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# Insights towards a better understanding and novel treatment modalities of Toxoplasmosis

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► **To cite this version:**

Maguy Hamie. Insights towards a better understanding and novel treatment modalities of Toxoplasmosis. Human health and pathology. Université Montpellier, 2019. English. NNT : 2019MONTT032 . tel-02464661

**HAL Id: tel-02464661**

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# THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Microbiologie, Maladies transmissibles et hygiène

École doctorale Sciences chimiques et biologiques pour la santé (CBS2)

Unité de recherche Institut des Biomolécules Max Mousseron IBMM UMR5247, Université de Montpellier

**Pistes pour une meilleure compréhension et de  
nouvelles modalités de traitement de la toxoplasmose**

**Insights towards a better understanding and novel  
treatment modalities for Toxoplasmosis**

**Présentée par Maguy Hamie  
Le 22 novembre 2019**

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**UNIVERSITÉ  
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# **ACKNOWLEDGMENTS**

First, I would like to express my special appreciation and thanks to my thesis director Professor Carine Deleuze-Masquéfa and thesis co-director Dr Hiba EL Hajj for their continuous support. It was fantastic to have the opportunity to work with both of you.

Dr. Hiba, you have been a tremendous mentor for me. I would like to thank you for encouraging my research and for allowing me to grow as a research scientist. Your advice on both research as well as on my career have been invaluable. I could not have imagined having a better advisor and mentor for my Ph.D. study. My words cannot describe enough how dear of a person you are to me!

I am also grateful to Professor Carine Deleuze-Masquéfa for her advice and help in the chemical part related to drugs synthesis, for her fast replies to my questions, for her support during the thesis committees and over the Skype conferences that shortened the long distance and made this work happen.

I am very thankful to Professor Pierre Antoine Bonnet for his patience, motivation, and immense knowledge. Thanks for being extremely helpful and supportive in the administrative and scientific procedures.

My sincere thanks also go to Dr Marwan EL Sabban who convinced me during our many discussions. His door office was always open whenever I ran into a trouble spot or had a question about my research. Many Thanks for this precious support.

My sincere gratitude goes to the Jury members Drs Mathieu Gissot, Florence Robert-Gangneux and Sébastien Bertout who gave me the great honor to evaluate this work. I am sure I will benefit from their expertise in the field and their constructive comments.

I would also like to extend my gratitude to all the Hiba El Hajj (HEH) lab members who all share responsibilities in all projects, discuss science in a healthy environment and have taught me that the lab can be a fun, educating and building experience.

My deep appreciation goes out to my friend Rita who has been a sister, that have supported me along the way and for that I am forever indebted for her. I would also like to say a heartfelt thank you to my other family members and friends: Berthe, Fatima, Batoul, Hala and Abdo who have provided me through moral and emotional support in my life.

I must express my very profound gratitude to my parents: Mom, Dad, Sisters, and my lonely brother “Mohamad” always believing in me and encouraging me to follow my dreams. Your prayer for me was what sustained me thus far. A special mention to my fiancé Abdallah for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. Finally, I thank my God, for giving me strength to overcome adversity, to do what’s right for the benefit of the greater good, to rise above negativity. Thanks for all your encouragement!

# ABSTRACT

*Toxoplasma gondii* is a prevalent parasite of medical and veterinary impact. In intermediate hosts, tachyzoites and bradyzoites are responsible for acute and chronic toxoplasmosis (AT and CT), respectively. In immunocompetent patients, AT evolves, due to the host immunity, into a persistent CT, which manifests as latent tissue cysts in the brain and skeletal muscles. CT correlates with several neuro-pathologies and cancers. In immunocompromised patients, CT may reactivate and poses a life threatening condition. Current treatments primarily target AT, are limited to general anti-parasitic/anti-bacterial drugs, and associate with several limitations. Here, we focused on targeting CT and understanding its molecular mechanisms. First, we explored the efficacy of Imiquimod against AT and CT. During AT, Imiquimod led to recruitment of T cells to peritoneum and spleen of treated mice and significantly decreased the number of brain cysts upon establishment of CT. Remarkably, gavage of mice with the remaining brain cysts from Imiquimod treated mice, failed to induce CT. Post-establishment of CT, we demonstrated that Imiquimod sharply reduced the number of brain cysts in chronically infected mice, and significantly increased Toll-Like Receptors 11 and 12. These TLRs are usually expressed by dendritic cells and monocytes, and bind a tachyzoite actin-binding protein, profilin. Concomitantly, TLR-7 was upregulated, likely by its agonist Imiquimod. Imiquimod induced interconversion as documented by the decreased protein levels of P21, and increased protein levels of P30, exclusively expressed in bradyzoites and tachyzoites respectively. Pathways downstream from TLR-11/12 were activated, through MyD88 dependent TLR signaling, which resulted in subsequent immune response induction. In vitro, *Toxoplasma* strain lacking profilin, does not respond to Imiquimod, suggesting a role through Profilin/TLR-11/12. Finally, Imiquimod treatment upregulated the transcript expression levels of Chemokine (C-X-C motif) ligand 9 (CXCL9) and 10 (CXCL10), known to induce T cell recruitment to reactivated *Toxoplasma* foci to clear the infection.

Then, we focused on molecular mechanisms involved in AT and notably CT. We characterized P18, a Surface-Antigen 1 (SAG-1) Related Sequence (SRS) superfamily member. When we deleted P18, the virulence was attenuated during AT. Indeed, P18 depletion led to a faster clearance of the parasites from the peritoneum of mice, paralleled by a substantial recruitment of dendritic cells, presumably a vehicle for tachyzoite

dissemination. Concomitantly, a lower number of tachyzoites was detected in the spleens while a higher number of parasites reached the brains of infected mice. P18 depletion increased the number of bradyzoite cysts, in vitro and in the brains of infected mice. An induced expression of cytokines/chemokines, including CXCL9 and 10 was also observed. Immunosuppression of infected mice with KO P18, delayed reactivation. Oral infection of Severe Combined Immunodeficiency (SCID) (with IFN- $\gamma$  secreting macrophages), and NOD/Shi-scid/IL-2R $\gamma$ null (NSG) mice (lacking IFN- $\gamma$ ), showed a significant prolonged survival in infected SCID but not NSG mice. This underlines a role for IFN- $\gamma$  in the conversion from bradyzoites to tachyzoites. Collectively, these data support a role of P18 in orchestrating the immune response, which ultimately facilitates tachyzoite trafficking to the brain and favors cyst formation. P18 plays also a central role in parasite reactivation and dissemination in an IFN-  $\gamma$  dependent fashion.

Altogether, we showed the promising therapeutic potential of Imiquimod against toxoplasmosis and characterized P18 role in immunomodulation to control dissemination and interconversion. Our study paves the path towards new therapeutic approaches against toxoplasmosis. It tackled key questions pertaining to establishment, maintenance and reactivation of CT and should result in a comprehensive solution to this endemic disease.

Keywords: chronic toxoplasmosis, Toll-like receptors 11, 12, 7, Interferon- $\gamma$ , reactivation, Imiquimod, p18.

# RÉSUMÉ



*Toxoplasma gondii* est un parasite répandu, ayant un impact médical et vétérinaire. Chez les hôtes intermédiaires, les tachyzoïtes et les bradyzoïtes sont responsables de la toxoplasmose aiguë (TA) et chronique (TC), respectivement. Sous la réponse immunitaire, la TA évolue en TC, se manifestant par des kystes latents dans le cerveau et les muscles squelettiques. De plus, une forte corrélation existe entre la TC et plusieurs neuropathologies et cancers. Chez les patients immunodéprimés, la TC peut être réactivée et conduire à une maladie potentiellement fatale. Les traitements actuels ciblent principalement les TA, et présentent plusieurs effets secondaires. Nous nous sommes concentrés sur la TC et la compréhension de ses mécanismes moléculaires. Nous avons d'abord étudié l'efficacité de l'imiquimod contre la TA et la TC. Au cours de la TA, l'imiquimod a entraîné le recrutement de cellules T dans le péritoine et la rate de souris traitées et a considérablement diminué le nombre de kystes cérébraux lors de l'établissement de la TC. Remarquablement, le gavage de souris avec les kystes cérébraux restants chez des souris traitées à l'imiquimod n'a pas pu induire de TC. Après l'établissement de la TC, nous avons démontré que l'imiquimod réduisait considérablement le nombre de kystes cérébraux chez les souris chroniquement infectées et augmentait les récepteurs Toll-Like 11 et 12, qui se lient à une protéine du tachyzoïte, la profiline. Parallèlement, l'expression de TLR-7 augmentait, probablement par son agoniste, l'imiquimod. L'imiquimod induit une interconversion, comme l'indiquent la diminution du taux de protéine P21 et l'augmentation du taux de protéine P30, exprimées exclusivement et respectivement chez les bradyzoïtes et les tachyzoïtes. Les voies en aval de TLR-11/12 ont été activées via la voie MyD88 de signalisation, entraînant une induction ultérieure de la réponse immunitaire. In vitro, l'imiquimod n'affecte pas la souche *Toxoplasma* dépourvue de profiline, suggérant un rôle via le complexe Profilin/TLR-11/12. Enfin, le traitement par l'imiquimod a régulé positivement les transcrits des ligands 9 (CXCL9) et 10 (CXCL10), connus pour induire le recrutement de lymphocytes T dans des foyers réactivés du *Toxoplasme* afin d'éliminer l'infection.

Ensuite, nous nous sommes concentrés sur les mécanismes moléculaires impliqués dans la TA et particulièrement dans la TC. Nous avons caractérisé P18, un membre de la superfamille SRS. Lorsque nous avons supprimé P18, la virulence était atténuée au cours de la TA, dû à un échappement plus rapide des tachyzoïtes du péritoine de souris,

parallèle à un recrutement significatif de cellules dendritiques. De manière concomitante, moins de tachyzoïtes étaient détectés dans la rate, tandis que plus de parasites ont atteint le cerveau de souris infectées. L'élimination de P18 a augmenté le nombre de kystes de bradyzoïtes *in vitro* et dans le cerveau de souris infectées. Une expression induite de cytokines, notamment CXCL9 et 10, a également été observée. L'immunosuppression de souris KO P18 infectées a retardé la réactivation. L'infection orale de souris immunodéficientes ayant des macrophages fonctionnels a montré un prolongement de survie, contrairement aux souris n'ayant pas de macrophage, soulignant un rôle de l'IFN- $\gamma$  dans l'interconversion. Collectivement, ces données confirment le rôle de P18 dans la modulation de la réponse immunitaire, facilitant le passage des tachyzoïtes dans le cerveau et favorisant la formation de kystes. P18 joue également un rôle central dans la réactivation et la dissémination de parasites de manière dépendante de l'IFN- $\gamma$ . Dans l'ensemble, nous avons montré le potentiel thérapeutique prometteur de l'imiquimod contre la toxoplasmose et caractérisé le rôle de P18 dans l'immunomodulation afin de contrôler la dissémination et l'interconversion. Notre étude ouvre la voie à de nouvelles approches thérapeutiques contre la toxoplasmose, sa persistance et sa réactivation.

Mots-clés : toxoplasmose chronique, récepteurs Toll-like 11, 12, 7, interféron- $\gamma$ , réactivation, Imiquimod, p18.

