTi 10.1 Megapixels camera, in most pictures colors were inverted and contrast enhanced and transformed in grayscale by Corel PhotoImpact X3 software. The relative intensity of the gelatin degradation by immunoprecipitated protein was quantified by densitometry, using the Gel Doc™ XR+ (Bio-Rad).

2.7. Statistical Analysis

Data are presented as mean ± Standard Deviation (SD), and unpaired two-tailed t-test or One-Way Anova were done for comparisons, using the GraphPad Prism software.

3. RESULTS

3.1. MMPs and CD147 and *In Vivo* Oral Infection

We have explored the expression of MMPs at the site of entry of *T. gondii* upon oral infection. IFN γ is a major controller of MMPs expression, maintaining its expression under tight regulation (Nosaka et al. 2011; He and Sun 2018). We used IFN $\gamma^{-/-}$ mice, in order to amplify the interference of *T. gondii* infection with the MMP-2, MMP-9 and CD147 expression at the site of entry ilea, and in the dissemination to deep tissues, such as lungs.

3.2. Enzymatic Activity at the Site of Entry after Oral *T. gondii* Infection

In situ zymography in FITC-gelatin was performed to determine if the enzymatic activity of proteases throughout the infected tissue increased due to the *T. gondii* infection. Intense cleavage activity was shown in both ileum and lungs obtained from infected IFN $\gamma^{+/+}$ and IFN $\gamma^{-/-}$ mice when FITC is freed from its quenched form by enzymatic activity in the tissue (Figure 1). We seek to identify the presence of circulating active forms of MMP-2 and MMP-9 enzymes and their zymogens (pro-MMP-2 and pro-MMP-9) at day 7 post-oral infection. Immunoprecipitation followed by the zymographic analysis of sera showed that both MMP-2 and MMP-9 are increased in *T. gondii* infected when compared to uninfected mice (Figure 2). Densitometric analysis showed that pro-MMP-2 and pro-MMP-9 forms are increased in infected C57BL/6 IFN $\gamma^{+/+}$ and IFN $\gamma^{-/-}$ animals, while active forms of MMP-2 and MMP-9 are only present in IFN $\gamma^{-/-}$ (Figure 2).

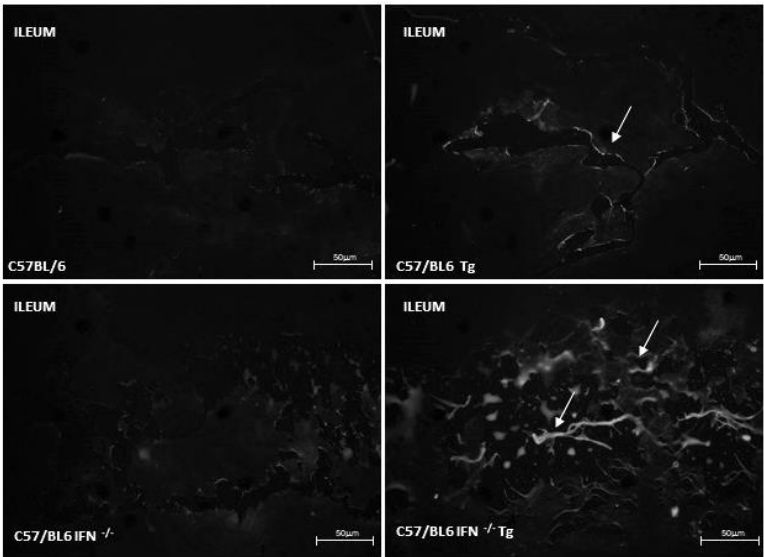


Figure 1. (Continued)

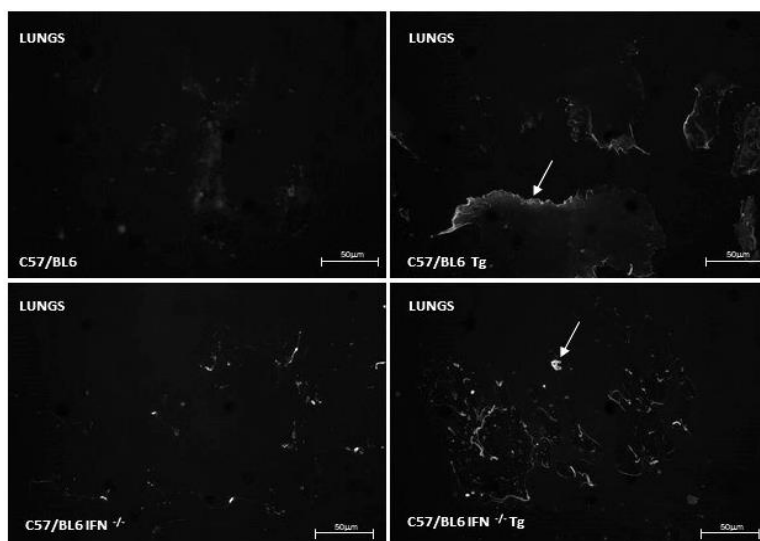


Figure 1. *In situ* zymography.

C57BL/6 or C57BL/6 IFN γ ^{-/-} mice (n=9 per group) were infected with 50 tissue cysts of *T. gondii* ME-49 strain. After 7 days post-infection (p.i) tissues were collected and frozen on OCT™. 8 μ m slices were obtained, covered with FITC-gelatin slides and left in humid chamber at 37°C for 48 h. Micrographs were obtained at AxioVision™ at 400x augmentation. Enzymatic activity is perceived as degraded gelatin liberating FITC (arrows) resulted from tissue contact. Color images were converted to grey images. Bars = 50 μ m.

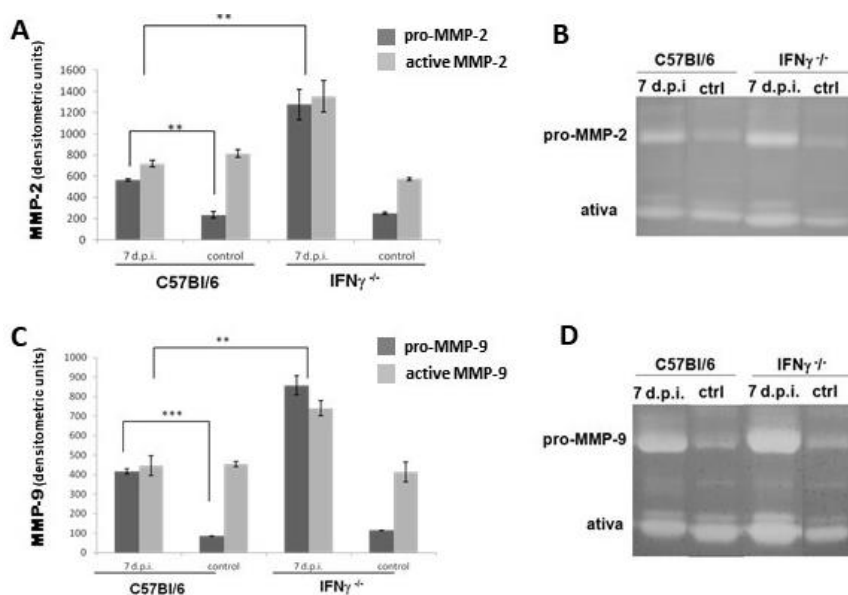


Figure 2. Presence of circulating zymogen and active forms of MMP-2 and MMP-9 after oral infection with *T. gondii*. Blood of 9 mice per group was collected and sera obtained. Serum was pooled and immunoprecipitated with anti-MMP-2 (A and B) or anti-MMP-9 (C and D) and submitted to zymography (n=4). Densitometry analysis were performed for four different zymograms obtained for each group by Gel Doc™ XR+ and results were plotted as histograms of densitometric units. One-way ANOVA statistics was performed: $p^{**} > 0.01$ or $p^{***} > 0.001$.

3.3. *In Situ* Expression of MMP-9 and MMP-2 at Ileum of *T. gondii* Infected C57BL/6 and C57BL/6 IFN γ ^{-/-} Mice

Uninfected or infected C57BL/6 mice did not express MMP-2 at ileum, while in IFN γ ^{-/-} infected mice MMP-2 expression was observed (Figure 3). Expression of MMP-9 was evident at ilea from infected C57BL/6 IFN γ ^{+/+} and IFN γ ^{-/-} mice (Figure 4). Uninfected mice did not show MMP-2 or MMP-9 expression.

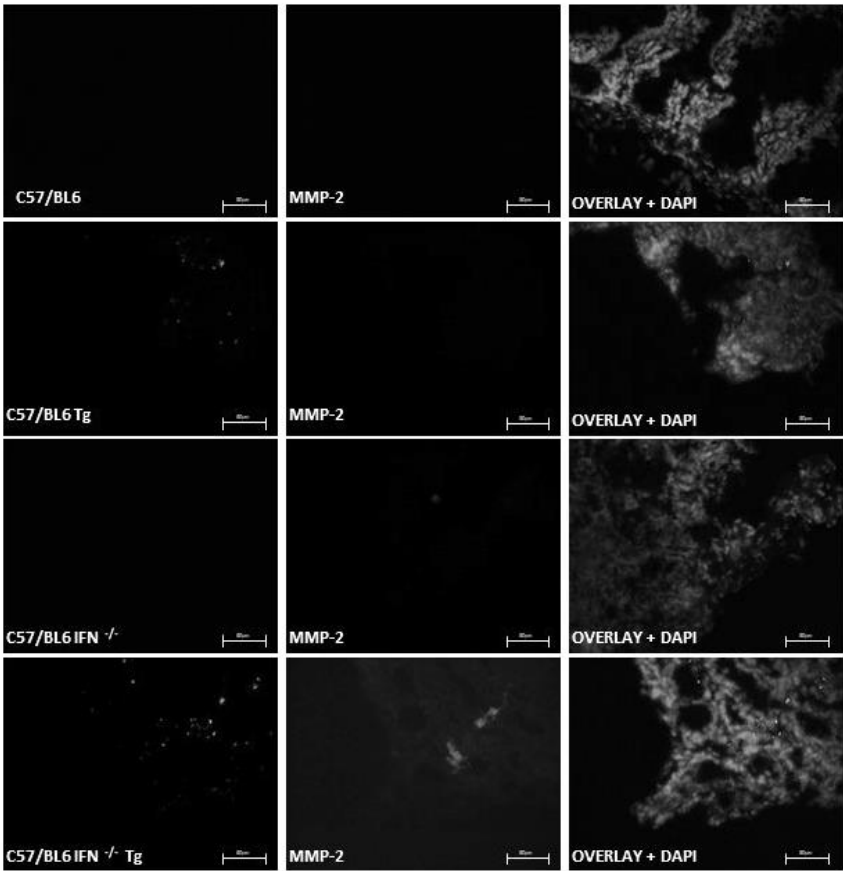


Figure 3. MMP-9 *in situ* expression at ilea of *T. gondii* infected mice.

C57BL/6 or C57BL/6 IFN γ ^{-/-} mice (n=9 per group) were infected with 50 tissue cysts of *T. gondii* ME-49 strain. After 7 days post-infection (p.i.) tissues were collected and frozen on OCTTM. 8 μ m slices were obtained, and slides were stained with anti-MMP-9 (red), *anti-T. gondii* (green) and DAPI (blue). Micrographs were obtained at AxioVisionTM at 400x augmentation. Image overlay was performed by Adobe PhotoshopTM. *T. gondii* stained in green noted by arrow heads. Color images were converted to grey tone images. Bars = 10 μ m.

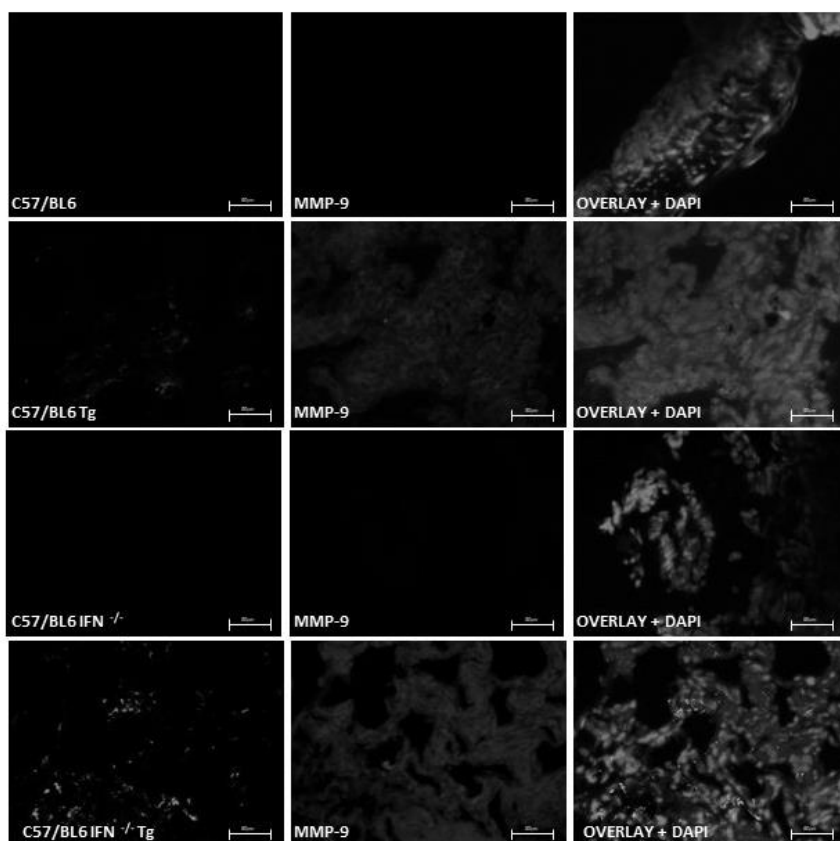


Figure 4. MMP-2 *in situ* expression at ilea of *T. gondii* infected mice.

C57BL/6 or C57BL/6 IFN $\gamma^{-/-}$ mice (n=9 per group) were infected with 50 tissue cysts of *T. gondii* ME-49 strain. After 7 days post-infection (p.i.) tissues were collected and frozen on OCTTM. 8 μ m slices were obtained, and slides were stained with anti-MMP-2 (red), anti-*T. gondii* (green) and DAPI (blue). Micrographs were obtained at AxioVisionTM at 400x augmentation. Image overlay was performed by Adobe PhotoshopTM. Color images were converted to grey tone images. Bars = 10 μ m.

3.4. *In situ* Expression of CD147 at Ileum of *T. gondii* Infected C57BL/6 IFN $\gamma^{+/+}$ and C57BL/6 IFN $\gamma^{-/-}$

Expression of CD147 was marked at ilea from infected C57BL/6 IFN $\gamma^{+/+}$ and IFN $\gamma^{-/-}$ mice (Figure 5). No CD147 expression was observed in uninfected mice. Parasite burden was evident in both mice strains and spots of co-localizing *T. gondii* and CD147 were apparent. The CD147 expression profile was cytoplasmic and interstitial.

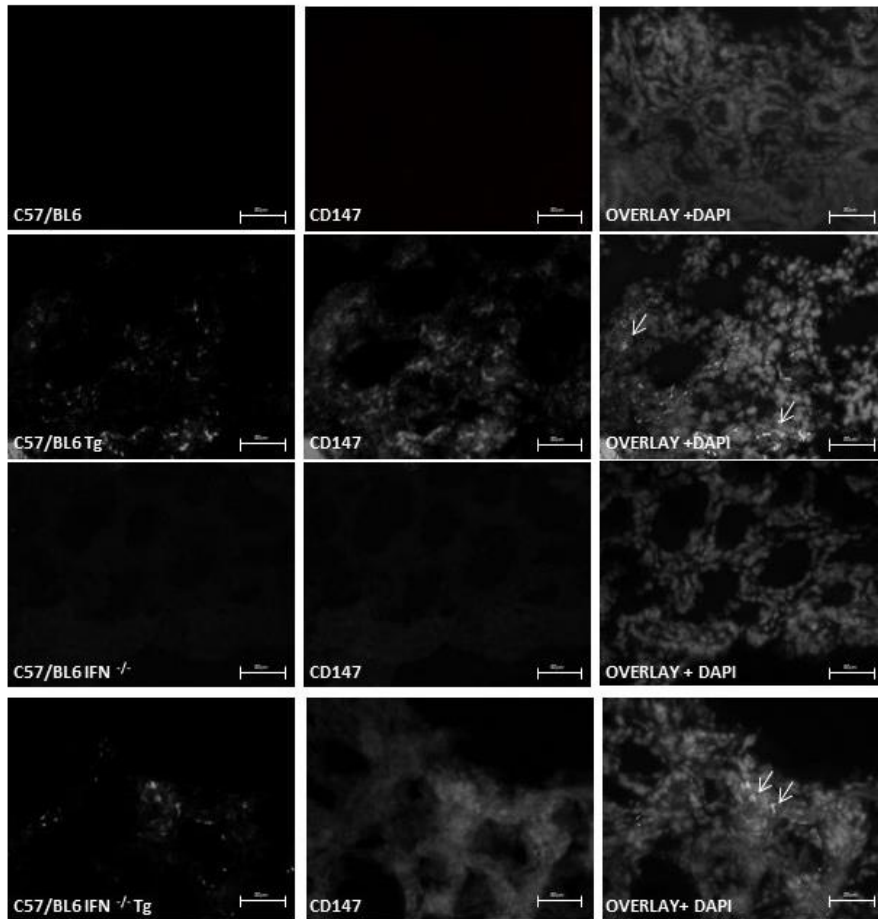


Figure 5. CD147 *in situ* expression at ilea of *T. gondii* infected mice.

C57BL/6 or C57BL/6 IFN $\gamma^{-/-}$ mice (n=9 per group) were infected with 50 tissue cysts of *T. gondii* ME-49 strain. 7 days post-infection (p.i.) tissues were collected and frozen on OCTTM. 7 μ m slices were obtained, and slides were stained with anti-EMMPRIN (red), anti-*T. gondii* (green) and DAPI (blue). Micrographs were obtained using AxioVisionTM at 400x augmentation. Image overlay was performed by Adobe PhotoshopTM. *T. gondii* stained in green noted by arrow heads. Color images were converted to grey tone images. Bars = 10 μ m.

3.5. *In Situ* Expression of CD147 and MMP-9 at the Lungs of *T. gondii* Infected C57BL/6 and C57BL/6 IFN $\gamma^{-/-}$

The expression of MMP-9 and CD147 at a secondary site of infection (lungs) was as intense as at the parasite entry site (ileum) (Figure 6). Expression of this molecule was higher than at ileum and more cells were stained even though fewer parasites were observed.

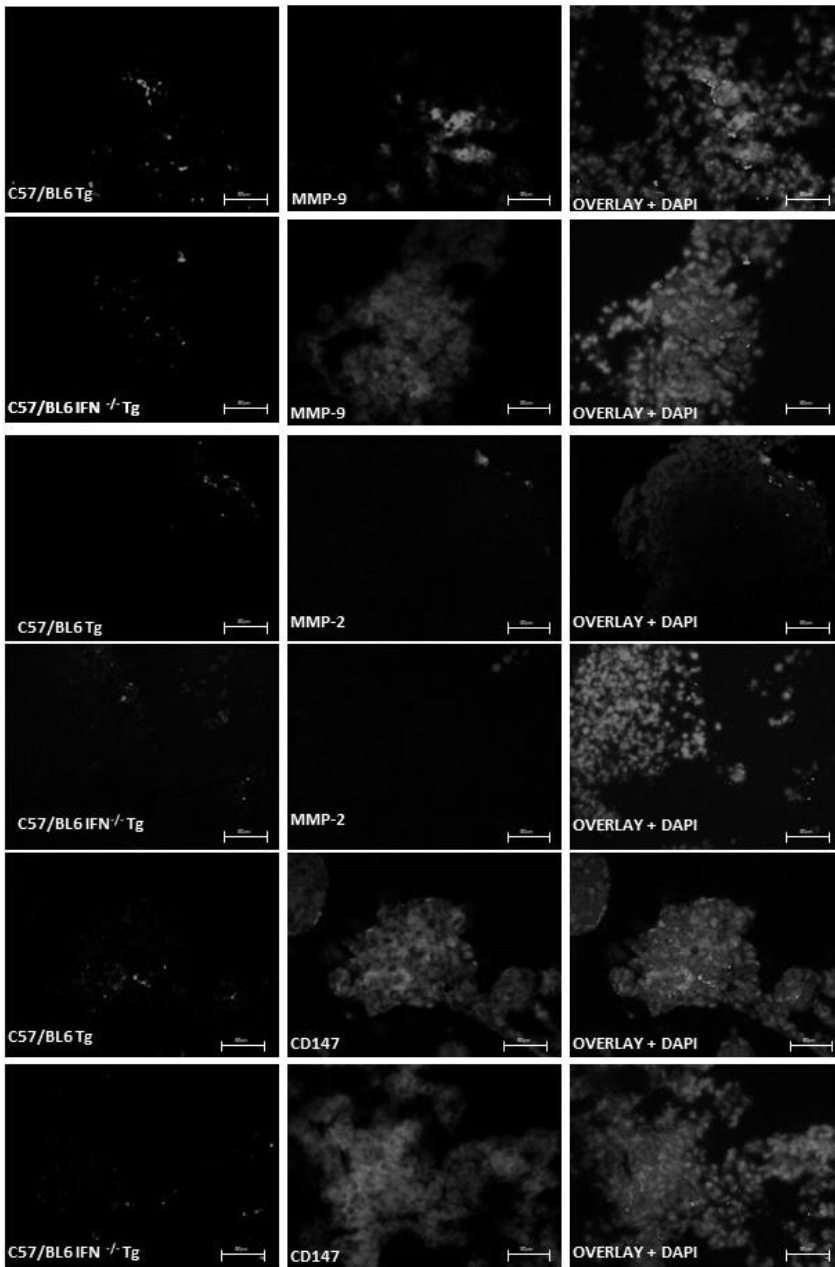


Figure 6. MMP-9, MMP-2 and EMMPRIN *in situ* expression in lungs of *T. gondii* infected mice. C57BL/6 or C57BL/6 IFN γ ^{-/-} mice (n=9 per group) were infected with 50 tissue cysts of *T. gondii* ME-49 strain. 7 days post-infection (p.i.) tissues were collected and frozen on OCT™. 7 μ m slices were obtained, and slides were stained with anti-MMP-9, MMP-2 or EMMPRIN (red), anti-*T. gondii* (green) and DAPI (blue). Micrographs were obtained at AxioVision™ using 400x augmentation. Image overlay was performed by Adobe Photoshop™. Color images were converted to grey tone images. Bars = 10 μ m.

CONCLUSION

T. gondii is a well-succeeded parasite since it is capable of disseminating from its primary site of entry, the intestinal epithelial barrier to distant sites such as CNS. Several mechanisms are proposed to explain the success of parasite dissemination throughout the host: i) gliding movement driving paracellular migration (Barragan and Sibley 2005; Weigh and Carding 2012); ii) leukocytes hitchhiking parasites in an integrin independent fashion (Drewry and Sibley 2019), and iii) leukocyte facilitated entry in a mechanism known as Trojan horse (DaGama et al. 2004; Lambert et al. 2009; Persson et al. 2009; Seipel et al. 2010; Schuindt et al. 2012, Brasil et al. 2018). However, none of those models were able to show feasible evidence of how the parasite cross blood-brain-barrier, and the precise picture of its entry into CNS is missing.

We dedicate the past few decades investigating the molecular bases of the leukocyte exploitation by *T. gondii*, mainly using *in vitro* infection of murine monocytes and macrophages. Data discussed here, suggest that the parasite triggers a metastatic program in infected cells, increasing expression of MMP-9, MT1-MMP, ADAM10, modulating CD44 expression and cleavage, and activation of uPA/uPAR pathway might be involved (Figure 7). Modification of pericellular space occurs and migration of infected monocytes/macrophages in 3D mimicry of extracellular matrix is increased and can be inhibited by MMP I inhibitor, suggesting that both ameboid and interstitial migration are dependent on this metastatic program (Seipel et al. 2010; Schuindt et al. 2012; Brasil et al. 2018). These findings do not overturn other proposed mechanisms such as GABA or Rho/ROCK-dependent signaling (Bhandage and Barragan 2019; Drewry et al. 2019).

The expression and/or contribution of MMPs in experimental toxoplasmosis were evaluated in two other oral infection models. Muñoz et al. (2009) showed that infection with 200 tissue cysts, promotes an increased in both pro and active forms of MMP-2 and MMP-9 in at ilea of C57BL/6 mice. Zymographic analysis indicates MMP-2 and MMP-9 augmentation by day 5 of infection. However, the authors associate ileum pathology with MMP-2 since MMP-2^{-/-} mice did not show pronounced pathology after 8 days of *T. gondii* infection. On the other hand, Cavalcanti et al. (2011) reported that in absence of MIF (macrophage migration inhibiting factor) less MMP-9 mRNA is observed at ilea of infected mice and no alteration of MMP-2 was detected, which was consistent with a drastic decrease in tissue damage by day 9 of infection. In this study, we observed increased expression of MMP-9 molecule in both ilea and lungs of mice after 7 days of oral infection. *In situ* zymography showed higher enzymatic activity at the site of entry, ileum, of orally infected animals. We also observed an increase of both active and zymogen forms of MMP-9 and MMP-2 at serum from infected mice. Similar to Cavalcanti et al. (2011) we observed only a slight increase in MMP-2 expression at ilea and lungs of infected C57BL/6 IFN γ ^{-/-} mice suggesting a major contribution of MMP-9 to the increased enzymatic activity. It has been shown that IFN γ down-regulates MMPs expression, particularly MMP-9 (Dhawan et al. 1995; Lai et al. 2003). In fact, we observed an increased expression of MMP-9 and CD147 even in presence of IFN γ .

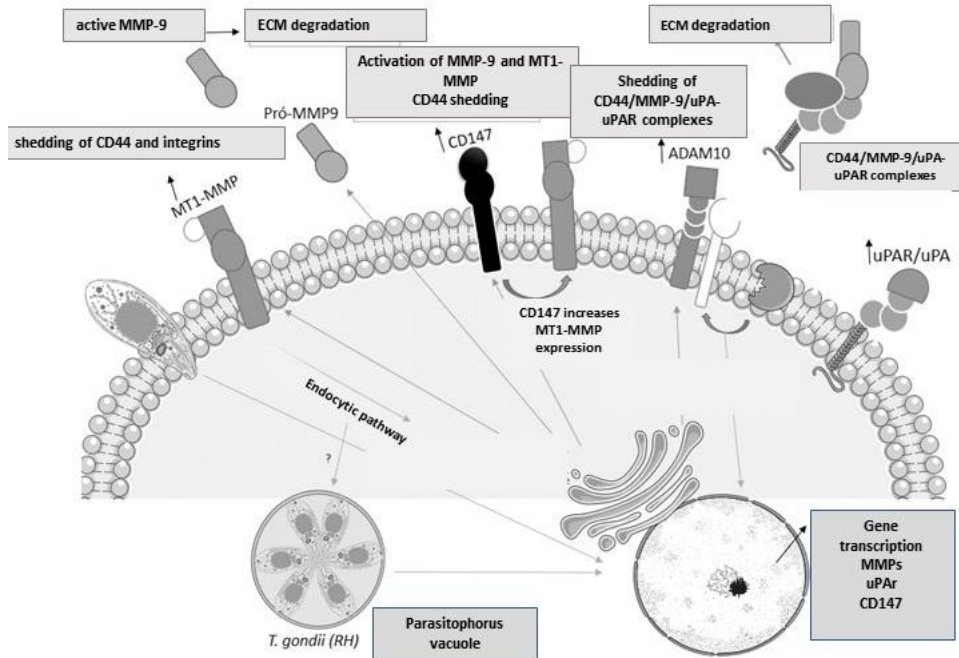


Figure 7. MMPs, uPA/uPAR and CD147 are involved in the metastatic-like phenotype of monocyte/macrophages infected with *T. gondii*. MMP-9, MT1-MMP and ADAM10 are increased at cell surface of infected cells. At the same time, CD44 serves as docking molecule to MMP-9 cleavage by uPA/uPAR, increasing both zymogen and active forms at pericellular space. On the other hand, CD44 is cleaved by ADAM10, promoting the shedding of CD44/MMP-9/uPAR complex. Increased expression of CD147 may interfere with signaling pathways leading to enhanced expression of MMP-9, MT1-MMP and ADAM10 genes. This model proposes the triggering of host cell metastatic-like program, involving these molecules to modify the pericellular space, and giving infected monocytes/macrophages a hypermotility phenotype.

Besides pro-inflammatory cytokine regulation of MMP-9 expression, two main upstream systems of control are known: the uPA/uPAR and the CD147 (extracellular matrix metalloproteinase inducer) pathways. The serine urokinase plasminogen activator (uPA) converts MMPs from its' zymogen into active forms when coupled to its receptor (uPAR) at the plasma membrane (Kanse et al. 2004; Czekay and Loskutoff 2009). Also, silencing of uPA or uPAR by siRNA inhibits MMP-9 secretion (Ahmad et al. 2009). We have shown that uPA/uPAR is involved in increased MMP-9 secretion in *T. gondii* infected murine macrophages (Schuindt et al. 2011). In the present work, we showed that CD147 is highly expressed at the site of entry and at one of the secondary sites of *T. gondii* dissemination. CD147 controls MMPs at the membrane level by favoring the cleavage of zymogens into active enzymes (Zhu et al. 2014; Grass and Toole 2015; Muramatsu 2016). Besides its role in protein-protein oligomerization, CD147 seems to be essential to MT1-MMP and MMP-9 synthesis, since its knockdown in monocytes by siRNA inhibits the expression of these molecules in response to *Chlamydia pneumoniae* (Schmidt ET AL., 2006). It has been shown that CD147 localizes at invasive structures called invadopodia, and its overexpression is able to bring non-transformed breast epithelial cells to an invasive phenotype (Grass et al. 2011). It is proposed that CD147 and LAT1 form heterotypic and homotypic interactions, especially

important for blood-brain barrier transmigration of leukocytes (Young et al. 2016) and regulation of innate response to pathogens at intestinal mucosa (Qiao et al. 2020).

In this chapter, we showed that MMP-9 and CD147 are overexpressed in oral infected mice, at the site of entry, the ileum and at deeper tissues during early dissemination, such as the lungs, as summarized in Figure 8. To our knowledge, this is the first time that CD147 is shown as part of the response to *T. gondii* infection.