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# **LIST OF ABBREVIATIONS**

AT: Acute Toxoplasmosis

CT: Chronic Toxoplasmosis

*T.gondii*: *Toxoplasma gondii*

IFN- $\gamma$ : Interferon gamma

HIV: Human Immunodeficiency Virus

MIC: Micronemes

ROP: Rhoptries

MAG-1: Merozoite Antigen-1

CW: Cyst Wall

AG: Amylopectin Granules

CDC: Centers for Disease Control

IgG: Immunoglobulin G

IgM: Immunoglobulin M

PCR: Polymerase Chain Reaction

DNA: Deoxyribo Nucleic Acid

HSCT: Hematopoietic Stem Cells Transplantation

DISC: Disrupted In Schizophrenia

DHFR: Dihydro Folate Reductase

SPFA: Sulfadiazine-Pyrimethamine-Folinic Acid

SAG-1: Surface Antigen-1

GPI: Glycosyl Phosphadityl Inositol

VLPs: virus-like particles

Th-1: T helper

LD<sub>50</sub>: Lethal Dose

ToxoDB: *Toxoplasma* Database

PCR-RFLP: Polymerase Chain Reaction-restriction Fragment Length Polymorphisms

DSB: Double Strand Break

NHEJ: Non-Homologous End-Joining

Pru: Prugiaud

SRS: SAG1-Related Sequences superfamily

EST: Expressed Sequence Tag

TLRs: Toll-like receptors

DC: Dendritic Cell

PAMPs: Pathogen-Associated Molecular Patterns

DAMPs: Damage-Associated Molecular Patterns

PRRs: Pattern Recognition Receptors

APC: Antigen Presenting Cell

AP-1: Activator Protein-1

MAPK: Mitogen-Activated Protein Kinase

ER: Endoplasmic Reticulum

ssRNA: Single Stranded Ribonucleic Acid

pDCs: plasmacytoid dendritic cells

TIR: Toll-Interleukin 1 Receptor

TIRAP: TIR Domain Containing Adaptor Protein

TRIF: TIR-domain-containing adapter-inducing interferon- $\beta$

TRAM: Translocation Associated Membrane Protein

SARM: Sterile Alpha And TIR Motif Containing

MyD88: Myeloid Differentiation primary response 88

MHC: Major Histocompatibility Complex

CCR5: C-C chemokine receptor type 5

IL: Interleukin

NK: Natural Killer

IRF8: Interferon Regulatory Factor 8

IM: Inflammatory Monocytes

CCL2: chemokine (C-C motif) ligand 2

Nrf2: Nuclear Factor Erythroid 2-Related Factor 2

NLRP: Nucleotide-binding domain Leucine-rich Repeats Protein family

IDO: Indoleamine 2,3- Dioxygenase

IRGs: Immunity-Related GTPases

NO: Nitric Oxide

Inos: Inducible Nitric Oxide Synthase

GBPs: Guanylate-Binding Proteins

ROS: Reactive Oxygen Species

NETs: Neutrophil Extracellular Traps

VCAM-1: Vascular Cell Adhesion Molecule 1

CNS: Central Nervous System

BBB: Blood Brain Barrier

TE: Toxoplasma Encephalitis

FDA: Food and Drugs Administration

HPV: Human Papilloma Virus

CL: Cutaneous Leishmaniasis

ELISA: Enzyme-linked immunosorbent Assay

FBS: Fetal Bovine Serum

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

# **INTRODUCTION**



## Chapter I: Generalities

### 1.1 Apicomplexa phylum

The phylum Apicomplexa includes the malaria-causing parasite (*Plasmodium* spp.), in addition to several major animal pathogens (e.g. *Eimeria* spp., *Theileria* spp., *Babesia* spp., *Neospora caninum*), as well as the causative agents of toxoplasmosis (*T. gondii*) and cryptosporidiosis (*Cryptosporidium* spp.) (Table 1). Some of these parasites are responsible for the most deadly parasitic diseases afflicting humans. They are also responsible for many diseases of veterinary and economic importance.

Class	Order	Species	Parasitosis
Hematozoa	Haemosporida	<i>Plasmodium</i>	Malaria
	Piroplasmida	<i>Babesia</i>	Animal and human Babesiosis
		<i>Theileria</i>	Animal and human Theileriosis
Coccidea		<i>EimeriaTenella</i>	Animal Coccidiosis
		<i>Sarcocystis</i>	Animal and Human Infection Cyst forms
		<i>Cryptospridium</i>	Animal and Human Infection Diarrhea
		<i>Toxoplasma</i>	Animal and Human Toxoplasmosis Infection by cysts/Oocysts
Perkinsidea		<i>Perkinsus sp.</i>	Oyster Parasite

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

Most apicomplexan parasites exhibit a complex life cycle and some require multiple hosts. All members of this phylum undergo several stages of development, restricted to one host or another. Being essentially intracellular, these parasites majorly benefit by gaining a considerably safe route to evade the host immune system (Blader & Saeij, 2009). In case of *T. gondii*, this is primarily achieved by infecting macrophages and dendritic cells, altering and down regulating the secretion of pro inflammatory cytokines such as IL-12 and activating anti-apoptotic machinery, hence securing a safe low profile vehicle to spread to various tissues (Melo, Jensen, & Saeij, 2011).

## **1.2 Toxoplasma gondii: Origin and Taxonomy**

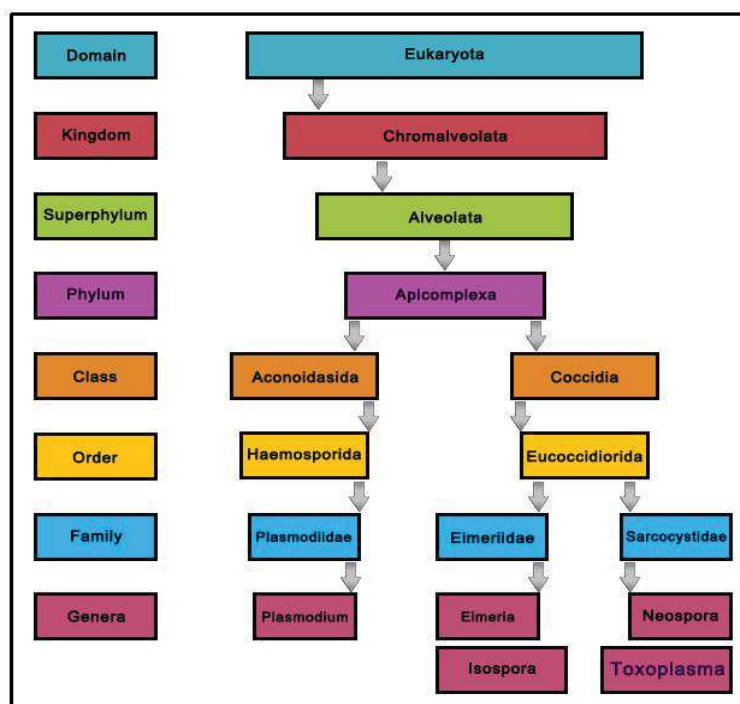
### **1.2.1. Origin**

In 1908, Nicolle and Manceaux were the first to describe *Toxoplasma gondii* (*T. gondii*) in an African hamster-like rodent called *Ctenodactylus gundi*, used primarily for *Leishmania* research at the Pasteur Institute in Tunis (Ferguson, 2009) (Nicolle & Manceaux, 1908). In parallel, Splendore discovered the same parasite in a rabbit in Brazil. The name *Toxoplasma* is based on the morphological appearance of the parasite whereby in Greek “Toxo” is equivalent to “arc” or “bow” and “plasma” means “Life”. The first viable specimen of *T. gondii* was isolated by Sabin and Olitsky in 1937 (Sabin & Olitsky, 1937), and the first case in humans was described in a neonate girl in 1938 who died at one month of age from an acute encephalomyelitis (Wolf, Cowen, & Paige, 1939). Later, many cases of acquired and congenital toxoplasmosis were described, and the development of a serological test, the “Dye” test, by Sabin and Feldman in 1948, further showed that *T.gondii* is widespread among humans and mammals (Sabin & Feldman, 1948). Over the next fifty years, more attention was given to the immune response against *T.gondii*, with the major discovery of the lymphoid cell mediated-immunogenicity against it. A crucial role of T-cells was confirmed when athymic nude mice infected with *T.gondii* failed to develop protective immunity against this parasite (Frenkel, 1988). In 1991, Gazzinelli showed that CD8<sup>+</sup> T lymphocytes producing interferon gamma (IFN- $\gamma$ ) are key players in the immune response to toxoplasmosis *in vivo* (R. T. Gazzinelli, Hakim, Hieny, Shearer, & Sher, 1991). Furthermore, the 80’s and

90's witnessed attempts to determine genetic differences between various *T. gondii* isolates from humans and animals (J. P. Dubey et al., 2008). The complete sequencing of the parasite's genome was accomplished in 2005 (A. Khan et al., 2005), aiming to set a basis for better understanding of the disease mechanisms, and subsequently for designing better treatment or even prevention (J. P. Dubey, 2008).

### 1.2.2. Taxonomy

The systematic classification of *T. gondii* goes back to 1980 (Levine et al., 1980) (Figure 1). Discoveries at the level of life cycle and advancements in electron microscopy yielded to the identification of a complex structure and a number of unique organelles. Similarities were shown between the previously considered unrelated parasites such as *Plasmodium* and *Eimeria* species (Scholtyseck & Mehlhorn, 1970). These led to a major change in the classification of Protozoa, to add a new Phylum, the Apicomplexa, accounting for all this relatedness, and to encompass the parasites of the genera *Babesia*, *Theileria*, *Plasmodium*, *Eimeria*, *Toxoplasma*, *Neospora*, and *Cryptosporidium* (Ferguson, 2009).



**Figure 1. Scientific classification of Apicomplexan parasites.** Retrieved from <http://www.ncbi.nlm.nih.gov/Taxonomy>.

### **1.3 Toxoplasma gondii life cycle**

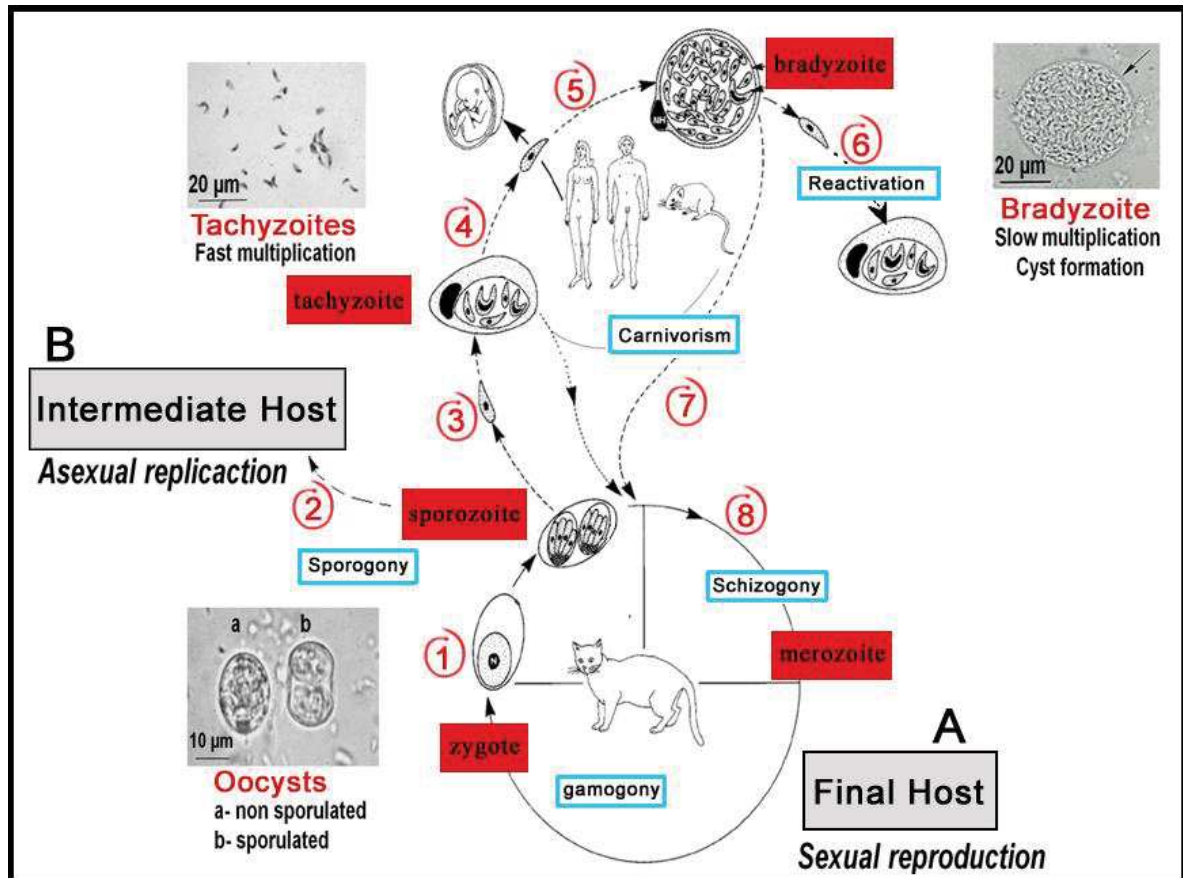
*T. gondii* is an obligate intracellular parasite that infects all warm-blooded animals, including approximately 30% of the human population worldwide (Tenter, Heckeroth, & Weiss, 2000). Its complete life cycle requires a sexual life cycle restricted to the feline intestinal epithelium and an asexual life cycle within any warm-blooded animal (Figure 2) (Hutchison, Dunachie, Siim, & Work, 1970; Sheffield & Melton, 1970). Intermediate hosts such as humans can acquire *T. gondii* by ingestion of meat contaminated with cysts. Ingestion of sporulated oocysts in contaminated salads also results in an infection with *T. gondii* (Fox et al., 2011; Weiss & Kim, 2000).

#### ***1. Sexual life cycle***

Oocysts are the result of the sexual cycle of *T. gondii*, which begins when a domestic cat or any other member of the *Felidae* family feeds on an infected prey, containing the latent bradyzoite stage of the parasite. The release of bradyzoites in the digestive tract, results in the initiation of the sexual development in the enterocytes of the cat's ileum. Approximately 2 days post infection, gametogony begins and gamonts appear throughout the ileum inside enterocytes within a span of 3 to 15 days (J. Dubey, 2009). Following gametogenesis, microgametes and macrogametes develop as the male and female gametes respectively. The female macrogamete has numerous organelles while the male microgamont harbors up to 21 microgametes which possess a top end perforatorium organelle, as well as flagella used to swim; these penetrate and fertilize mature female macrogametes (Speer & Dubey, 2005).

After fertilization of a macrogamete by a microgamete, a zygote is formed in the intestinal lining cells of the infected feline definite host. Intestinal epithelial cells then rupture and oocysts are shed into the intestinal lumen as immature oocysts about 3-7 days following ingestion of the infected prey. Upon defecation, unsporulated oocysts are released in nature. Sporulation occurs in the outside environment. This process starts with a single, relatively amorphous zygote and ends after 1 to 5 days post excretion and under appropriate environmental conditions with a sporulated oocyst containing 2 sporocysts

harboring 4 sporozoites each and ready to start a new cycle (Dabritz & Conrad, 2010; Dabritz et al., 2007; J. P. Dubey, 1998).



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

## ***2. Asexual life cycle***

Upon oral ingestion of contaminated food with sporulated oocysts by any warm-blooded animal, the asexual or intermediate life cycle begins (Figure 2). The digestive enzymes of the gastro-intestinal tract lead to the liberation of sporozoites which will rapidly transform into tachyzoites in the blood. These tachyzoites are responsible for the acute phase of infection and are capable of invading any nucleated cell as well as crossing the blood-placental barrier. Approximately 5 days after ingestion, and under the immune response, these tachyzoites transform into bradyzoites, which encyst in the brain and skeletal muscles. These tissue cysts may remain dormant for years, until a predator eats an intermediate host and a new cycle starts (J. P. Dubey *et al.*, 1998; J. P. Dubey, 2007). The reactivation of bradyzoites into tachyzoites can be encountered in immunocompromised patients (HIV patients or patients with organ or bone marrow transplantation), where it can become life threatening (Mele, Paterson, Prentice, Leoni, & Kibbler, 2002).

## **Chapter II: Morphology and stages of *Toxoplasma gondii***

Although *T. gondii* was discovered in 1908, the full life cycle remained unravelled until 1970, whereby only the asexual section of the life cycle and its namely tachyzoites and bradyzoites stages were known (J. P. Dubey, 2009). Uncovering the sexual part of the life cycle, was initiated by the work of Dr. J.K. Frenkel who investigated the screening on felids and several potential host species for shedding of oocysts (J. Dubey & Frenkel, 1972). During its life cycle, *T. gondii* exhibits three morphologically distinct infectious stages: tachyzoites, bradyzoites, and sporozoites.

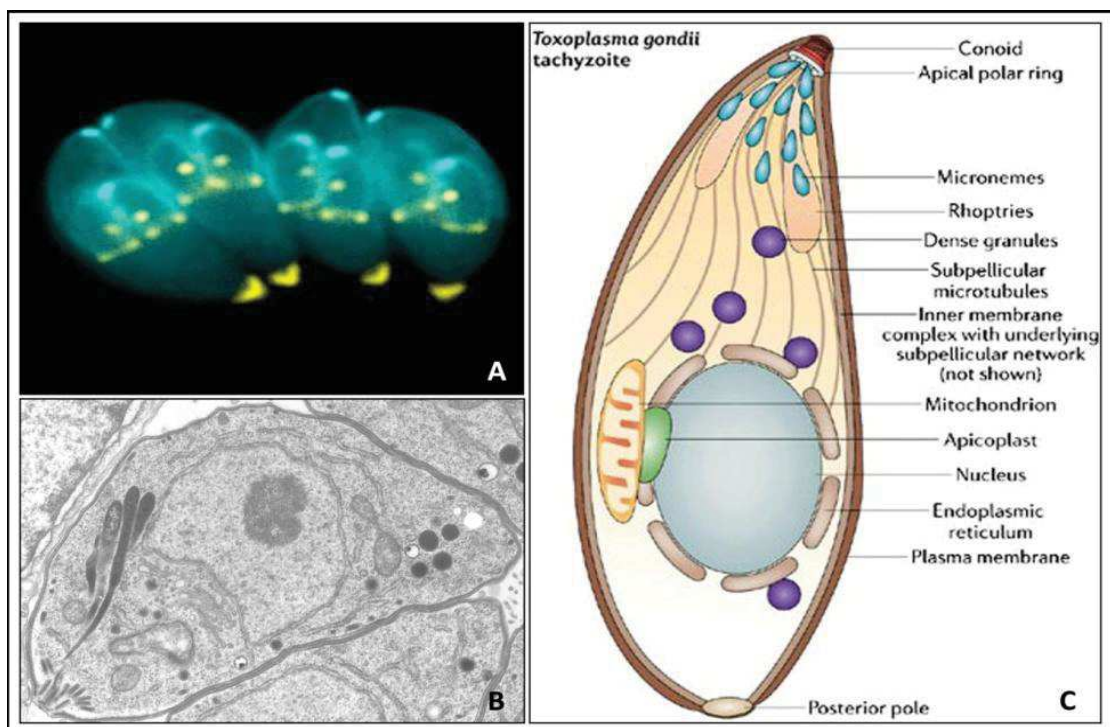
### **2.1. Tachyzoites**

In Greek, ‘tacos’ stands for ‘speed’. Tachyzoites are so called by Frenkel (Frenkel, J.K., 1973) and represent the anteriorly pointed and posteriorly rounded proliferative intracellular forms, responsible for the acute phase of the infection. After endodyogeny inside a host cell, each tachyzoite divides into two daughter tachyzoites, lysing the mother parasite (Figure 3). The process continues until the host cell can no longer embrace the huge number of proliferating tachyzoites, and simply bursts releasing these stages to infect neighboring cells. At the nano-structural level, the tachyzoite is an assembly of several organelles, inclusions, an outer covering (pellicle), sub-pellicular microtubules, polar rings, apical rings, rough and smooth endoplasmic reticula, mitochondrion, conoid, rhoptries, micronemes, dense granules, micropore, Golgi complex, a plant inherited plastid-like organelle called apicoplast, in addition to a centrally localized nucleus mainly featuring a central nucleolus surrounded by chromatin clumps (Figure 3) (J. P. Dubey, 2009; McGovern & Wilson, 2013a).

Invasion by *Toxoplasma gondii* tachyzoites is a multistep process that begins upon contact with the host cell and apical reorientation of the parasite. This results in a burst of microneme secretion, immediately followed by the discharge of rhoptry organelles content (Dubremetz, 2007). *T. gondii* possesses 8–12 rhoptries that cluster together at the apical pole of the parasite and occupy 10%–30% of the total cell volume. Data from stereological analysis suggest that only one organelle can discharge at a time (Paredes-Santos, de Souza, & Attias, 2012). Micronemes (MICs) are rice-grain-like



structures, usually fewer than 100 in number, situated at the conoidal end of *T. gondii*. Upon secretion and redistribution on the parasite surface, transmembrane MICs are thought to connect external receptors to the submembranous actin–myosin motor that provides the power for parasite gliding and host cell invasion (Cerede et al., 2005; Dubremetz, 2007; Joiner & Dubremetz, 1993). In addition to the rhoptries and the micronemes, the parasite contains dense granules which play a major role in the structural modifications, stability and maintenance of the parasitophorous vacuole (Gold et al., 2015). In addition, some dense granule proteins that are released regulate host cell gene expression (Bougdour et al., 2013; Bougdour, Tardieux, & Hakimi, 2014; Braun et al., 2013), and immune response (Pernas et al., 2014; Rosowski, Nguyen, Camejo, Spooner, & Saeij, 2014; Shastri, Marino, Franco, Lodoen, & Boothroyd, 2014), making them an essential component in parasite survival and disease pathogenesis.



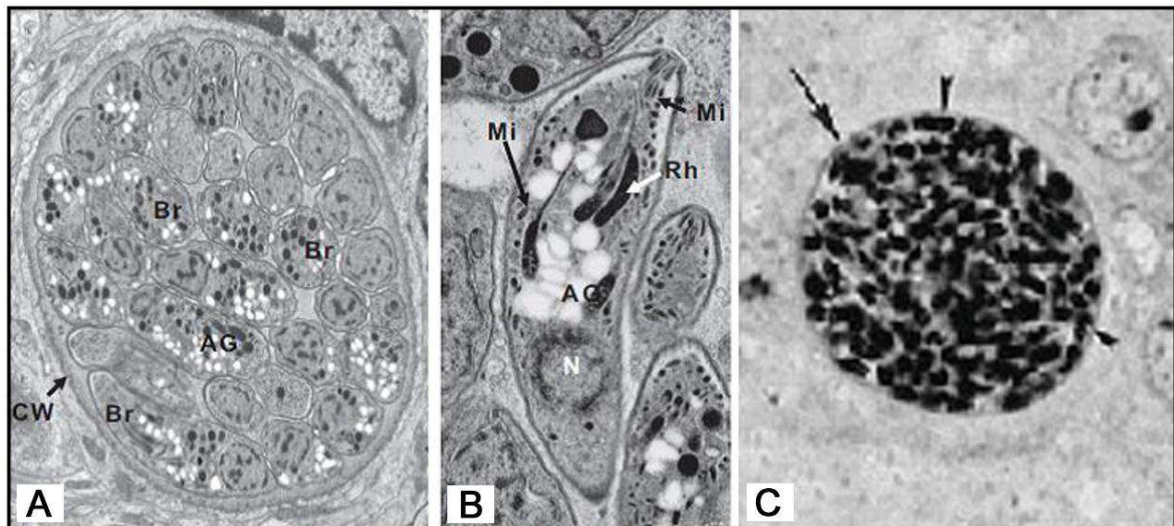
**Figure 3. A. Endodyogeny process.** Daughter *Toxoplasma gondii* parasites form inside the mother parasite (Murray, J., PLoS Pathogens Issue Image, 2006. 2006). B. Clicher of Jean-François Dubremetz: An intracellular *T. gondii* tachyzoite inside a parasitophorous vacuole C. Schematic drawing showing the ultrastructure of *T. gondii* tachyzoite (Ajioka, Fitzpatrick, & Reitter, 2001).



## **2.2. Bradyzoïtes**

In 1973, Frenkel proposed the term “Bradyzoïte”, with “brady” meaning “slow” in Greek, to describe the slow growing developmental stage of *T. gondii*, encysting in tissues. Dubey and Beattie (1988) proposed that cysts should be called tissue cysts to avoid confusion with oocysts. The first characterization of these cysts appeared when they were found resistant to digestion by gastric enzymes, in contrast to tachyzoïtes which were readily destroyed. This highlights the importance of encysted forms of *T. gondii* as key part for the life cycle’s continuity. In the same year, isolation of viable bradyzoïtes from *T. gondii* tissues cysts of chronically infected animals, was performed using pepsin digestion. Tissue cysts (Figure 4) vary in size; young tissue cysts may be as small as 5 µm in diameter and contain only two bradyzoïtes, while older ones may reach an average of 50 to 70 µm in diameter, with around one thousand bradyzoïtes in mature cysts formed in the brain, and up to 100 µm with a more elongated form and definitely larger bradyzoïte content (Knoll, L.J., T. Tomita, and L.M. Weiss, 2014). Bradyzoïtes (Figure 4) are responsible for the chronic phase of toxoplasmosis. They are formed after the immune system reacts against the virulent tachyzoïte stage (J. P. Dubey, 2009). In immunocompromised patients, bradyzoïtes reactivate back to tachyzoïtes and may become life threatening (Bannoura, El Hajj, Khalifeh, & El Hajj, 2018; Rajapakse, Weeratunga, Rodrigo, de Silva, & Fernando, 2017; Z. D. Wang et al., 2017).

Ultrastructure analysis showed that bradyzoïtes differ from tachyzoïtes in several features (Table 2), including the posterior position of the nucleus in bradyzoïtes, in comparison to its central location in tachyzoïtes. Amylopectin granules in these latent forms are abundant, as compared to their rarity or absence in tachyzoïtes (J. P. Dubey, 2008). Furthermore, in comparison to the labyrinthine rhoptries in tachyzoïtes, these organelles are electron dense in mature bradyzoïtes and often appear convoluted and looping back on themselves (J. Dubey, 1998). Micronemes are rather numerous in bradyzoïtes and more slender than those of tachyzoïtes. Lipid bodies that are occasionally present in tachyzoïtes, are virtually completely absent in bradyzoïtes. The cyst wall has an average thickness of less than 0.5 µm (L. J. Knoll, Tomita, & Weiss, 2014).



**Figure 4: Structure of bradyzoites.** **A.** Transmission electron micrographs of bradyzoites (Br) within a tissue cyst. Note the presence of cyst wall (CW) and numerous amylopectin granules (AG) in the cytoplasm of bradyzoites. **B.** Higher magnification of ultrastructural morphology of bradyzoites. Note the presence of rhoptries (Rh) and micronemes (Mi) **C.** Tissue cysts of *T. gondii* in mouse brains with numerous bradyzoites (arrow heads) enclosed in a cyst wall (arrow) (J. P. Dubey, 1998).