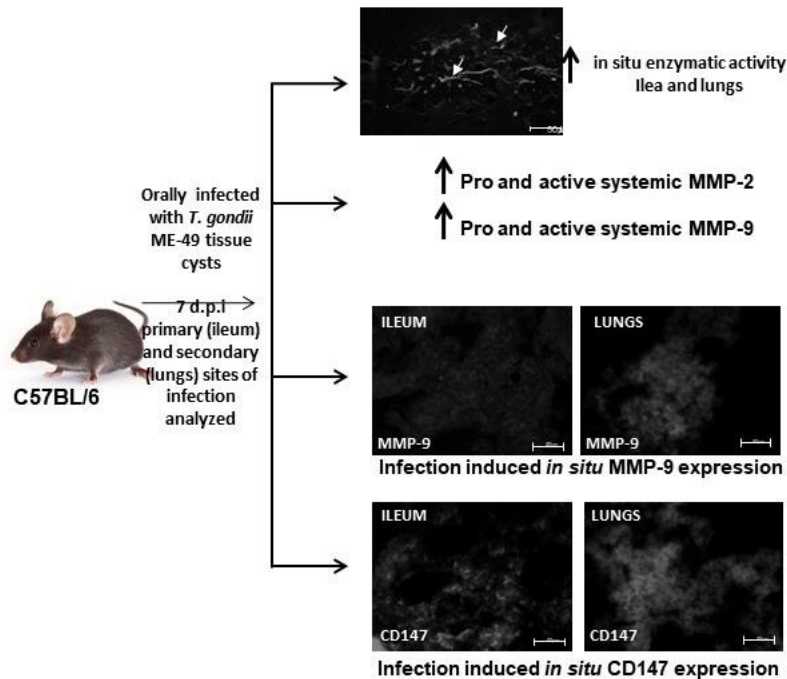


Figure 8. Increased expression of MMP-9, MMP-2 and CD147 at site of entry and early dissemination upon oral murine infection with *T. gondii*. C57Bl/6 mice orally infected with 50 cerebral tissue cysts show increased enzymatic activity at ileum, augmented circulating zymogen and active forms of MMP-2 and MMP-9. Expression of MMP-9 and CD147 at ileum is observed only in infected mice. Expression of MMP-9 and CD147 is also induced upon infection at distant sites of infection, lungs representing here the dissemination of *T. gondii* to deeper tissues.

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Chapter 10

**OVER THE TOP:
ARMWRESTLING BETWEEN *TOXOPLASMA GONDII*
AND HOST SKELETAL MUSCLE TISSUE**

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ABSTRACT

Toxoplasma gondii is a worldwide-occurring parasite that infects all nucleated cells and numerous hosts. Among the different tissues infected by this parasite, muscle tissue requires special attention, as it is critical for *T. gondii* host permanence and transmission. However, beyond simply comprising a route for tissue cyst consumption, muscle cell interaction with this parasite is relevant for tachyzoite to bradyzoite stage conversion. On the other hand, *T. gondii* infection can cause myositis, both in immunocompetent or immunocompromised hosts, as well as malformations in congenitally infected embryos and post-natal myogenesis and tissue regeneration down-regulation or interference. The muscle-*T. gondii* interaction is unique, and this chapter aims to describe this important biological event in a context of intracellular *T. gondii* parasite development.

Keywords: *Toxoplasma gondii*, congenital toxoplasmosis, skeletal muscle, myogenesis, myocytes, myokines

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1. INTRODUCTION: MUSCLE TISSUE. BIOLOGICAL CHARACTERISTICS

The skeletal muscle (SkM) tissue, one of the largest muscles in the body, is essential for locomotion and strength and is considered an endocrine organ (Pedersen and Febbraio 2008). The SkM is very well organized, comprising myofibers surrounded by connective tissue and a complex differentiation process. Groups of fibers are involved by the perimysium and the entire muscle is associated with the epimysium (Frontera and Ochala 2014). Myofibers are large post-mitotic multinucleated cells with contractile capacity. The motor unit is the junction of a motoneuron and the associated muscle fiber that, in response to a certain stimulus, will execute movement (Heckman and Enoka 2012). For contraction, muscle fibers display very well-organized structures, termed sarcomeres, within myofibrils. These contractile units contain mostly (but not only) two proteins, actin and myosin. The actin-myosin interaction is ATP-dependent and results in muscle movement. Many other proteins are present in sarcomeres involved in the contractile process, such as tropomyosin, titin, nebulin and desmin. Sarcomeres consist in units from one Z line to another, with a central M line as visualized in electron microscopy. A dark A band in contrast to the light I band is also observable (Frontera and Ochala 2014).

SkM fibers differ from each other regarding metabolism, contractile speed and calcium processing, and are named according to their myosin heavy chain isoforms (Westerblad et al. 2010). Type I fibers comprise the slow-twitch type, rich in mitochondria and displaying a predominant oxidative metabolism (Spangenburg and Booth 2003), which makes them more resistant to fatigue. Type II fibers (IIa, IIb and IIx) are considered fast-twitch types, although type IIa exhibits an intermediate profile. These fibers present fast contraction and oxidative capacity and are relatively resistant to fatigue (Spangenburg and Booth 2003). Type IIb is expressed in rodents, but not in humans (Smerdu et al. 1994). Type IIb/IIx fibers contain less mitochondria and display a lower oxidative capacity, relying on anaerobic pathways (Westerblad et al. 2010). They also, however, exhibit high glycolytic enzyme and myosin ATPase activity and are more prone to fatigue than slow-twitch fibers (Spangenburg and Booth 2003).

Myogenesis is a highly complex process regulated by numerous factors. During embryonic myogenesis, the expression of certain transcription factors *Paired box genes*, Pax3 and Pax7, initiates muscle commitment, followed by the expression of Myogenic Regulatory Factors (MRFs) (Zammit 2017). Pax3 is essential in early embryonic myogenesis, present since the pre-somitic mesoderm, while Pax7 acts during perinatal development (Tajbakhsh 2009). Pax7 remains activated in all quiescent satellite cells, whereas Pax3 appears to be present only in certain muscle types (Buckingham and Relaix 2015). After dermomyotome formation, MRFs act in promoting differentiation. The MRF family is formed by MyoD, Myf5, myogenin and Mrf4 helix-loop-helix transcription factors. Myogenic cells expressing MRFs Myf5, Mrf4 and MyoD migrate to limb formation sites (Buckingham and Relaix 2015). The process continues with the differentiation of progenitor cells into myoblasts, committed muscle cells that are still able to proliferate. Myoblasts then differentiate into myocytes, which are able to fuse (and no longer divide) and form myotubes and myofibers (Yaffe and Feldman 1965).

Regenerative myogenesis is dependent on satellite cells. Described in 1961, satellite cells comprise stem cells adjacent to myofibers committed to the muscular lineage (Mauro 1961). Although different subpopulations may exist, they are able to proliferate and differentiate based on myogenic factor expressions in response to damage, following the myogenic program, as part of the population remains quiescent (Biressi et al. 2013). After injury, it is estimated that satellite cell proliferation occurs in five days, when myoblast differentiation has already begun (Tajbakhsh 2009).

Muscle tissue has been shown to be a secretory tissue (Pedersen and Febbraio 2008) that, in response to exercise or damage, can secrete a variety of cytokines and other molecules, termed myokines (Pedersen et al. 2003). Although a vast array of SkM-secreted molecules exists, some are especially noteworthy due to their systemic effects. Interleukin-6 (IL-6), for example, is an important myokine that affects muscle tonus, angiogenesis, inflammation pathways and the insulin metabolism, among others (Steensberg et al. 2000; Forcina et al. 2018).

2. MUSCLE AS A VEHICLE FOR *TOXOPLASMA GONDII* TRANSMISSION

Muscle tissue plays a significant role in the *T. gondii* life cycle and in its transmission. It is widely known that *T. gondii* preferably establishes cysts in post-mitotic cells, mostly neurons and muscle cells, in different tissues, *i.e.*, heart, SkM, tongue and diaphragm. One of the main forms of *T. gondii* transmission occurs through the ingestion of meat from a previously infected animal carrying tissue cysts, transferring the parasite to the next host. Carnivory is, therefore, an important route for all hosts, either in the wild, through hunting and predation, or due to human feeding habits, the parasite's intermediate hosts. Definitive hosts, felids, exhibit predatory behavior, and many other animal groups also display carnivore habits, thus enabling the spread of *T. gondii* transmission. In some countries, raw and undercooked meat consumption is responsible for most *T. gondii* infection cases, leading to either congenital or acquired toxoplasmosis (Wohlfert et al. 2017; Djurković-Djaković et al. 2019). Food-associated toxoplasmosis outbreaks have also been reported in several countries, such as Canada, Australia and Brazil (Tenter et al. 2000). Depending on cultural habits, this transmission route may be more or less relevant (Hampton 2015).

2.1. Commercial Importance of *T. gondii* Infection in Skeletal Muscle

As noted previously, meat consumption can be an important source of *T. gondii* infection. Therefore, it is expected that *T. gondii* infection in farm animals affects meat production and commercial meat values, due to either acquired infections leading to human or livestock contamination or congenital infections that cause animal losses and consequent financial losses to producers. Thus, *T. gondii* monitoring is paramount, although still under-reported in many countries.

Infection risks are also associated to regional meat consumption habits. In southern Italy, for example, a study on wild boars indicated a high prevalence of *T. gondii* tissue cysts in the masseter muscles, heart and brain of 23.8, 22.6 and 22% of the investigated animals,

respectively (Sgroi et al. 2020). The masseter muscle is used in southern Italy to prepare raw meat products, such as sausages and a local dish named *guanciale* and is, therefore, relevant as a potential infection source. Different genotypes were detected in the animals, where most (9/11) were atypical isolates, one was identified as a type II and another, as type III (Sgroi et al. 2020). Atypical genotypes must also be taken into account when evaluating infection risks. The high prevalence and variety of *T. gondii* genotypes in Brazil, for example, is well described (Dubey et al. 2012; Dardé et al. 2014). In the state of Rio de Janeiro, an assessment of naturally infected pigs indicated new genotypes with high infective potential (Frazão-Teixeira et al. 2011), and five novel genotypes were isolated from the hearts and brains of the assessed pigs (TgPgBr1–5). Four of the five isolated genotypes (TgPgBr1, TgPgBr2, TgPgBr3 and TgPgBr5) were revealed as highly virulent in mice (Frazão-Teixeira et al. 2011).

In another study conducted in Italy, 28.6% *T. gondii* muscle prevalence in sheep and 27.5% in goats were observed through antibody assessments. Molecular evaluations, however, detected *T. gondii* DNA in 5.9% of goats and 6.6% of sheep, with more infected adults compared to young animals (Gazzonis et al. 2020). All detected parasites comprised the type II genotype, the most abundant genotype in Europe. Different host species should be considered even within the same country, as different handling protocols and associated risks are noted for different animals, while parasite-host interactions can also differ between species.

In northern India, poultry is one of the preferred meats for consumption. *T. gondii* infection assessments in these animals indicate that different handling techniques results in prevalence rate variations (Thakur et al. 2020). Backyard poultry, for example, exhibited higher *T. gondii* prevalence (24.4%) compared to deep litter poultry (16.2%), and no infection was detected in caged poultry, while other regions in the same country exhibited higher poultry infection rates. In China, a similar study indicated higher infection predominance in free-range chickens (18.8%) compared to caged chickens (with 5.6%) (Xu et al. 2012).

Surveillance assessments are an important tool in the prevention of *T. gondii* transmission. In France, for example, some studies have been carried out to estimate *T. gondii* infection in ovine meat and prevent human contamination (Halos et al. 2010). One investigation, for example, took into account both national and imported sheep meat consumption, the latter representing a significant amount of total consumed ovine meat. The investigation revealed 15% infection prevalence in locally-produced lambs and 81% in adult animals, similar to imported meat infection rates, of 15% in lamb and 79% in adults (Halos et al. 2010), indicating no difference between imported and locally-produced meat, with a much higher overall prevalence in adult animals.

Experimental infection assays have been carried out to assess potential infections regarding commercial pig cut consumption in Brazil. Concerning six commercial cuts, *coppa* and tongue cuts were the most infected, as 73% of mice inoculated with *coppa* homogenates and 67% of mice inoculated with tongue homogenates developed infections (Alves et al. 2019). As pig meat is highly consumed in Brazil, a high risk of infection is associated with this type of meat. Sheep meat consumption in Brazil is also high. Our research group characterized the prevalence of *T. gondii* infection in sheep from the state of Rio Grande do Sul (Consalter et al. 2019), reporting a high prevalence of 70.2% when evaluating 319 sheep, indicating that all of the 14 evaluated herds contained *T. gondii*-positive animals. This prevalence was also assessed by different groups in various Brazilian states, *i.e.*, São Paulo,

Rio de Janeiro and Sergipe, reported at 24.2, 28.22 and 53.3% respectively (Ragozo et al. 2008; Mendonça et al. 2013; Cosendey-KezenLeite et al. 2014).

Congenital toxoplasmosis can be a threat to certain farm animal groups. In this regard, it has been suggested that pigs are resistant to congenital toxoplasmosis, whereas ovines are susceptible (Vargas-Villavicencio et al. 2016). In fact, congenital infection has been pointed as a major cause of abortion in sheep and goats (Sah et al. 2019), and a study carried out in Iran described the abortion and death of lambs 4 to 5 days after birth following congenital toxoplasmosis outbreaks (Movassaghi et al. 2016). In Bangladesh, a recent evaluation in aborted cattle, sheep and goats revealed *T. gondii* DNA in four sheep fetuses and three goat fetuses out of five from each specie, and no detection in two cattle fetuses (Sah et al. 2019). In Brazil, congenital ovine and caprine toxoplasmosis has been described in the states of Rio Grande do Sul and Pernambuco, with and without abortion cases (Dubey et al. 2012). Similar to human congenital toxoplasmosis, congenital infection in sheep and goats causes more losses during early pregnancy stages (Innes et al. 2009).

Cattle meat contains very low *T. gondii* DNA rates and viable parasites (Dubey 2010). A recent study analyzed samples from different European countries, i.e., Italy, the Netherlands, Romania and the United Kingdom, comparing their detection rates in the diaphragm muscle by magnetic capture quantitative PCR (MC-PCR) and by bioassays and antibody investigations in the liver (Opsteegh et al. 2019). An infection prevalence of 4.6% was detected by MC-PCR and 1.6% by bioassays. Different animals tested positive by different direct techniques, and no agreement between the serological and direct tests was observed. This lack of correlation was discussed as being due to the low performance of serological methods, which are, therefore, not recommended to indicate consumer infection risks. However, as direct detection methods are not executable on a large scale, the authors suggest the development of new screening strategies (Opsteegh et al. 2019).

3. TOXOPLASMOSIS AND MYOSITIS – CLINICAL EVIDENCE

More than an illness that threatens newborns and immunocompromised individuals, toxoplasmosis can also lead to polymyositis and myocarditis in immunocompetent subjects. Clinical reports have reported muscle tissue compromises in patients presenting different ages (Samuels et al. 1976; Matsubara et al. 1990; Paspalaki et al. 2001; Plonquet et al. 2003). The affected individuals generally displayed muscle weakness and myalgia, accompanied or not by myocardial compromising, as well as other features, such as weight loss, fever and hepatosplenomegaly. Since these symptoms are not *T. gondii*-specific and toxoplasmosis tests are not routine, one can only assume that many myositis cases caused by *T. gondii* infection are non-diagnosed and that cases are underestimated.

The first study directly correlating toxoplasmosis as a polymyositis cause dates from 1961 (Rowland and Greer 1961), 53 years after the first description of this protozoan. Although human toxoplasmosis and encephalomyelitis cases had been described since 1923 (Jankû 1923, Prague), parasite identification in muscle tissue as a tool for clinical diagnostics was only suggested in 1946, by Callahan, Russel and Smith. The term “toxoplasmic polymyositis” was only used in 1961, by Rowland and Greer. At that time, little or no muscle pain and polymyositis were associated with toxoplasmosis cases. Karasawa and others, for

example, presented a case of a housewife with polymyositis and other unspecific symptoms in 1981, but did not identify the condition as toxoplasmosis. This case, like many others described during the same period, were only confirmed as toxoplasmosis cases in *postmortem* analyses (Rowland and Greer 1961). Since then, some studies have focused on describing polymyositis and discussing its pathogenesis (Whitaker 1982; Hay et al. 1988). More recent studies have presented successfully resolved polymyositis cases after etiologic agent identification and specific therapy application (Cuomo et al. 2013; Hassene et al. 2008), reinforcing the need for toxoplasmosis screening in similar patients.

Congenital toxoplasmosis is a relevant disease manifestation with a high destructive potential. Myositis and myocarditis, for example, have been identified in a case reporting a congenitally-infected baby presenting various visceral lesions (Garcia et al. 1985). Initial studies have also demonstrated myositis in a murine congenital toxoplasmosis model (Hay et al. 1988). Muscle pain and weakness have been well described in immunocompromised individuals, caused either by acute infection or tissue cyst reactivation (reviewed by Wohlfert et al. 2017). A pioneer study in the field described myositis in AIDS patients, demonstrating the presence of *T. gondii* cysts in muscle during biopsies (Gherardi et al. 1992). As in other *T. gondii*-myositis related assessments, that study also suggested parasite identification in patients presenting multiorgan dysfunction of unknown origin and high serum creatine kinase levels. It is important to note that toxoplasmosis is considered a noteworthy food-borne disease to date, especially due to potential encephalic compromises.

4. TOXOPLASMOSIS AND MYOSITIS – MYOGENESIS AND REGENERATION

For the past decade, our research group and others have studied the effects of experimental *T. gondii* infection on SkM cells. We have demonstrated that *T. gondii* infection of primary SkMC cultures with the RH strain (Type I) is able to down-regulate M-cadherin expression, leading to molecular modifications in the host cell surface that interfere with membrane fusion and, consequently, affect the myogenesis process (Gomes et al. 2011). Accordingly, our experimental congenital infection results in mice indicate malformed fetuses with compromised muscle development, confirmed by histological analyses (Gomes and Barbosa 2017). Different malformation degrees were observed between littermates and the presence of immune infiltrating cells in muscle tissue was noted (Gomes and Barbosa, 2017). Myoblast infection prior to myogenesis induction can deregulate MRF expression, leading to defective differentiation (Vieira et al. 2019), comprising increased proliferation, smaller myotube formation, increased IL-6 and Monocyte Chemoattractant Protein-1 (MCP-1) secretion and a compromised Wnt/ β -catenin pathway. *T. gondii* stimulates the secretion of soluble factors, which can cause the same deleterious effects as those observed by direct SkMCs infection (Vieira et al. 2019). The same effect was demonstrated when a conditioned medium from infected rat myoblasts (L6 cell line) was used to treat fresh myoblasts, leading to altered proliferation status (Kim et al. 2016). It remains unclear which specific factors, *i.e.*, protein, microRNA, cytokine, among others, are responsible for these effects. However, the pro-inflammatory milieu induced by infection seems to be directly involved in myogenesis impairment.

In vivo *T. gondii* infection results in inflammation and muscle damage, disrupting macrophage responses to damage (Jin et al. 2017). M1 macrophages are known as proinflammatory macrophages and promote an inflammatory, non-resolutive stage. On the other hand, M2 are known as pro-regenerative macrophages. In response to damage, M1 macrophages first act in the acute phase, followed by a switch, where M2 macrophages exceed M1. These compounds play an important role in muscle regeneration and myogenesis. Mice infection by *T. gondii* impairs the macrophage switch between M1-M2 profiles, thus preventing tissue regeneration (Jin et al. 2018b). It has, however, been demonstrated that regulatory T cells (Treg)-derived factors can ameliorate inflammation and muscle damage after *in vivo* *T. gondii* infection (Jin et al. 2018b). Moreover, treatment with IL-10 and amphiregulin can improve muscle regeneration after *T. gondii* infection.

Chronic *T. gondii* infection also impairs muscle regeneration. Following induced damage, the muscle tissue from infected mice takes longer to regenerate, presenting reduced satellite cell proliferation and altered macrophage transcription heterogeneity (Jin et al. 2018a).

More recently, *T. gondii* infection has been shown as able to cause severe cachexia in mice experimental infection model (Hatter et al. 2018), with 20% body weight loss, including muscle mass wasting of the extensor digitorum longus, tibialis anterior, gastrocnemius and quadriceps muscles. Adipose tissue was also decreased in this model. The canonical cachexia cytokines IFN- γ , IL-1 β , TNF- α and IL-6 were also upregulated in infected animals. Interestingly, the infection also led to sustained commensal dysbiosis in the infected mice. The cachexia observed following *T. gondii* infection appears to be dependent of IL-1R pathway (Melchor et al. 2020), as infected IL-1R knockout animals exhibited improved body weight, less muscle loss and fibrosis, unrelated to parasite load reduction (Melchor et al. 2020). These studies highlight the importance of the immune component in *T. gondii* infection and SkM pathology.

This pathological profile can also contribute to locomotor abnormalities in infected mice, as it has been reported that the step lengths in both acute and chronically infected mice is different from control animals (Galván-Ramírez et al. 2019). Concerning SkM evaluations, half of the animals presented muscle damage, most displaying dystrophic calcification, in different degrees, and mild to focal myositis. In one of the cases a sarcoplasm cyst was identified (Galván-Ramírez et al. 2019). According to histological findings, myositis and myocarditis were more intense in chronically infected mice compared to those suffering acute infection. The authors called attention to the possible continuous liberation of bradyzoites from tissue cysts and bradyzoite-tachyzoite conversion with lytic cycle establishment, resulting in cell rupture, which could be responsible for decreased locomotion. *T. gondii*-infected mice also presented body weight decreases (Bortolini et al. 2016; Jin et al. 2017; Hatter et al. 2018; Melchor et al. 2020) and reduced food consumption (Hatter et al. 2018; Melchor et al. 2020), which was also observed by our group (Paloma C. Vieira & Daniel Adesse, personal observations). It is not clear whether these locomotion pattern changes are associated to diminished food consumption. However, nighttime activity is also reduced in infected animals (Melchor et al. 2020). These observations bring a different perspective to previously reported locomotor changes in *T. gondii*-infected mice (Gullinelo et al. 2008), and reinforce the role of SkM pathology in acquired toxoplasmosis.

T. gondii-induced locomotion infection effects can aid in understanding some functional impairments that may affect infected individuals, and an increasing number of studies have indicated that exercise can improve memory, anxiety, diabetes, rheumatic diseases and

Alzheimer's disease, among others (Benatti and Pedersen 2015; Leal et al. 2018; Lourenço et al. 2019). Bortolini and collaborators demonstrated the effects of two distinct exercise protocols in *T. gondii* infection cases (Bortolini et al. 2016), demonstrating that a combination of strength and aerobic exercise prior to *T. gondii* infection can increase animal survival. The authors also verified that exercised animals displayed a stronger IFN- γ response to a *T. gondii*-soluble tachyzoite antigen (STAg) at different concentrations (50, 25, and 10 $\mu\text{g mL}^{-1}$) and a higher IFN- γ / IL-10 ratio (Bortolini et al. 2016). The infection was also shown to decrease exercise performance, even during the chronic phase (Bortolini et al. 2016). A previous assessment using swimming as an aerobic exercise beginning on day 1 of *T. gondii* infection did not identify mice survival alterations (Chao et al. 1992). However, weight and appetite improvements were observed with exercise. Additionally, TNF levels increased by infection were attenuated with exercise (Chao et al. 1992). These results indicate that exercise could act as a tool to improve deleterious *T. gondii* effects. This is a promising study area, as exercise can induce the secretion of a series of immunologic effectors and acts in attenuating inflammatory-related diseases (Leal et al. 2018).

5. TOXOPLASMA – SKELETAL MUSCLE CELL INTERACTIONS

5.1. The Influence of Muscle on Parasite Stage Conversion

Tachyzoite-bradyzoite conversion is a critical step for the establishment of host latency, when tissue cysts are formed in host cells and the parasites become immunologically dormant (Jeffers et al. 2018). Many groups have investigated the molecular mechanisms that determine stage conversion and have applied different experimental approaches to increase *in vitro* cyst formation, including extracellular medium pH changes and the use of recombinant cytokines (reviewed in Ferreira-da-Silva et al. 2008). However, the host cell type may also influence parasite fate. Cystogenesis was first demonstrated in *in vitro* skeletal muscle cells in 2008, following infection by an avirulent strain Me49 (Guimarães et al. 2008). Later, cyst formation was demonstrated to a higher extent in myotubes, followed by fibroblasts (Ferreira-da-Silva et al. 2008). The virulent strain RH (type I) can undergo spontaneous stage conversion in skeletal muscle cells in *in vitro* conditions (Ferreira-da-Silva et al. 2009b). More recently, it was demonstrated that parasites in skeletal muscle cells and neurons express higher amounts of bradyzoite antigen 1 (BAG1) mRNA than those in fibroblasts and astrocytes (Swierzy et al. 2017). In addition, parasites in fibroblasts and astrocytes exhibit a higher expression of mRNAs from cell cycle regulators compared to skeletal muscle and neurons (Swierzy et al. 2017).

Parasite-host interactions can diverge within the same cell type during different differentiation stages. Tachyzoite replication appears to be higher in myoblasts than in fully differentiated myotubes, whereas bradyzoite conversion is higher in myotube infection cases (Gomes et al. 2011; Swierzy and Lüder 2015). In this case, the cell cycle withdrawal required for the myogenic process was identified as a trigger for stage conversion (Swierzy and Lüder 2015).

5.2. *T. gondii* Induces Host Cell Cycle Changes

Toxoplasma gondii is capable of remodeling the host cell cycle (Brunet et al. 2008; Molestina et al. 2008; Lavine and Arrizabalaga 2009; Kim et al. 2016). Trophoblast infection leads to cell cycle arrest in the G2/S phase (Brunet et al. 2008). Molestina and collaborators demonstrated an induction towards the S phase followed by G2/M arrest in infected fibroblasts (Molestina et al. 2008). Not only can the parasite modify the cell cycle in infected cells, but secreted factors released by parasitized cells can also trigger the same effect (Lavine e Arrizabalaga 2009), as first demonstrated in fibroblasts. In myoblasts, exosomes secreted by *T. gondii*-infected cells also led to an arrest of the S or G2/M phases (Kim et al. 2016). *T. gondii* effector proteins have been identified as responsible for host gene expression manipulation (Hakimi et al. 2017). ROP and GRA proteins, secreted by rhoptries and dense granules, respectively, are especially relevant (Hakimi et al. 2017). Numerous novel roles are routinely discovered concerning the functions of these proteins (Rastogi et al. 2020). For example, GRA16 has been implicated in host cell cycle regulation, positively modulating host proteins involved in cell cycle progression and p53 regulation (Bougdoor et al. 2013). On the other hand, it was recently demonstrated that ROP16 kinase could silence the cyclin B1 gene promoter (Sabou et al. 2020). Different pathways may, therefore, be involved in cell cycle manipulation by *T. gondii*.

Interestingly, infection can also differentially alter the transcriptional profile of each cell type (skeletal muscle, fibroblasts, neurons and astrocytes) 24 hours after infection (Swierzy et al. 2017). Even with a generic infection profile, the *T. gondii*-host interaction is very particular, and this specific interaction will determine the parasite's fate.

5.3. Mitochondrial Interaction

Since the description of the molecular mechanisms by which *T. gondii* recruits host cell's mitochondria to parasitophorous vacuole (PV) proximity (Pernas et al. 2014), the relationship between the parasite and this organelle has been further explored. SkM is one of the richest mitochondria sites, and this organelle plays an essential role in energetic metabolism, which makes this an interesting model to explore the *T. gondii*-mitochondria interaction phenomenon. The *in vitro* association between mitochondria and PV has been described in different cell types and was initially suggested as mediated by *T. gondii* ROP2 (Sinai and Joiner 2001; Sinai 2008). However, a subsequent study demonstrated that mitochondrial association was independent of ROP2/8 (Pernas and Boothroyd 2010). More recently, Pernas and collaborators demonstrated that MAF1 is responsible for mitochondria recruitment (Pernas et al. 2014).

Concerning SkM, our group proved that the PV membrane could be associated with mitochondria both directly and through endoplasmic reticulum mediation (Figure 1, de Oliveira, 2014, dissertation). This event has been observed in both tachyzoite-bearing vacuoles and bradyzoite-bearing cysts, with a radial distribution in the latter (Andrade et al. 2001; Guimarães et al. 2008; de Oliveira 2014; Gomes et al. 2014). This interaction seems to be also involved with the cystogenic process (Guimarães et al. 2008; de Oliveira 2014). In one study, ISA-34, a compound with high affinity for cytochrome bc1 and with an inhibitory

effect on oxidative phosphorylation, was used to treat infected SkMCs. The applied treatment induced a drastic reduction in cyst formation (66%) and maintenance (72%), indicating the important role of the host's metabolism in cystogenesis (de Oliveira 2014). As mitochondria play important roles in muscle metabolism and regeneration, it seems likely that this relationship may directly impact muscle processes and physiology. However, no knowledge if this association is associated or not to *T. gondii* myogenesis and myositis effects is available to date.

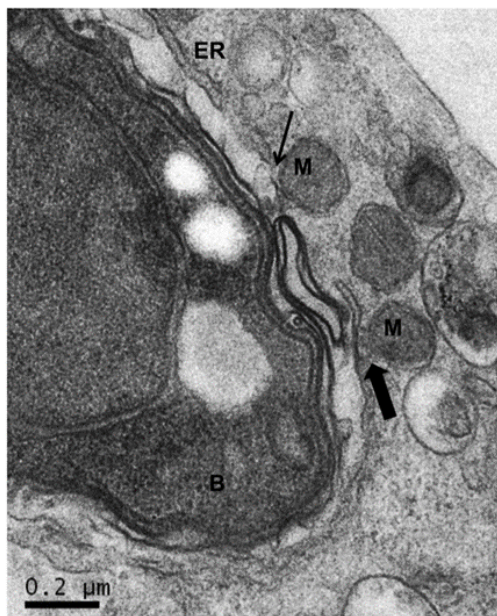


Figure 1. Mitochondria-parasitophorous vacuole association in SkMC. The mitochondria (M) can be associated both directly (thin arrow) or indirectly (thick arrow) with a parasitophorous vacuole containing a bradyzoite form (B) of the Me49 strain. In the latter case, the association is mediated by the endoplasmic reticulum (ER). Reproduced from de Oliveira (2014).

5.4. Structural Skeletal Muscle Cell Alterations

Infected SkMCs, as well as other cell types, present cytoskeleton organization modifications induced by *T. gondii*. During cyst formation, the host cell organelles are reorganized in fibroblastic cell lines (Paredes-Santos et al. 2018). The parasite stage can also influence cytoskeleton protein rearrangement in muscle cells. Myoblast infection results in β -tubulin remodeling near the cyst wall, although this was not observed when myotubes were infected (Garcia 2016). Microtubules were one of the first cytoskeletal filaments described around the PV during *T. gondii* infection in muscle cells (Andrade et al. 2001). The parasite, thus, seems to sequester growing microtubules and microtubule-organizing centers (Walker et al. 2008), and it has been suggested that microtubule recruitment and association with PV could prevent phagolysosomal fusion, thus protecting *T. gondii* parasites (Andrade et al. 2001).