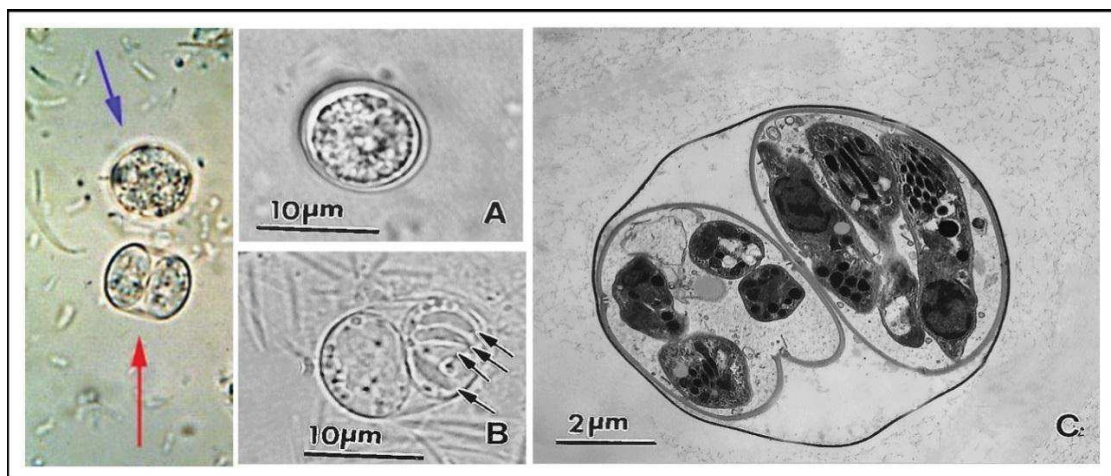
Tachyzoites	Bradyzoites
Fast replicating forms	Slowly replicating forms
Intracellular and divide by endodyogeny	
More centrally located nucleus	Posteriorly located nucleus
Labyrinthine rhoptries	Solid rhoptries
Absent or few amylopectin granules	Numerous amylopectin granules
Occasionally presence of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

Table 2. Comparison between tachyzoites and bradyzoites.

### **2.3 Sporozoites**

Felids are the only definitive hosts for *T. gondii* and harbor sexual reproduction occurs in their intestine, which culminates in the production of oocysts subsequently shed

to the environment in feces. An oocyst is essentially a cytoplasm containing a nucleoplasm, surrounded and protected by a double-layered wall, which makes it resistant to environmental stress; the inside content of a cyst is known as a “sporont”. Upon excretion, oocysts are unsporulated (Figure 5). Sporulation occurs outside the cat within 1 to 5 days of excretion depending upon aeration and temperature conditions (J. P. Dubey, 1998). During the initial phase of the sporulation, the nucleus divides twice and gives rise to 4 nuclei. Then the cytoplasm divides and 2 spherical sporoblasts are formed, each harboring 2 nuclei (Figure 5). As the sporulation continues, the sporoblasts elongate and the sporocysts are formed. Later, each nucleus divides into two, and following cytoplasmic cleavage, four infective sporozoites form within each sporocyst. It is worth noting that an infected cat may shed as many as one billion oocysts, which are resistant to chemical and physical methods of inactivation (waste-water and sewage) (Fritz, Bowyer, Bogyo, Conrad, & Boothroyd, 2012). They can survive and remain infective for years in fresh water and for at least 24 months in salt water (Lindsay & Dubey, 2009).



**Figure 5. *Toxoplasma gondii* oocysts.** An unsporulated oocyst (blue arrow) and a sporulated oocyst (red arrow) seen at high power (40 X) (University of Pennsylvania School of Veterinary Medicine, 2008). **(A)** Unsporulated oocyst. Note the central mass (sporont) occupying most of the oocyst. **(B)** Sporulated oocyst with two sporocysts. Four sporozoites (arrows) are visible in one of the sporocysts. **(C)** Transmission electron micrograph of a sporulated oocyst. Note the thin oocyst wall (large arrow), two sporocysts (arrowheads), and sporozoites, one of which is cut longitudinally (small arrows) (J. P. Dubey, 2009).

## **Chapter III: Toxoplasmosis**

### **3.1. Epidemiology of Toxoplasmosis**

Toxoplasmosis is one of the most common zoonotic diseases. Sero-prevalence varies from one country to another, from 7 to 80%. Low sero-prevalence ranging between 10 and 30%, was observed in North America, South-East Asia, and Northern Europe. Moderate sero-prevalence ranging between 30 and 50%, was found in Central and Southern Europe, while high prevalence is common in Latin America and tropical African countries (Robert-Gangneux & Darde, 2012).

*T. gondii* is capable of infecting approximately 30% of the world's human population, and more than 40 million people in the United States (CDC 2019), in addition to warm-blooded vertebrates and avian species (Skariah, McIntyre, & Mordue, 2010). At the American University of Beirut, Bouhamdan *et al.* realized a retrospective study targeting information related to IgG and IgM anti-*T. gondii* antibodies. They found that the overall *Toxoplasma* seropositivity was 62.2% for IgG and 6.8% for IgM (Bouhamdan, Bitar, Saghir, Bayan, & Araj, 2010), reflecting a high exposure of the Lebanese population to *Toxoplasma* infection. In France, we estimated seroprevalence to be 41 to 53% and this prevalence increased with age (Fromont, Riche, & Rabilloud, 2009). Surprisingly, the USA present with a high parasite burden, yet with a low awareness among physicians and citizens at both the prevalence of the disease, and the costs associated with it. Hence, the Centers for Disease Control and Prevention (CDC) considered recently toxoplasmosis as a neglected parasitic infection, requiring public health action (Ben-Harari & Connolly, 2019).

### **3.2. Modes of Transmission**

The mechanism of transmission of *T. gondii* remained a mystery until its life cycle was unraveled in 1970. Intermediate as well as definitive hosts can contract an infection by *T. gondii* mainly *via* one of two main modes of transmission:

(1) Horizontal mode of transmission either following oral ingestion of infectious sporulated oocysts in food contaminated by cats feces, or upon and ingestion of tissue cysts that can be potentially present in raw or partially raw meat.

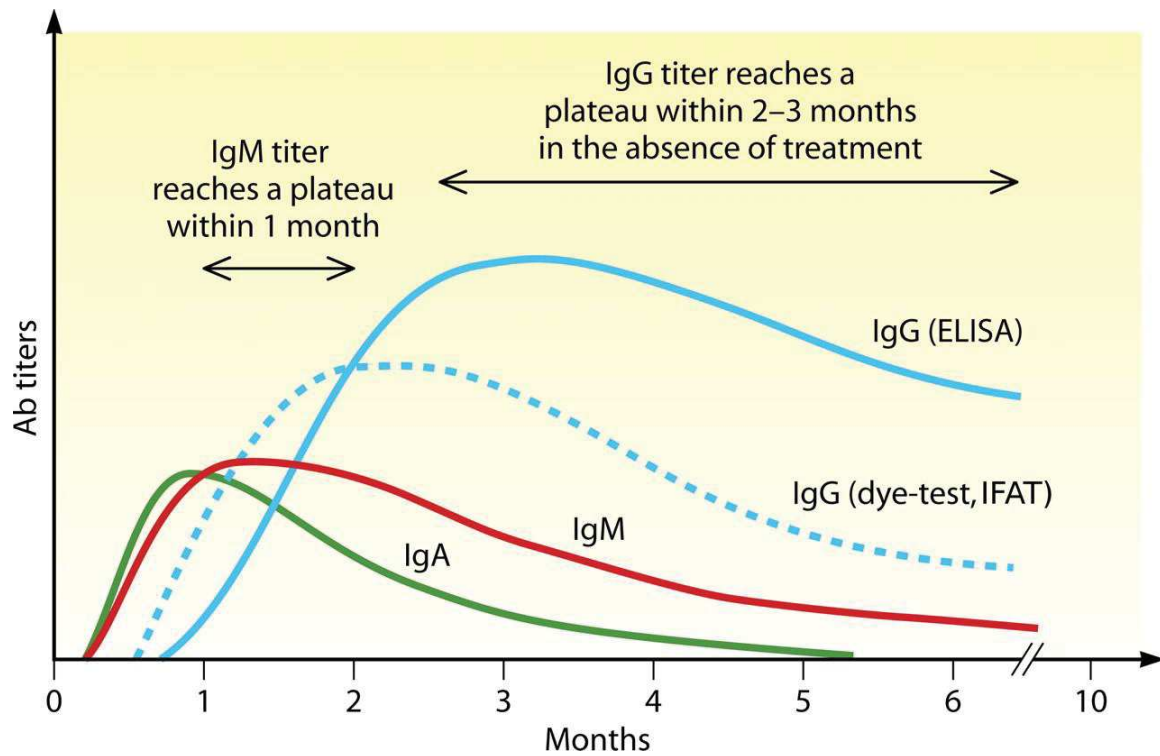
(2) Vertical mode of transmission *via* the placental spread of tachyzoites from a pregnant primo-infected mother to her fetus/baby.

These routes of transmission allow a high level of sustainability of *T.gondii* in the environment. Indeed, transmission is not limited by any means; infection can move back and forth between definitive and intermediate hosts with all the needed routes available. Thus, carnivores can acquire *T. gondii* infection *via* carnivorism, herbivores *via* ingestion of infectious oocysts, and humans, being omnivores, are prone to infection *via* both means. Furthermore, the asexual life cycle can sustain the parasites indefinitely *via* the transmission of tissue cysts in-between intermediate hosts (Pereira, Franco, & Leal, 2010; Tenter et al., 2000).

### **3.3. Diagnosis**

*T. gondii* infection can be diagnosed using serologic tests, ultrasound scans, PCR and amniocentesis (Chaudhry, Gad, & Koren, 2014). Results of serologic tests measure immunoglobulin IgM and IgG (Figure 6). Following acute infection, IgM antibody titers rise starting on day 5 and reach the maximum level at 1 to 2 months. In contrast, IgG antibodies are usually detectable within 1 to 2 weeks after acute infection, reach their peak within 12 weeks to 6 months, and generally remain detectable throughout life (Liesenfeld et al., 1997). Serological surveys alone do not provide information about the prevalence of viable parasites. PCR-based methods were developed to detect parasite DNA especially in pregnant women (Robert-Gangneux & Darde, 2012), and made a significant improvement in both the prenatal diagnosis of congenital toxoplasmosis, as well as the detection of acute disease in the immunocompromised patient. A positive PCR requires urgent initiation of treatment in these patients (Bastien, 2002; Bourdin et al., 2014). In addition, when *Toxoplasma* DNA is detected in the amniotic fluid, the treatment is required, with postnatal clinical follow-up (Villard et al., 2016).





**Figure 6. Kinetics of the antibody (Ab) response (Robert-Gangneux & Darde, 2012).**

### **3.4. Pathogenesis of *Toxoplasma gondii***

More and more associations are being made between various medical conditions and *T. gondii* infections (Flegr, Prandota, Sovickova, & Israili, 2014). Some reports show that elevated levels of immunoglobulin G in pregnant ‘toxoplasma-positive’ women are linked to prenatal anxiety and depression (Abo-Al-Ela, 2019; Okusaga et al., 2011), others associate infection with *T. gondii* to behavioral disorders (Fekadu, Shibre, & Cleare, 2010; McConkey, Martin, Bristow, & Webster, 2013), and yet others correlate various mental health disorders like schizophrenia, depression and even suicide attempts with seropositivity to *T. gondii* (Fuglewicz, Piotrowski, & Stodolak, 2017; Hsu, Groer, & Beckie, 2014). The manifestations of toxoplasmosis differ between immunocompetent and immunocompromised patients.

### **3.4.1. Toxoplasmosis in immunocompetent patients**

A recently acquired infection, corresponds to the acute phase of infection, and is asymptomatic in more than 80% of immune-competent individuals (Montoya & Liesenfeld, 2004; Reza Yazdani et al., 2018). In these patients, toxoplasmosis occurs after an incubation of a few days, and manifests with mononucleosis-like symptoms, with cervical posterior adenopathies, myalgias, and asthenia. In addition, various non-specific clinical signs are encountered and include skin rash, fever, and elevation of monocytes with hyper-basophilic lymphocytes (Montoya & Liesenfeld, 2004). The severity of infection is also related to the genotype of the infecting parasite strain. Acquisition of toxoplasmosis during childhood or adulthood may account for high levels of visual impairment, and toxoplasmosis is a leading cause of blindness in South America (de Boer, Wulfraat, & Rothova, 2003). Furthermore, “Atypical” strains, which caused lethal infections in immune-competent individuals, were isolated in French Guiana. These subjects developed fatal pneumonitis, myocarditis, meningo-encephalitis, or polymyositis (inflammatory myopathies) (Carne et al., 2002; Robert-Gangneux & Darde, 2012).