As stated above, M-cadherin is also affected by *T. gondii* muscle cell infection. This junctional protein is anchored on the cytoplasmic side by catenins and connected to actin microfilaments and microtubules (Meng and Takeichi 2009; Wang et al. 2013). Our group has demonstrated that infection of primary SkMCs decreases M-cadherin expression and leads to protein disorganization in infected cells, with the presence of cadherin aggregates in different cell sites (Gomes et al. 2011).

Plectin is an important protein for cytoskeleton stability, since it can bind to desmin intermediate filaments and organelles, stabilizing these connections and positioning organelles as required for cell integrity maintenance (Castañón et al. 2013). This protein appears to be subtly rearranged surrounding the cyst wall and parasitophorous vacuole in both myoblasts and myotubes (Garcia 2016). The *T. gondii* protein toxofilin can sequester actin molecules, binding to actin monomers *in vitro*, and also seems to participate in the rearrangement of the actin filaments of the *T. gondii* parasite itself as it invades the host cell (Poupel et al. 2000). This process may contribute to decreased polymerization of actin monomers and, therefore, the response of invaded cells (Czimbalek et al. 2015). Muscle cells, which are rich in actin filaments, may be directly affected by this activity. Actin filament disorganization was observed 24 hours following RH strain infection in primary skeletal muscle cultures (Gomes 2011). Myosin heavy chain expression is decreased when C2C12 cells are infected in the myoblast stage (Vieira et al. 2019). It is tempting to speculate that, collectively, these evidences may indicate that *T. gondii* has a detrimental effect on the entire contractile apparatus.

5.5. Autophagy and Host Cell Death

The relation between *T. gondii* infection and the host's autophagic process has not yet been thoroughly explored in SkM compared to other cell types. Our group has demonstrated that autophagy induced by rapamycin or nutrient depletion may inhibit *T. gondii* proliferation in primary mouse SkMCs (Souto et al. 2016). Furthermore, *T. gondii* may increase this process in SkMCs and take advantage of host cell autophagy components to replicate (Souto et al. 2016). An association of structures suggestive of autophagy (SSA) and the parasitophorous vacuole have also been described. On the other hand, other studies have demonstrated that *T. gondii* proliferation may impair host autophagy in HeLa cells (Lee et al. 2013). Conversely, RH strain parasites induce autophagy (and apoptosis) in human umbilical cord mesenchymal stem cells (Chu et al. 2017). The autophagic process in *T. gondii* infection may also affect stage conversion. Parasite autophagy induction by rapamycin has been proven to inhibit tachyzoite to bradyzoite conversion (Li et al. 2016).

Autophagy in SkM plays an important role in muscle homeostasis regulation. Removal of damaged cellular components is important for muscle function and energy supply. However, if dysregulated, as in certain conditions like cachexia, autophagy can be a serious problem, leading to muscle atrophy (Zhang et al. 2019). The AKT-mTOR (mechanistic target of rapamycin) signaling pathway, a known autophagy regulator, is essential for muscle regeneration and growth (Bodine et al. 2001; Dunlop and Tee 2014). The relationship between this pathway and *T. gondii* infection in muscle tissue is not yet understood and may be important to understand the effects of this infection.

Apoptosis manipulation by *T. gondii* infection is a controversial theme. On the one hand, some authors defend a protective effect caused by this parasite (Contreras-Ochoa et al. 2013; Wu et al. 2016), while, on the other, some assessments indicate apoptosis induction in the presence of *T. gondii* (Chu et al. 2017). Recently, we demonstrated that *T. gondii* infection in myoblasts is not capable of inducing apoptosis as revealed by the detection of activated caspase-3 (Vieira et al. 2019). From an evolutionary-based point of view, one may suggest that host cell death induction would not be beneficial for any parasite life cycle. However, parasite genotype variability, as well the host's genetic background, may partially explain the different infectivity and cytopathologic profiles observed to date.

5.6. Skeletal Muscle Cell Activation

T. gondii infection stimulates the secretion of immune mediators and can manipulate the host cell for its own survival. In this regard, our group has verified that infected SkMCs display increased interleukin-12 (IL-12), interferon-gamma (IFN- γ) and prostaglandin E₂ (PGE₂) secretion (Gomes et al. 2014). PGE₂ synthesis is stimulated by positive cyclooxygenase-2 (COX-2) expression modulation as described in macrophages, and may inhibit Th1 response, tumor necrosis factor (TNF) and nitric oxide (NO) synthesis (Peng et al. 2008; Gomes et al. 2014).

Lipid droplet (LD) biogenesis, increased in *T. gondii* infected SkMC from primary cultures but not in fibroblasts, plays a role in parasite survival (Gomes et al. 2014). LDs and the sarcoplasmic reticulum (SR) were found to be associated with the PV. Moreover, LD contents may be a source of lipids and other parasite nutrition components. Therefore, new approaches to eliminate the parasite using LDs as targets have been developed for several cell types. Using a feline renal epithelial cell line (CRFK), our group investigated whether fatty acid synthase inhibition would modulate *T. gondii* infection (Rosa 2019, dissertation). C75, a fatty acid synthase inhibitor was capable of inducing a discrete reduction in the replicative capacity in this model, but resulted in a drastic effect on cyst wall maturation and is a potential pharmacological candidate for chemotherapy (Alvarez-Rosa 2019, dissertation). Nolan and collaborators evaluated the effects of T863 treatment, a pharmacological inhibitor of acyl coenzyme A: diacylglycerol O-acyltransferase 1 (DGAT1), in infected cells, reporting that this treatment affects RH strain tachyzoites and leads to malformed Me49 strain cysts (Nolan et al. 2018). These findings can serve as a rationale for the design and prospection of anti-*T. gondii* drugs and therapies.

CONCLUSION

The study of SkM in toxoplasmosis is paramount due to several reasons. This chapter highlighted some of the most important aspects and proposed additional questions. As stated previously, this tissue is extremely relevant for *T. gondii* transmission and host survival. The parasite can affect SkMCs and their functions in different ways and, in turn, suffer differential effects, as the host cell state is also able to influence intracellular parasite fate. *T. gondii* infection effects on myoblasts and myotubes are summarized in Figure 2.

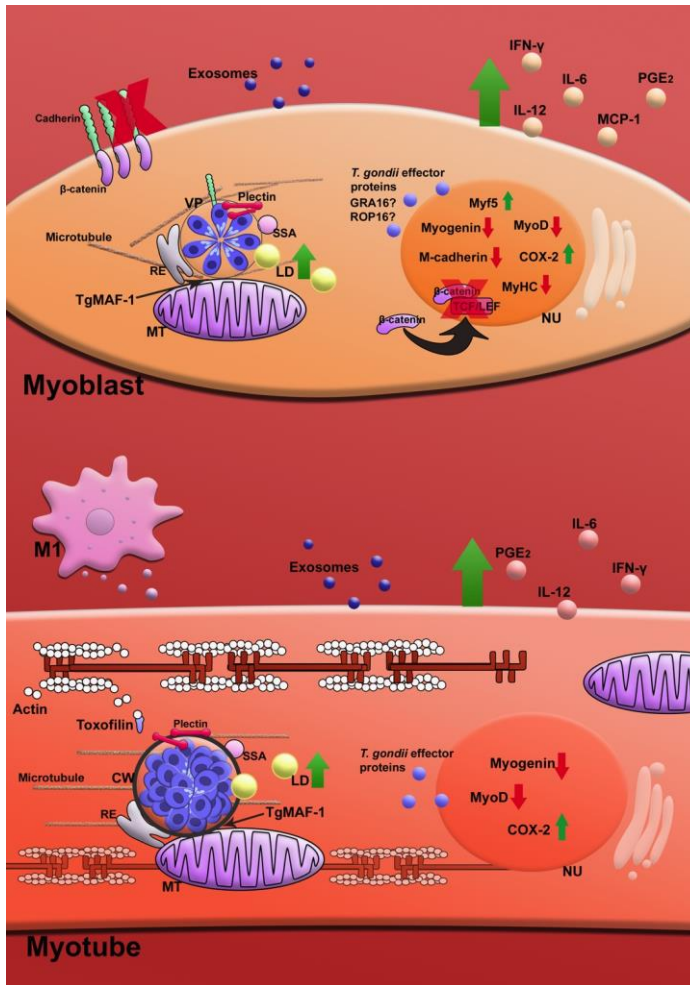


Figure 2. Schematic representation of the main findings describing how *T. gondii* infection affects myoblasts and myotubes. Myoblast infection stimulates COX-2 expression, increasing PGE₂ secretion. MRFs (Myf5, MyoD, MyoD and myogenin) are decreased, indicating myogenesis impairment; M-cadherin is disarranged and the Wnt/β-catenin signaling pathway is impaired. Intracellular parasites recruit host organelles including the endoplasmic reticulum (RE) and mitochondria (MT, either directly or through the reticulum) and lipid droplets (LD). Structures suggestive of autophagy (SSA) synthesis are stimulated and recruited to the vacuole. Cytoskeleton proteins are rearranged to surround the PV, including plectin and tubulin. Infected cells release exosomes that can induce paracrine effects in neighboring cells. Myotube infection alters the parasite transcriptome and stimulates tachyzoite-bradyzoite conversion, with cyst wall (CW) protein secretion, resulting in tissue cyst formation. Pro-inflammatory molecule secretion is stimulated by *T. gondii* in both myoblasts and myotubes, i.e., MCP-1, interleukin-6 and -12 (IL-6, IL-12) and IFN-γ. Recruitment of LD, SSA, plectin, microtubules, RE and MT have also been described in infected myotubes. Toxofilin recruits and disarranges actin molecules. Acquired infection leads to macrophage polarization towards a pro-inflammatory M1 profile, impairing muscle regeneration. Decreased myogenin and MyHC expression can contribute to muscle atrophy in infected tissue. N = nucleus.

Even with important advances concerning SkM-*T. gondii* interaction assessments, several questions remain unanswered, including the following:

1. What are the effects of *T. gondii* infection on muscle metabolism?
2. Is a relationship between exercise and infection in place regarding pathogenesis and parasite control, among others?
3. What is the role of *T. gondii* effector proteins in SkM remodeling?

ACKNOWLEDGMENTS

This study was supported by Instituto Oswaldo Cruz (Ações Estratégicas para o Desenvolvimento e Fortalecimento dos Laboratórios de Pesquisa do Instituto Oswaldo Cruz (PAEF), Fundação Oswaldo Cruz (Edital INOVA Geração de Conhecimento 2018, grant number 3231984391), Conselho Nacional de Pesquisa e Desenvolvimento Tecnológico (CNPq, grant numbers: 401772/2015-2 and 444478/2014-0 for DA), Fundação Carlos Chagas Filho de Amparo à Pesquisa do Rio de Janeiro (FAPERJ), Projetos Temáticos (grant number SEI-26 260003/001351/2020) and Redes em Saúde (grant number E-26-211.570/2019) for D.A and H.S.B. D.A. has support from FAPERJ – Jovem Cientista do Nosso Estado (E-26/201.336/2021) and H.S.B. Edital APQ1 (grant number E-26-210.310/2019).

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Chapter 11

CURRENT TREATMENT OF TOXOPLASMOSIS

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ABSTRACT

The research for a treatment for toxoplasmosis started after *Toxoplasma gondii* was recognized as a human pathogen in 1939. Several studies with different sulfonamides identified sulfadiazine as one of the most effective in mice models of toxoplasmosis. Soon later, studies with a series of 2:4-diaminopyrimidines identified pyrimethamine (Daraprim®) as the most effective drug for the treatment of toxoplasmosis so far studied. However, the most important discovery in the search for an effective treatment for toxoplasmosis came from the demonstration of the synergistic effect of sulfadiazine and pyrimethamine against *T. gondii*, providing then the first effective therapy for the treatment of this disease in humans in 1954. Currently, even after the introduction of other drug options for the treatment of toxoplasmosis, the combination of sulfadiazine and pyrimethamine remains the first-option therapy for this disease. This chapter will present the drugs and discuss the current therapies recommended for the treatment of different manifestations of toxoplasmosis.

Keywords: *Toxoplasma gondii*, Sulfadiazine, Pyrimethamine, acute disease

INTRODUCTION

Although first described in 1908 (Splendore 1908), *Toxoplasma gondii* was not recognized as a human pathogen until 1939. The infection in humans was first conclusively identified in the autopsy tissues from an infant who presented severe encephalomyelitis after few days after birth and died at the age of 31 days (Wolf, Cowen and Page 1939a; 1939b). A review of similar outcomes previously described in the literature and the subsequent diagnosis

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of new cases in infants showed that *T. gondii* was a common pathogen related to congenital infections (Janku 1923; Torres 1927; Richter 1936; Wolf and Cohen 1937; Sabin 1941; 1942). After the first conclusive demonstration of a congenital *T. gondii* infection in humans, Pinkerton and Weinman (1940) reported the first fatal case of acute toxoplasmosis in an adult from Peru. Some years later, another important clinical manifestation of the disease related to blindness (known as ocular toxoplasmosis) was reported (Hogan 1951; Wilder 1952).

After *T. gondii* was recognized as a human pathogen, the search for treatments for toxoplasmosis became urgent. Initial screenings with several known antimicrobials active in protozoan and bacterial infectious diseases, such as aureomycin, chloramphenicol, fumagillin and neomycin, have not resulted in the identification of a suitable promising drug for human treatment (Weiman 1952; Eyles and Coleman 1953a). However, in 1942, two independent studies from Sabin and Warren in the USA (Sabin and Warren 1942) and Biocca and Pasqualin in Brazil (Biocca and Pasqualin 1942) showed the *in vivo* effect of sulfonamides (sulfanilamide, sulfapyridine and sulfathiazole) against *T. gondii*. In 1946, Biocca and Nobrega showed the superior effect of sulfadiazine (SDZ; Figure 1) compared to sulfathiazole. The superior effect and safety of sulfadiazine were later confirmed by Eyles and Coleman (1953b). However, the use of sulfonamides in the clinics for the treatment of acute acquired toxoplasmosis did not result in benefits for patients (Kass et al. 1952; Sexton et al. 1953).

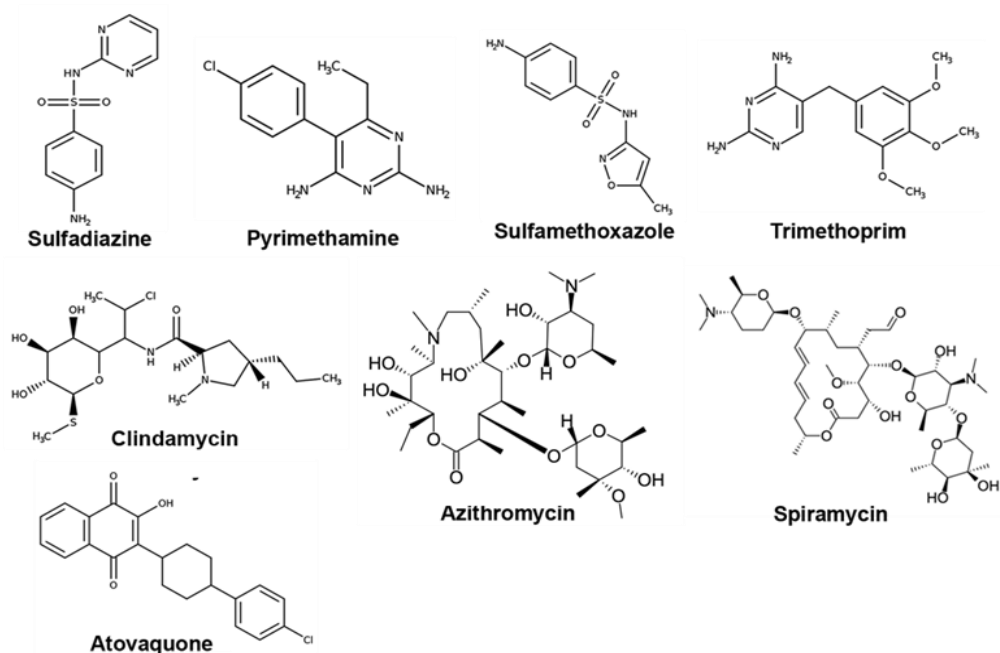


Figure 1. Drugs used for the treatment of Toxoplasmosis.

Studies with a series of 2:4-diaminopyrimidines developed by the Wellcome Research Laboratories identified the compound pyrimethamine (PYR; Figure 1) ((2:4-diamino-6-(p-chlorophenyl)-S-ethyl pyrimidine), known as trade name Daraprim®, as the most active against *T. gondii* (Eyles and Coleman 1952). However, PYR effective dose in animal models of toxoplasmosis was close to the maximally tolerated dose and the possible therapeutic use

of PYR was questioned (Eyles 1953). The most important discovery in the chemotherapy of toxoplasmosis came then from the demonstration of a strong synergism between PYR and SDZ (Eyles and Coleman 1953c). Compared to other tested drugs, the treatment with the combination of low doses of PYR and SDZ resulted in higher survival rates of infected mice (Eyles and Coleman 1953c). Daraprim® was approved for human use by the U.S. Food and Drug Administration (FDA) in 1953. Soon after, in 1954, the therapeutic efficacy of the combination of SDZ and PYR was demonstrated in patients who presented the ocular (Ryan et al. 1954) and acute systemic forms of the disease (Wettingfeld et al., 1956).

Posterior studies with macrolide and lincomycin antibiotics provided valuable options for the treatment of toxoplasmosis. The anti-*T. gondii* effect of the macrolide antibiotic spiramycin (Figure 1) in mice model of toxoplasmosis was first reported in 1956 by Bogacz and later confirmed by others (Garin and Eyles 1958; Bonaduce 1960; MasBakal and In't Veld 1965). Spiramycin showed a valuable effect in preventing the congenital transmission of *T. gondii* during pregnancy without showing side effects and causing any evidence of harmful effects on fetal development (Couvreur, Desmonts and Thulliez 1988; Chang and Pechere 1988) and it is still used for this condition (discussed below). Studies using the antibiotic lincomycin clindamycin (CLN; Figure 1) have shown its effectiveness in *vivo* models of ocular toxoplasmosis in mice and rabbits (McMaster et al. 1973; Tabbara et al. 1974). The efficacy of CLN in human ocular toxoplasmosis was confirmed (Tate and Martin 1977; Tabbara and O'Connor 1980). Besides, CLN also demonstrated efficacy for the treatment of toxoplasmic encephalitis in AIDS patients, becoming a second-line therapy for this condition (Dunay *et al.*, 2018). The advent of AIDS in the 1980s and the association of *T. gondii* as one of the main opportunistic pathogens, increased the need and interest in finding more options of active drugs, resulting then in the identification and introduction of macrolide antibiotics clarithromycin and azithromycin (Figure 1), the combination of trimethoprim (TMP) and sulfamethoxazole (SMX) (co-trimoxazole® or Bactrim®) and atovaquone (ATV) (Wellvone®, Mepron®) for the treatment of toxoplasmosis (Georgiev, 1994; Figure 1).

2. CURRENT TREATMENT OF TOXOPLASMOSIS

Since 1954 the therapy of choice for toxoplasmosis has been based on the combination of PYR and SDZ and this regimen is administered for all forms of the disease. PYR is the most effective drug against *T. gondii* infection and its administration combined with other drugs is used as a second option in cases of SDZ adverse effects by patients (Table 1). FDA recently approved the first generic formulation of PYR (FDA 2020), which might be an alternative to replace Daraprim® after its scandalous overpricing in 2015 in the USA. To reduce the potential side effects of PYR, the addition of folinic acid (FA) is recommended (Table 1). The combination of SDZ plus PYR plus FA is referred to as triple therapy. In the case of intolerances to SDZ, the replacement by CLN is preferable over other options in both immunocompetent and immunosuppressed patients (Dunay et al., 2018). Alternatively, SMX and TMP can be administered in regions where the SDZ-PYR regimen is not available but cannot be a substitute for the cases of sulfa intolerance (Goswami et al., 2014; Zhang et al., 2018). Treatment is usually administered for 4–6 weeks in immunocompetent individuals.

Table 1. Drugs and dosages used for the treatment of toxoplasmosis in immunocompromised and immunocompetent individuals^a

Regimen	Condition	Posology
SDZ plus PYR plus FA ^b (Triple therapy)	Immunocompetent individuals	<u>PYR</u> : 100-200 mg for 1-2 days, followed by 25 or 50 mg daily PO <u>SDZ</u> : 1g (< 60 kg) – 1.5g (≥ 60 kg) qid (total 4-6 g per day) PO <u>FA</u> : 10-20 mg daily PO
	Immunocompromised patients	<u>PYR</u> : 100-200 mg one day, followed by 50mg (< 60 kg) or 75 mg (≥ 60 kg) daily P.O. <u>SDZ</u> : 1g (< 60 kg) – 1.5g (≥ 60 kg) qid (total 4-6 g per day) P.O. <u>FA</u> : 10-50 mg daily
CLN plus PYR plus FA ^b	Immunocompetent individuals	<u>PYR</u> : 100-200 mg for 1-2 days, followed by 25 or 50 mg daily <u>CLN</u> : 300 mg qid <u>FA</u> : 10-20 mg daily
	Immunocompromised patients	<u>CLN</u> : 600 mg qid <u>PYR</u> : 200 mg once, followed by 75 mg daily <u>FA</u> : 10-50 mg daily
TMP-SMX	Immunocompetent individuals	<u>SMX</u> – 800 mg / <u>TMP</u> – 160 mg bid PO or IV
	Immunocompromised patients	<u>SMX</u> – 50 mg/kg / <u>TMP</u> – 10mg/kg/day in two divided doses PO or IV
TMP-SMX	Prophylaxis in HIV-infected person (including pregnant women)	-Adults: <u>SMX</u> – 800 mg/ <u>TMP</u> – 160 mg daily -Children (3-34.9 kg): <u>SMX</u> – 20-32 mg/kg/day <u>TMP</u> – 4 – 6.5 mg/kg/day
Atovaquone alone or plus PYR plus FA ^b	Immunocompetent individuals	<u>ATV</u> : 1.5g bid <u>PYR</u> : 100-200 mg (divided in two doses q12h) for 1-2 days, followed by 25 or 50 mg daily; <u>FA</u> : 10-20 mg daily
	Immunocompromised patients	<u>ATV</u> : 1.5g bid <u>PYR</u> : 200 mg once, followed by 75 mg daily <u>FA</u> : 10-50 mg daily
Atovaquone plus SDZ	Immunocompromised patients	<u>ATV</u> : 1.5g bid (total 3g per day) <u>SDZ</u> 1.5g qid (total 6g per day)
Azithromycin plus PYR plus FA ^b	Immunocompetent individuals	<u>AZT</u> : 250-500mg daily <u>PYR</u> : 100-200 mg for 1-2 days, followed by 25 or 50 mg daily; <u>FA</u> : 10-20 mg daily
	Immunocompromised patients	<u>AZT</u> : 1000 mg daily <u>PYR</u> : 200 mg once, followed by 75 mg daily <u>FA</u> : 10-50 mg daily

^aSource: WHO (2014); Brasil (2018); Dunay et al., (2018); Montoya (2018).

^bFolinic acid should not be substituted by folic acid. PO – oral administration; bid – twice a day; qid – four times a day.

In patients showing active cerebral toxoplasmosis, the treatment of choice is the combination of SDZ plus PYR, and in the cases of intolerance to sulfonamides, the replacement by CLN plus PYR is recommended (Katlama et al., 1996). Alternatively, SMX and TMP also showed efficacy and safe for cerebral toxoplasmosis and is an option in places where SDZ and PYR are not available (Goswami et al., 2014; Pellegrino et al., 2019). Some advantages of the combination of TMP and SMX compared to PYR and SDZ is that the first does not require the inclusion of FA, it is available in a single combined formulation and can also be administered intravenously.

In HIV-infected patients and others showing severe immunosuppression with CD4 lymphocyte counts $<100/\text{mm}^3$, prophylactic treatment with SMX and TMP is recommended to prevent the reactivation of *T. gondii* chronic infection (Table 1) (WHO 2014). The reactivation of the disease in these individuals shows a high index of morbidity and mortality. Thus, the treatment should be maintained until they become clinically stable, recover their immune status, and in the case of HIV patients, show viral suppression on antiretroviral therapy (WHO 2014). SMX and TMP combination is also effective for preventing *Pneumocystis carinii* pneumonia (PCP) in HIV-infected persons, making this regimen suitable as prophylaxis in these patients. In cases of intolerance to TMP and SMX, the administration of ATV is an option, as it is also effective against *P. carinii* (Hughes et al., 1993; Mendorf et al., 2015).

Pregnant women with gestational age <14 -18 weeks and with primary *T. gondii* infection should be treated with spiramycin (Table 2). PYR is potentially teratogenic and therefore cannot be used in the first trimester of gestation. Spiramycin accumulates in the placenta and acts in preventing *T. gondii* transmission to the fetus, but has a poor transplacental passage and does not treat the congenital infection (Dunay et al., 2018). The effectiveness of spiramycin in preventing fetal transmission seems to be limited to the early diagnosis of *T. gondii* infection and immediate initiation of anti-*T. gondii* therapy (ideally within three weeks of seroconversion) (Mandelbrot 2020; Picone et al., 2020). Treatment with SDZ and PYR plus FA is then recommended when the gestation age is ≥ 14 -18 weeks. The administration of Sulfadoxine-PYR (Fansidar®) plus FA could also be used (Peyron et al., 2019). Treatment is usually administered until the end of pregnancy; alternatively, SDZ plus PYR can be replaced or alternated with spiramycin when the fetal infection is ruled out (Table 2) (Montoya 2018). Compared to non-treated, infected fetuses treated with SDZ plus PYR show lower incidence and severity of clinical manifestations, such as retinochoroiditis and brain sequels (Avelino et al., 2014; Foulon et al., 1999; Mandelbrot 2020; Strang et al., 2020).

Table 2. Drugs and dosages used for the treatment of pregnant individuals and congenitally infected newborns^a

Regimen	Condition	Posology
SDZ plus PYR plus FA ^b (Triple therapy)	Pregnant women with primary or active infection Only if > 14 -18 weeks gestation	PYR: 100 mg for 1-2 days, followed by 50 mg daily SDZ: 1g every 6 or 8h (total 3-4g per day) FA: 10-20 mg daily Until the end of gestation
	Congenitally infected newborns	PYR: 1mg/kg q12h for two days, followed by 1mg/kg/day for 2-6 months and then 1mg/kg/day 3 times a week (Monday, Wednesday and Friday) until 1year SDZ: 50mg/kg q12h FA: 10mg 3 times a week (Monday, Wednesday and Friday)
Spiramycin	Only used in pregnant woman	1g (3 million units) every 8h in < 14 -18 weeks of gestation Alternatively to SDZ plus PYR until the end of gestation when fetal infection is ruled out
	Children presenting acute bone marrow toxicity due to PYR treatment	50 mg/kg q12h (Total 100 mg/kg/day)

^a Sources: Mitsuka-Breganó (2010); Dunay et al., (2018); Montoya (2018).

^b Folinic acid should not be substituted by folic acid.

Table 3. Drugs and dosages used for the treatment of Ocular Toxoplasmosis^a

Regimen	Posology
SDZ plus PYR plus FA	Same doses as in Table 1
SMX plus TMP	-PO SMX 800 mg + TMP 160 mg bid -Intravitreal (<i>off-label use</i>) TMP – 1.28-1.6 mg /SMX – 6.8-8 mg in a single dose ^b
CLN alone or combined	- Alone PO or IV: 300 - 600 mg q6h - Intravitreal: CLN 1mg plus DXM 400 µg in a single dose, weekly or biweekly - Combined with SDZ plus PYR plus FA (Quadruple therapy): Same as table 1 for triple therapy plus CLN 300 mg q6h PO - Combined with SDZ: same as table 1 for SDZ above for CLN - Combined with PYR plus FA: same as table 1 for PYR and FA and above for CLN. PO -Combined with SMX plus TMP: same as above. PO
Azithromycin alone or combined	-Alone: 500 mg/day PO -Combined with PYR plus FA: same doses shown in table 1 for PYR and FA and above for AZT. PO
Atovaquone alone or combined	-Alone: 1500mg twice daily and same doses shown in table 1 for PYR and FA (<i>off-label use</i>) -Combined with PYR plus FA: 1500mg ATV twice daily and same doses shown in table 1 for PYR and FA -Combined with SDZ: 1500mg ATV twice daily and same doses shown in Table 1 for SDZ

^a Sources: Cortés et al., (2019); Butler et al., (2012);

^b Choudhury et al., (2015); Souza et al., (2017).

The treatment of congenitally infected newborns with SDZ plus PYR and FA is recommended whether symptomatic or not at birth (reviewed in Dunay et al., 2018). The early treatment of both symptomatic and non-symptomatic newborns decreases the severity of existing symptoms or prevents disease recurrence and its consequent sequelae during the first year of life (reviewed in Dunay et al., 2018). A Brazilian cohort with 77 patients with confirmed congenital toxoplasmosis showed that children who initiated treatment within the first four months of life, especially within the first two months, were less susceptible to the late development of retinochoroiditis (Lago et al., 2020). In newborns and children, the treatment with SDZ plus PYR is usually well tolerated (Ben-Harari et al., 2017), but in cases of acute bone marrow toxicity, it should be replaced by spiramycin until recovery (Mitsuka-Breganó 2010) (Table 2).

For ocular toxoplasmosis, the treatment with SDZ plus PYR and FA is the classic therapy (Table 3). However, the combination of SMX plus TMP is also commonly used for this condition (Butler et al., 2012; Soheilian et al., 2005; Zhang et al., 2018). Long-term use of SMX plus TMP has proved to be an alternative to prevent recurrence of toxoplasmic retinochoroiditis (Fernandes Felix et al., 2016; 2020; Silveira et al., 2015). Other options for the treatment of ocular disease include SDZ, PYR, FA plus CLN (quadruple therapy), CLN alone or combined with TMP and SMX or SDZ, azithromycin and Atovaquone (Butler et al., 2012; Harrell and Carvounis 2014; Reis et al., 2015) (Table 3). In the case of ocular lesions, the prescription of systemic corticosteroids (mostly prednisone) may be associated with the treatment, however, should not be prescribed for immunosuppressed patients (Butler et al., 2012; de-la-Torre and Gomez-Marin, 2020). The treatment of ocular disease should be

maintained until the eye lesion is no longer active. Intravitreal treatment with CLN plus dexamethasone (DMX) is used when macule or optic disc are threatened or involved, or as an alternative when there is a limited response, or patients show intolerance to systemic therapy (Cunningham Jr et al., 2015; Zamora et al., 2015; Lasave et al., 2010; Soheilian et al., 2011).