### **3.4.2. Toxoplasmosis in immunocompromised patients: Neurotoxoplasmosis**

The host immune response plays a key role in the control of parasite replication and maintenance of tissue cysts. With the growing number of individuals receiving therapies against immune-mediated inflammatory diseases and malignancies, clinicians are aware of the potential occurrence of *Toxoplasma* encephalitis, not only during reactivation of latent infection, but also presenting as a primary infection (Gharamti et al., 2018). Reactivation of the latent phase of the infection can occur in immunocompromised patients, due to various factors impairing the protective cellular immune response such as HIV infection, immunosuppressive therapies administered in the context of hematopoietic stem cells- (HSCT) or solid organ transplant, or chemotherapy against cancer (Kollu, Magalhaes-Silverman, Tricot, & Ince, 2018; Robin et al., 2019; Roche, Rowley, Brett, & Looby, 2018). In HIV patients, toxoplasmic encephalitis is the predominant manifestation of the disease, while pulmonary or disseminated

toxoplasmosis is more characteristic of transplant patients (Rajapakse et al., 2017; Robert-Gangneux & Darde, 2012). Despite the availability of prophylactic and treatment options, reactivation of chronic toxoplasmosis still occurs and can become life threatening (Bannoura et al., 2018; Kodym et al., 2015; Montoya & Liesenfeld, 2004). These patients present with neurologic symptoms, most frequently diffuse encephalopathy, meningoencephalitis, cerebral mass lesions, headaches, confusion, poor coordination, and seizures. At advanced stages, the recrudescence of chronic toxoplasmosis can lead to pneumonia and encephalitis, and thus to fatality (Bannoura et al., 2018; Hunter & Remington, 1994; Montoya & Liesenfeld, 2004). Our group recently reported a case of a child with a ten-day history of upper respiratory tract illness, vomiting, and headaches. Unfortunately the child died and postmortem brain autopsy revealed a wide reactivation of cerebral toxoplasmosis, with both tachyzoite and bradyzoite stages of the parasite detected in his brain (Bannoura et al., 2018). Acute toxoplasmic encephalitis was also reported in a 65-year-old male while receiving treatment of pseudo-lymphoma. Analysis of his cerebrospinal fluid demonstrated mild lymphocytic pleocytosis and the presence of *T. gondii* by PCR (Gharamti et al., 2018). Furthermore, several cases of reactivation of cerebral toxoplasmosis following rituximab therapy were reported (Safa & Darrieux, 2013) (Morjaria et al., 2016) (Holland et al 2015).

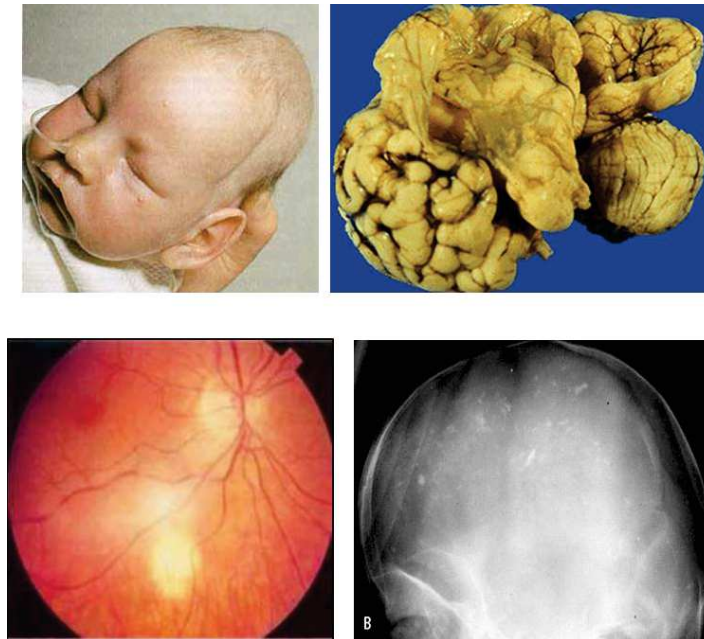
### **3.4.3. Congenital toxoplasmosis**

In sero-negative pregnant women, primary infection with *T. gondii* occurs following the placental transmission of the parasite to the fetus. The degree of severity of congenital toxoplasmosis is inversely related to the gestational trimester at which the infection is contracted (Dunn *et al.*, 1999) (L. Yamamoto et al., 2017). Infection of the fetus during the first trimester of pregnancy, often leads to abortion, stillbirth or a child born with severe abnormalities of the brain and eyes, such as hydrocephalus, intracranial calcifications, deafness, mental retardation, seizures, retinochoroiditis, and even blindness (Figure 7) (Robert-Gangneux & Darde, 2012; Singh, 2016; Swisher, Boyer, & McLeod, 1994). Transmission to the fetus in the second or third trimester is less likely to



cause such severe clinical manifestations, but may result in subclinical disease, which may lead to retinochoroiditis or learning difficulties after birth (Weiss & Dubey, 2009). Fortunately, only few pregnant women acquire the infection during pregnancy (Nahouli, El Arnaout, Chalhoub, Anastadiadis, & El Hajj, 2017; Nowakowska et al., 2006).

Overall, the screening and its correct timing, the appropriate treatment, and the development of novel modalities are still badly required in case of this neglected disease.



**Figure 7. Clinical manifestations of congenital toxoplasmosis.** Hydrocephalus (upper panels), retinochoroiditis (left lower panel) and intra-cranial calcifications (right lower panel).

#### **3.4.4. Toxoplasmosis and other associated diseases**

Until recently, parasite persistence in healthy individuals was regarded as clinically asymptomatic. However, an increasing number of reports indicates that chronic toxoplasmosis is associated with aberrant host behavior (Luft & Remington, 1992). Chronic toxoplasmosis influences also the progression of several neurological disorders such as schizophrenia, and Parkinson disease among others (Gaskell, Smith, Pinney, Westhead, & McConkey, 2009; Severance et al., 2016; Webster & McConkey, 2010). Recently, it was proved that a major mental illness-related susceptibility factor, the

“Disrupted in schizophrenia” (DISC1), is involved in host immune responses against *T.gondii* infection, and that certain genotypes of DISC1 correlate with higher serology against this parasite (Kano et al., 2018). Epidemiologic studies indicate that *Toxoplasma* infection can alter behavior and neurotransmitter function causing an increase in the level of dopamine, a condition widely observed in schizophrenics (Webster & McConkey, 2010). Additionally a survey approach revealed that individuals with schizophrenia were more exposed to cats in comparison to control non-schizophrenic groups (Yolken, Dickerson, & Fuller Torrey, 2009). Some medications used to treat schizophrenia inhibit the replication of *T. gondii* in cell culture (Torrey & Yolken, 2003). Altered dopamine levels have been also associated with this infection, along with several neurological conditions and imbalances; these are related to mood control, sleep patterns, Parkinson’s disease, and even attention deficit disorder, in addition to several others (Gaskell et al., 2009). Furthermore, the prevalence of anti-*T. gondii* antibodies was reported to be higher in different types of cancer patients (e.g. lung, cervix, brain and endometrial cancers) (Cong et al., 2015). A positive association between seropositivity for *T. gondii* and brain cancer incidence was associated with the ability of the parasite to interfere with the brain cells miRNAome (Thirugnanam, Rout, & Gnanasekar, 2013). It was also reported that the incidence of adult brain cancers is higher in countries where the infection with *T.gondii* is common (Jung et al., 2016; Thomas et al., 2012).

### **3.5. Treatment of toxoplasmosis**

Common treatments for toxoplasmosis remain limited to general anti-parasitic/anti-bacterial drugs. These include spiramycin, azithromycin, atovaquone, pyrimethamine-sulfadiazine, pyrimethamine-clindamycin and trimethoprim-sulfamethoxazole (Dard et al., 2018). Indeed, unlike its mammalian host, *T. gondii* is unable to use preformed dietary folates and synthesizes folates *de novo* (Katlama, Mouthon, Gourdon, Lapierre, & Rousseau, 1996). Thus, the recommended first-line therapy remains the synergistic combination of pyrimethamine, an inhibitor of the dihydrofolate reductase (DHFR) enzyme, with sulfadiazine, an inhibitor of the dihydropteroate synthase (Lapinskas & Ben-Harari, 2019; Montoya & Liesenfeld, 2004).

This combination is usually administered with folinic acid (leucovorin) (Katlama, Mouthon, et al., 1996) and blocks the biosynthesis of parasite folate, thus nucleic acid synthesis and parasite replication. However, this combination associates with several limitations, including, hematological side effects such as neutropenia severe drop of platelet count, thrombocytopenia, leucopenia (Ben-Harari, Goodwin, & Casoy, 2017), elevation in serum creatinine and serum liver enzymes, hypersensitivity or allergic reactions (Katlama, De Wit, O'Doherty, Van Glabeke, & Clumeck, 1996) and emergence of resistant parasites (Dupouy-Camet, 2004; Montazeri et al., 2018), especially in immunocompromised patients (Rodriguez-Diaz, Martinez-Grueiro, & Martinez-Fernandez, 1993). Pyrimethamine can be teratogenic and should not be used during the early months of pregnancy (Rodriguez-Diaz et al., 1993).

In addition, these drugs, whether given as prophylactic or therapeutic agents, target only the acute phase of the infection and remain useless against the tissue cysts characterizing the chronic form of toxoplasmosis (Schmidt et al., 2006). Indeed, to date, there is no approved therapy that eliminates tissue cysts responsible for chronic infections (Alday & Doggett, 2017; Montazeri et al., 2016; Montazeri et al., 2017). Degerli et al. evaluated the effectiveness of azithromycin, a protein synthesis inhibitor in both *T.gondii* tachyzoite and bradyzoite stages. The main limitation is the needed prolonged period of treatment with the drug (Degerli, Kilimcioglu, Kurt, Tamay, & Ozbilgin, 2003). In pregnant women, treatment is based on the administration of spiramycin or sulfadiazine-pyrimethamine-folinic acid (SPFA) if fetal infection is confirmed (Degerli et al., 2003). A summary of the currently used treatment of toxoplasmosis in immunocompetent, in immunocompromised patients and in pregnant women is provided in tables 3, 4 and 5 respectively.

Regimen <sup>b</sup>	Comments
Pyrimethamine (100 mg daily for 1 or 2 days and then 25–50 mg daily) plus sulfadiazine (1g every 6 h [q6h]) plus folinic acid (10–20 mg daily)	Blood counts, creatinine, and liver function should be monitored regularly Adequate hydration should be ensured to prevent renal damage from crystalluria
Pyrimethamine plus folinic acid (dosing as described above) plus clindamycin (300 mg q6h)	Blood counts should be monitored regularly Clindamycin may cause diarrhea, including <i>Clostridium difficile</i> infection
TMP-SMX (5/25–10/50 mg/kg/day in divided doses)	Blood counts, creatinine, and liver function should be monitored regularly Adequate hydration should be ensured to prevent renal damage from crystalluria
Atovaquone (1,500 mg twice daily) ± pyrimethamine plus folinic acid (dosing as described above)	Blood counts and liver function should be monitored regularly Atovaquone should be taken with a high-fat diet
Pyrimethamine plus folinic acid (dosing as described above) plus azithromycin (250–500 mg daily <sup>c</sup> )	Blood counts should be monitored regularly Azithromycin may cause hearing problems and a prolonged QT interval
Intravitreal clindamycin (1 mg) plus dexamethasone (400 µg)	Only for ocular toxoplasmosis; may need to be repeated 1 or 2 times if response is suboptimal

<sup>a</sup>Treatment should be considered for immunocompetent patients with severe or persistent symptoms, ocular involvement, or laboratory-acquired infection. For ocular infection, concomitant steroids (prednisone at 0.5 to 1 mg/kg/day) with gradual tapering can be used; this decision is best made by the ophthalmologist.

<sup>b</sup>Folinic acid is different from folic acid.

<sup>c</sup>A higher dose of azithromycin (1,000 mg) should be considered for severe nonocular systemic disease.

Table 3. Treatment of toxoplasmosis in immunocompetent patients (Dunay, Gajurel, Dhakal, Liesenfeld, & Montoya, 2018).



Induction therapy	Maintenance therapy	Comments
For those with body wt of $\geq 60$ kg, pyrimethamine (200 mg once and then 75 mg daily) plus sulfadiazine (1.5 g q6h) plus folinic acid (10–50 mg daily)	Pyrimethamine (50 mg daily) plus sulfadiazine (1 g q6h) plus folinic acid (10–25 mg daily)	Blood counts, creatinine, and liver function should be monitored regularly Adequate hydration should be ensured to prevent renal damage from crystalluria
For those with body wt of $< 60$ kg, pyrimethamine (200 mg once and then 50 mg daily) plus sulfadiazine (1 g q6h) plus folinic acid (10–50 mg daily)	Pyrimethamine (25 mg daily) plus sulfadiazine (0.5 g q6h) plus folinic acid (10–25 mg daily)	Blood counts, creatinine, and liver function should be monitored regularly Adequate hydration should be ensured to prevent renal damage from crystalluria
Pyrimethamine plus folinic acid (dosing as described above) plus clindamycin (600 mg q6h)	Pyrimethamine plus folinic acid (dosing as described above) plus clindamycin (600 mg q8h)	Blood counts should be monitored regularly Clindamycin may cause diarrhea, including <i>Clostridium difficile</i> infection
TMP-SMX (10/50 mg/kg/day <sup>a</sup> in divided doses)	TMP-SMX (5/25 mg/kg/day in divided doses)	Blood counts, creatinine, and liver function should be monitored regularly Adequate hydration should be ensured to prevent renal damage from crystalluria
Atovaquone (1,500 mg twice daily) $\pm$ pyrimethamine plus folinic acid (dosing as described above)	Atovaquone (750–1,500 mg twice daily) $\pm$ pyrimethamine plus folinic acid (dosing as described above)	Blood counts and liver function should be monitored regularly Atovaquone suspension should be taken with a high-fat diet to optimize bioavailability
Atovaquone plus sulfadiazine (dosing as described above)	Atovaquone plus sulfadiazine (dosing as described above)	Blood counts, creatinine, and liver function should be monitored regularly Atovaquone suspension should be taken with a high-fat diet to optimize bioavailability
Pyrimethamine plus folinic acid (dosing as described above) plus azithromycin <sup>b</sup> (1,000 mg daily)	Pyrimethamine plus azithromycin not recommended due to a high relapse rate; one of the above regimens should be used instead	Blood counts should be monitored regularly Azithromycin may cause hearing problems and a prolonged QT interval

<sup>a</sup>Higher doses of 15/75 or 20/100 mg/kg/day can be used.

<sup>b</sup>Clarithromycin can be used instead of azithromycin but is associated with more GI intolerance and drug interactions. If used, a lower dose of 500 mg twice a day is preferred, especially in HIV-infected patients; the efficacy of this dosing regimen is not clear (see the text for details).

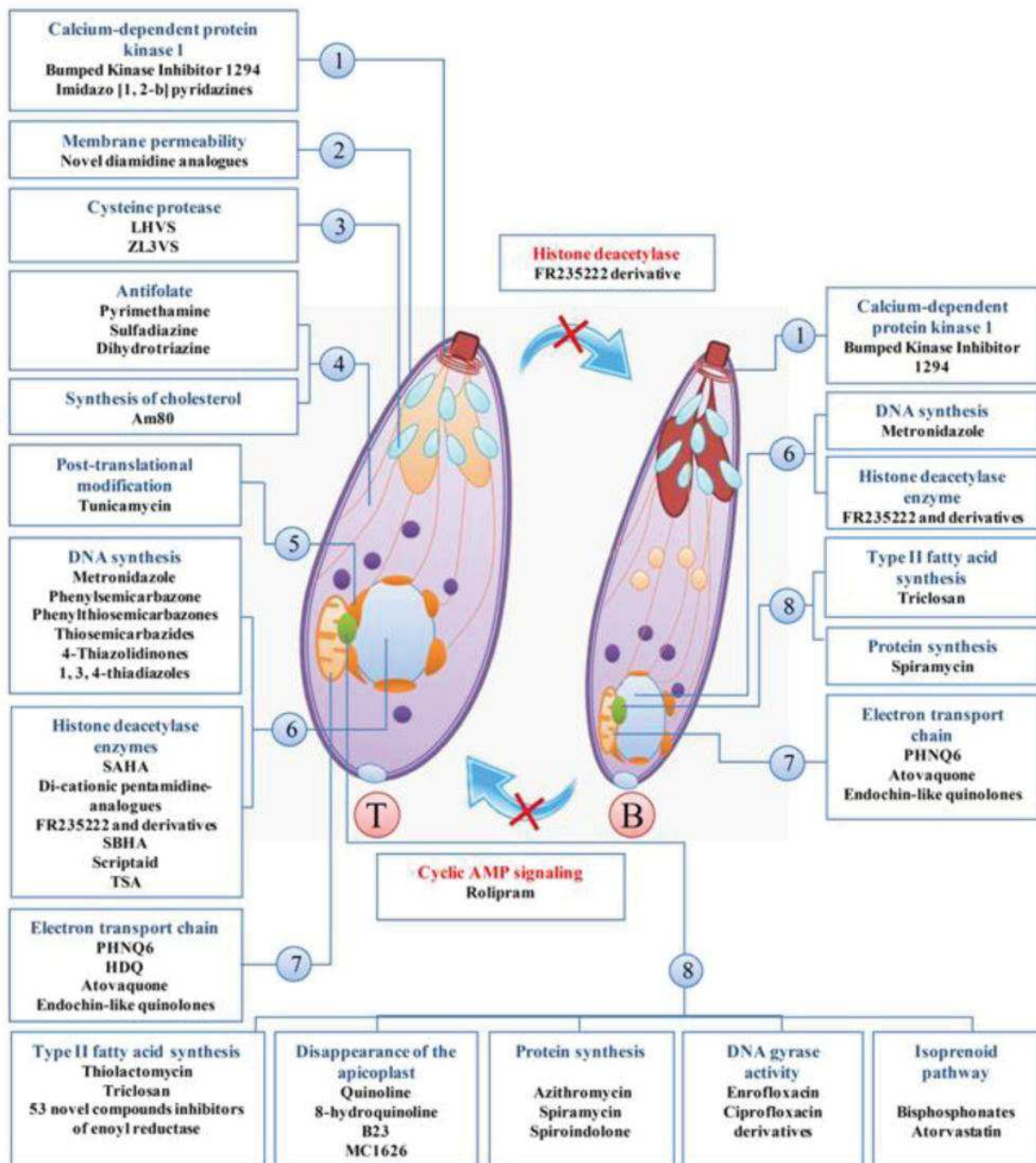
Table 4. Treatment of toxoplasmosis in immunocompromised patients (Dunay et al., 2018)

Infection stage	Regimen	Comments
Maternal infection at $< 14$ weeks of gestation, no fetal infection	Spiramycin (1 g [3 million units] every 8 h until delivery)	Spiramycin is not effective for treating established fetal infection and hence should be used only for prevention of vertical transmission Amniocentesis and fetal ultrasound should be performed when feasible to rule out fetal infection
Maternal infection at $> 14$ weeks of gestation <sup>a</sup>	Pyrimethamine (100 mg daily for 2 days and then 50 mg daily) plus sulfadiazine (1 g q8h [body wt of $< 80$ kg] or 1 g q6h [body wt of $\geq 80$ kg]) plus folinic acid (10–20 mg daily pending fetal USG and amniocentesis) If fetus is confirmed to be infected (abnormal USG and/or positive amniotic fluid PCR), continue pyrimethamine plus sulfadiazine plus folinic acid until delivery If fetus is not infected (e.g., negative USG and amniotic fluid PCR), pyrimethamine plus sulfadiazine plus folinic acid may be switched to spiramycin Alternatively, pyrimethamine plus sulfadiazine plus folinic acid can be continued until delivery or alternated with spiramycin on a monthly basis	Pyrimethamine is teratogenic and should not be used in early pregnancy Serial fetal USG and amniotic fluid PCR should be performed at 18 weeks of gestation
Congenital infection in newborns	Pyrimethamine (1 mg/kg q12h for 2 days and then 1 mg/kg/day for 2–6 mo and then 1 mg/kg/day 3 times a week) plus sulfadiazine (50 mg/kg q12h) plus folinic acid (10 mg 3 times a week)	Treatment should be started as soon as feasible after birth and continued for at least 1 year

<sup>a</sup>The 14-week cutoff period for starting pyrimethamine and sulfadiazine in pregnant women may vary in different countries.

Table 5. Treatment of acute toxoplasmosis in pregnant women and newborns (Dunay et al., 2018).

In a systematic review, Montazeri et al. evaluated the *in vitro* and *in vivo* activities of anti-*Toxoplasma* drugs and compounds during the period 2006-2016. Eighty clinically available drugs and a large number of new compounds with more than forty mechanisms of action were summarized. The known mechanism of action of select drugs is described in Figure 8. Several target based drug screens were also identified including mitochondrial electron transport chain, calcium-dependent protein kinase 1, type II fatty acid synthesis, DNA synthesis, DNA replication among several others (Montazeri et al., 2017) (Figure 8). Most of these drugs are effective against tachyzoites, and only very little trigger bradyzoites or the back and forth switch between both stages (Montazeri et al., 2017). It is worth noting that an ideal drug against toxoplasmosis, should not only be effective against the proliferative tachyzoite stage of the parasite, but it should also exert an activity against the tissue cyst stage especially that the chronic form of the disease is the most prevalent one. In addition, these drugs should be capable to cross the blood brain barrier and to penetrate the cysts targeting bradyzoites (Benmerzouga et al., 2015). Hence, new effective drugs against toxoplasmosis are still needed.



**Figure 8: Drugs/compounds with known mechanisms of action on life stages of *T.gondii* tachyzoites (T), and bradyzoites (B).** 1: apical end; 2: Cell membrane; 3: microneme; 4: cytosol; 5: endoplasmic reticulum; 6: core; 7: mitochondria; 8: apicoplast (Montazeri et al., 2017).

### 3.6. Vaccination against toxoplasmosis

Due to the high burden of toxoplasmosis and the failure/adverse effects of the currently used treatment strategies against the parasite, several attempts were made to

develop vaccines against *T. gondii*. In 1995, the first commercial vaccine for toxoplasmosis, Ovilis Toxovax, was developed and used against congenital toxoplasmosis in sheep. It consisted of an injectable suspension of attenuated parasites of the strain S48, an originally isolated strain from a case of ovine abortion in New Zealand. Following approximately 3,000 passages in mice, this strain lost its ability to differentiate into tissue cysts in mice and into oocysts in cats (Buxton & Innes, 1995; Innes, Bartley, Buxton, & Katzer, 2009). This strain was used to prevent abortions due to toxoplasmosis in sheep but cannot be used in humans because of the high risk of parasite reactivation in this live vaccine. Other vaccine candidates were tested, including in particular apical complex proteins from *T. gondii* (rhoptries, micronemes and dense granules), multi-antigen vaccines, and other adjuvants (Dodangeh et al., 2019; Faridnia, Daryani, Sarvi, Sharif, & Kalani, 2018). However, these searches could not yield to a proper prevention of toxoplasmosis in humans (M. Zhang et al., 2013).

Some classes of antigens were proposed to be potential vaccine candidates; these include:

- i. Recombinant Surface Antigen-1 (SAG-1), which is a GPI-anchored and highly immunogenic surface marker of the tachyzoite stage of *T. gondii* and which may protect against acute toxoplasmosis (Siachoque, Guzman, Burgos, Patarroyo, & Gomez Marin, 2006; Yang, Wu, & Morrow, 2004), and thus brain cyst formation (Bonenfant et al., 2001; Letscher-Bru et al., 1998).
- ii. Recombinant GRA4 and ROP2 given with Alum adjuvant, which provided protection against brain cysts in C57BL/6 mice (Martin et al. 2004).
- iii. A mixture of SAG1, GRA1 and Merozoite Antigen-1 (MAG1), given with Freund's complete Adjuvant, which reduced brain cyst burden by 90% in BALB/c mice.
- iv. A mixture of GERBU: an adjuvant based on cationic lipid solid nanoparticles and N-acetylglucosaminyl- N-acetylmuramyl-l-alanyl-d-isoglutamine, a glycopeptide derived from *Lactobacillus bulgaricus* cell walls ( Biochemicals GmbH, Gaiberg, Germany) with GRA7 and a MIC2-MIC3-SAG1 chimeric protein which provided an 80% reduction in brain cysts in outbred SWISS mice following challenge with *T. gondii* 76K (Jongert, Verhelst, Abady, Petersen, & Gargano, 2008).



- v. The double Knock out of MIC1-MIC3 genes, which markedly impaired virulence and conferred protection against *T. gondii* challenge (Cerede et al., 2005).

An ideal vaccine must have different antigens in all three infected stages of *T. gondii*, high pathogenicity, and capacity to induce strong immune responses (Rezaei et al., 2019). Some studies related to vaccine development proposed multi-epitope DNA vaccines composed of CD8<sup>+</sup> T cell-eliciting, rhomboid protease 4 and GRA14 of the *Toxoplasma gondii* RH strain, as well as CD4<sup>+</sup> helper T lymphocyte epitope(s) administered with lipid adjuvant (El Bissati et al., 2016), coated with calcium phosphate nanoparticles (Rahimi et al., 2017) or recombinant proteins formulated in Poly (DL-lactide-co-glycolide microspheres (Allahyari et al., 2016), or virus-like particles (VLPs) (Lee et al., 2016) to increase both the cellular and humoral responses by augmentation of memory CD8<sup>+</sup> T cells, to induce a higher IFN- $\gamma$  production. These multi-epitope vaccines protected mice against parasite burden when challenged with *T. gondii*.

In light of the absence of an effective treatment, mostly against the chronic phase of the infection, there is an urgent need for seeking effective strategies against *T. gondii* tissue cysts, to prevent disease relapse in immunocompromised patients and to reduce the risk of progression of neuro-pathologies or brain tumors in seropositive immunocompetent patients.

## **Chapter IV: Clonal strains**

### **4.1. Virulence**

Many parameters of virulence are associated with the success of *T. gondii* to invade the host cells. Host cells have immunity related transcription signaling factors that can be modulated by the parasite (Hunter & Sibley, 2012; Rosowski et al., 2014) (Melo et al., 2011; Saraf, Shwab, Dubey, & Su, 2017). Virulence of *T. gondii* was always associated with the amount of tachyzoites needed to infect and kill a mouse (Saeij, Boyle, & Boothroyd, 2005). However, when it comes to humans, the case is different since it is not possible to know the initial count of parasites infecting a human host, thus the virulence is partly based on the organ location and septicemia (Dubremetz & Lebrun, 2012). In addition, virulence of *T. gondii* is not only related to the parasite itself, but also to factors pertaining to the host it is infecting, and the nature of the immune responses it triggers (Dubremetz & Lebrun, 2012; Melo et al., 2011; Saraf et al., 2017; Tait et al., 2010). More importantly, virulence is still shifting towards the crucial genes responsible for drawing the final image of the severity of the infection (Dubremetz & Lebrun, 2012).

### **4.2. Clonal strains**

A combination of phylogenetic and statistical analyses indicates that *T. gondii* has a highly clonal population structure. It was accepted for a long period of time that *T. gondii* belongs to one of the three widespread clonal lineages *i.e.* types I, II, or III (Table 6). These differ by less than 1% at the DNA level (Howe & Sibley, 1995; Weilhammer & Rasley, 2011). Type II strains are the most commonly isolated from patients (Ajzenberg et al., 2002; Ferreira et al., 2011).