3. MECHANISMS OF ACTION OF DRUGS USED FOR THE TREATMENT OF TOXOPLASMOSIS

3.1. Folic Acid Antagonists

The Folate pathway involves the sequential synthesis of dihydrofolate (folic acid) and tetrahydrofolate (folinic acid) by the enzymes dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR), respectively. The tetrahydrofolate is an important co-factor for the synthesis of deoxythymidine by the enzyme thymidylate synthase. Thus, inhibition of tetrahydrofolate synthesis directly affects cell proliferation (Rustum 2004; Martins-Duarte et al., 2020).

The synthesis of folates is the target of the drugs used as the first-line therapy for toxoplasmosis: SDZ and PYR. Indeed, folate synthesis is also the target of SMX and TMP. The sulfonamides, such as SDZ and SMX, are structurally analogous to the *para*-aminobenzoic acid (PABA) and act as competitive inhibitors of folic acid synthesis by the enzyme DHPS. PYR and TMP (2,4-diaminopyrimidines) mimic a region of pteridine in folic acid and are competitive inhibitors of the enzyme DHFR (van Bambeke et al., 2017). As sulfonamides and 2,4-diaminopyrimidines inhibit two sequential enzymes in the same pathway, the combination of both classes of drugs exerts a synergistic effect (Hitchings 1952; Georgiev 1994). While humans do not have DHPS and acquire folic acid from their diet, DHFR is essential to synthesize tetrahydrofolate in humans. FA administration decreases the adverse effects caused by the residual inhibition of PYR on humans' DHFR (Allegra et al., 1987; Hopper et al., 2019). However, FA is not required for trimethoprim administration.

3.2. Macrolides and CLN Antibiotics

Antibiotics inhibitors of prokaryotic protein synthesis are also active against *T. gondii*. Both macrolides (azithromycin and spiramycin) and CLN bind to the larger 50S subunit of bacterial ribosomes but act by different mechanisms. While azithromycin inhibits the growth of nascent peptides by binding in the polypeptide exit tunnel close to the peptidyl transferase center on the 23S rRNA (Jelic and Antolovic, 2016), spiramycin binds to the peptidyl transferase center and blocks the formation of the peptide bond and extension of the peptide chain, leading to dissociation of peptidyl-tRNA from ribosome (Brisson-Noel et al., 1988). CLN binds the 23S rRNA molecule of the 50S subunit and inhibits peptidyl transferase, blocking the transfer of the new amino acid onto the peptide growing chain (Kostopoulou et al., 2013). In *T. gondii*, macrolides and CLN inhibit the protein synthesis of the apicoplast, a non-photosynthetic secondary plastid that originated from the chloroplast of a red alga

engulfed by an apicomplexan ancestor (Beckers et al., 1995; Fichera and Roos 1997; Pfefferkorn and Borotz 1994).

3.3. Atovaquone (ATV)

ATV is a naphthoquinone derivative developed by Wellcome Research Laboratories for the treatment of malaria (Gutteridge, 1987). ATV is a structural analogue of coenzyme Q (ubiquinol) and exerts its effect inhibiting the electron transport chain in mitochondria. More specifically, the mechanism of action involves the inhibition of the cytochrome *bc1* complex (complex III), localized at the mitochondrial inner membrane. The complex *bc1* contains two active sites: one site oxidizes the ubiquinol (Qo) and the second reduces the ubiquinone (Qi). ATV binds to the oxidative site of ubiquinol between the domain Qo in cytochrome *b* and the iron-sulfur protein, inhibiting the oxidation of ubiquinol to ubiquinone (Kessl et al., 2003).

4. ADVERSE EFFECTS AND PROBLEMS RELATED TO THE TREATMENT

The occurrence of adverse effects is frequently reported for the drugs used for the therapy of toxoplasmosis. Administration of SDZ requires cautiousness, as about 3-8% of people are allergic to sulfonamides, and cannot be treated with SDZ or even SMX (Giles et al., 2019). Besides, SDZ is also commonly associated with several adverse effects, from mild such as skin reactions, nausea, vomiting and diarrhea, to severe, as kidney crystalluria, toxic epidermal necrolysis and Stevens-Johnson syndrome (Iaccheri et al., 2008; Arantes et al., 2017). SDZ should also be avoided for people with G6PD deficiency (Richardson and O'Malley 2020). PYR administration could cause gastrointestinal upset, dermatologic side effects, and bone marrow suppression, so blood cells count monitoring is advisable. FA administration minimizes PYR side effects. Another limitation of PYR is its teratogenic potential, which prevents its administration in the first trimester of pregnancy (Dunay et al., 2018). In AIDS patients the occurrence of adverse effects related to SDZ are more common, thus, the rate of treatment discontinuation is higher than in immunocompetent patients (Giles et al., 2019; Katlama et al., 1996).

In the case of allergy or discontinuation of SDZ and PYR treatment, the combination of PYR and CLN is the second option. The treatment with CLN and PYR also shows adverse effects but less severe (Katlama et al., 1996). The side effects related to CLN administration are usually skin reactions and gastrointestinal disorders (nausea, vomiting, anorexia and diarrhea). Allergy to CLN might also occur. CLN administration increases the risk of colitis due to *Clostridium difficile* infection and requires attention (Vardakas et al., 2016). Gastrointestinal upsets are also reported for azithromycin and atovaquone (Taylor and White 2004; Firth and Prathapan 2020). Azithromycin and spiramycin administration require attention, as macrolides are associated with QT interval prolongation and torsades de pointes. The risk of fatal heart rhythms during macrolides administration should be considered in patients with a prolonged QT interval (including congenital long QT), hypokalemia, hypomagnesemia, bradycardia, and patients with a history of torsades de pointes, arrhythmias, or uncompensated heart failure (Firth and Prathapan 2020).

In addition to the adverse effects, the current treatment of toxoplasmosis only treats the acute phase of the disease and does not eradicate the chronic infection, which persists as a potential risk of reactivation for the whole life of infected individuals.

CONCLUSION

For almost 70 years, the combination of SDZ and PYR remains the first-line therapy for toxoplasmosis. Although effective in the acute phase of the disease, this therapy is associated with several side effects leading to a high index of abandon, mainly in AIDS patients. Besides, treatment with SDZ and PYR does not eradicate chronic infection. As substitutes for SDZ and PYR, there are few options, which are also associated with side effects and are also ineffective against the chronic phase of the disease. Thus, future studies in the search for new treatments for toxoplasmosis should focus on new drugs with low toxicity and efficacy for both the acute and chronic phases of the disease.

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Chapter 12

ADVANCES IN THE DEVELOPMENT OF TREATMENTS FOR THE CHRONIC PHASE OF TOXOPLASMOSIS

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ABSTRACT

It is estimated that one-third of the world population is chronically infected with *Toxoplasma gondii*. Even after 80 years of the conclusive identification of *T. gondii* as an important human pathogen, there is no available treatment for the chronic phase of toxoplasmosis. In general, chronic infection is considered harmless and without risk for most immunocompetent individuals, however, it is a major concern in immunocompromised patients due to the potential emergence of serious outcomes, such as toxoplasmic encephalitis. Indeed, recent evidence point that, even in immunocompetent individuals, the parasite persistence in the host organism leads to relevant negative changes in the central nervous system (CNS) functioning. The growing concern that both immunocompromised and immunocompetent individuals may suffer the deleterious effects of long-lasting *T. gondii* infection, makes the search and discovery of active drugs for the treatment of chronic toxoplasmosis necessary. This chapter will present the advances and improvements in experimental models *in vitro* and *in vivo* used in the discovery of compounds with activity against encysted bradyzoites, and highlight targets and compounds recently identified as promising for the development of future treatment for latent toxoplasmosis.

Keywords: *Toxoplasma gondii*, Toxoplasmosis, Latent phase, Bradyzoite

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1. INTRODUCTION

An effective treatment against the latent/chronic phase of toxoplasmosis has been advancing slowly, mainly due to the complexity of *T. gondii*. The establishment of the chronic phase involves the conversion of the fast-growing stage tachyzoite (responsible for the acute phase of the disease) into the slow-growing stage bradyzoite, which is sheltered by a cyst wall (Saeij and Frickel 2017). The cyst wall is believed to limit the parasite recognition and elimination by the immune system and protect the parasite from access to drugs (Ildiko Rita Dunay et al. 2018; Hill and Dubey 2002; Weiss 2000). For the active stage of *T. gondii* infection, the combination of pyrimethamine and sulfadiazine can control the proliferation of the parasite, however, so far, this combination, or any other drug approved for the treatment of toxoplasmosis, is not capable of eliminating the latent form of the parasite (Moncada and Montoya 2012; Konstantinovic et al. 2019).

Until recently, it was believed that in general, the chronic infection was clinically innocuous, especially immunocompetent individuals without a medical history of toxoplasmosis symptoms. However, due to the parasite neurotropic characteristic, recent evidence point that, even in immunocompetent individuals, the parasite persistence in the host organism leads to relevant negative changes in the CNS functioning. A positive correlation between *T. gondii* and schizophrenia or depressive disorders, for example, was suggested (Stock et al. 2017; Dalimi and Abdoli 2012). Moreover, the reactivation of chronic toxoplasmosis in the CNS (toxoplasmic necrotizing encephalitis) is a concern in immunocompromised patients, as it is one of the main causes of morbidity and mortality in these patients. The growing concern that both immunocompromised and immunocompetent individuals may suffer the deleterious effects of long-lasting *T. gondii* infection, makes the search and discovery of active drugs for the treatment of chronic toxoplasmosis necessary.

The search for effective compounds against the chronic stage of toxoplasmosis is a difficult task, as some fundamental characteristics of bradyzoites include the lower permeability of cyst wall compared to tachyzoite parasitophorous vacuole membrane and a considerably slower metabolism and proliferation when compared to tachyzoite (Tu et al. 2019; Jeffers et al. 2018). The differences among parasite stages explain the lack of activity of the current drugs against the chronic disease, as they act directly in inhibiting the metabolism and proliferation of *T. gondii*. Other limitations that possibly hampered the study and identification of selective compounds against bradyzoite were the lack of good *in vitro* models for drug screening and the limited knowledge about the biology and specific proteins essential to the bradyzoite stage. However, recently the development of *in vitro* models, as well as the growing knowledge of essential proteins for the development and viability of bradyzoite has provided opportunities for the discovery and development of selective compounds directed to the chronic infection of toxoplasmosis.

This chapter will address the advances and improvements in the *in vitro* and *in vivo* experimental models used in the discovery of compounds with activity against encysted bradyzoites, and highlight targets and compounds recently identified as promising for the development of future treatment for latent toxoplasmosis.

2. EXPERIMENTAL MODELS FOR STUDYING THE CHRONIC PHASE OF TOXOPLASMOSIS

The development and use of *in vitro* and *in vivo* models of *T. gondii* infection have become alternatives in an attempt to mimic the infection that occurs in humans and enable the discovery and selection of promising new drugs for the treatment of toxoplasmosis.

2.1. *In Vitro* Models

The experimental use of animals has been raising ethical questions about the quantity and the resulting death of them in the experiments. Besides, the use of animals does not provide the necessary throughput for large-scale evaluation of compounds. Thus, the development of *in vitro* models that reproduce, even partially, the *in vivo* conditions would reduce the use of animals in drug testing and also the cost and time of preliminary studies of new drugs, especially in the pre-clinical trials phase (Kolesnikova and Moiseeva 2017). Besides, *in vitro* methods could also be a complement to *in vivo* assays.

Mimicking *in vivo* conditions in an *in vitro* model presents several challenges. The formation of cyst *in vivo* is influenced by the *T. gondii* strain, the type of host and its immune response, thus both the cell line and the parasite strain should be carefully chosen (Ghanemi 2015). A review carried out by Montarezi and col. showed that between the years 2006-2016, the main cell types used to evaluate therapeutic approaches in the context of toxoplasmosis involved: Human Foreskin Fibroblast (HFF), Rhesus Monkey Kidney Epithelial Cells (LLC-MK2), African green monkey kidney carcinoma (Vero), human cervix carcinoma (Hela), mouse macrophage cell line (J774A.1) and fibroblasts MRC-5 (Montazeri et al. 2017). Concerning *T. gondii* strains, during the years 1987 to 2017, ME49 (type II), Prugniald (type II), EGS (atypical) and VEG (type III) strains were the most used in *in vitro* experiments (Montazeri et al. 2018).

A few years ago, due to the lack of *in vitro* models for the chronic phase, researchers suggested an *ex-vivo* model as a method for evaluating the activity of new compounds against cysts. This model consisted of isolating cysts from the brain of chronically infected mice and incubating them *in vitro* with compounds for different periods of hours. The effect of compounds on cyst viability after the *ex-vivo* assay was evaluated through morphological analysis and the inoculation of the treated cysts in a new mouse (Huskinson-Mark, Araujo and Remington 1991; Ferreira et al. 2012). However, the *ex-vivo* model departs considerably from the natural condition of the infection, since the cysts are exposed directly to the compounds. In the *in vivo* environment, the drug must cross the host cell membrane to reach the intracellular cyst. Besides, an *ex-vivo* assay would demand a considerable amount of animals to obtain cyst, limiting the large-scale screening of compounds.

Studies that evaluated the effect of external conditions in triggering the *in vitro* interconversion between acute and chronic phases of *T. gondii* contributed to the establishment of an *in vitro* model to study the chronic phase of toxoplasmosis. The incubation of infected cells with a high temperature (43 °C) or with an alkaline pH medium (pH 8.2) resulted in the increase of cysts-like structures in fibroblasts cultures and other cells type. Besides, the use of nitric oxide donors or cytokines as gamma interferon also

contributed to *in vitro* cystogenesis in macrophages culture (Soête, Camus, and Dubrametz 1994; Böhne, Heesemann, and Gross 1994; 1993). However, the rate of conversion showed to be strain-dependent and low-virulent strains, such as ME49, showed a higher rate of cyst formation *in vitro* than virulent strains, such as RH (Soête, Camus, and Dubrametz 1994).

The use of the alkaline condition (pH 8.2) has been widely explored for the induction of cystogenesis *in vitro* and the evaluation of drugs against the chronic stage of toxoplasmosis. Di Cristina and col. have demonstrated that 16h after *T. gondii* infection in HFF culture, the incubation with a limited CO₂ and alkaline (pH 8.2) cell medium induced a high rate of bradyzoite conversion (Di Cristina et al. 2017). Drug screening using this approach should initiate 7-days after the induction and can be studied for 1-3 weeks after infection (Mayoral et al. 2020). For this experiment the use of the HFF cell-type is required, since these cells stop growing after the monolayer formation, allowing more time to cultivate and better experiment control.

The *in vitro* characterization of the atypical strain EGS also contributed to a valuable model for the study of cyst biology and the screening of molecules against bradyzoites. EGS strain showed a considerable rate of spontaneous conversion to bradyzoite in different cell types, such as LLC-MK2, HFSF and HFF fibroblasts, and C6 glial cells. Cultures infected with the EGS strain could be maintained for two months or more, functioning as a tool for long-term experiments on cyst formation and development (Paredes-Santos et al. 2013; 2018). The construction of an EGS strain expressing stage-specific fluorescent proteins (tachyzoites express RFP and bradyzoites express GFP) (Paredes-Santos et al. 2016) helped to improve the use of this strain as a model for the development of a high-throughput screening assay. The use of the stage reporter EGS strain allows rapid real-time quantification of the tachyzoite and bradyzoite load in just one assay (M. McPhillie et al. 2016; Portes and De Souza 2019).

A high rate of spontaneous differentiation *in vitro* has also been observed in cells for which the parasite has tropism *in vivo*, such as CNS cells and the cardiac and skeletal muscle cells (Ferreira Da Silva et al. 2008; Paredes-Santos et al. 2013). The current knowledge of *T. gondii* infection is largely based on two-dimensional (2D) cell cultures. However, 2D cell cultures may not recapitulate the main infection conditions *in vivo*, as they introduce artificial pressures and tensions, which can later alter infectious processes that depend on spatiality, for example, invasion, replication, and egress (Danielson et al. 2018). To avoid these two limitations (non-human cell and 2D culture utilization), recently, Corrêa Leite and col. explored a methodology for culturing *T. gondii* in an *in vitro* three-dimensional CNS model derived from human pluripotent stem cells (iPSC), named BrainSpheres. In this system, many aspects of *T. gondii* infection in the human CNS, such as cyst formation, neural cell death, alteration in neural gene expression and the triggering of release of several inflammatory mediators, could be mimicking (Corrêa Leite et al. 2020). Thus, this system constitutes an interesting model for new chemotherapies evaluation of against quiescent phase of toxoplasmosis.

2.2. *In Vivo* Models of Chronic Infection

In vivo models are essential tools for understanding the processes that occur during *T. gondii*-host interaction, such as parasite cell cycle, host immune response, parasite

evasion/virulence, and to validate the potential antiparasitic activity of the new compounds. Thus, since the first descriptions of *T. gondii* in 1908, different laboratories have established *in vivo* models to elucidate the main aspects in the interaction between *T. gondii* and its hosts.

Most of the *in vivo* models established so far have involved the use of animals that are easier to handle, such as mouse, rat, cat, pig, chicken and *Calomys* (DaMatta, Arnholdt, and Miranda 2019; Montazeri et al. 2017), and among these, mice are the most studied model. The advantage of using a mouse is that it has a small size, requiring low amounts of compounds for drug tests. Concerning mice, the common lineages used for drug tests are BALB/c, Swiss-Webster and C57BL/6 (Montazeri et al. 2017). While C57BL/6 is more susceptible to *T. gondii* infection, BALB/c and Swiss can control cerebral *T. gondii* infection, mimicking the chronic disease found in immunocompetent humans (Yasuhiro Suzuki et al. 2000; Y. Suzuki, Yang, and Remington 1995; Brown et al. 1995). The use of non-isogenic mouse-strains, such as Swiss, could mimic the variation generally observed in a heterogeneous population of individuals, who have considerable genetic variability in susceptibility to infections and different responses to treatments (Vommaro, Martins-Duarte and Souza 2014). Females are usually chosen for *in vivo* experiments, as they are more susceptible to acute infection of *T. gondii* and show greater brain-cyst formation when compared to males. Since hormones can influence the activation of the immune response, it is suggested that the hormonal difference between the genera acts as one of the factors for differences in the protective response against *T. gondii* (Roberts, Cruickshank, and Alexander 1995).

The main routes for drug administration in animal models are oral-gavage, intravenous, subcutaneous, intradermal, intramuscular, epidural, intraperitoneal, and intranasal. The choice of the route of administration is based on the desired effect, whether local or systemic (Turner et al. 2011). Studies have shown that the choice of a particular route of administration can alter the treatment outcome, since parameters such as absorption, distribution, metabolism and excretion may be involved in the drug effectiveness. For example, drugs that are rapidly cleared by hepatic processes will show a decreased extent of availability following oral administration due to the metabolism of the drug on its first pass through the liver (Benet 1978). In this sense, testing different administration routes is recommended to confirm the antiparasitic activity of a compound before discarding it. However, it is important to emphasize that oral administration is the preferred route for the treatment of infectious diseases, as it offers greater ease, safety and little invasiveness, leading to greater adherence to treatment by patients.

Concerning the *T. gondii* strain, the avirulent ME49 strain is the most used for studying chronic toxoplasmosis in the mouse model. Unlike the virulent RH strain, which lost its ability to tissue cysts formation in mice, the ME49-strain reproduces a more attenuated infection allowing the establishment of a considerable number of brain cysts. The choice of the infection route is also a variable to be considered, as it may alter the outcome of the infection (Johnson 1984). Oral infection with cysts is preferable, as it reproduces the natural transmission of *T. gondii*. For studying the effect of drugs against the chronic phase, mice are inoculated with a non-lethal amount of non-virulent parasites (eg. ME49 strain). Generally, the presence of brain cysts is observed after thirty days of infection. At the end of treatment, the effect of drugs against cysts is evaluated by counting the number of brain cysts or quantifying the presence of parasitic DNA (Vommaro, Martins-Duarte and Souza 2014).

The reactivation of chronic disease is a risk for immunocompromised patients, thus the discovery of drugs that could be used for this condition is also required when considering the development of new treatments at the interface of chronic and acute toxoplasmosis. Experimental reactivation of toxoplasmosis can be stimulated by treating chronically infected animals with immunosuppressive drugs. The reactivation of infection is confirmed by evaluating the active infection in the serum, organs, and tissues. The treatment is initiated few days after the confirmation of reactivation, (Djurković-Djaković and Milenković 2001).

An alternative model of *T. gondii* infection using fish was recently described (Sanders et al. 2015). Murine models present limitations due to the cost and the inability to perform high throughput assays. Thus, to develop an improved *in vivo* model, zebrafish (*Danio rerio*) was adapted for a 37°C environment and infected intraperitoneally with *T. gondii* cysts. A 7-day observation period showed that the infection pattern was similar to that found in mammalian infection, with parasites developing in the somatic muscle, heart, liver, spleen, kidney, and brain. According to the authors, this approach could provide a novel and robust platform for the high-throughput screening of compounds with the potential to treat the chronic (bradyzoite) form of *T. gondii*.

The most common methods to monitor and evaluate the effectiveness of drugs against the latent stage of toxoplasmosis *in vivo* are (Vommaro, Martins-Duarte and Souza 2014):

1. Counting of cerebral cysts: after the end of the treatment, the animals are euthanized and the brain is removed. The number of cysts is evaluated by examining small aliquots of the macerated brain suspension on an optical microscope.
2. Histopathological or immunohistochemical assays: after treatment, the animals are euthanized and organs are removed for histological processing. Tissue sections obtained from processed organs are stained or incubated with anti-*T. gondii* antibodies, allowing the identification of the parasite.
3. Bioassay: This test indicates the presence of a residual infection with viable parasites in the treated mice. At the end of treatment, the animals are euthanized, and the brain, liver, heart and lung are removed. Organ homogenates are inoculated in healthy mice and the presence of infection is accompanied by mice mortality, the presence of anti-*T. gondii* antibodies.
4. Quantitative polymerase chain reaction (qPCR): allows detecting and quantifying the presence of infection even in a tissue sample with a small number of parasites. Real-time PCR is faster and more sensitive and reduces false positives. It is a useful technique for checking the presence of a residual infection and proving the cure of surviving animals after treatment.
5. Bioluminescence: allows the real-time mapping of the spatial progression of the infection in the same animal. For this assay, it is necessary to use transgenic parasites expressing the luciferase enzyme gene in a stable form.

It is worth mentioning that despite many compounds shown *in vitro* activity, most of them when challenged in an *in vivo* system fail to protect the animal from infection (Payne, Neal, and Knoll 2013; Vigerust and McCullers 2007). Thus, the use of animals is still required to confirm the effectiveness of the new compounds.

3. INHIBITORS SHOWING ACTIVITY AGAINST BRADYZOITES

The advancements in the knowledge of bradyzoite biology leading to the discovery and functional characterization of specific proteins allowed the identification and modeling of specific inhibitors against important proteins for bradyzoite survival. In this way, we will address some results describing parasitic targets and inhibitors that have aroused interest in the development of new therapies against chronic toxoplasmosis.

3.1. Cytochrome *bc1* Inhibitors

Cytochrome *bc1* (Cyt *bc1*) is a component of the mitochondrial electron transport chain and exerts an important part of the energetic metabolism of eukaryotic cells. Electron transport involves the sequential transfer of electrons through multiprotein complexes (complex I to complex IV) in a process that depends on the ubiquinone (Coenzyme Q) (Painter, Morrissey, and Vaidya 2010; Nixon et al. 2013; Monzote and Gille 2010). Cyt *bc1* or complex III is the target of atovaquone (ATO), a drug that is approved for the clinical treatment of human toxoplasmosis. ATO is a hydroxy-1,4-naphthoquinone (an analogous of ubiquinone) and exerts its activity as a competitive inhibitor of Cyt *bc1* through the binding to its Qo-site (Alday and Doggett 2017; Konstantinovic et al. 2019; Secrieru et al. 2020). Data from animal experimentation showed that ATO exerts activity against the chronic toxoplasmosis (Ildiko R. Dunay et al. 2004; Djurkovic-Djakovic et al. 2002), however, this effect was not observed in humans. It is also suggested that electron transport chain interruption by ATO would impair the pyrimidine biosynthesis in Apicomplexan and, thus, preventing the DNA replication of the parasite (Esser, Yu, and Xia 2014).

Doggett and col. (2012) demonstrated the potential therapeutic use of an endochin-like quinolone (ELQ) class of compounds against acute and latent phases of toxoplasmosis. ELQs are also inhibitors of the Cyt *bc1* complex and bind to the Qi-site (Alday et al. 2016; Doggett et al. 2012). Inhibition of Qi site by ELQs increased the production of reactive oxygen species (ROS) from Cyt *bc1* (Menna-Barreto and De Castro 2014; Dröse and Brandt 2008) and since ROS production has been reported as an antiparasitic agent (Schirmer et al. 1987; Sorci and Faivre 2009), the increase in the production of ROS after Cyt *bc1* inhibition could contribute to the observed antiparasitic activity. Within 50 ELQs compounds, ELQ-271 and ELQ-316 were selected as the most promising. Intraperitoneal administration of ELQ-271 and ELQ-316 against mice model of chronic toxoplasmosis showed a superior effect in reducing the brain cyst load when compared with ATO. To improve the activity of ELQs against *T. gondii*, McConnell and col. (McConnell et al. 2018) evaluated a series of 4(1H)-Quinolones with 3-position substituents against wild-type (RH strain) and drug-resistant strains of *T. gondii*, leading to the identification of compounds able to inhibit *T. gondii* proliferation at picomolar concentration. *In vivo* experiments showed that oral administration of compound ELQ-400, active against the Qo site, was superior to ATO and ELQ-316 in an animal model of infection using the RH strain. Differences in the *in vivo* effect of ELQ-400 and ELQ-316 when orally administrated could be due to the high crystallinity of ELQ-316, which compromises its oral bioavailability. Continuing to explore the effectiveness of ELQ-316 and improve its oral absorption and plasma concentrations, Doggett and col. designed a carbonate ester prodrug of

ELQ-316 (ELQ-334). The prodrug ELQ-334 was challenged against models of acute and chronic toxoplasmosis in mice and showed that ELQ-334, when given orally, improved ELQ-316 bioavailability. Treatment with oral ELQ-334 showed efficacy in reducing the number of *T. gondii* cysts in the same order as treatment with intraperitoneal ELQ-316 (Doggett et al. 2020).

To improve the low solubility and toxicity of quinolone-based compounds McPhillie and col. designed and synthesized a series of tetrahydroquinolinones (THQs) inhibitors of Qi-site in Cyt bc1. Among seven THQs compounds initially synthesized, two (JAG50 and JAG21) showed activity against tachyzoites of RH strain in concentrations below 1 μ M and without toxicity to host cell. These compounds also showed high efficiency *in vitro* against encysted bradyzoites of EGS strain (M. J. McPhillie et al. 2020). Moreover, JAG21 (a Qi inhibitor) showed a synergist activity *in vitro* when associated with ATO (a Qo inhibitor). The effect of JAG21 in *in vivo* models of acute and chronic infection showed that low doses of this compound were able to protect mice from acute infection and also resulted in a remarkable reduction in the brain cyst load in chronically infected mice (M. J. McPhillie et al. 2020).

3.2. Factor IF2- α

The molecular mechanisms involved in the interconversion between tachyzoite to bradyzoite have also been explored as targets for the development of new chemotherapies against chronic toxoplasmosis. As mentioned above, stress conditions (alkaline pH, thermal stress, exposure to sodium arsenite, and arginine starvation) are associated with the resistant parasite form development.

In eukaryotes, the phosphorylation of initiation factor-2 α (eIF2 α) by a family of protein kinases (including eIF2 kinases) is involved in the response to stress and adaptation to the environment (Sullivan et al. 2004; Narasimhan et al. 2008). In this scenario, it was shown that phosphorylation of an eIF2 α found in *T. gondii* is important for the development and maintenance of latent parasite forms. To evaluate if the TgIF2 α phosphorylation process could be explored as a novel antiparasitic drug target, the effect of guanabenz (GA), an alpha-adrenergic receptor agonist approved by FDA to treat hypertension and an inhibitor of TgIF2 α (Baum and Shropshire 1976), was evaluated against *T. gondii*. Treatment with GA induced bradyzoite formation and inhibited the reactivation of latent bradyzoites *in vitro* (Konrad et al. 2013). GA effect was demonstrated to be directly related to the maintaining of TgIF2 α phosphorylation in parasites, without host eIF2 α involvement. The authors suggested that the anti-*T. gondii* activity was due to a mechanism involving the translational control that promotes and stabilizes the latent bradyzoite form (Konrad et al. 2013). Besides, GA was able to alter the cyst morphology in HFF cell culture *in vitro* and reduce the cyst burden when administrated to acute and chronic infected mice (Benmerzouga et al. 2015). As GA has already been approved for human use, it could be rapidly repurposed as a novel anti-*T. gondii* agent that could dually inhibit the replicative and latent stages of the parasite.

3.3. Calcium-Dependent Protein Kinase 1 (CDPK1)

CDPK is a family of serine-threonine protein kinases only found in plants and protists, including ciliates and apicomplexans. CDPK activity is regulated by intracellular calcium oscillations and is involved in important events for *T. gondii*-host cell interaction, such as motility, invasion, differentiation and egress (Kieschnick et al. 2001; Nagamune and Sibley 2006; Billker, Lourido, and Sibley 2009). Considering that these events are essential for the establishment of *T. gondii* infection, CDPK aroused interest in the search for molecular targets for the development of therapies for toxoplasmosis. Moreover, CPDKs are not found in mammalian host cells, allowing then the design of selective inhibitors (Lourido et al. 2010).

Exploiting the natural occurrence of a small gatekeeper residue in TgCDPK1 as a potential target, bulky pyrazolo-pyrimidine (PP) inhibitors analogues were identified as selective inhibitors of CDPK1 activity *in vitro* (Lourido et al. 2013). Based on these results, a series of PP analogue derivatives were synthesized and tested against models of acute infection and disease reactivation, showing to be able to reduce mice mortality (Lourido et al. 2013; Rutaganira et al. 2017). Interestingly, using an *ex vivo-in vitro* assay, when bradyzoites were incubated with PP analogue derivatives, their invasion capacity was impaired (Rutaganira et al. 2017).