Although these strains are genetically quite similar, they show strong phenotypic differences in the laboratory mouse models, whereby virulence in mice is associated with the parasite genotype. Infection by type I strains is fatal ( $LD_{50} = 1$ ) in all strains of laboratory mice, whereas infection with type II ( $LD_{50} \sim 10^2$ ) or type III ( $LD_{50} \sim 10^3$ ) strains generally result in controlled persistent infections (Sibley & Boothroyd, 1992) (Table 6).

Strains	Type I	Type II	Type III
Genetic	98 % similar		
virulence	High	Intermediate	low
Examples	RH	ME49, Pru (Prugniaud), Pru $\Delta KU80$ , 76K	NED
Characteristics	<ul style="list-style-type: none"> <li>• unable to make cysts of bradyzoites because it lyses cells before making cysts</li> <li>• grows faster than types II &amp; III</li> <li>• completely lyses a flask of cultured cells much faster than types II &amp; III</li> <li>• Extracellular parasites remain infectious for a longer time compared with the types II &amp; III</li> </ul>	<ul style="list-style-type: none"> <li>• able to make bradyzoites cysts</li> <li>• grow slower than type I</li> <li>• completely lyses a flask of cultured cells much slower than types I</li> <li>• Extracellular parasites remain infectious for a shorter time compared with the types I</li> </ul>	<ul style="list-style-type: none"> <li>• able to make bradyzoites cysts</li> <li>• grow slower than type I</li> <li>• completely lyses a flask of cultured cells much slower than types I</li> <li>• Extracellular parasites remain infectious for a shorter time compared with the types I</li> </ul>

Table 6. Comparison between Type I, II and III strains of *T. gondii* (Saeij et al., 2005).

Recently, more genotypes were included and differ in their DNA sequences and geographical distribution. Updates in ToxoDB following many molecular techniques, such as polymerase chain reaction-restriction fragment length polymorphisms analysis (PCR-RFLP) (Howe & Sibley, 1995), microsatellite DNA analysis (Ajzenberg et al., 2002; Ajzenberg, Collinet, Mercier, Vignoles, & Darde, 2010), and multi-locus DNA sequence typing of introns (A. Khan et al., 2011) allowed to refine the population structure of *T. gondii* into 189 ToxoDB PCR-RFLP genotypes from 1457 samples

(Shwab et al., 2014). The 10 most frequently identified are genotypes #2, #3, #1, #5, #4, #9, #6, #7, #8 and #10, accounting for 13.8, 12.6, 12.2, 5.0, 4.5 3.8, 3.3, 2.6, 2.3 and 2.1% of the samples, respectively. Genotypes #1 and #3, which differ only at the Apico locus, compose the conventional Type II lineage and account for 24.8% (362/1457) of the population. Genotype #1 is also referred to as Type II clonal, whereas #3 as Type II variant (Table 7). Genotype #2, also known as Type III, account for 13.8% (201/1457) of the samples. Genotypes #4 and #5, which differ only at the SAG-1 locus and are collectively known as Type 12, account for 9.5% (139/1457) of the population. Genotypes #1, #2 and #3 (Type II clonal, Type III and Type II variant) are identified worldwide, and are highly prevalent in Europe. Genotypes #1, #2, #3, #4 and #5 dominate in North America. Genotypes #2 and #3 (Types III and II variant) dominate in Africa, and genotypes #9 and #10 (Chinese 1 and Type I) are prevalent in East Asia (Shwab et al., 2014).

Conventional genotype designations	ToxoDB PCR-RFLP Genotypes	Representative isolates	References
Type I, type 1	#10	GT1	Su <i>et al.</i> (2012)
Type II, type 2 (type 2 clonal)	#1	PTG	Su <i>et al.</i> (2012)
Type II, type 2 (type 2 variant)	#3	PRU	Su <i>et al.</i> (2012)
Type III, type 3	#2	VEG	Su <i>et al.</i> (2012)
Type 12, atypical, exotic	#4	B41	Khan <i>et al.</i> (2011); Su <i>et al.</i> (2012)
Type 12, atypical, exotic, includes Type X and Type A	#5	ARI	Khan <i>et al.</i> (2011); Su <i>et al.</i> (2012)
Type BrI, atypical, exotic, Africa 1	#6	FOU, TgCatBr2	Pena <i>et al.</i> (2008); Mercier <i>et al.</i> (2010); Su <i>et al.</i> (2012)
Type BrII, atypical, exotic	#11	TgCatBr1	Pena <i>et al.</i> (2008); Su <i>et al.</i> (2012)
Type BrIII, atypical, exotic	#8	P89 (TgPgUs15), TgCatBr3	Pena <i>et al.</i> (2008); Su <i>et al.</i> (2012)
Type BrIV, atypical, exotic	#17	MAS, TgCkBr147	Pena <i>et al.</i> (2008); Su <i>et al.</i> (2012)
Chinese 1, atypical, exotic	#9	TgCtPRC4	Dubey <i>et al.</i> (2007b); Chen <i>et al.</i> (2011); Su <i>et al.</i> (2012)

Table 7. Genotypes of major *Toxoplasma gondii* lineages (Shwab et al., 2014).

### **4.3. Effective genetic model of type II: Pru $\Delta$ KU80**

Double Strand Break (DSB) repair in most eukaryotes occurs primarily *via* two different recombination pathways (Haber, 2000). The homologous recombination pathways repair a DSB using mechanisms that recognize highly homologous DNA sequences, while the non-homologous end-joining (NHEJ) pathway does not rely on

DNA sequence homology. Instead, NHEJ involves direct ligation of the ends of broken DNA strands. KU70 and KU80 proteins form a heterodimer that tightly binds the DNA ends at the DSB, an early and essential step of NHEJ (Walker, Corpina, & Goldberg, 2001; Wu et al., 2008). Many eukaryotes preferentially use the NHEJ pathway to repair a DSB, and exogenous targeting DNA can be integrated anywhere into the genome independent of DNA sequence homology (Haber, 2000). The NHEJ pathway also appears to be preferentially used by *T. gondii* based on the high rates of non-homologous recombination and low gene targeting frequencies observed experimentally (Donald & Roos, 1998; Fox & Bzik, 2002; Gubbels, White, & Szatanek, 2008). Fox *et al.* relied on this NHEJ pathway and developed *KU80* knockouts ( $\Delta ku80$ ) in type II *T. gondii* (Fox, Ristuccia, Gigley, & Bzik, 2009). These  $\Delta ku80$  are void of the dominant pathway mediating random integration of targeting episomes and hence allow for increased efficiency of double-crossover homologous recombination at targeted loci (Fox *et al.*, 2009). Fox *et al.* targeted the deletion of four parasite antigen genes (*GRA4*, *GRA6*, *ROP7*, and *tgd057*) that encode characterized CD8<sup>+</sup> T cell epitopes that elicit corresponding antigen-specific CD8<sup>+</sup> T cell populations associated with control of infection (Fox *et al.*, 2011), thus highlighting the importance and value of such knock-outs.

Since then, this strain has been widely used to generate several parasite knock-outs, and it still used despite the introduction of the crispr-cas9 technology to the genetic manipulation of the parental Pru strain. This mainly due to technical drawbacks and impossibility to generate knock-outs/tagging of certain genes using the Pru strain. We used this strain to deplete *P18* encoding gene (introduced in the following chapter) and characterize its function (Second article of the results section).

## **Chapter V: Bradyzoite expressed SAG1-related sequences superfamily of proteins (SRS)**

Studies have shown that the various *T. gondii* developmental stages can be quite different in their molecular marker expression profiles; such molecular markers can be those pertaining to metabolic iso-enzymes, secreted proteins, or surface and cytoplasmic molecules (D. Ferguson, 2004) (Boothroyd, 2009). Along with stage conversion that *T. gondii* undergoes, changes in morphology and biomolecular changes mainly represented by stage-specific antigen expression occur (Luder & Rahman, 2017; Lyons, McLeod, & Roberts, 2002). Studies in this area have yielded the identification of several stage-specific surface antigens, enzymes and even heat shock proteins specific to either one of the developmental stages.

**In this thesis, we are interested, not only in targeting chronic toxoplasmosis by identifying effective immunomodulatory drugs against this phase of the infection, but also in understanding molecular and cellular mechanisms related to the establishment, persistence and reactivation of cerebral toxoplasmosis. One of the markers we studied is P18, belonging to the SRS family. This chapter will elaborate on this family of proteins, with an attention to those expressed in the bradyzoite stage.**

### **5.1. SAG1-related sequences superfamily of proteins (SRS)**

The surface of *T. gondii* is coated with developmentally expressed, glycosylphosphatidylinositol (GPI)-linked proteins. Collectively, these surface antigens are known as the SRS (SAG1-related sequences) superfamily of 144 proteins (Wasmuth et al., 2012). An SRS gene typically contains one or two domains, each with four to six cysteines (4-Cys or 6-Cys) that participate in disulfide bonds and a glycosylphosphatidylinositol (GPI) anchor for attachment to the parasite cell surface. SRS proteins are expressed in a stage-specific manner. SAG1, SAG2A, SAG2B, SAG3, SRS1, SRS2, and SRS3 are mainly expressed on the tachyzoite surface. Contrary to

SAG2A and SAG2B, which are expressed in tachyzoites, SAG2C, -2D, -2X, and -2Y appeared to be expressed exclusively on the surface of bradyzoites (Saeij, Arrizabalaga, & Boothroyd, 2008). Comparing SRS gene expression between strains revealed 44 differentially expressed SRS genes; 8 genes were downregulated in type I, 2 genes were downregulated and 25 genes were upregulated in type II, and 7 genes were downregulated and 8 genes were upregulated in type III (Wasmuth et al., 2012). The SRS superfamily segregated into two families and eight distinct domain subfamilies, subfamilies 1 to 6 (which includes SRS34A) and subfamilies 7 and 8 (which includes SRS29B) (Wasmuth et al., 2012). Some SRS proteins are very closely related, while others share less than 30% sequence identity. It is known that SRS29B and SRS34A (formerly SAG2A) are highly immunogenic during infection, whereas SRS29B and SRS57 function as adhesins (Dzierszinski, Mortuaire, Cesbron-Delauw, & Tomavo, 2000). An interaction with cellular ligands is supported by structural evidence that SRS29B forms a homodimer with a deep, positively charged groove capable of docking sulfated proteoglycans (Boulanger, Tonkin, & Crawford, 2010).

Moreover, SRS25 possesses a degraded SRS domain containing only three cysteines, is expressed in both *Neospora* and *Toxoplasma*, and is not strictly stage specific. SRS33 is the only gene with evidence of strong expression across all coccidian parasites. SRS54 is highly polymorphic and differentially expressed among *Toxoplasma* strains (Wasmuth et al., 2012).

A pellicular surface antigen, P36 was extensively studied, and belongs to the SRS family and was used as a bradyzoite marker. Indeed, Knoll and Boothroyd found that the BSR4 protein, a member of the SAG1 family, encodes the P36-reactive protein (Knoll & Boothroyd, 1998). Surprisingly, the BSR4 transcript was found to be equally abundant in tachyzoites and bradyzoites, suggesting post-transcriptional regulation of this gene (Knoll & Boothroyd, 1998). Investigation of other proteins detected by this antibody revealed SRS9 as the primary target of the P36 mAb. SRS9 encodes a bradyzoite-specific protein with high similarity to members of the SRS family of surface antigens and is located immediately downstream of BSR4 (Van, Kim, Camps, Boothroyd, & Knoll, 2007). Kim *et al.* created a bioluminescent strain lacking the *SRS9* gene and revealed that during an acute infection; wild-type and  $\Delta srs9$  strains replicated at similar rates and could



disseminate systemically following similar kinetics, and initially yielded similar brain cyst numbers. However, during a chronic infection,  $\Delta srs9$  cyst loads substantially decreased compared to those of the wild type, suggesting that SRS9 plays a role in maintaining parasite persistence in the brain. When chronically infected mice were treated with the immunosuppressant dexamethasone, however, the  $\Delta srs9$  strain reactivated in the intestinal tissue after only 8 to 9 days, versus 2 weeks for the wild-type strain. Thus, SRS9 appears to play an important role in both persistence in the brain and reactivation in the intestine (S. K. Kim, Karasov, & Boothroyd, 2007). Furthermore, a bradyzoite specific cyst wall, CST1, a 250 kDa protein localized to the granular material in the cyst wall was identified as a key contributor to cyst wall robustness and hence cyst persistence (Tomita et al., 2013; Weiss, LaPlace, Tanowitz, & Wittner, 1992; Y. W. Zhang, Halonen, Ma, Wittner, & Weiss, 2001). Parasites deficient of CST1 were shown to result in reduced cyst number and fragility of brain cyst wall along with thinning and easy disruption of the cyst wall underlying granular region (Tomita et al., 2013). Recently, Tu et al. expanded their functional analysis to two other cyst wall proteins, CST2 and CST3. The generation of the respective knock out strains revealed a normal phenotype with respect to growth or cyst formation *in vitro*, yet, CST2-KO parasites were markedly less virulent during the acute infection in mice (Tu et al., 2019). In addition, Tomita et al. identified in the cyst wall, a novel mucin domain containing SRS protein, SRS13 and found that it is upregulated in bradyzoites. SRS13 localized to the cyst wall, but showed to be dispensable for normal cyst wall formation (Tomita, Ma, & Weiss, 2018).