CDPK1 is also inhibited by bumped kinase inhibitors (BKIs) compounds, which are ATP-competitive inhibitors of CDPK1 (Sánchez-Sánchez et al. 2018), BKIs are inactive against mammalian kinases and show a broad-spectrum activity against protozoan parasites (Hanke et al. 1996). Sánchez-Sánchez and col. (2019) showed the safety and efficacy of using the BKI-1294 inhibitor in preventing vertical transmission in sheep experimentally infected with *T. gondii*. Treatment with BKI-1294 decreased fetal mortality and abortion after challenge with a lethal inoculum of *T. gondii* for fetuses (Sánchez-Sánchez et al. 2019). Dogget and col. (2014) demonstrated the oral efficacy of BKI-1294 against toxoplasmosis using type I RH strain. BKI-1294 reduced the number of tachyzoites by more than 93% in the mice peritoneal fluid (Doggett et al. 2014). Hulverson and col. (2019) evaluated the effectiveness of new BK1 molecules from original Pyrazolopyrimidine and 5-aminopyrazole-4-carboxamide compounds against systemic and brain infection by *T. gondii* in mice. The new compounds were effective in reducing tachyzoites in the peritoneal fluid and the parasitic load in the brains of mice (Hulverson et al. 2019).

3.4. Cathepsin L Inhibitors

The peptidases/proteases, enzymes play a critical role in protein catabolism. These enzymes are classified into seven categories based on the principal catalytic residue in the active site: Aspartic, Cysteine, Glutamic, Serine, Threonine, Metallo, and Mixed. Cathepsins (CP) are found within the cysteine-protease family and are widely distributed in eukaryotic organisms. The CPs have aroused interest because, during *T. gondii* invasion and survival processes, the release of some key proteins by secretory organelles (micronemes, rhoptries, and dense granules) require proteolytic processing by these enzymes (Huang et al. 2009; Dou and Carruthers 2011).

Cathepsins L and B are the major cysteine proteases found in protozoans. In *T. gondii*, five members of cysteine proteases are expressed: cathepsin B or Toxopain-1 (TgCPB), cathepsin L (TgCPL), and cathepsin C's (TgCPC 1, 2, and 3) (Dou and Carruthers 2011; Dou et al. 2014). TgCPL is distributed in the vacuolar compartment of the parasite and exerts its activity mainly in bradyzoite (Zwicker et al. 2020). In support of this, cysts that expressed a catalytically inactive CPL (Di Cristina et al. 2017) or were treated with a CPL inhibitor (Larson et al. 2009) had their viability compromised. Thus, TgCPL is a promising target for the development of new treatments against the chronic stage of toxoplasmosis.

Screening of a small library of cathepsin inhibitors against *T. gondii* identified the compound morpholinourea-leucyl-homophenolalaninyl-phenyl-vinyl-sulfone (LHVS) as a specific inhibitor of TgCPL. LHVS belongs to the class of vinyl sulfones and was able to inhibit 70% of the *T. gondii* invasion in HFF cells when parasites were pretreated with this compound. The effect of LHVS in inhibiting invasion was associated with a deficiency in the secretion of micronemal proteins MIC2 and M2AP due to TgCPL inhibition (Chin et al. 2007). Besides LHVS also killed encysted bradyzoites in cell culture, confirming the potential of TgCPL inhibitors as promising agents against the chronic stage of toxoplasmosis (Zwicker et al. 2018). However, despite the activity against *T. gondii*, LHVS showed a non-brain penetrant feature (Barclay et al. 2007), limiting its use against *in vivo* chronic toxoplasmosis.

Aiming at leading compound better than LHVS, Zwicker and col. (2018) researched within several structures of known human CPL inhibitors, leading to the selection of a dipeptide nitrile scaffold as the leader to design new selective structures against TgCPL with potency and physicochemical properties predictive of CNS permeability. Although the dipeptide series showed potency *in vitro* and could penetrate CNS, its physicochemical properties were still far from desirable and showed a poor pharmacokinetic *in vivo* (Zwicker et al. 2018). Looking again for scaffolds that could exhibit better physicochemical properties with improved *in vivo* pharmacokinetic, Zwicker and col. performed an HTS for TgCPL and, among several classes of potential inhibitors discovered, the triazine nitrile was selected as the leading candidate based on its precedent to inhibit human CPL and physical properties consistent with CNS-active compounds. Through a rational design, the triazine nitrile scaffold was optimized until obtaining analogues with improved potency and selective against TgCPL. Most importantly, non-peptidic triazine nitrile inhibitors reduced the viability of bradyzoite cysts viability *in vitro* with effectiveness equivalent to a TgCPL genetic knockout (Zwicker et al. 2020). These findings support TgCPL as a promising target to be explored for the development of a treatment for the chronic phase of toxoplasmosis.

CONCLUSION

The discovery of a treatment for chronic toxoplasmosis has been challenging. The neurotropism of *T. gondii per se* makes the development of drugs against the chronic phase of toxoplasmosis a difficult task, as a compound aimed for the treatment of this stage of disease should combine the following characteristics: i) High activity against encysted bradyzoite; ii) Target specificity and selectivity against *T. gondii*; iii) Low toxicity for the host, decreasing the occurrence of unexpected side effects that cause treatment interruption; iv)

physicochemical properties predictive of CNS permeability and v) Favorable *in vivo* pharmacokinetics.

The lack of good *in vitro* models of encysted bradyzoite also hindered the discovery of active compounds. For a long time, the search of active compounds against bradyzoite was based on *in vivo* tests, using compounds selected based on their activity against the tachyzoite stage *in vitro*. Besides, the little knowledge of bradyzoite biology also contributed to the slow progress in finding selective targets in this stage. As shown in this chapter, a promising target against the bradyzoite stage will not necessarily be a good target for the tachyzoite stage. Thus, to succeed in discovering a future treatment for the chronic phase of toxoplasmosis, research must focus on the identification of compounds directed to the bradyzoite stage and not to tachyzoite.

The considerable enhancement in the comprehension of the mechanisms involved in the parasite interconversion and the biology of bradyzoite, together with the recent development of *in vitro* models for obtaining encysted bradyzoites will contribute to the identification of stage-specific targets and selective compounds, allowing the design of compounds combining potency, low toxicity, and desirable physicochemical characteristics.

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Chapter 13

PREVALENCE OF *TOXOPLASMA GONDII* INFECTION IN SOUTH AMERICA

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ABSTRACT

In this chapter, we will present data about the prevalence of toxoplasmosis in South America. Toxoplasmosis is an important public health problem worldwide, affecting one-third of the world population. A high prevalence of infection by *Toxoplasma gondii* is found in South America. Besides numerous outbreaks of acute toxoplasmosis also occur in South America. In this region, the infection incidence is higher in low socioeconomic status and education individuals and may be related to greater exposure to the parasite, reduced access to health services, and delays in acquiring appropriate treatment for infection control.

Keywords: Brazil, Argentina, Colombia, Chile, Ecuador, epidemiology

1. INTRODUCTION

Toxoplasmosis is an important public health problem worldwide, affecting one-third of the world's population (Montoya and Liesenfeld 2004). However, the infection rate varies according to regions. The incidence of toxoplasmosis in developing and underdeveloped

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nations is very high, particularly in parts of tropical South America, Southeast Asia and Africa (Pappas, Roussos and Falagas 2009). Besides, numerous outbreaks of acute toxoplasmosis occur in South America, and the largest global outbreak was recently reported in 2018 in the municipality of Santa Maria (Rio Grande do Sul state, Brazil), which affected more than 900 people.

Through a systematic review with meta- and modeling-analysis, Bigna et al., 2020 estimated a worldwide seroprevalence of anti-*T. gondii* IgG and IgM antibodies in pregnant women of 32.9% and 1.9%, respectively. The highest incidence of IgG antibodies was observed for the American region (45.2%), followed by Eastern Mediterranean (30.7%), Africa (36.5%), Europe (30%), Southeast Asia (24.6%) and Western Pacific region was the lowest (11.2%) (Bigna et al. 2020). However, while the prevalence in North American countries was lower than 10%, in South American countries a prevalence of at least 30% was found, and some countries showed more than 50% (Bigna et al. 2020).

Differences in the prevalence of *T. gondii* infection among countries could be due to various factors such as cultural, climatic, ethical practices, and cats' presence (Dabritz and Conrad 2010). In European countries in cold climatic conditions, the prevalence is very low but increases in warm climatic conditions (Pappas, Roussos and Falagas 2009). Habits in preparing foods such as thoroughly cooked foods (meat) or half-cooked were the critical factors influencing transmission in this region (Tenter, Heckeroth and Weiss 2000). Socioeconomic status has been an influencing factor in toxoplasmosis infection in South America. In Brazil individuals with a low level of scholarly or literacy and socioeconomic status showed a higher risk of ocular infection and exposure to the parasite, respectively (Ferreira et al. 2014; Mareze et al. 2019). In another study in northeastern Brazil with students aged between 5 and 21, the socioeconomic conditions were determinants in the prevalence of systemic and ocular toxoplasmosis (De Amorim Garcia et al., 2004). Low socioeconomic status may be associated with greater exposure, reduced access to health services, and delays in acquiring appropriate treatment for infection control (Barrera-Dussán et al. 2018; Velasco-Velásquez et al. 2020).

2. EPIDEMIOLOGY IN SOUTH AMERICA

T. gondii infection is widely spread in South America. High prevalence has been reported in Chile (Contreras et al. 1996; Munoz-Zanzi et al. 2016), Argentina (Durlach et al. 2008; Carral et al. 2013) Colombia (Gómez Marín 2002; Ramírez et al. 2019), Venezuela (Díaz-Suárez and Estevez 2009), Ecuador (Velásquez Serra et al. 2020) and Brazil (Dubey et al. 2012).

Brazil shows one of the highest world rates of infection. In Brazil, serological studies indicate that 50-80% of the population is seropositive for *T. gondii*, which means that that are four times more infected individuals in Brazil (56%) than in the United States of America (13%) (Dubey et al. 2012). The incidence of *T. gondii* infection in pregnant women and children less than one year of age in Brazil is 36-92% and 53%, respectively (Dubey et al. 2012). Epidemiological studies show that the prevalence varies by region within Brazil, for example, an incidence of 56% was reported in individuals from riverside communities in the Amazon, North of Brazil (Vitaliano et al. 2015), 52.6% in the population of the city of Pontal

do Paranapanema (Prestes-Carneiro et al. 2013), 64.9% in women of childbearing age in the metropolitan region of São Paulo, South-East region of Brasil (Fernandes et al. 2009), 80% in HIV-positive patients in the city of Pelotas, South Region of Brazil (Xavier et al. 2013), 89% in pregnant women in the city of Goiania, Central-West region of Brazil (Avelar et al. 2018), 68.3% in pregnant women in the city of Gurupi, North region of Brazil (Gontijo da Silva et al. 2015), and 50-60% of pregnant women in cities in the southern region of the country (Reis et al. 2006; Cademartori et al. 2008; Dias et al. 2011; Lopes-Mori et al. 2013).

Concerning congenital disease, studies show that in Brazilian children, eye lesions are larger, more numerous, and more likely to reach the posterior pole of the retina than in European children (Gilbert et al. 2008).

Serological studies in Argentina showed a *T. gondii* infection of 18.33% in pregnant women and 21.2% in blood donors in Buenos Aires (Carral et al. 2013; Kaufer et al. 2017). The prevalence of anti-*T. gondii* antibodies in pregnant women from urban and peri-urban areas of Buenos Aires were 26.8% and 36.4%, respectively, suggesting undeveloped peri-urban environments as a risk factor (Rivera et al. 2019). Another report in Argentina indicated a transplacental transmission rate of 5.4% (Carral et al. 2008). In Ecuador the prevalence of infection varies according to region and incidences of 18.8-74% were reported (Velásquez Serra et al. 2020).

An observational cross-sectional study in Lambayeque Peru showed that 35.5% of pregnant women were positive for anti-*T. gondii* IgG or IgM. In this study older maternal age, urban origin and consumption of non-drinking water were identified as risk factors associated with infection (Silva-Dias et al. 2020). A study conducted in Riberalta Bolivia, showed a prevalence of 86% of anti-*T. gondii* antibodies in pregnant women, proximity with cats and ingestion of pork meat were identified as risk factors (Soto Sanchez et al. 2014).

In Colombia, toxoplasmosis is a major public health problem (Tenter 2009; Ertug et al. 2005; Gómez-Marín 2002). The seroprevalence of *T. gondii* antibodies is heterogeneous between regions. While dry regions have lower frequencies of congenital disease (0-1 per 1000 newborns), humid regions have a higher prevalence (2.5-3.8 per 1000 newborns) (Gómez-Marín et al. 2011). As indicated by Gómez-Marín in 2002, the prevalence rate according to the 1980 national health study was 47%, associated with a progressive increase with age; the highest rates are found in the Atlantic Region (63%), in contrast to the central zone (36%). Recently, Ramírez et al., (2019) demonstrated that the seroprevalence of IgG antibodies against *T. gondii* in blood donors of a blood bank in the city of Cúcuta, in Colombia, was 38.5% (134/348), which is higher than the global average prevalence in blood donors, which is 33% (Forountan-Rad et al., 2016). Besides, the same study showed higher seropositivity for *T. gondii* over 26 years (Ramírez et al., 2019). In Colombia, individuals with a low-income level showed a higher frequency of larger lesions and bilateral ocular toxoplasmosis due to congenital infection (Velasco-Velásquez et al. 2020).

Toxoplasmosis is endemic in Chile, Munoz-Zanzi et al. (2016) examined the epidemiology of toxoplasmosis in distinct community types from a highly endemic area in the South of the country. Around 40% of children aged 13-17 have already displayed serologic evidence of past infection, evidencing that a significant risk of exposure starts early in life (Munoz-Zanzi et al. 2016). A seroprevalence of 55.9% was found, which is higher than the 47% estimate obtained from a large-scale survey carried out with 6,438 people in a greater southern region of Chile in 1990 (Schenone et al. 1990).

In Venezuela, studies on *T. gondii* infection are scarce. However, some studies have determined the toxoplasmosis prevalence in several urban groups and communities. A serological investigation with 335 individuals ranging from 1 to 65 years old from six communities at San Carlos Island, west of Venezuela, showed a prevalence of 49.8% (Chacin-Bonilla et al. 2003). Seroprevalence rates of 41.8-53.5% in 0-60- year-old individuals and 57.7-66.7% in children aged 15 years (Bonfante-Garrido et al. 1984) have been reported. Chacin-Bonilla et al. (2001) reported a seroprevalence of 49.7% (222 of 447) in Amerindians aged 1-69. Seroepidemiology investigation in childbearing age women from a marginal community of Maracaibo showed a prevalence of 33% (Diaz-Suárez and Estevez 2009).

In other countries like Uruguay, high levels of infection are found in animals intended for human consumption, such as 38.5% in sheep (Freyre et al. 1999), which demonstrates the importance of meat consumption as one of the sources of human contamination (Dubey 1996). *T. gondii* has been identified as the main causative agent of reproductive losses in sheep in Uruguay (Freyre et al. 1995). It is estimated a loss of 1.4-3.9% of sheep in gestation, with a loss of around US\$ 1.4-4.7 million (Freyre et al. 1999). Animals intended for human consumption when infected with *T. gondii*, besides serving as sources of contamination, also represent economic losses due to abortions and neonatal deaths (Buxton 1998).

There are also numerous outbreaks of acute toxoplasmosis described in South America. The first outbreak of toxoplasmosis was recorded in 1967 in Bragança Paulista, São Paulo, Brazil, and since then, more than 35 cases were reported worldwide, especially in Brazil. Outbreaks due to the consumption of contaminated meat occurred in Paraná (Bonametti et al. 1997) and Rio Grande do Sul (Brasil 2006). Cases due to consumption of contaminated vegetables and fruits occurred in São Paulo (Ekman et al. 2012) and Pará (Morais et al. 2016).

Between November 2001 and January 2002, a large outbreak of toxoplasmosis was reported in the city of Santa Isabel do Ivaí, Paraná state, Brazil, with 294 residents presenting serology suggestive of acute infection by *T. gondii*. Among these, 155 showed symptoms compatible with acute disease. The source of infection was the drinking water of the city, which was contaminated with oocysts. Of the 408 patient cases examined for ophthalmic conditions, 10% had eye injuries and 4.4% had necrotizing retinal lesions (De Moura et al. 2006).

In 2015, an outbreak of toxoplasmosis was reported in the municipality of Gouveia, the state of Minas Gerais in southeastern Brazil (Malta et al., 2019). Several cases of fever, malaise, weight loss and lymphadenopathy have been reported and recent toxoplasmic infection was eventually confirmed in 52 cases. All patients had the disease after drinking water from a single, presumably contaminated, source (Malta et al., 2019). Of these, 12 (23%) patients had retinochoroiditis; 4 patients had bilateral and 2 had macular lesions. Five patients had recurrences during follow-up, a rate of 22% per person-year. Two patients had late ocular involvement that occurred >34 months after the initial diagnosis (Brandão-de-Resende et al. 2020).

The largest worldwide outbreak of toxoplasmosis recorded up to date, occurred in Santa Maria, in the Rio Grande do Sul state, with peak cases reported between March and April 2018. Investigation of the outbreak began when doctors reported a significant increase in the number of cases of a clinical syndrome characterized by fever, myalgia, headache, rash, and mild gastrointestinal and respiratory signs. As of November 30, 2018, the laboratory exams confirmed 902 cases, and 260 were still under investigation. Of the confirmed cases, 135

(15%) were pregnant women, of whom three fetal deaths, nine abortions, and 28 cases of congenital toxoplasmosis were diagnosed. The contaminated water was confirmed as a source of infection (Minuzzi et al. 2020).

Outbreaks of acute toxoplasmosis caused by atypical virulent strains were reported in the border region of Suriname with French Guiana in the Amazon rainforest. Severe cases with ocular and multi-visceral involvement in immunocompetent individuals were observed, including death records. In this case, it was not possible to conclude the source of infection, as the infected individuals were exposed to the following risk factors: contact with soil, vegetation, and water in the Amazon rainforest, presence of domestic and wild cats around the village, in addition to hunting and consumption of wild animals (Demar et al., 2007). Recently, Blaizot et al. (2020) reported an outbreak of acute toxoplasmosis in a remote Amerindian village in French Guiana that occurred in 2017.

Pino et al. (2009) described an epidemic outbreak of acute toxoplasmosis in immunocompetent patients from Colombian Armed Forces during jungle operations. Eighteen cases were confirmed by indirect immunofluorescence for anti-*T. gondii* IgG antibodies. The most important symptoms in these patients were prolonged fever, lymphadenopathy, and pulmonary and gastrointestinal involvement. One patient developed a severe myopericardium involvement. The consumption of water contaminated with oocysts and, probably, infection with an atypical strain of the parasite was suggested as the probable cause of the epidemic outbreak.

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Chapter 14

CEREBRAL TOXOPLASMOSIS IN PEOPLE LIVING WITH HIV/AIDS

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ABSTRACT

The availability of combined antiretroviral therapy reduced the incidence of cerebral toxoplasmosis in people living with HIV/AIDS (PLWHA). However, cerebral toxoplasmosis continues to be the most common cause of expansive brain lesions and to cause high morbidity and mortality in PLWHA, particularly from low and middle-income countries. The typical manifestations are focal sub-acute neurological deficits and ring-enhancing brain lesions with a predilection for the corticomedullary junction, basal ganglia and thalami. However, the spectrum of clinical and neuroradiological manifestations is broad. Pyrimethamine-based regimens and trimethoprim-sulfamethoxazole (TMP-SMX) present similar efficacy but TMP-SMX shows potential practical advantages. This chapter reviews current literature on cerebral toxoplasmosis and emphasizes on host immune responses to the parasite antigens; host immunosuppression in HIV and cerebral toxoplasmosis; advances in diagnostic methods; and the main issues about clinical and radiological manifestations and treatment.

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1. INTRODUCTION

After infection in humans, tachyzoites multiply rapidly by endodyogeny within intracellular parasitophorous vacuoles. When the cells become packed with tachyzoites, the host cell plasma membrane ruptures and parasites are released into the extracellular milieu. Then, free tachyzoites can infect any nucleated cell, and continue intracellular replication, spreading throughout host tissues. If the immune system is unable to control the infection, tachyzoites are highly virulent and cause generalized toxoplasmosis, which is always fatal (Carruthers 2002; Dubey 2008 and 2009; Montoya and Liesenfeld 2004). Normally, host T-cell-mediated immune response plays an important role in the suppression of tachyzoite replication and resistance to *T. gondii*. This event can result in chronic infection or the clearance of parasites in most asymptomatic cases. Variations on the course of infection depend on the number of inoculated parasites, parasite genetic background, host genetics and immunological status (Dardé 2004; Dubey 2009; Pereira-Chioccola, Vidal and Su 2009).

In the chronic phase, the tissue cysts can persist indefinitely in the brain and muscle, developing life-long protective immunity against re-infection (Carruthers 2002; Montoya and Liesenfeld 2004). As different *T. gondii* genotypes were shown in the same patient, re-infections are possible (Dardé 2004). In the chronic phase, tissue cysts are periodically ruptured, but the bradyzoite released are normally destroyed by the host immune response. Nevertheless, around 10-20% of the infected immunocompetent individuals, during the chronic infection are reactive by the rupture of a tissue cyst in the eyes, brain, or muscles causing local necrosis accompanied by inflammation. Hypersensitivity plays a major role in such reactions; however, the infection usually collapses, with no local converted multiplication of tachyzoites (Carme et al. 2002; Glasner et al. 1992; Pereira-Chioccola, Vidal and Su 2009).

When asymptomatic infected individuals develop some immunodeficiency, results in reactivation of latent infection that culminates in the conversion of bradyzoites to the active and rapidly replicating tachyzoites that may result in the often-fatal tissue injury. As the cysts have a predilection for neural and muscle tissue as well as the eye, most cases of reactivation disease presenting chorioretinitis or, more frequently, cerebral toxoplasmosis that is a life-threatening condition (Carme et al. 2002; Glasner et al. 1992; Pereira-Chioccola, Vidal and Su 2009). The reactivation of latent infection is an immunodeficiency such as severely immunosuppressed HIV-infected patients in absence of highly active antiretroviral therapy and prophylaxis results more frequently in cerebral toxoplasmosis. In rare cases, disseminated toxoplasmosis can occur, which involves at least two organs, in failure of an efficient Th1 immune response (Pereira-Chioccola, Vidal and Su 2009; Schluter and Barragan 2019; Vidal 2019).

2. *TOXOPLASMA GONDII* ANTIGENS AND HOST DEFENSES

In the classical infection, the invasion process initiates with the lytic cycle leading to cell and tissue destruction that causes the *Toxoplasma* pathology. The parasite induces the formation of a parasitophorous vacuole in the host cell cytoplasm. This vacuole contains secreted parasite and host proteins that, normally promote phagosome maturation, thereby

preventing lysosome fusion (Dubet 2008; Sibely and Ajioka 2008; Zhou et al. 2005). This process is rapid, dynamic, and relies on the secretion of numerous secretory proteins from micronemes, rhoptries, and dense granules (Sibley and Ajioka 2008). The secretion of these antigens is an essential component of stimulation or boosting of the immune system including antibody production, as well as the T-cell response (Cesbron-Delaw and Capron 1993; Sibley and Ajioka 2008). These molecule groups are parasite excretory/secretory antigens (ESA), which represent the majority of circulating antigens in sera from infected hosts. ESA are expressed at tachyzoite, sporozoite and encysted bradyzoite stages (Cesbron-Delaw and Capron 1993).

The secretion by the bradyzoite cysts is a mechanism maintaining long-lasting immunity to the parasite (Zenner et al. 1999). On the other hand, ESA released by tachyzoites are highly immunogenic and induce protective immunity, either antibody-dependent or cell-mediated (Cesbron-Delaw and Capron 1993; Costa-Silva et al. 2008; Prigione et al. 2000; Rachinel et al. 2004; Rezaei et al. 2019; Zenner et al. 1999). CD4⁺ T cells specific for ESA are involved in the maintenance of long-term immunity in healthy chronically infected individuals. In this way, immunocompetent infected individuals had low anti-ESA antibodies titers whereas patients with cerebral toxoplasmosis, and consequently with circulating blood tachyzoites develop high titers of anti-ESA antibodies (Meira et al. 2008).

T. gondii infection induces a strong type 1 polarized immune response, engaging both innate and adaptive immune systems. The major mechanism of host resistance to toxoplasmosis is mediated by the production of pro-inflammatory cytokines including interleukin 12 (IL-12), interferon γ (IFN- γ), and tumor necrosis factor α (TNF- α) (Denkers and Gazzinelli 1998; Gazzinelli et al. 1992). The major sources of IFN- γ , which is an important key in the response to infection, are CD4⁺ Th1 lymphocytes, CD8⁺, natural killer (NK) cells and T-cells responding to IL-12 (Denkers 2003; Yap and Sher 2000). These mechanisms prevent rapid replication of tachyzoites and subsequent pathological changes (Daryani et al. 2014; Denkers 2003; Denkers and Gazzinelli 1998; Khan, Matura and Kasper 1994; Schlüter et al. 2003; Yap and Sher 2000).

After two-three weeks of infection, the extracellular tachyzoites are cleared from host tissues by the combined action of cytokines, IgG, IgM, IgA, and IgE antibodies against *T. gondii* proteins. Intracellular parasites differentiate into latent bradyzoite forms, which are surrounded by the parasitophorous vacuole that is enclosed in a cyst wall. The tissue cysts preferentially reside in neural and muscular tissues. This differentiation can be increased by exposure of the organism to stress conditions such as in immune response to the tachyzoites. T lymphocytes are a critical source of IFN- γ during this stage. In some cases, tachyzoites may persist longer in the spinal cord and brain because immune responses are less effective in these organs. The control of intracerebral parasites is dependent, also, on IFN- γ producing CD4 and CD8 T cells, which are recruited to the brain (Daryani et al. 2014; Denkers 2003; Denkers and Gazzinelli 1998; Gazzinelli et al. 1992; Khan, Matura and Kasper 1994; Schlüter et al. 2003; Yap and Sher 2000). The ability of bradyzoites to escape the host immune response and persist in a quiescent form within the host is another event in the *T. gondii* life cycle.

3. CEREBRAL TOXOPLASMOSIS IN ACQUIRED IMMUNODEFICIENCY SYNDROME

Before the AIDS epidemic, cerebral toxoplasmosis was a rare complication observed in immunosuppressed patients. The first cases of cerebral toxoplasmosis were described between 1982 and 1983, at the beginning of the AIDS epidemic (Luft et al. 1983). As the number of people living with HIV and AIDS (PLWHA) (Lebech et al. 1992; CDC 2021) increased in the world, cerebral toxoplasmosis became one of the most frequent opportunistic infections and a common cause of focal brain lesions in this population (Luft and Remington, 1992). However, the introduction of the combination antiretroviral therapy (cART) reduced the incidence of cerebral toxoplasmosis in PLWHA, particularly from high-and middle-income countries (Sacktor 2002; Antinori et al. 2004; D'Arminio Monforte et al. 2004; Coelho et al. 2014).

The most accepted explanation for the strong association between reactivated cerebral toxoplasmosis and AIDS is that this parasite infection is highly endemic in various world regions and immunocompromised patients have an alteration in anti-parasitic T-cell response and, therefore, fail to control the intracellular parasites. Low rates of opportunistic diseases, including neurological infections, have been reported in countries with access to cART. Despite of decline in both morbidity and mortality in these countries, the occurrence of cerebral toxoplasmosis still represents a poor prognostic determinant in the natural history of PLWHA with special emphasis in low-and middle-income countries (Ford et al. 2015; Vidal et al. 2005; 2013).

The prevalence of cerebral toxoplasmosis varies by countries and depends on the prevalence of *T. gondii* infection in the general population. Differences in genotypes of *T. gondii* isolates, races and ethnicities and the mode of transmission seem also to account for influencing the occurrence of the infection (Pereira-Chioccola, Vidal and Su 2009; Vidal 2019). Low-income countries, middle-income countries, and high-income countries have a prevalence of toxoplasmosis of 55%, 34%, and 26%, respectively (Ammassari et al. 1996; Pappas, Roussos and Falagas 2009; Nissapatorn and Sawangiaroen 2011; Miranbo et al. 2016; Azovtseva et al. 2020; Karshima and Karshima 2020). Globally, *T. gondii* is the most common cause of focal brain lesion in PLWHA (Ammassari et al. 1996; Vidal and Oliveira 2013; Ford et al, 2015; Vidal 2019). In addition, other variables to explain the persistent elevated incidence of cerebral toxoplasmosis in some settings include late HIV diagnosis, non-adherence to cART, failure of retention in care, and antiretroviral drug resistance (Antinori et al. 2004; Vidal and Oliveira 2013; Ford et al. 2015).

4. DIAGNOSIS APPROACH

The initial approach requires the presence of a compatible clinical and radiological syndrome in a PLWHA in order to identify two diagnostic categories of cerebral toxoplasmosis (Vidal, Penalva and Oliveira 2008; Vidal 2019): (1) Confirmed criteria: (i) *histopathology-confirmed*: requires the identification of brain biopsy (or postmortem examination) showing *T. gondii*. Hematoxylin-eosin or immunoperoxidase stains are used to demonstrate *T. gondii* (tachyzoites); or (ii) *laboratory-confirmed* cerebral toxoplasmosis

requires evidence of *T. gondii* DNA in cerebrospinal fluid (CSF) or blood by molecular diagnosis; and (2) Presumptive criteria: unequivocal radiological response after 10 to 14 days of empiric anti-*Toxoplasma* therapy.

5. CLINICAL AND RADIOLOGICAL MANIFESTATIONS

Cerebral toxoplasmosis usually presents neurological sub-acute manifestations. However, the disease can show a rapidly progressing and fatal diffuse encephalitis or ventriculitis without evidence of focal brain lesions in imaging studies or even a stroke-like presentation (Figure 1) (Gray et al. 1989; Luft and Remington 1992; Mauhin et al. 2016).

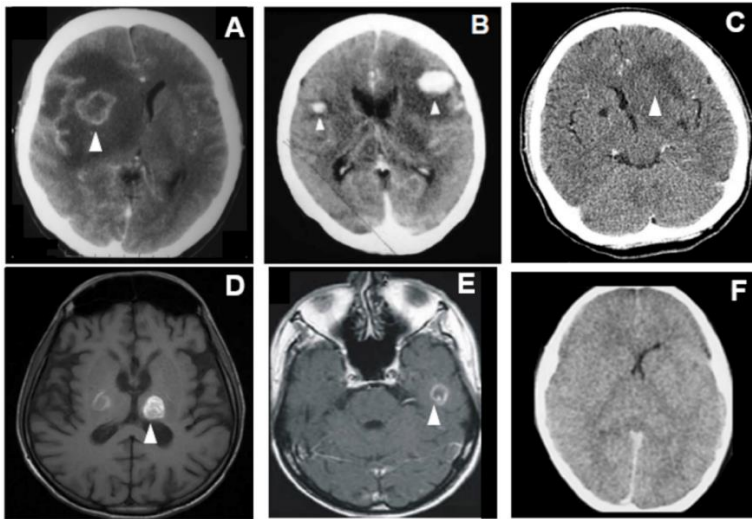


Figure 1. Neuroimages showing the main findings of cerebral toxoplasmosis in people living with HIV/AIDS. Computed tomography (CT) showing a hypodense lesion with ring-enhancing and perilesional edema (A); CT showing a nodular-enhancing and perilesional edema (B); CT showing an expansive hypodense lesion without contrast enhancing but with mass effect (C); T1-weighted magnetic resonance imaging (MRI) without gadolinium showing a spontaneous hyperdense lesion compatible with hemorrhagic toxoplasmosis (D); T1-weighted MRI with gadolinium showing a ring enhancing brain lesion with a small, enhancing asymmetric nodule along the wall of the lesion (the “eccentric target sign of cerebral toxoplasmosis) (E); and contrast enhanced CT scan showing diffuse edema compatible with toxoplasmic diffuse encephalitis (F). The arrows show the abnormalities.

Clinical manifestations of cerebral toxoplasmosis depend mainly on topography and the number of lesions. The most common signs and symptoms are headache (38-93%), fever (35-88%), focal neurological deficit (22-80%), mental confusion (15-52%), seizures (19-58%), cranial nerve palsy (12-28%), psychomotor or behavioral changes (37-42%), ataxia (2-30%), and visual abnormalities (8-19%). Patients may also present intracranial hypertension syndrome and involuntary movements (Mamidi et al. 2002; Rosembaum, Levy and Pomerantz 2002; Skiest 2002). Patients can also have any pattern of headache with or without other manifestations. Uncommon, isolated and nonspecific manifestations are observed as hemichorea, behavioral modification must be well evaluated. In the absence of treatment, progression of neurological abnormalities results in stupor, coma, and death. As expected,

abnormal mental status and need to intensive care units constitute independently associated variables with a poor outcome. In cerebral toxoplasmosis, meningismus is rare, since predominantly produces encephalitis with little or no meningeal involvement (Luft and Remington 1992; Sonnevile et al. 2012; Luma et al. 2013).

This broad spectrum of clinical manifestations in PLWHA indicates urgent brain imaging in order to decide an early introduction of empirical therapy (Vidal et al. 2004; Colombo et al. 2005; Mesquita et al. 2010a 2010b; Foudrinier et al. 2016). Thus, imaging studies are essential for the presumptive diagnosis of cerebral toxoplasmosis. The initial image is usually a computed tomography (CT) (Cingolani et al. 1996; Antinori et al. 1997). However, magnetic resonance imaging (MRI) is more sensitive, particularly to identify little lesions and those located in the posterior fossa. Single lesion is observed in about 30% of the patients by CT, but this proportion is lower in MRI. Radiological findings can be classified as typical and atypical (Ammassari et al. 1996). The typical findings, observed in around 80% of the cases, include hypodense lesions with ring-enhancing and perilesional edema or hypodense lesions with nodular enhancing and perilesional edema. The atypical findings are shown in around 20% of the cases and include hypodense lesions without contrast enhancing and with expansive effect, CT without focal lesions and MRI demonstrating focal lesions, and diffuse cerebral encephalitis without visible focal lesions. A highly suggestive imaging for toxoplasmosis, although unusual, is the “eccentric target sign,” which is a small asymmetric nodule along the wall of the enhancing ring (Antinori et al. 1997).

6. DIFFERENTIAL DIAGNOSIS

There are no *pathognomonic* clinical or radiological findings of cerebral toxoplasmosis. Differential diagnosis of extensive brain lesions in PLWHA is essential and two factors should be always considered: the local neuroepidemiology and the degree of immunosuppression in the host. Expansive brain lesions present geographic particularities. In developed countries, primary lymphoma of central nervous system (PLCNS) constitutes the main differential diagnosis of cerebral toxoplasmosis (Foudrinier et al. 1996; Vidal et al. 2004; Colombo et al. 2005). In developing countries, focal forms of cerebral tuberculosis (tuberculomas and rarely tuberculous brain abscess) are the main alternatives diagnosis. PLCNS usually presents with CD4 cell counts below 50 cells/mm³, cerebral toxoplasmosis frequently with below 100 cells/mm³, and cerebral tuberculosis usually with below 200 cells/mm³ (Foudrinier et al. 1996; Vidal et al. 2004; Colombo et al. 2005). Of these three etiologies, cerebral tuberculosis is more likely to present with CD4 cell counts above 200 cells/mm³ (Foudrinier et al. 1996; D'Arminio Monforte et al. 2004; Vidal et al. 2004; Colombo et al. 2005; Mesquita et al. 2010a; 2010b). In addition to these more frequent neurologic complications, the differential diagnosis of expansive brain lesions in PLWHA is broad and includes other infections as Chagas disease and bacterial or fungal brain abscess; AIDS and non-AIDS associated tumors as metastases of disseminated lymphomas and glioblastoma multiform, respectively; and vascular diseases. For these reasons, more invasive approach such as stereotactic biopsy should be considered in all PLWHA with expansive brain lesion empirically treated for cerebral toxoplasmosis that does not respond to anti-parasitic treatment after 10-14 days (Schluter and Barragan 2019; Vidal 2019).

7. LABORATORIAL DIAGNOSIS

7.1. Molecular Diagnosis

PCR has shown to be an important diagnostic tool since is used in all forms of symptomatic toxoplasmosis. Such diagnosis is fast and has provided, also, a rapid and specific treatment, avoiding or reducing serious damage caused by *T. gondii*, particularly in HIV infected patients (Foudrinier et al. 1996; Vidal et al. 2004; Colombo et al. 2005; Mesquita et al. 2010a; 2010b).

Studies have demonstrated the applicability of conventional PCR or real-time PCR in toxoplasmosis diagnosis using different PCR targets, such as the tachyzoites genes encoding for specific proteins, B1 gene; P30, ribosomal DNA genes and 529-bp (Burg et al. 1989; Cingolani et al. 1996; Foudrinier et al. 1996; Antinori et al. 1997; Homan et al. 2000; Vidal et al. 2004; Colombo et al. 2005; Mesquita et al. 2010a; 2010b; Murat et al. 2013; Anselmo et al. 2014; Robert-Gangneux and Belaz 2016; Camilo et al. 2017). Among them, two are more frequently used by the good sensitivity and specificity. One is the 529-bp sequence, which has 200-300 copies in the genome of *T. gondii*. The other is the B1 gene that has 35 copies in the genome and is conserved in different parasite strains (Burg et al. 1989; Homan et al. 2000).

Sensitivity, specificity and reproducibility of PCR depend on factors including the standardization of reagents and protocols for DNA extraction; the adequate storage of the clinical samples; and the time elapsing between the start of specific therapy and collection. For cerebral toxoplasmosis diagnosis, clinical samples (blood or CSF) must be collected before or until the third day of the anti-*Toxoplasma* therapy (Burg et al. 1989; Cingolani et al. 1996; Antinori et al. 1997; Homan et al. 2000; Mesquita et al. 2010b; Murat et al. 2013; Anselmo et al. 2014; Robert-Gangneux and Belaz 2016; Camilo et al. 2017). The use, in parallel, of a second amplification reaction using as target a human sequence guarantees the quality of the DNA extraction and PCR inhibitors, avoiding false results (Lee et al. 2001).

7.2. Serological Diagnosis

The high prevalence of *T. gondii* infection in healthy individuals in different geographical areas hinders the use of serologic tests to identify symptomatic infections. In this setting, physicians often face conflicting results and disagreements about interpretations of results. This often leads to incorrect information being provided by the laboratories to the physicians as well as by the physicians to their patients. In the case of cerebral toxoplasmosis, a positive serology is small useful in settings where the *T. gondii* seroprevalence in population is very high (Vidal 2019).

As most PLWHA with any focal brain lesion can have positive serological diagnosis for toxoplasmosis, the identification of negative serological result presents a high negative predictive value. Most of these patients with positive serological tests have high IgG titers with high-avidity (Colombo et al. 2005; Vidal et al. 2011). These findings support the idea that the reactivation of the latent infection occurs in the secondary immune response. However, these antibodies may not be detected in up to 5% of patients due to immune suppression (Mechain et al. 2000; Colombo et al. 2005; Vidal et al. 2011).

Anti-*T. gondii* antibodies were unable to define a reactivation or to follow the course of cerebral toxoplasmosis. However, as these antibodies are usually present in cerebral toxoplasmosis, some studies suggested that, statically, high titers might be indicative of the active disease or higher risk to develop it (Mechain et al. 2000; Colombo et al. 2005; Vidal et al. 2011). Thus, a negative serological result or low titers do not exclude a positive diagnosis for cerebral toxoplasmosis and must not delay the start of empiric treatment to cerebral toxoplasmosis in PLWHA with compatible clinical and radiological findings (Lamoril et al. 1996; Antinori et al. 1997; Franzen et al. 1997; Ajzenberg 2010; Vidal 2019).

Anti-*T. gondii* antibodies are determined in conventional serology as ELISA and immunofluorescence, using total extract of tachyzoites as antigen, which consists of cytoplasmic and membrane components or recombinant antigens (Pietkiewicz et al. 2004; Pfrepper et al. 2005; Beghetto et al. 2006; Holec et al. 2008). However, these antigens failed for distinguishing sera from patients with cerebral toxoplasmosis and asymptomatic infected immunocompetent individuals, with no or a low number of circulating tachyzoites, as they have similar reactivity.

Studies have shown that ESA constitutes an excellent serological marker for the diagnosis of cerebral toxoplasmosis in HIV-infected patients by the fact of they are produced by tachyzoites, the form responsible for disseminating the infection plays an important role in the stimulation of the humoral and cellular immune responses to control infection. Numerous tachyzoites are released from the quiescent cists and a considerable proportion of ESA elicited the specific immune response. Different from asymptomatic individuals, these patients have antibodies for both, ESA and total crude tachyzoites antigens. When ESA are used as antigen in ELISA or in immunoblot distinguish sera or CSF from patients with the active disease. Normally, these clinical samples are three times more reactive than those from seropositive individuals (Pereira-Chioccola, Vidal and Su 2009; Meira et al. 2008; Meira et al. 2011; Meira et al. 2013; Meira et al. 2014).

As HIV-related cerebral toxoplasmosis usually has little or no meningeal involvement, basic CSF characteristics are usually not relevant and only subtle abnormalities such as cell counts and protein values normal or mildly elevated. Eosinophil cells are occasionally found in the CSF (de Almeida et al. 2019).

Anti-ESA IgG antibodies are also detected in CSF in HIV-related cerebral toxoplasmosis and the values were different from those HIV-related with AIDS, seropositive for toxoplasmosis but with other neurological diseases (Meira et al. 2011; Meira et al. 2013).

7.3. Histopathological Diagnosis

Stereotactic brain biopsy was a classical procedure for the etiological identification of focal brain lesions in PLWHA in the first two decades of the HIV epidemic (Gildenberg, Gathe and Kim 2000; Skiest 2002). After the cART era, brain biopsies are uncommon. Primary central nervous system lymphoma and progressive multifocal leukoencephalopathy are the most frequent diagnosis in PLWHA underwent to brain biopsy after an empirical treatment with anti-*Toxoplasma* therapy. Interestingly, around 10-20% of brain biopsies of focal brain lesions in PLWHA confirm the diagnosis of cerebral toxoplasmosis. The rates of morbidity and mortality of PLWHA underwent stereotactic brain biopsy are 1% and 5%,

respectively (Rosenow and Hirschfeld 2007; Lee et al. 2016; Zhan et al. 2017). Usually, the outcomes of brain biopsy are better in centers with higher technical expertise. Barriers to the use of biopsy include the clinical condition of the patients, the topography of the lesion, its invasiveness, elevated costs, and structural requirements (Antinori et al. 1997; Levy et al. 1992).

Nowadays, brain biopsy is uncommon in the management of focal brain lesions in PLWHA but this procedure is still relevant in some scenarios. The main indications to perform brain biopsy in PLWHA with focal brain lesion are: (i) failure to respond to anti-*Toxoplasma* therapy, after 10 to 14 days, showed by persistence or worsening of initial clinical and neuroradiological findings; and (ii) cases with a high probability of alternative diagnosis to cerebral toxoplasmosis. It is important to consider that in places with a high prevalence of *T. gondii* infection in the general population, patients with cerebral toxoplasmosis may present a single lesion and negative anti-*T. gondii* antibodies (Vidal 2019). Therefore, in this situation, each case must be evaluated individually.

8. TREATMENT

The case fatality rate of HIV-related cerebral toxoplasmosis significantly decreased in the cART era. During pre-cART, the fatality rate was around 90% in the first months after the diagnosis of this opportunistic disease. In recent years this rate is around 15-30% (Vidal and Oliveira 2103; Ford et al. 2015; Vidal 2019). Thus, approximately 80% of patients treated with anti-*Toxoplasma* drugs demonstrate clinical and radiological improvement. These results show important improvement in the management of cerebral toxoplasmosis, including more availability of appropriate resources as imaging and laboratory exams and better intensive care strategies (Sonnevile et al. 2012; Vidal and Oliveira 2013). Nevertheless, the long-term outcome is challenging and the rate of neurological sequelae due to cerebral toxoplasmosis continues to be high in the cART era (30-40%). These aspects cause an important impact on the quality of life of PLWHA (Sacktor 2002; Vidal and Oliveira 2013; Martin-Iguacel et al. 2017).

The median time of neurological response to HIV-related cerebral toxoplasmosis is 5 days. Of the patients who improve, 86% show clinical improvement on the seventh day of treatment and 95% show radiographic improvement on the 14th day (Poder and Sande 1992; Luft et al. 1993). Pyrimethamine plus sulfadiazine (P-S) has been used since the first described cases of cerebral toxoplasmosis in the AIDS epidemic. In experimental models, P-S has shown, significantly, more active in kill *T. gondii* than trimethoprim-sulfamethoxazole (TMP-SMX) (Tan et al. 2012). P-S acts synergistically inhibiting *T. gondii* proliferation and survival through inhibiting the folate metabolic pathway. Both drugs inhibit dihydrofolate reductase and dihydropteroate synthase, respectively, and consequently block the synthesis of tetrahydrofolate, which is required by the parasite for DNA synthesis (Grossman and Remington 1979).

Clindamycin is a lincomycin, which inhibits *T. gondii* by an unknown mechanism that involves the parasite apicoplast (Holliman 1989). The sulfa desensitization should be attempted in patients with a history of sulfa allergy.

If sulfa desensitization is not possible, pyrimethamine plus clindamycin (P-C) has been a classical alternative regimen in most guidelines, although, it is less effective in preventing relapses compared to P-S (Niyongabo, Leport and Vilde 1991; Dannemann et al. 1992; Georgiev 1994; Bedos et al. 1995; Katlama et al. 1996; Nelson et al. 2011; Gilbert et al. 2015).

TMP-SMX has a similar action mechanism of P-S. However, trimethoprim, unlike pyrimethamine, is a highly selective inhibitor of dihydrofolate reductase from *T. gondii*. This feature explains the lower hematological toxicity caused by trimethoprim compared to pyrimethamine and why the use of folic acid is only necessary with the latter drug (Niyongabo, Leport and Vilde 1991; Dannemann et al. 1992; Georgiev 1994; Bedos et al. 1995; Nelson et al. 2011). Trimethoprim-sulfamethoxazole usually appears as an alternative in international guidelines, but other recommendations include TMP-SMX in the first-line therapies (WHO 2017; Ben-Harari et al. 2017; Ministry of Health of Brazil 2018). In clinical practice, however, TMP-SMX is infrequently used when P-C and P-S are available. In Africa and in other low- and middle-income countries, TMP-SMX is the first choice particularly where pyrimethamine-based regimens are not available or where there is experience with TMP-SMX. Pyrimethamine-based regimens are beset by important limitations, as adverse events, poor tolerability, complex posology, and the absence of parenteral formulations. These situations are a serious problem in patients with alterations in mental status (Ben-Harari et al. 2017; Hernandez et al. 2017). Studies have shown that TMP-SMX is similar to pyrimethamine-based regimens in terms of relative efficacy and safety for the treatment of cerebral toxoplasmosis. In addition, TMP-SMX presents potential practical advantages: (i) convenience of use of lower pill burden and dosing frequency and the availability of intravenous formulations, particularly important in severely ill patients; (ii) availability of several generic TMP-SMX formulations with the consequent impact on cost-effectiveness and increased accessibility; and (iii) convenience of use simplifying the early initiation of cART.

9. PHARMACOLOGIC PROPHYLAXIS

9.1. Primary Prophylaxis

The current recommendation indicates the primary prophylaxis for cerebral toxoplasmosis in cases of positive serological diagnosis for toxoplasmosis and lymphocyte CD4 counts below 100-200 cells/mm³ (Cingolani et al. 1996; Oliveira et al. 2006; Saadani Hassani and Marston 2015). The recommendation to discontinue the primary prophylaxis is the lymphocyte CD4 counts below 200 cells/mm³ for 3-6 months in response to cART or with lymphocyte CD4 counts below 200 cells/mm³ and HIV viral load below limits of detection. The primary prophylaxis should be reintroduced when HIV load above detection limits even though the lymphocyte CD4 counts are below 200 cells/mm³ (Furrer et al. 2000; Mussini et al. 2000; Miro et al. 2006). TMP-SMX is the preferred regimen (Girard et al. 1993; Opravil et al. 1995; Furrer et al. 2000; Mussini et al. 2000; Miro et al. 2006).

9.2. Secondary Prophylaxis

As all anti-*Toxoplasma* therapies used in clinical practice are active against tachyzoites, but not against tissue cysts, the therapy discontinuation after the induction phase of treatment can result in reactivation of the disease (Vidal 2015). Classical recommendations for secondary prophylaxis of cerebral toxoplasmosis consist of a combination of P-S and leucovorin. P-C is commonly used for patients with intolerance to sulfa. Typically, sulfadiazine should be taken every 6 hours. However, in patients with adherence difficulties, an alternative regimen includes the same daily total dose of sulfadiazine every 12 hours, but the clinical experience of this scheme is limited. On the other hand, a dose of 600 mg clindamycin every 8 hours is recommended because of the high failure rate observed with lower doses (Katlama et al. 1996; Podzamczar et al. 2000). TMP-SMX is an interesting alternative to secondary prophylaxis for cerebral toxoplasmosis.

Previous studies analyzing the secondary prophylaxis for the prevention of HIV-related cerebral toxoplasmosis relapse using pyrimethamine-based therapy and TMP-SMX found similar relapse rate (Connolly et al. 2017a, 2017b). The recommended dose of TMP-SMX for secondary prophylaxis is 50% of a daily initial dosage taken every 12 hours. Patients with lymphocyte CD4 counts below 200 cells/mm³ for until 6 months in response to cART are recommended to discontinue secondary prophylaxis (Connolly et al. 2017a, 2017b). Patients with lymphocyte CD4 counts below 200 cells/mm³ should restart secondary prophylaxis regardless of the HIV viral load (Connolly et al. 2017b).

10. IMMUNE RECONSTITUTION INFLAMMATORY SYNDROME (IRIS)

The spectrum of IRIS consists of two patterns. The paradoxical IRIS: the worsening of symptoms of a previously diagnosed opportunistic infection for which the patient is receiving treatment. The unmasking IRIS: diagnosis of a new opportunistic infection with inflammatory characteristics after the initiation of cART (Bahr et al. 2013; Manzardo et al. 2015). Unmasking IRIS is difficult to differentiate from a classical presentation of cerebral toxoplasmosis. In paradoxical IRIS, alternative explanations for the worsening include failure of opportunistic disease treatment, failure of cART due to lack of adherence or drug resistance, and the onset of other diseases.

The incidence of IRIS varies with each pathogen, and its influence on the immune system is specific. *T. gondii* is an unusual cause of IRIS probably due to the mechanisms of immune evasion by this parasite (Farkash et al. 1986; Blader and Saeij 2009). The first clinical case of cerebral toxoplasmosis-related IRIS was published in 2001 (Tsambiras, Larkin and Houston 2001) and the first pathological-proven case was reported in 2009 (Pfeffer et al. 2009). Considering the abovementioned information, cerebral toxoplasmosis-related IRIS is rare but should be considered in the appropriate clinical and laboratory context. The low frequency of IRIS associated with this opportunistic disease facilitates the early introduction of cART, in contrast to other diseases as tuberculous meningitis or cryptococcal meningitis.

CONCLUSION

After the introduction of cART, the incidence of cerebral toxoplasmosis decreased in PLWHA. However, this disease remains the most common cause of expansive brain lesions and causes high morbidity and mortality in persons with advanced immunosuppression, particularly those living in low-and middle-income countries. Cerebral toxoplasmosis is characterized by a wide spectrum of clinical and radiological manifestations. The timely introduction of anti-*Toxoplasma* therapy is key in the management of focal brain lesions in PLWHA. TMP-SMX can be used for primary prophylaxis, initial therapy, and secondary prophylaxis of HIV-related cerebral toxoplasmosis. Currently, early initiation of cART is possible in cerebral toxoplasmosis because IRIS is uncommon and TMP-SMX simplifies the posology.

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Chapter 15

TOXOPLASMA GONDII: OCULAR DISEASE

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ABSTRACT

Toxoplasmosis is the most important and frequent cause of blindness secondary to inflammatory retinal diseases. *T. gondii* infects up to a third of the world's population and is responsible for the majority of infectious uveitis (intraocular inflammation) cases. In some countries, up to 50% of all cases of posterior uveitis in a given population are attributable to toxoplasmosis (Soheilian et al. 2004, Vallochi et al. 2005). Toxoplasmic retinochoroiditis is often seen in settings of congenital or postnatally acquired disease as a result of acute infection or recurrence. The retina is the primary site of *T. gondii* infection in the eye, but the choroid, vitreous and anterior chamber are also involved (Nussenblatt and Belfort 1994). The population structure of *T. gondii* is highly clonal. There are three predominant clonal lineages, namely I, II, and III found in Europe and North America, but the genetic makeup of *T. gondii* is more complex than this three-strain population, and unusual genotypes, known as atypical, may contribute to various clinical outcomes of toxoplasmosis in different localities (Vallochi et al. 2008). An effective vaccine against human *T. gondii* infection is a desirable but elusive target, and therefore, toxoplasmosis will remain an essential blinding disease for many years to come.

1. INTRODUCTION

The obligate intracellular protozoan *Toxoplasma gondii* can infect a broad spectrum of warm-blooded vertebrates, including humans (Blader and Saeij 2009). *T. gondii* infection is often acquired through ingestion of water or food contaminated by infective oocysts,

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consumption of meat products containing viable cysts, or vertical transmission of tachyzoites (Tenter, Heckeroth, and Weiss 2000). In humans, the clinical presentations of *T. gondii* infection vary from asymptomatic infection, or non-specific mild symptoms, to severe toxoplasmosis (Montoya and Liesenfeld 2004, Boothroyd and Grigg 2002).

Howe and Sibley (Howe and Sibley 1995) initially found a highly clonal population structure of the parasite, which fell into three predominant lineages (Types I, II, and III) by a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis, employing 106 *T. gondii* isolates from Europe and North America. PCR-RFLP study employing about 1,500 samples worldwide revealed 189 different genotypes (Shwab et al. 2014). The clonal lineages have several different phenotypes, such as growth, migration, and transmigration (Barragan and Sibley 2003). Several studies suggested that the type of *T. gondii* strain might be responsible for the variability, or at least part of the variability, on the outcomes of human infection, as it has observed in laboratory mice (Boothroyd and Grigg 2002, Xiao and Yolken 2015, Sibley et al. 2009).

It has long been known that the Type I strains of *T. gondii* are highly virulent, whereas Type II or Type III strains are less virulent or non-virulent in mice (Sibley and Boothroyd 1992, Sibley and Ajioka 2008). Different types of *T. gondii* could cause illness in humans at different severity levels, indicating different *T. gondii* might present different virulence. Xia et al. (Xia et al. 2017), in a meta-analysis study, found a robust association between Type II strains and human infection because the highest proportions of Type II strains presented in groups of asymptomatic infection and toxoplasmosis.

Xia et al. (Xia et al. 2017) performed a multiple comparison analysis of Type I strain infection, revealing a significantly higher proportion of patients with toxoplasmosis and mild symptoms than those with asymptomatic infection, which suggested that Type I strains mainly caused apparent diseases or mild symptoms, potentially with a medium to a high level of virulence in humans. Previously, a cross-sectional study reported a predominance of recombinant Type I/III or atypical parasite infections in patients presenting severe ocular disease (de-la-Torre et al. 2013). In another study, Type I strains were reported to be more frequently associated with congenital toxoplasmosis in humans (Vallochi et al. 2005). Type I strains could generate significantly higher parasitemia levels and increase both the risk of transplacental transmission and the severity of infection in the fetus or newborn (Howe and Sibley 1995, Derouin and Garin 1991). Type III strains infection proportions of patients with mild symptoms and toxoplasmosis were significantly higher than those with asymptomatic infection, similar to the Type I strains infection (Xia et al. 2017).

In North America, Type II strains were most commonly associated with human infection, including in patients with congenital toxoplasmosis and patients with AIDS (Howe and Sibley 1995, Howe et al. 1997). In Europe, the same trend that Type II strains predominate in human infection was observed (Ajzenberg et al. 2002, Honoré et al. 2000).

Despite being a ubiquitous parasite, its prevalence in the human population varies according to geographic location. It is considered a neglected disease of poverty, given its significant association with low-income populations. People with socioeconomic vulnerability are at high risk of *T. gondii* infection due to inadequate sanitary conditions, which favors environmental contamination (Jones and Dubey 2010). Evidence in the literature suggests that *T. gondii* can modify humans' behavior, increasing its impact on public health.

The number of articles that reported on the possible associative of chronic and latent *T. gondii* infection in humans with behavioral changes is relatively scarce; however, some evidence showed the relationship of this infectious agent with the occurrence of neuropathological disorders, especially in the psychiatric sphere, such as schizophrenia (Martinez et al. 2018). A systematic review with a meta-analysis, which evaluated reports from 23 studies, showed that individuals with schizophrenia have an increased prevalence of antibodies against *T. gondii* (Torrey et al. 2007). Currently, the neurodevelopmental theory of schizophrenia includes infectious factors; among these, toxoplasmosis is the most studied and documented (Fuglewicz, Piotrowski, and Stodolak 2017).

The vision of the patient with toxoplasmosis is decreased for several reasons. The vitreal inflammation by itself may be so great as to significantly reduce vision. Also, the lesion may be situated in the posterior pole (although not in the fovea per se), with edema and probable inflammatory by-products of the reactions affecting central vision; however, the fovea may be relatively far from the center of the retinitis. This may be like the phenomenon seen in pars planitis.

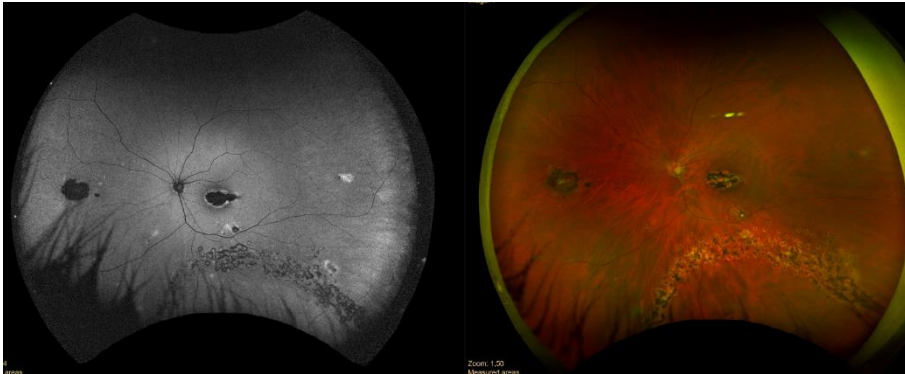


Figure 1. Red-free and color fundus imaging a 35-years-old male patient presented with a macular retinochoroiditis due to toxoplasmosis associated with a retinal tear causing a flat retinal detachment. Photocoagulation was chosen as the therapeutic approach, as the patient was monocular due to total retinal detachment in the contralateral eye.

Another cause of decreased vision is an infection involving the macula (Figures 1 and 2). Once the fovea is involved in the actual infection itself, the potential for a significant return of good vision is poor, and all efforts must be made to prevent this from occurring. The most important sequela of ocular toxoplasmosis is loss of vision due to direct involvement of the fovea by the infection. However, choroidal neovascularization can also occur as a late complication of the disease (Fine et al. 1981, Skorska, Soubrane, and Coscas 1984). Rose reported on a patient with ocular toxoplasmosis with a retinal vein occlusion, papillitis, and florid disc neovascularization (Rose 1991). Pakalin and Arnaud observed an occurrence that manifested with an arteriolar branch occlusion at a site passing through an area of necrosis (Pakalin and Arnaud 1990). They emphasized the need to include toxoplasmosis in the differential diagnosis of even arterial inflammatory phenomena, something not usually done. Bosch-Driessen and associates reported that nine of 150 patients (6%) with ocular toxoplasmosis had retinal detachments, and another seven (5%) had retinal breaks (Bosch-Driessen et al. 2000). Retinal detachment was seen more commonly in myopes, and the visual

prognosis is guarded: five of nine eyes with retinal detachment were left with 20/200 visual acuity or worse. As with any disorder that disrupts Bruch's membrane, choroidal neovascularization, usually at the rim of an old lesion, needs to be looked for. One report describes multiple choroidal neovascularization lesions in one eye with punctate outer retinal *Toxoplasma* lesions (Fujiwara et al. 2006).



Figure 2. Color fundus imaging of a 35-years-old male patient presented with a macular retinochoroiditis due to toxoplasmosis.

Serologic evidence of the *Toxoplasma* organism is of immense importance in helping the clinician make the diagnosis. The presence of anti-*Toxoplasma* immunoglobulin M (IgM) titers suggests a recently acquired infection. Observations by Rubens Belfort Jr, MD, in Brazil (personal communication, 1993) suggested that, at least in some cases, the IgM peak may be highly ephemeral and thus easily missed. Although the ELISA technique has been recommended by many, the most reliable type of serologic testing is still under debate. The diagnosis of ocular toxoplasmosis by serologic means alone may not always be reliable (Rothova et al. 1986). Rothova and coworkers noted that even though IgG antibody positivity was seen in 100% of their patients with clinically apparent ocular toxoplasmosis, 58% of the control subjects also showed positive results. Although seven of 25 patients (28%) with toxoplasmosis had detectable circulating immune complexes with IgG and *Toxoplasma* antigen, two of 12 control subjects (16%) did as well. In reviewing findings from normal control subjects and patients with suspected ocular toxoplasmosis, Phaik and coworkers estimated that at least 77% of those with ocular toxoplasmosis had serotiters $\geq 1:256$, somewhat higher than those found in control subjects (Phaik et al. 1991). Nonetheless, we believe that the diagnosis is very much a clinical one, with serologic findings being supportive but not definitive.

CONCLUSION

Toxoplasmosis is endemic through most of the world. It is generally assumed that approximately 25 to 30% of the world's human population is infected by *Toxoplasma* (Montoya and Liesenfeld 2004). The prevalence varies widely between countries (from 10 to 80%) and often within a given country or between diverse communities in the same region (Pappas, Roussos, and Falagas 2009). Low seroprevalences (10 to 30%) have been observed in North America, South East Asia, Northern Europe, and Sahelian countries of Africa. Moderate prevalence (30 to 50%) has been found in Central and Southern European countries, and high rates have been found in Latin America and tropical African countries.

The diagnosis of retinochoroiditis relies primarily on an ophthalmological examination. The presence of typical lesions (white focal lesions often associated with a vitreous inflammatory reaction) (Figure 2), together with *Toxoplasma* seropositivity, calls for specific anti-*Toxoplasma* treatment, further confirmed by an excellent clinical response.

Substantial advancements in the field of *Toxoplasma* research have occurred in the past 20 years. Basic research in molecular and cell biology benefited from developing high-throughput genomic and proteomic technologies and new imaging techniques. However, in the meantime, epidemiological studies also gained more importance. The emergence of *Toxoplasma* as a waterborne disease in several countries has stimulated environmental research. An ecological and integrated approach was developed to understand better the complex circulation of *Toxoplasma* between its multiple hosts and the environment and, finally, of the risk factors for human infection (Robert-Gangneux and Dardé 2012).

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Chapter 16

IMAGING TECHNIQUES IN THE STUDY OF *TOXOPLASMA GONDII*

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ABSTRACT

From simple slide observation to cutting edge methods (e.g., electron tomography and super-resolution microscopy), Imaging and Image Analysis are important steps in any research project. The use of light and electron microscopy techniques have been used extensively to study *Toxoplasma gondii* cell biology and its interaction with the host cell, leading to recent discoveries and characterizations. Here we will discuss these techniques and how they have been applied in the *Toxoplasma* field. We will also discuss sample preparation and Image Analysis set-ups. We will conclude by presenting newly developed approaches that we think are the new areas that will be important for novel discoveries.

1. INTRODUCTION

1.1. Microscopy and *Toxoplasma gondii* – Brief History to Latest Advances

The history of imaging approaches in *Toxoplasma gondii* starts with the misidentification of a parasite in the North African rodent, *Ctenodactylus gundi* in 1908. Initially believed to be a *Leishmania* spp. parasite, researchers Nicolle and Manceaux realized they had discovered a new organism they named *Toxoplasma gondii*, due to its morphology and the hosts it was

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isolated initially (Nicolle and Manceaux 1908). The first known *Toxoplasma* photography was from the retina of an infant suffering from congenital Toxoplasmosis, published by Josef Janku in 1923 (Janku 1923). Janku described a parasite nucleus that was stained with a pale blue color using both hematoxylin and Giemsa staining (Figures 1A, B). He also studied tissue cysts containing bradyzoites, describing *Toxoplasma* parasites that strongly stained with hematoxylin and eosin. Many reports identified *Toxoplasma*-like organisms in the tissues of different animal species, humans included. In 1934, a major advance in the field of microscopy was done by Frederick Zernike, who invented the phase-contrast microscope (Zernike and Stratton 1934), an achievement that earned him a Nobel Prize in 1953. This invention paved the way for microbiology research, allowing *Toxoplasma* researchers to identify further details on the morphology of the parasite. In 1937, during studies involving viruses, *Toxoplasma* parasites were identified and studied using the same techniques employed in virology at the time (Sabin and Olitsky 1937). From 1937 to 1950, studies involving tissue observations using light microscopes elucidated more information on this parasite. During this period, *Toxoplasma* parasites were identified in brain and eye tissue in mice, rabbits and rats (Wolf et al. 1939) and later humans (Wolf et al. 1939). Although Nicolle, Manceaux and Splendore (Nicolle and Manceaux 1908; Splendore 1908) noted the parasites could reproduce by division, it was later demonstrated by silver staining that parasites reproduce by endodyogeny (Goldman et al. 1958).

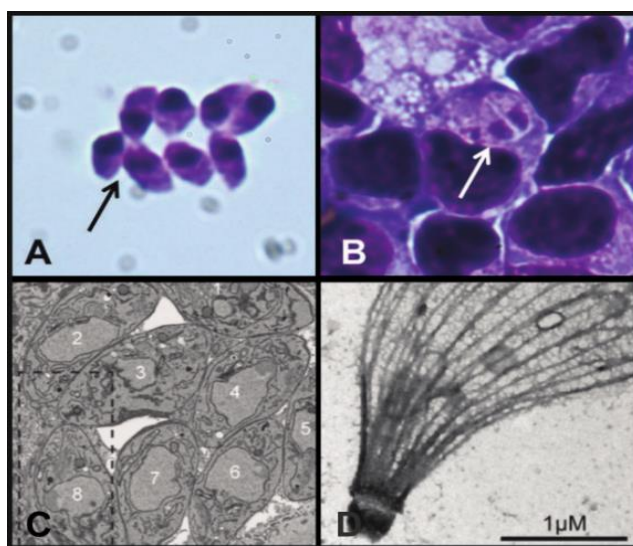


Figure 1. Observations of *Toxoplasma* under microscopes. A. Extracellular tachyzoites from mice peritoneum seven days post infection. Stained with H&E. B. Replicating parasites. Stained with H&E. C. General view of a host cell infected with intracellular *Toxoplasma gondii* in an Electron Microscope. The cell nucleus, rhoptries and micronemes are present. Each individual parasite is numbered. D. An electron micrograph showing the anterior end of *Toxoplasma gondii* showing the cytoskeletal features of the parasite along with structures of the conoid after the removal of the plasma membrane. The pellicular microtubules (MT) originate from the more posterior end of the polar ring (arrows). A and B were adapted from McGovern and Wilson (2013). C was adapted from Hunt et al. (2019). D was adapted from Tosetti et al. (2020). All images under Creative Commons Attribution CC BY license.

During the 1950s, another critical development occurred in the shape of Differential Interference Contrast (DIC) (Nomarski 1960). DIC microscopy allowed greater contrast in transparent samples. Using a combination of staining, and advances of light microscopy such as DIC, the complete life cycle of *Toxoplasma* was identified in 1972 (Dubey and Frenkel 1972).

From the decade 1950 and 1960, electron microscopy revolutionized biological studies of microorganisms. In 1954, transmission electron microscopy (TEM) was first used in *Toxoplasma* ultrastructure morphology research (Gustafson et al. 1954). Several studies involving *Toxoplasma* uncovered complex polarized structures and organelles across the parasite life cycle (Figures 1C, D) (Dubey and Frenkel 1972; Ferguson et al. 1975; Sheffield and Melton 1970). The first mention of endodyogeny, which is a form of asexual proliferation was done via TEM, as fine structures were analyzed revealing that bradyzoites presented few structural differences when compared to tachyzoites (Sheffield and Melton 1968; Vivier 1970). Comparative electron microscopy played a critical role during this time, showing how microorganisms are related by associating morphology and ultrastructure. Combining studies involving other parasites that were thought to be unrelated at the time such as *Plasmodium* and *Eimeria* (Scholtyseck and Mehlhorn 1970) and the advances in comparative electron microscopy, Levine and colleagues created a new classification phylum called Apicomplexa, comprising of several human and animal parasites such as *Theileria*, *Cryptosporidium*, *Plasmodium*, *Eimeria*, *Neospora*, *Babesia* and *Toxoplasma* (Levine et al. 1980). Scanning Electron Microscopy (SEM) also played a role in *Toxoplasma* research, allowing researchers to observe the ultrastructure of tachyzoites invading host cells (Chiappino et al. 1984; Klainer et al. 1973).

At the end of the decade of 1970, the discovery of the Green Fluorescent Protein (GFP) (Prendergast and Mann 1978) brought a turning point in biological research, as GFP and its derivatives revolutionized microscopy by using fluorescence. Advances in light microscopy later revealed the formation of the parasitophorous vacuole (PV) (Suss-Toby et al. 1996) and later paved the way together with video microscopy to determine parasite motility, host cell invasion and to dissect the actors responsible for these phenomena (Gaskins et al. 2004; Håkansson et al. 1999; Wetzel et al. 2003). For the first time, individual proteins could be visualized in tissues without using antibodies or dyes. Using this technique, antibodies conjugated with GFP demonstrated that active invasion and formation of the PV did not require cytoskeletal remodeling from the host cell (Morisaki et al. 1995). Using fluorescein-tagged monoclonal antibodies that are exclusive to bradyzoites allowed to determine differences between bradyzoite and tachyzoite parasites, showing the stage conversion process was asynchronous and can occur at the same time in the same PV (Bohne et al. 1993). *Toxoplasma* is a parasite that can be easily manipulated genetically, explaining its popularity as a model organism. Combining this remarkable quality with the in-cell expression of GFP and other fluorescent reporters (Striepen et al. 1998) permitted a new era of live-cell imaging. This brought forth the dissection of the function of many molecular actors in parasite biology such as micronemes and rhoptries during invasion (Carruthers 2002).

Electron microscopy has provided great advances in the study of *Toxoplasma* biology. Using techniques that range from thin section observation and immunocytochemistry to electron tomography and serial section reconstruction, different publications have provided characterization of novel organelles (Köhler et al. 1997; Miranda et al. 2010; Parussini et al. 2010), have shown the fine structure of cellular components (Lemgruber et al. 2011; Magno

et al. 2005; Melo et al. 2000; Nichols et al. 1994; Sibley et al. 1995) and even the cyst form of the parasite (Lemgruber et al. 2011). Recent advances in EM, cryo-tomography and 3D reconstruction from fixed, serially sectioned samples allowed the visualization of sub-cellular structures in a 3D setting, providing a full spatial image of the parasite and its organelles (Kudryashev et al. 2010; Paredes-Santos et al. 2012).

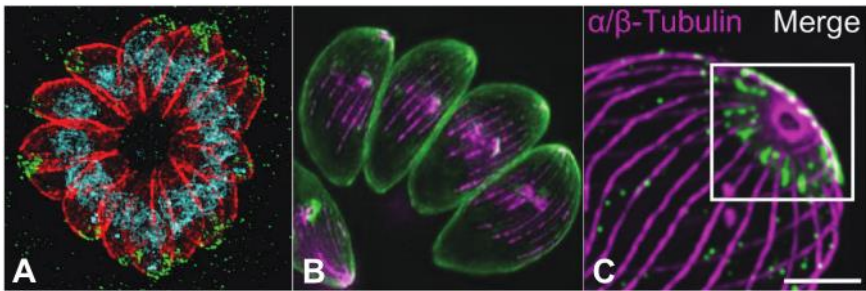


Figure 2. Current microscopy applications including SRM and ExM. A. Super-Resolution Structured Illumination Microscopy of the characteristic *Toxoplasma* rosette, with DNA stained in cyan, plasma membrane in red and an apical protein in green. B. Represent live Super-resolution Structure Illumination Microscopy (SR-SIM) on parasites labeled with GAPM1a (green) and cortical microtubules (TubA1, magenta). C. Ultrastructure Expansion microscopy (U-ExM) combined with STED, presenting that Apical Cap proteins AC9 - AC10 are arranged in rows that are localized between the subpellicular microtubules. B was adapted from Harding et al. 2019 and C was adapted from Tosetti et al. 2020. Figures B and C are licensed under a Creative Commons Attribution 4.0 International License.

Light microscopy also went through an important revolution with the development of Super-resolution Microscopy (SRM), a group of techniques capable of resolving images with sub-diffraction of light resolution (Moerner 2006). Structures that previously required EM to observe were suddenly resolvable with light microscopy together with fluorescent molecules both from natural and artificial sources (Figure 2). Among the first studies to exploit this technology, stimulated emission depletion (STED) was used to study microneme transport by overexpressing Rab-GTPases. Rab5A and Rab5C overexpression revealed their importance in the traffic regulation of micronemes and rhoptries and more importantly, STED microscopy revealed how micronemal organelles are organized in specific subcompartments inside the parasites (Kremer et al. 2013). Structured Illumination Microscopy (SR-SIM) in live and fixed samples have contributed to the advance of our understanding in the PV arrangement (Figure 2A) and cytoskeleton arrangement (Figure 2B).

More recently, Expansion Microscopy (ExM) is revolutionizing the field as it allows the physical expansion of a sample, allowing standard light microscopy techniques to perform as super-resolution approaches (Figure 2C) (Chen et al. 2015; Gambarotto et al. 2019). This technique has already been shown to work with *Toxoplasma* parasites, as it was used to determine the structure and localization of the Apical Cap proteins (AC9-10) intercalated between sub-pellicular microtubules (Tosetti et al. 2020).

Most of the modern techniques described in this short summary are routinely used in *Toxoplasma* research. These techniques usually present advantages and disadvantages that need to be gauged to decide the best tool suited for the type of research needed. In the next

section, light microscopy techniques will be discussed alongside their advantages and disadvantages.

1.2. Choosing the Right Tools for the Job – Applying Microscopy to Suit Research Needs

We are living in exciting times for microscopy in *Toxoplasma* research. Currently, several techniques are available with advantages and disadvantages depending on the use and the needs of the researcher. To begin this chapter, we will start with a brief description of the most common light microscopy techniques that are found and how they have been employed (Figure 3).

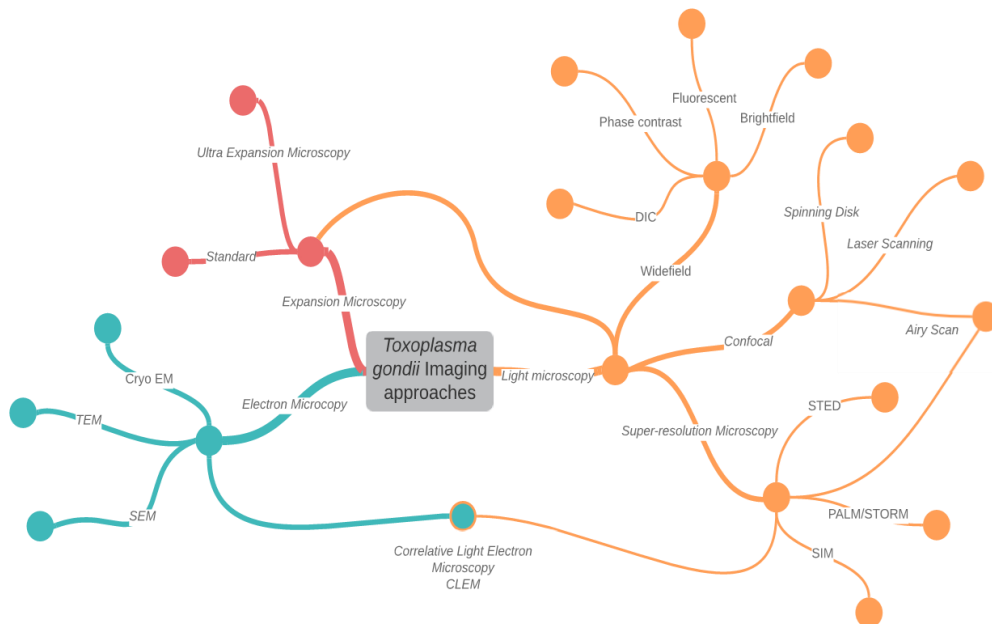


Figure 3. Mind map showing different approaches to image *Toxoplasma gondii* parasites. The main current methods include light and electron microscopy. Light microscopy can be divided into standard and super-resolution. Correlative Light Electron Microscopy (CLEM) is a combination of both. Expansion Microscopy is a method that uses light microscopy.

Widefield microscopy is perhaps the most widely available microscopy set up in the field. Widefield uses a basic illumination principle where light is permanently illuminating the whole sample. The light source typically is halogen, metal halide lamps or LED with the detection being done through digital cameras, light receptors and oculars. Since the sample is constantly illuminated, it often provides poor contrast, requiring additional tools to increase its imaging capabilities. This is done by employing Phase Contrasts, Differential Interference Contrast (DIC) and fluorescence. Due to the nature of having an image captured from a permanently illuminated sample, it is prone to background signal that decreases the quality of the data you can obtain. For microscopy applications using fluorescence, computer-based deconvolution can be employed to increase the image quality and allowing simple 3D

reconstruction. However, as we will learn further in this chapter, deconvolution can also be problematic, as it can introduce image artifacts into your output data. The most common drawbacks of widefield microscopy are a high background noise from unspecific light sources, fast photo-bleaching due to the amount of light used, difficulties on light penetrating samples and low spatial resolution due to light scattering. Widefield microscopy is perhaps the most applied and widespread microscopy technology used in *Toxoplasma gondii* research and is commonly used for standard fluorescent microscopy experiments where simplicity and reliability are required.

Confocal microscopy is also known as Laser scanning confocal microscopy is a more complex microscopy technique that mitigates many shortcomings of widefield techniques with some drawbacks, particularly a considerable increase in equipment complexity and high cost. The light sources of confocal microscopes are lasers coupled with optical fiber, focusing the light. This property of lasers is ideal for penetrating light techniques as the high energy density of lasers allows light penetration in the sample. Confocal microscopy use pinholes as a defining principle to select photons based on the origin of the focal plane. For example, during wide-field microscopy the sample is illuminated as a whole, exciting all possible fluorophores, which will then emit photons in return. These photons are captured by the objectives and funnel into the camera and light sensors. Confocal microscopes, in contrast, concentrate most of the light in a very specific area in a focal plane. This small and specific spot is then manipulated to fully scan the sample, illuminating it in sections. This principle allows the peak of excited fluorophores to be on the same focal plane and photons emitted by other focal planes (e.g., background noise) are not captured, reducing unspecific background noise. This means that the pinhole blocks all the out-of-focus light from reaching the light detectors. Since the illumination is focused in a small area, photobleaching is also reduced greatly, allowing better sample handling. Confocal microscopy approaches in *Toxoplasma* are too many to count. Generally, both confocal and widefield are used depending on the availability of researchers as both can perform the same basic functions but confocal is particularly useful in cases where deep sample penetration of light is required, reduction in photobleaching and speed. A good example is perhaps a live study of *Toxoplasma* invasion in zebrafish as a test model for infection, where confocal microscopy was used to visualize macrophage infection and posterior migration on zebrafish live larvae (Yoshida et al. 2020).

Two-photon excitation microscopy (TPEF) is a type of fluorescent technique that provides superior tissue penetration of up to 1 mm in thickness. In regular fluorescent microscopy, a single photon is used to excite a fluorescent molecule commonly in the visible excitation wavelength (390–700 nm). Following excitation, the electron will return to its stable state, releasing a light photon with less energy than the excitation photon in the process, resulting in the excitation wavelength being shorter than the emission one. In TPEF, excitation requires the simultaneous excitation of two-photons with a longer wavelength than the emission. To do this, two-photon usually requires near-infrared (NIR) excitation light that can excite fluorescent dyes. By using NIR, light scattering is minimized and due to the multiphoton absorption, the background signal is suppressed. The combination of effects allows deep tissue penetration that can surpass confocal microscopy. This approach has been tested in living mice brain tissue to analyze the interactions between CD8 T-cells and parasites from infected mice (Schaeffer et al. 2009).

These key differences between microscopy techniques affect the possible resolution of the final image. Theoretically speaking, light microscopy has a resolution limit of around 250

nm. However, this is counting on perfectly aligned equipment, high numerical aperture optics and low background noise. Since both widefield and confocal microscopes are based on light, their resolution is hard limited by it. However, since widefield microscopes provide high background noise, their resolution capabilities are greatly reduced while confocal microscopes are able to resolve images close to the theoretical light diffraction limit.

Toxoplasma research can require resolution that goes beyond this physical limit in some cases. Often, techniques such as electron microscopy would be used but in the last decade, a new microscopy branch was developed called Super-Resolution Microscopy (SRM) (Jacquemet et al. 2020). In standard microscopy, a fluorophore appears as a single point of light in a blurry state. The size of this blur is called the point-spread function (PSF) and when two molecules are closer than 250 nm, both blurs appear as one, making separating these two molecules impossible. SRM is capable of achieving sub-diffraction resolution, managing to resolve structures of up to 20 nm in some cases, allowing to resolve individual molecules.

SRM comprises multiple techniques that rely on different principles with different applications. The most common SRM types are Stimulated Emission Depletion (STED) Microscopy, Structured Illumination Microscopy (SIM) and Stochastic Optical Reconstruction Microscopy (STORM)/Photoactivation Localisation Microscopy (PALM).

Stimulated Emission Depletion (STED) Microscopy uses a technique named spatially patterned excitation. This technique uses two lasers on a focal plane (excitation laser and STED laser) that when used together, reduces the effective PSF and the lower the PSF, the higher the resolution (Brücker et al. 2011). Additionally, STED used a process called stimulated emission where the lasers deplete fluorophores in excited states near the excitation focal point causing them to return to the ground state. This can be visualized as a suppressed doughnut-shaped ring of fluorophores around the center of the image field, while the image field itself is not suppressed. The signal from the center is then captured by a highly sensitive single-photon detector and used to generate a high-resolution image. The resolution can also be enhanced by applying time gates to the collected data called gated STED or gSTED. Other more complex methods using a similar process are Saturated Structured-Illumination Microscopy (SSIM) and Reversible Saturable Optically Linear Fluorescence Transitions (RESOLFTs). STED is a deterministic functional technique in which exploitable non-linear fluorophore responses are employed to image samples below the light diffraction limit. Other techniques we are going to revise further in this chapter such as SIM, PALM and STORM use mathematical modeling to reconstruct images, presenting different routes to circumvent the light diffraction limit. STED is a fantastic SRM approach for *Toxoplasma* to produce an extremely high-resolution image. For example, STED has been used to analyze Centrin-associated proteins during replication where Peripheral annulus proteins were resolved as a ring shape in a multi-layer structure that is responsible for centrin localization during replication (Lentini et al. 2019).

Structured Illumination Microscopy (SIM) is another technique used to increase the resolution to sub-diffraction levels that use striped illumination patterns to excite the fluorescent sample multiple times. During each of these times, the position of these stripes rotates to obtain a signal from different angles. The resulting data from this process is analyzed by computer software that reads the Moiré patterns that are formed and reconstructs an image based on the aggregate of all the images that were captured. The stripes used in the sample interact with high-frequency light that is produced by the sample. This interaction generates a third pattern that is then analyzed, improving by using multiple images to increase

details further up to twice the original light resolution. SIM provides some of the best benefits among SRM techniques, which include live and 3D imaging. This has been used in *Toxoplasma* research where SIM revealed punctate ERK7 concentration in the basal end of the apical complex ring in mature and daughter parasites (O'Shaughnessy et al. 2020). Live SIM has also been used to assess live actin dynamics upon Ca^{+} dependent pathway stimulation along with actin disruption (Del Rosario et al. 2019).

Stochastic Optical Reconstruction Microscopy (STORM) works by activating only a small number of fluorophores at a single time to reduce potential spatial overlaps, reaching resolutions of up to 5 nm. This process combines photo-switchable dyes with a low power activation laser that allows fluorophore blinking from the ground state to excitation. STORM uses this blinking over time, allowing the identification of precise spatial locations of single molecules. Typically, STORM uses an activator dye that activates fluorophores and a reporter dye that allows a signal to appear. In contrast, direct STORM (dSTORM) only uses a reporter dye, that is combined with buffers and lasers to trigger photo-switching. STORM has been used alongside SIM to assess *Toxoplasma* protein trafficking along actin filaments and microtubules during replication, where live SIM was used to show trafficking and STORM was done to assess single-molecule distribution and recycling during mature and daughter cells (Periz et al. 2019).

In recent years, another method to obtain a high resolution that is compatible with both standard and super-resolution microscopy approaches has the potential to change the field entirely. Expansion microscopy (ExM) is a tool capable of increasing the effective resolving capacity of any microscope by physically expanding the sample. This process is done by using a swellable hydrogel that binds the biomolecules in the sample covalently in situ, which is then expanded by incubating the sample in water. This process has a clear advantage in that it can be done with minimal tools, only requiring reagents that can be obtained with ease and forgoing the need for complex microscopy equipment. The process requires a hydrogel to be formed within fixed cells and tissues. This is done by fixing the cell and diffusing monomers and crosslinkers in the sample. Once the sample is incubated with crosslinkers that will bind to the sample own molecules, polymerization is triggered by the addition of polymerization starters and initiators. Since the sample biomolecules all have different physical properties, a process called homogenization is needed where proteinase K or heat treatments are used to disrupt complex protein networks, allowing isotropic expansion of the sample. Since the proteolysis step can be damaging to antibodies and other fluorescent molecules, several protocols are employed, where some stainings are done following homogenization. Expansion microscopy has been used to localize Apical Cap proteins (AC9 and AC10) in the alveolin network, intercalated between sub-pellicular microtubules (Tosetti et al. 2020).

2. FACTORS INVOLVED IN CAPTURING MICROSCOPY DATA

2.1. Using the Right Subjects

2.1.1. *Toxoplasma gondii* Strains

Toxoplasma has been used in laboratory settings for over 100 years. In the northern hemisphere, three distinct clonal lineages are found (Type I, II and III) while the southern

hemisphere presents a richer distribution of heterogeneous lineages, often called aberrant or exotic. *Toxoplasma* research has focused on these three clonal lineages, with type I as the most commonly used due to its fast-growing speed and adaptation to lab settings.

Among type I parasites, the RH strain is the most widely used in the laboratory. Originally isolated in 1939 (Sabin 1941) from a 6 years old boy, the prolonged culture of this parasite over the next decades caused the strain to lose its stage conversion capabilities, becoming unable of producing oocysts in cats (Frenkel et al. 1976). The type I strain is considered as the more virulent strain in mice, with type II and III strains as less virulent and slow (Sibley and Boothroyd 1992).

Choosing a *T. gondii* strain for microscopy work will require a good understanding of the main biological question that needs to be answered, as type I RH *Toxoplasma* strains will not be useful to study tachyzoite to bradyzoite stage conversion and type II would produce different dynamics inside the host cell (Frenkel et al. 1976), such as the absence of association to the host's nucleus due to the lack of the MAF1 protein (Pernas et al. 2014). Luckily, genetic manipulation is similar between strains and doesn't require adaptation, facilitating the process of adapting the parasite to the needs of particular studies. *Toxoplasma* genomic manipulation is relatively straightforward, representing a key model organism in the study of Apicomplexan parasites (Szabo and Finney 2017). Over the years, many different *Toxoplasma* reporter strains have been created exploiting the capabilities of different fluorescent proteins and molecules (e.g., GFP fused to a protein of interest). Choosing between these molecules is vital to our research and is one of the most important aspects of planning microscopy approaches.

2.1.2. Fluorescent Proteins

The original GFP was discovered at the end of 1970 (Prendergast and Mann 1978) and it took almost 20 years until GFP was used as a genetically encoded fluorescent tag (Chalfie et al. 1994). This was followed by in-deep engineering into optimizing the fluorescent protein and expand the variety of colors (Figure 4) (Cubitt et al. 1995; Heim and Tsien 1996). By this time, *Toxoplasma* research started to incorporate Fluorescent Proteins (FP) into its research tool repertoire, resulting in studies characterizing parasite motility, host cell invasion and the molecular elements involved (Gaskins et al. 2004; Håkansson et al. 1999; Wetzel et al. 2003).

Work on FP continued with many other Fluorescent Proteins appearing over the years including the red fluorescent protein DsRed from the anemone *Discosoma striatamushroom* (Campbell et al. 2002; Shaner et al. 2004); RFP from the anemone *Entacmaea quadricolor* (Shaner et al. 2004) among others. One of the most important aspects of FP engineering is to develop brighter, more stable molecules. This was done to reduce the intracellular concentration of the FP, allowing less interference with the tagged protein while increasing the contrast for imaging. Achieving this goal required improving gene expression, codon optimization and making structural changes in the protein. Another important aspect is that most of the original FPs were naturally dimeric or tetrameric, which requires the union of two or three molecules to achieve fluorescence. Naturally, this hampered the dissection of biological phenomena where the concentration of the FP was high, directly altering the native biological functions of the tagged proteins. To solve this, monomeric variants were engineered (Campbell et al. 2002) that do not need to oligomerize, managing to be bright as monomers. These protein variants are usually denoted by the "m" prefix before the name (e.g., mCherry) and should be preferentially used when choosing FP for research.

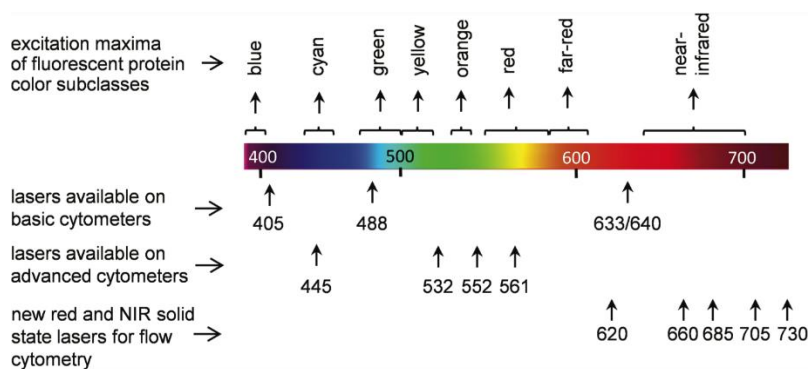


Figure 4. Excitation maxima of the fluorescent proteins and probes wavelengths used in *Toxoplasma gondii* research. Fluorescent probes, both organic and inorganic can be used to label proteins and structures in *Toxoplasma gondii*. Re-used from Telford et al. 2015 (Creative Commons Attribution CC BY license).

Additionally, photo-switchable fluorescent proteins have also been developed (Chudakov et al. 2004) that are capable of transition from cyan to green by using 405 nm light. Another example that is invaluable for live imaging is green to red FP photoconversion. This is done by using a 405 nm laser for excitation, resulting in emission at the range of 580-670 nm (Sattarzadeh et al. 2015). These advances in fluorescent proteins add to the increasing repertoire available for microscopists, allowing better and more elegant approaches for the imaging needs that are required.

2.1.3. Calcium Ion Indicators

Another example of fluorescence in microscopy is fluorescent calcium indicators. They work by producing a fluorescent signal as a result of calcium (Ca^{2+}) concentration-dependent changes (Miyawaki et al. 1997; Persechini et al. 1997). Calcium indicators have been used to assess calcium-dependent functions such as the case of host-cell invasion and egress (Pace et al. 2014). In this case, evidence that Ca^{2+} influx is critical for virulence as extracellular Ca^{2+} influx to the cytosol of the parasite leading to an increase of Ca^{2+} , improving gliding, conoid extrusion, microneme secretion and invasion. Calcium sensor proteins were used to measure Ca^{2+} influx using Fura-2 (Miyawaki et al. 1997) fluorescent signal during critical parts of the lytic cycle of the parasite.

2.1.4. Artificial Fluorescent Probes

Many options for fluorescent proteins exist, however they have limitations. When compared to organic dyes, the photophysical properties are not as good, are less bright, can blink and can be unstable. Protein-based fusion tags, on the other hand, are capable of covalently attach to organic fluorophores inside living cells (Lin and Wang 2008). Generally, these fluorescent proteins are the size of a fluorescent protein but can be smaller. Additionally, these fluorescent tags are usually not fluorescent on their own but become so when exposed to a fluorescent ligand, amplifying the range of experiments they are capable of performing (Los et al. 2008). The utility of these molecules offers sequential labeling schemes, for example, pulse-chase experiments can be done where an initial fluorophore is

added followed by a second different fluorophore for the same ligand. This can result in labeling the same target and temporally separating the target.

Artificial fluorescent probes are particularly useful in SRM, as they often present better chemical and physical characteristics in terms of photobleaching, excitation, emission and size. Techniques such as SIM are the closest to standard fluorescent microscopy approaches but artificial probes are always better suited for it (Galbraith and Galbraith 2011). For other super-resolution techniques, the excitation state of the fluorescent probe is critical, as they often need to present a photo-switchable state either reversibly or irreversibly from a light to a dark state.

Single-molecule super-resolution techniques require precise location on single fluorophores, requiring special probes. Although photoactivable fluorophores can be used for this, inorganic dyes can present up to tenfold higher photon yields, providing superior precision in localization (Shtengel et al. 2009). Traditionally, these probes were attached to an antibody and couple with the size of the inorganic dyes, can present sizes up to threefold the size of a genetically expressed fluorescent protein. To solve this problem, new tagging methods for proteins of interest are used such as the Halo, SNAP and CLIP tag that covalently attaches to a probe carrying a specially devised dye, providing superior performance in virtually any single-molecule super-resolution application (Dellagiacoma et al. 2010; Hein et al. 2010; Lee et al. 2010).

In a more practical example, Periz and colleagues devised an elegant pulse-chase experiment for *Toxoplasma* recycling during parasite division, where micronemal proteins were labeled by an Oregon Green fluorescent ligand for a HALO-tag, followed by using a TMR-based ligand for the same tag 18 hours later (Periz et al. 2019). This resulted in the separation of two groups of micronemal proteins, where both micronemal populations were present in both daughter cells following endodyogeny (Periz et al. 2019).

Another example in *Toxoplasma* research is the use of SiR Tubulin to label the microtubules in live parasites prior to fixation to study microtubule deformation following invasion (Del Rosario et al. 2019) and microtubule protein trafficking during replication (Periz et al. 2019). SiR Tubulin is a fluorophore based on silicon rhodamine (SiR) and the microtubule-binding drug Docetaxel. This reagent can label microtubules in live cells in a highly specific manner without high background noise.

Small fluorescent molecules provide a wide range of utility for microscopy. This utility includes the detection of specific metabolic elements, biomolecules, proteins, structures and even whole tissues. Generally, these fluorophores are modified to act as fluorescent tags that react and bind to the target molecule by either biological mechanisms such as a self-labelling protein tag (Encell 2012; Keppler et al. 2003; Klein et al. 2011; Los et al. 2008) and chemical-based (Kapuscinski 1995). Classical examples of chemical-based fluorescent labels are DAPI (Kapuscinski 1995) and Hoechst (Latt et al. 1975). In this case, both are nuclear-specific dyes that are used to stain fixed and live cells depending on the situation. DAPI and Hoechst are structurally similar and bind to the grooves of DNA, particularly with a high affinity for A/T-rich regions. These dyes are not fluorescent on their own but become bright upon DNA binding. Additionally, Hoechst is permeable, meaning it can be used without a permeabilization step.

2.2. Sample Processing

Sample processing is perhaps, the most important part to obtain quality data. Incorrect sample preparation often leads to poor analysis of the data generated by imaging and thus, incorrect interpretation and conclusions. Processes such as sample fixation, permeabilization and the immunofluorescence protocol used have a large impact on the final sample and should not be overlooked. Selecting the best fluorescent probes, using the most advance microscopy techniques become completely useless unless the sample is properly prepared to provide the best quality possible as the resulting imaging is only as good as the sample (Figure 5).

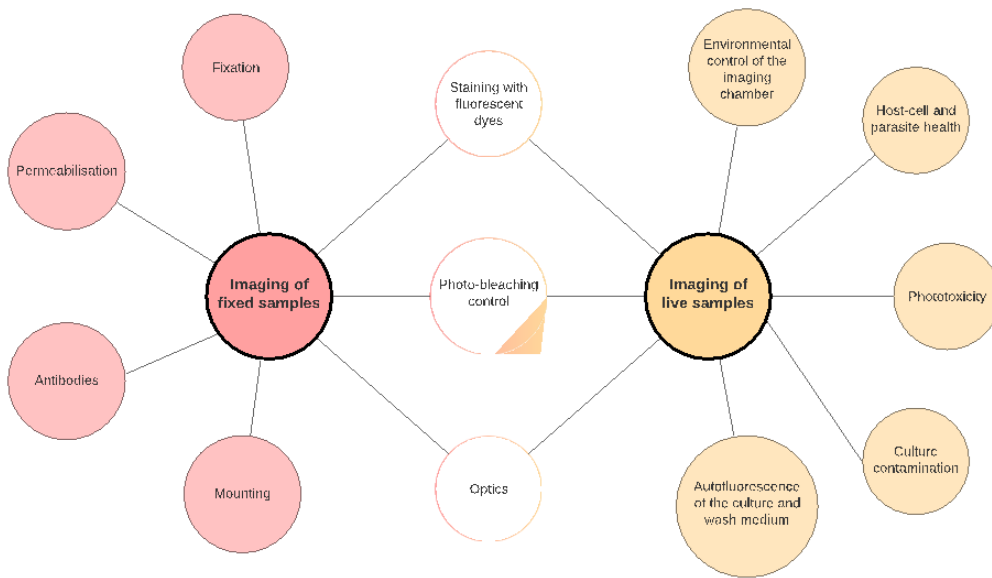


Figure 5. Factors affecting imaging quality. Factors that affect imaging quality in both fixed and live samples. Although several factors are not shared between both methods, a few are common.

For this reason, sample preparation is a critical step requiring a plan to acquire good quality data. The workflow for this step goes from: Experimental design -> Fluorescent probes and microscopy techniques -> sample preparation -> fixation -> permeabilization -> immunofluorescence -> imaging (Figure 6).

2.2.1. Sample Fixation

Generally, fluorescent microscopy experiments are classified as either live or fixed experiments. For fixed experiments, the cells to be imaged require to be fixed and killed prior to the sample preparation process and are usually paired with immunofluorescence. Experiments using fluorescent microscopy require the use of a fixative agent that kills the cells but maintains cellular structure as much as possible. Maintaining cellular structure is critical, as partially preserved structures or worse often leads to bad imaging data. Cell fixatives are generally divided into two groups: aldehyde and alcohol fixatives. These fixatives work by cross-linking and preserving protein structures that will allow the posterior use of antibodies for immunofluorescence (Leyton-Puig et al. 2016).

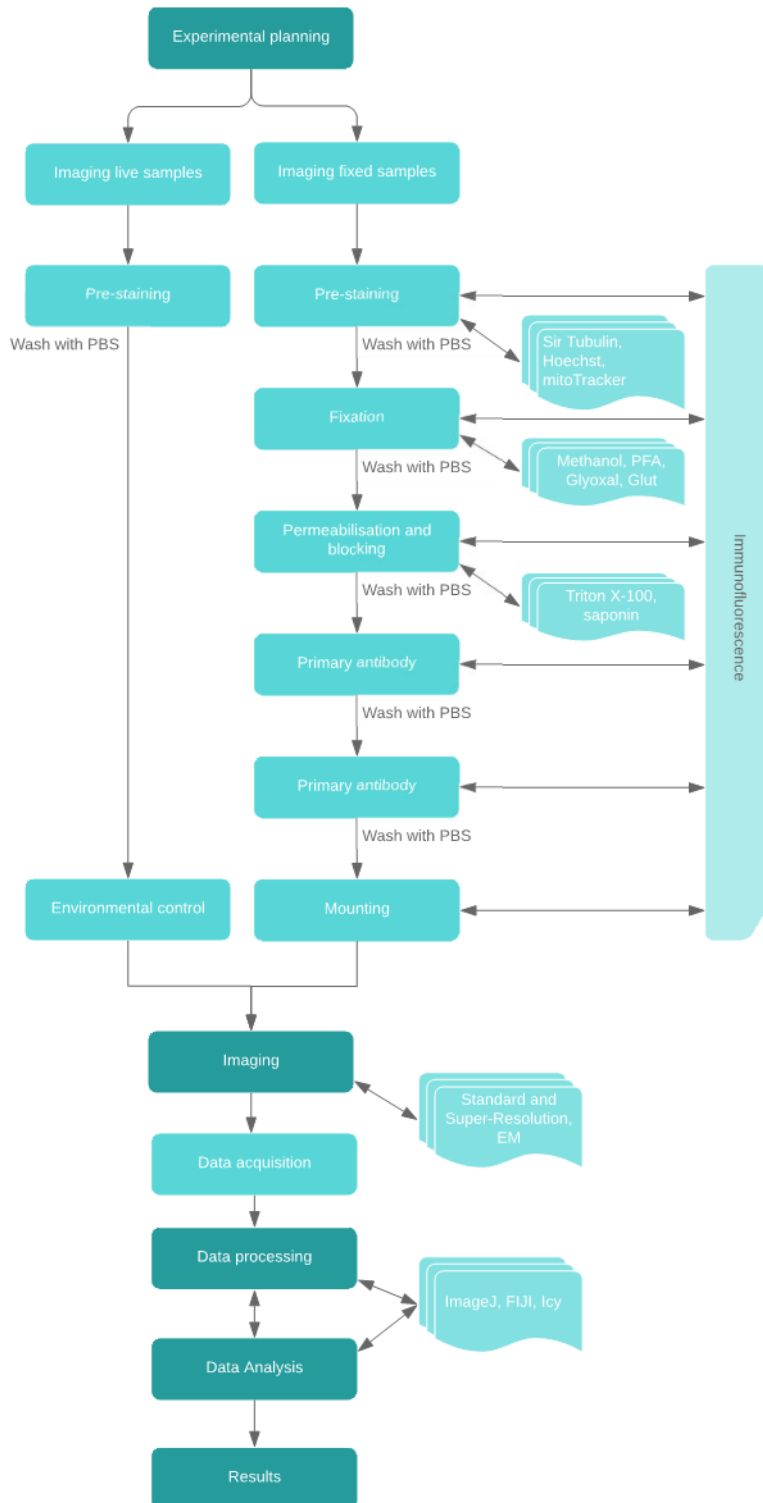


Figure 6. Workflow of microscopy experiments. Flow of microscopy experimentation that includes live and fixed imaging.

Aldehydes work by crosslinking proteins, maintaining cell morphology. Aldehydes are generally slower than alcohol-based fixatives, but they represent the most commonly used fixatives due to their ability to preserve cellular structures, cell morphology and protein features. From this group, the most commonly used aldehyde-based fixative is paraformaldehyde (PFA) at 4%. This is done by leaving the fixative from 10 to 20 minutes and washing away the sample afterward. This fixation speed can be improved by combining PFA with glutaraldehyde, increasing the fixation speed and thus, obtaining better preservation of molecules, though glutaraldehyde is autofluorescence and can give some background during imaging. Another fixative that is becoming more common is glyoxal. Glyoxal is considered less toxic than PFA but fixates samples faster than PFA and PFA + glutaraldehyde. Additionally, it has been found to crosslink proteins more effectively, improving antigenicity (Richter et al. 2018). Traditionally, *Toxoplasma* research has used PFA as the fixative of choice. However, glyoxal is an attractive alternative that should be evaluated more deeply as PFA fixation can lead to changes in morphology and protein structure.

Alcohols, on the other hand, fixate samples faster than aldehydes while also maintaining structures, particularly cytoskeletal structures. At the same time, alcohol-based fixatives, such as methanol, don't precipitate small molecules during fixation, masking them and making difficult their imaging. Methanol is also incompatible with fluorescent-tagged proteins, as their fluorescent is abolished due to this type of fixation. Additionally, methanol fixation is known to cause morphological changes in *Toxoplasma* tachyzoites, changing them from a crescent shape to a more circular one.

A critical and often overlooked aspect of fixation is buffer selection. Ideally, buffers should be used with fixatives to maintain cellular structures. This should be done by using isotonic buffers that maintain the pH as close to physiological as possible. For example, this becomes more apparent with the amount of finer actin structures preserved on samples fixed with 4% PFA with the addition of a cytoskeleton buffer (CB-Buffer – 4% PFA 5 mM Tris-HCl pH 8.0 and 0.2 mM CaCl₂) designed to maintain the structural integrity of cytoskeletal proteins, while samples fixed only with 4% PFA shows a small reduction of the aforementioned actin structures.

2.2.2. Permeabilization

Permeabilization methods allow fixatives (if necessary), antibodies, dyes and other elements to access cell structures in a rapid way by the removal of lipids using detergents. Detergents are polar lipids with a hydrophilic end, capable of turning hydrophobic structures into hydrophilic by binding into the hydrophobic moieties. Other detergents exist, such as non-ionic detergents that possess a methyl group able to solubilize membranes without destroying protein structures. This group is quite diverse and comprises well-known detergents that include Triton X-100 and Tween 20. On the other hand, ionic detergents possess strong membrane solubilization properties, as they contain highly charged hydrophilic groups. However, they are harsh on the sample and often disrupt native proteins conformations. This group includes SDS, CHAPS and deoxycholate. Saponins are also detergents but present low protein disruption qualities. This is often quite desirable for permeabilization but due to their mild effect, they cannot permeabilize cells completely, particularly those containing low cholesterol.

Permeabilization methods are also important, as commented in the last example, different methods can alter protein structures depending on the severity of the used compound. This can be clearly seen when treating two equal samples with either Saponin or Triton-X. Triton-X is an organic solvent that permeabilizes by dissolving unspecific lipids from cell membranes that can also affect protein structures, while Saponin works by interacting and selectively removing cholesterol, leaving holes in the cell membrane. Being Triton-X a harsher non-specific permeabilizer, the GRA7 structure around the parasite vacuole are lost, leaving unspecific debris; while Saponin maintains the structural integrity as it selectively maintains protein structures, leaving structures that resemble membranes (Jamur and Oliver 2010).

2.2.3. Blocking

If immunofluorescence is required, blocking is vital to reduce the non-specific binding of antibodies to the sample. Unspecific antibody binding can occur due to many reasons, but cross-linking antibodies to unspecific structures is the most important one. Usually, this blocking step is done by incubating the sample with a solution of bovine serum albumin (BSA), gelatine, casein or serum. These solutions are usually employed in concentrations between 1 to 10% and are used following permeabilization or used in conjunction.

2.2.4. Antibodies

To correctly label the proteins or structures that are required, antibodies divided into primary and secondary are used. Primary antibodies bind the target protein directly and the secondary bind to the primary antibody and are usually fused to a fluorescent molecule. It is also possible to fuse the primary antibody directly to a fluorescent molecule to achieve direct immunofluorescent. This method is faster than using the indirect method, but it can lead to unspecific labeling and should be avoided unless the antibodies used are well characterized.

Primary antibodies look for a specific epitope in the sample and are classified as monoclonal and polyclonal. Monoclonal antibodies present a homogenous population of antibodies that all bind to a single epitope. These types of antibodies are usually generated *in vitro* by using a single B-cell clone. Polyclonal antibodies, on the other hand, are a heterogenous mixture that can target multiple epitopes of the same protein. They are generated from many B-cell clones. Both options are valid when performing immunofluorescence, but monoclonal antibodies are usually preferred when doing single-molecule super-resolution microscopy.

The concentration of antibodies varies greatly from batch to batch, especially if sourced from non-commercial origins. This type of antibody usually requires testing and often degrade when stored for long times. Commercially sourced antibodies are usually more stable and are optimized to use standard concentrations (usually 1:1000). The concentration of antibodies is very important as low concentrations result in low signal due to no target saturation and high concentration leads to unspecific binding of antibodies into other structures. Independently of the concentration, this step is usually done by incubating the sample for 60 minutes at room temperature and followed by washing with phosphate-based buffers such as PBS.

Secondary antibodies target the first primary antibody directly and provide a significant advantage to direct immunolabeling since they provide increased sensitivity. Additionally, they are more specific and can be tinkered with, as they often target antibodies of specific

animal origins. Additionally, since they only require to be specific against other antibodies, they are sold as ready-to-use commercially, fused to fluorescent molecules. However, when working with this type of antibody, it is important to use secondary antibodies raised against the same animal species of the first antibody, as different ones are generally incompatible. There are exceptions to this rule, as some animal species cross-react against other (e.g., mice with rat or rabbit). Secondary antibodies are usually incubated for another 60 minutes and washed with a phosphate-based buffer such as PBS.

2.2.5. Counter Stains

Counter stains in *Toxoplasma* immunofluorescence assays are usually done to identify cellular organelles such as the nucleus. The nucleus is usually identified by DNA-binding artificial dyes such as DAPI or Hoechst. Other molecular probes such as phalloidin can be used to label actin, as they provide high F-actin affinity. However, the affinity is often limited in *Toxoplasma* actin and initially believe to be due to a low amount of F-actin filaments in the parasite (Poupel et al. 2013) but later shown to be due to unstable actin filaments that can be visualized when paired with another element such as anti-actin nanobodies (Periz et al. 2017).

Another benefit of these counter-staining methods is that they don't usually have cross-reactivity with one another and can be used in conjunction with methods such as immunolabelling, providing much-needed versatility to imaging experiments.

2.2.6. Mounting Media

Mounting media is a good method for sample storage and preserving the fluorescence for longer time periods, as they have chemical agents that scavenge free radicals, reducing photobleaching. The mounting media is used to treat sample slides and not only allows preservation but also increases the refractive index of the sample, improving imaging. When mounting the sample in practice, it is important to use distilled water prior to sealing, as the salts from the PBS buffers will precipitate after drying, making imaging much harder. Testing different mounting media is advisable since some can cause cell shape deformation/compression.

2.3. Live Sample Preparation

Live imaging is often challenging, and successful imaging depends on keeping the specimen in a stress-free environment, maintaining optimal conditions as much as possible. These considerations comprise several factors that include the culture medium, CO₂ levels, temperature, osmolarity and light toxicity.

Toxoplasma is an obligate intracellular parasite and thus, the host cell health will largely impact imaging during intracellular live imaging. Additionally, free-living parasites often used for assays such as gliding (Frénal et al. 2010; Keeley and Soldati 2004; Whitelaw et al. 2017) still require similar conditions, as they grow using favorable conditions for their host-cells. These conditions are maintained by supplementing the growth media with 10% Fetal Bovine Serum (FBS) and maintaining 5% CO₂ in the culture chamber. Generally, growth media uses phenol red as a pH indicator. Although useful for routine cultures, it can increase the background noise during imaging. Simple corrections such as changing the medium used

during image capturing can improve data quality. However, if the sample is washed with PBS and then the original media is replaced with a non-fluorescent option, such as a phenol-free medium, the Signal to Noise Ratio (SNR) can increase to better levels, easing posterior analysis of the obtained data.

The temperature used is also crucial, since temperature changes will affect cell growth (Sheffield and Melton, 1970). For this reason, when imaging live samples, a chamber incubator for the microscope is essential. Failure to provide one will trigger a non-physiologic behavior of the cells, resulting in data that is not useful. Osmolarity is also important, as the medium will evaporate during capturing. To solve this, the environment needs to be humidified via CO₂ gas that is bubbled through a water source. This must be done with caution, as the humidified area should be localized to avoid mechanical or electrical damage to the microscope.

Another factor to take into account is cell health, as it will affect any results obtained from the experiment. Monitoring both host cells and parasite cellular morphology is critical and can be done by using transmitted-light techniques such as DIC or bright-field. Generally, plasma membrane blebbing or formation of bulge-like structures are cues that cell health is not ideal (Tinevez et al. 2009). The fluorescent proteins in the sample are also important to monitor, particularly in the host-cells but not limited to, as clustering of fluorescent proteins are an indication of stress and protein overexpression (Link et al. 2006).

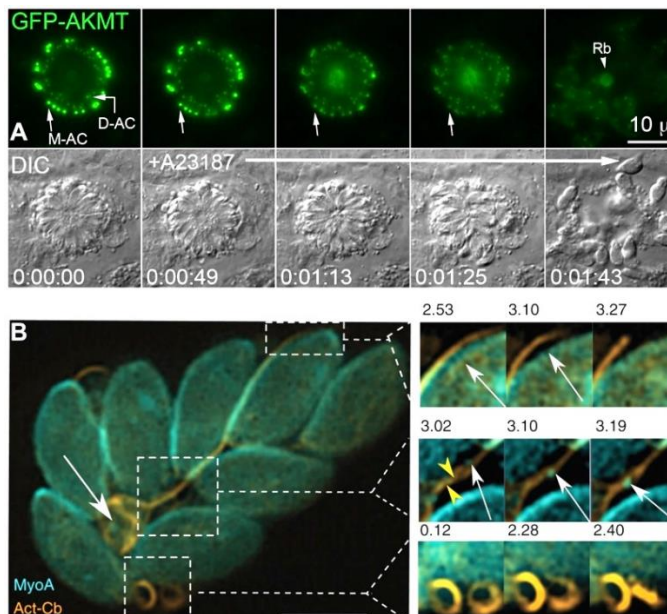


Figure 7. Live imaging examples of *Toxoplasma gondii*. A. *Toxoplasma* lysine methyltransferase, AKMT (for Apical complex lysine (K) methyltransferase). AKMT is crucial for the lytic cycle and is present across parasite division and egress (white arrows). Depletion of AKMT greatly inhibits motility activation, compromising invasion and egress. The residual body (Rb) is left behind after egress. B. Using live SR-SIM, the mobile and interconnected F-actin network is shown to support vesicular transport between daughter cells. Stills show MyoA vesicles associated with F-actin filaments (arrow). Recycled MyoA is transported from the daughter to the residual body. A was adapted from Heaslip et al. 2011 and B was adapted from Periz et al. 2019; both re-used under the Creative Commons Attribution CC BY license.

Cellular contamination is also an important factor that can affect imaging and cell cultures in general. For this reason, routine maintenance protocols should be employed to regularly test both the *Toxoplasma* parasites and host-cells for mycoplasma, yeast, bacteria and fungus (Khan and Grigg 2017).

Lastly, it is important to take into account the type of live experiments and their potential individual requirements in terms of reagents. For example, when performing gliding assays, unique buffers are needed to trigger parasite motility called gliding buffers (Frénal et al. 2010; Keeley and Soldati 2004; Münter et al. 2009). Other assays such as egress assays require the use of egress triggers, usually by employing BIPO and calcium ionophores (Arrizabalaga et al. 2004; Caldas et al. 2010; Lourido et al. 2012) that provide an influx in the calcium ion concentration inside and outside the parasite, trigger rapid egress. Protocols for each type of experiment vary greatly and the general recommendation is to check previous literature (Figure 7).

2.4. Capturing

Image acquisition during both fixed and live fluorescent microscopy requires the collection of fluorescent light, maximizing the amount of captured light while minimizing phototoxicity effects as much as possible.

Filters for excitation and emission and beam-splitting dichroic mirrors are required for any fluorescence-based microscope. Excitation and emission filters are often localized in either fluorescent filter cubes or filter wheels that allow automatically changing the filters for specific excitation and emission wavelengths. Single-color experiments require an excitation and emission filter and a dichroic mirror for each specific fluorophore. These filters need to be as specific as possible, as often even similar fluorophores are not necessarily compatible between them (e.g., RFP and rhodamine-based dyes) (Sanderson et al. 2014). Multi-color experiments also use excitation and emission filter and a dichroic mirror for specific, individual dyes but images need to be captured sequentially, using different light conditions and filters depending on the case. Situations such as this, require careful mirror mounting, as misalignment results in x, y, or z variations between filters, making co-localization experiments unreliable. Multichroic mirrors can improve these variations and are particularly useful for fast-speed imaging, however, light detection is less efficient (Piper 2010).

During practical applications using multiple channels for capturing images, it is important to use controls such as capturing images using all available channels, even if only one staining/color is used. This is done to assess crosstalk, which is when one non-specific dye is excited by the target dye due to similarities, and bleed-through, which is when the emission of one dye can be detected into another channel belonging to a different dye (Kraus et al. 2007).

Another important aspect is the light collection of the lenses, which is a value proportional to the magnification (Mag) and numerical aperture (NA). High numerical aperture is critical, as it can collect light more efficiently, improving image quality and accuracy. Higher magnifications, on the other hand, decrease the efficiency of light-collection. This can be illustrated by comparing a 100x objective with a 1.4 NA and a 60x 1.4NA. The 60x objective will collect considerably more light and thus, will provide far better image quality (Frigault et al. 2009).

During capturing, light exposure and the number of fluorescent probes should be maintained to a minimum, as over-exposure can result in the creation of image artifacts, can mask other signals and produce non-physiological responses. To also reduce phototoxicity, it is advisable that initial sample focusing should be done using transmitted light. The results of using fluorescent dyes or proteins vary by the structures that need to be visualized. The dim signal often results in low SNR and is more common in live imaging. To improve the SNR, non-specific background fluorescence should be reduced by employing phenol red-free medium in the sample at least 12 hours prior to imaging (Frigault et al. 2009). In addition to capturing good quality both live and fixed data, it is possible to improve the signal post-imaging. This is done by post-processing techniques that will be discussed next.

2.5. Post-Processing/Analyzing Data

Post-processing imaging data is absolutely necessary to put into context the information we are acquiring. This can take the shape of “cleaning” the captured data via deconvolution or other computational approaches and processing the data with specialized software that allows the interpretation and contextualization of the information. Image deconvolution considerably improves the SNR and even spatial resolution of images after capturing. To perform image deconvolution, a collection of z-stacks is necessary to increase the effectivity of distortion correction. Live-cell imaging often does not require it, unless 3D structures are necessary for visualization.

The other side of post-processing is both commercial and free software that can be used for image analysis. Obtaining data from microscopy images is, perhaps, the most important aspect of microscopy in general. This is particularly useful depending on the context of the data that is available. For example, image processing is vital to analyze, associate and quantify fluorescent signals that can occur in tagged proteins, structures or cells. It is also critical for high-throughput imaging applications, where hundreds, or thousands of images require processing and analysis. Regardless of the type of data at hand, image analysis provides highly accurate, unbiased and reproducible results far superior to manual analysis.

Image analysis software has dramatically increased over the years, with many different software packages and algorithms that possess either general applications or very specific functions. Since the sheer amount of commercial and open-source software available makes it difficult to choose the right tool, this chapter will present an overview of some of the choices and spend more time with the most popular imaging analysis package, ImageJ/FIJI image (Schindelin et al. 2012, 2015).

There is a plethora of image analysis software, both commercial and open-source. Commercial software usually provides more streamlined and user-friendly interfaces for image analysis. They are often highly expensive, can include a wide variety of functions and are often bundled with imaging instruments. The most common include Volocity, Imaris (Bitplane Scientific Software), NIS-Elements (Nikon), SlideBook, MetaMorph and ZEN (Zeiss). Open-source software on the other hand, freely available online and provide a wider variety of functions with the downside of being less user-friendly. Examples include Icy (De Chaumont et al. 2012), CellProfiler (Carpenter et al. 2006), Vaa3D (Peng et al. 2010), ImageJ (Schindelin et al. 2015) and FIJI (Schindelin et al. 2012).

ImageJ/FIJI is perhaps the most popular and well-known image analysis software. It has been in circulation for a long time while being completely free, allowing it to become highly popular and praised (Schindelin et al. 2015). The main advantage is that a large number of researchers have developed individual applications (plugins and macros) that expand the original capabilities and allowing unparalleled versatility to this software. Due to its popularity, also serves as a central hub of sorts, allowing researchers to focus on the development of tools without worrying about the software infrastructure and dissemination. This flexibility in design has allowed it to become the standard for image analysis software, becoming the baseline for other image analysis software such as Icy. A major challenge ImageJ currently faces is the overabundance of plugins and options, making choosing the right tool difficult, as many solutions are available. To solve this issue and make ImageJ more specialized, FIJI was created; tailored to the microscopy community (Schindelin et al. 2012). FIJI provides new functions for microscopy analysis, as well as an automated updating function that simplifies plugin management. In addition, FIJI also provides support for several scripting languages that can be paired with libraries of algorithms that promote productivity.

CONCLUSION

Toxoplasma gondii research and microscopy have historically gone hand-by-hand, with several major advances in the biology of this apicomplexan parasite have been accompanied by imaging. From the early days of characterization in *Ctenodactylus gundi* samples by Nicolle and Manceaux, to current Super-resolution techniques, microscopy has proven to be a vital tool for parasitologists. However, limitations in the field still hamper the integration of new technologies, limiting the potential and quality of research. These limitations include distribution, complexity and accessibility of equipment, with accessibility as the biggest hindrance in the field. Currently, accessibility of technology is limited, as high-end microscopes require specialized training and are expensive to buy and maintain.

There is a growing movement in the field of microscopy to reduce limitations in terms of accessibility, as 3D printing is slowly becoming more prevalent in several manufacturing industries. Briefly, 3D printing or additive manufacturing is a type of manufacturing where a 3D object designed using specialized software is “printed” by a machine in an additive manner. This means that the object is printed by layers-upon-layers using a wide variety of materials such as polymers, resins, metals and even concrete (Gu et al. 2016; Ligon et al. 2017; Weisgrab et al. 2019). Commercial 3D printers have become cheap and accessible enough that anyone can print 3D objects downloaded from an online repository without prior training. Although 3D printing is still in its infancy, several projects involving 3D printed microscopes already exist where a researcher can download and print almost all the parts of a microscope. These advances will allow a wider set of researchers to access complex microscopy techniques, increasing research output.

Besides imaging techniques, image processing is also suffering a revolution with the adaptation of AI-based computational approaches for data analysis. These pipelines rely on Machine Learning algorithms that can learn to recognize patterns and shapers, classifying cells and detecting events beyond what the human eye is capable of. These systems are

particularly useful for high-throughput experiments but can be employed for tasks as simple as cell counting. Luckily, *Toxoplasma* is already been used with such a system to allow parasite and host quantification, host cell segmentation and classification; and host-pathogen interactions using co-localization of host proteins recruited the parasite during host cell response to *Toxoplasma* infection (Fisch et al. 2020, 2019).

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Toxoplasma gondii

BIOLOGY AND ROLE IN HEALTH AND DISEASE

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ISBN 978-1-68507-229-2



9 781685 072292