## **5.2. SAG-4 or p18: discovery and cloning**

For quite a long while, research in the field of toxoplasmosis remained centered around the readily available tachyzoite developmental stage. However, with the emerging interest in stage conversion, many studies were directed towards the characterization of stage specific developmentally regulated molecules. Antibodies against stage-specific markers for bradyzoites and tachyzoites were developed (Tomavo et al., 1991). These antibodies were selected by differential immunofluorescence assays aiming to isolate



them from hybridomas produced against these organisms. Antigenically reactive markers to these antibodies were found on human isolates of *T. gondii*, as well as on laboratory strain bradyzoites obtained from either brain cysts or *in vitro*-grown parasites (Tomavo et al., 1991). These monoclonal antibodies are T<sub>8</sub> 4A<sub>12</sub> recognizing the previously mentioned 36 kDa protein called P36, T<sub>8</sub> 2C<sub>2</sub> recognizing a 34 kDa protein called P34, T<sub>8</sub> 4G<sub>10</sub> recognizing a 21 kDa protein called P21 and T<sub>8</sub> 3B<sub>1</sub> recognizing an 18 kDa protein called P18 (Tomavo et al., 1991). These proteins represent four pellicular antigens, three of which are exposed on the surface of the organism (Tomavo et al., 1991). Among the three bradyzoite markers (P34, P21 and P18), only the gene encoding for P18 was sequenced and published (Odberg-Ferragut et al., 1996). Real time using P18-specific primers demonstrated the stage expression of this gene in the bradyzoites transcripts. The sequenced gene showed no substantial homology to any of the known genes, hence it was recognized as novel and in accordance to the nomenclature proposed by Sibley, the *P18* gene was given the name *SAG4* (Odberg-Ferragut et al., 1996). Later on, *SAG4* or P18 was attributed to the SRS family and its encoding gene was *SRS35*. Although antibodies against P18 were generated against bradyzoites, Expressed Sequence Tag (EST) data revealed a low expression of P18 transcripts in tachyzoites, while in the bradyzoites, it is one of the most abundantly expressed SRS (Wasmuth et al., 2012). Insights towards a functional characterization of P18 are still absent. **In the results section, the second article will be allocated to functionally characterize this protein *in vitro* and *in vivo*.**

## **Chapter VI: Toll-like receptors and downstream signaling pathways**

Innate immunity is the first line of host defense against pathogen infection (Sasai & Yamamoto, 2013). Following infection with *T. gondii*, innate immune cells migrate to the site of infection where they detect the parasite, mainly *via* Toll-like receptors (TLRs) (Pifer & Yarovinsky, 2011; Yarovinsky, 2014). Before covering the innate and adaptive immune responses triggered by *T. gondii* infection, we will dedicate this chapter to TLRs and their context in case of this parasitic infection.

### **6.1. Overview**

The mammalian immune system consists of innate and adaptive branches, which cooperate to eliminate infectious pathogens (Akira, Uematsu, & Takeuchi, 2006). The “rapidly sensing” innate immune system is the first line of host defense against pathogens and is mediated by phagocytes including macrophages and dendritic cells (DCs) (Akira et al., 2006). These cells recognize pathogen-associated molecular patterns (PAMPs) or Damage-Associated Molecular Patterns (DAMPs) *via* germ line-encoded pattern recognition receptors (PRRs) present in their extracellular milieu and intracellular compartments (Amarante-Mendes et al., 2018; Kawai & Akira, 2009). Adaptive immunity involves a tightly regulated interplay between antigen-presenting cells (APC) and T and B lymphocytes, which facilitate pathogen-specific immunologic effector pathways, generation of immunologic memory, and regulation of host immune homeostasis (Bonilla & Oettgen, 2010).

### **6.2. Pattern Recognition Receptors**

Pattern Recognition Receptors (PRRs) are denoted by common characteristics that define their role in the immune system. They are non-clonal, germline encoded receptors expressed on all cells of a given type, independent of the immunologic memory. PAMPs recognized by PRRs are essential for the survival of microorganisms and are therefore difficult to be altered. PAMPs recognition initiates transcriptional responses along with non-transcriptional responses, such as the induction of phagocytosis, autophagy, cell death, and cytokine processing (Brubaker, Bonham, Zanoni, & Kagan, 2015). The signal

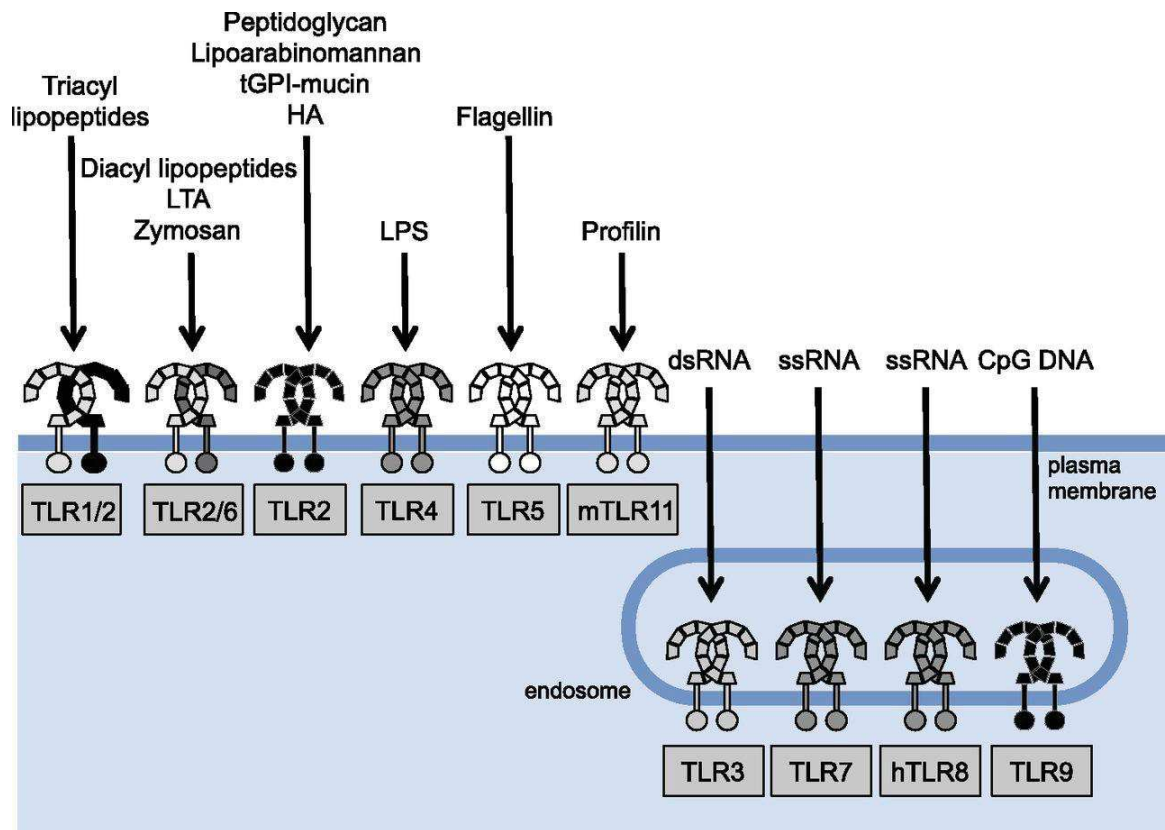
transduction pathways that are activated *via* PRRs coincide on a common set of signaling modules including NF- $\kappa$ B, activator protein-1 (AP-1), and mitogen-activated protein kinase (MAPK). These modules activate microbicidal and pro-inflammatory responses required to eliminate or, at least, to contain the infectious agents by initiating innate and adaptive immune responses (Amarante-Mendes et al., 2018).

### **6.3. Toll-like receptors**

Toll-like receptors (TLRs) represent a unique category of pattern recognition receptors that recognize distinct pathogenic components, often utilizing the same set of downstream adaptors (Patra, Kwon, Batool, & Choi, 2018). These are type I transmembrane receptors localized on the cytoplasmic membrane and endosomal/lysosomal cellular compartments, that play a role in the coupling of innate to adaptive immunity (Gay & Gangloff, 2007). Thirteen different types of TLRs (TLR1-TLR13) were discovered and described in mammals (Vijay, 2018) after the first discovery of TLR-4 in humans, in late 1997 (Medzhitov, Preston-Hurlburt, & Janeway, 1997).

#### **6.3.1. Classification**

There are 10 human and 12 mouse TLRs. TLRs 11, 12 and 13 are exclusively expressed in mice, whereas TLR 10 is expressed only in human. The rest of the TLRs are commonly expressed in both (Dominic De Nardo, 2015). These can be broadly subdivided into two groups: the ones localized on the plasma membrane and the ones localized within the acidified endo-lysosomal compartments (Figure 9). The former group includes TLRs 1, 2, 4, 5, and 6, whereas the latter comprises of TLRs 3, 7, 8, 9, 11, 12 and 13 (Table 8). Plasma membrane TLRs interact directly with components of microbial pathogens that contact the host cell. On the contrary, endolysosomal TLRs sense nucleic acid of endocytosed pathogens after their breakdown in these organelles. This compartmentalization is crucial to avoid contact with host or self-nucleic acid present in the extracellular milieu, protecting from autoimmune responses (Barton & Kagan, 2009).



**Figure 9: Cell surface and intracellular Toll-like receptors (TLRs) and their ligands (Goulopoulou, McCarthy, & Webb, 2016).** TLRs are divided into two groups based on their cellular localization when sensing their respective ligands. TLRs 1, 2, 4–6, and 11 localize to the cell surface (cell surface TLRs) and TLRs 3 and 7–9 reside at endosomal compartments (intracellular TLRs). Cell surface TLRs respond to microbial membrane materials such as lipids, lipoproteins, and proteins, whereas intracellular TLRs recognize bacteria- and virus-derived nucleic acids.

TLR expression, localisation and ligands.

TLR	Species	Localisation	Microbial ligands	Endogenous ligands	Synthetic ligands
TLR1	Human and mouse	Plasma membrane	Triacyl lipoproteins	Unknown	Pam3CSK4
TLR2	Human and mouse	Plasma membrane	Lipoproteins, zymosan, mannan, peptidoglycan, lipoteichoic acid,	Versican	Pam2CSK4, Pam3CSK4
TLR3	Human and mouse	Endolysosomal membrane	Viral dsRNA	mRNA	PolyI:C, polyA:U
TLR4	Human and mouse	Plasma and endolysosomal membrane	LPS	Oxidised low-density lipoprotein, Amyloid-beta	Lipid A derivatives
TLR5	Human and mouse	Plasma membrane	Flagellin	Unknown	Recombinant flagellin
TLR6	Human and mouse	Plasma membrane	Diacyl lipoproteins, lipoteichoic acid, zymosan	Oxidised low-density lipoprotein, Amyloid-beta, versican	Macrophage-activating lipopeptide 2, synthetic diacylated lipoproteins, Pam2CSK4
TLR7	Human and mouse	Endolysosomal membrane	Viral and bacterial ssRNA	Immune complexes, self RNA	Thiaziquinoline and imidazoquinoline compounds (e.g. R848, imiquimod)
TLR8	Human and mouse	Endolysosomal membrane	Viral and bacterial ssRNA	Immune complexes, self RNA	Thiaziquinoline and imidazoquinoline compounds (e.g. R848, imiquimod)
TLR9	Human and mouse	Endolysosomal membrane	Viral and bacterial CpG DNA, DNA:RNA hybrids	Chromatin IgG immune complexes, self DNA	Class A, B and C CpG oligodeoxynucleotides
TLR10	Human	Plasma membrane	Unknown	Unknown	Unknown
TLR11	Mouse	Endolysosomal membrane	Profilin and flagellin	Unknown	Unknown
TLR12	Mouse	Endolysosomal membrane	Profilin	Unknown	Unknown
TLR13	Mouse	Endolysosomal membrane	Bacterial 23S ribosomal RNA (rRNA)	Unknown	23S rRNA derived oligoribonucleotide

Table 8. Toll-like receptors classification, localization and ligands (Dominic De Nardo, 2015).

The below two sections will focus mostly, on four TLRs: TLR-11, 12, and 7. Indeed TLR-11 and 12 are well described and very important in the context of *Toxoplasma* infection. TLR-7 is well characterized in the context of the treatment with the immunomodulatory drug, Imiquimod being the drug of focus in the first manuscript of our results section.

#### **6.4. TLR-11 and TLR-12**

TLR-11 is plasma membrane TLR, known to recognize uro-pathogenic *Escherichia coli* and *T. gondii* parasite. The bacterial ligand binding this receptor is still unknown (Anders & Patole, 2005), however *T.gondii* ligand binding to it, is a motor protein called profilin (Plattner et al., 2008). Profilin is an actin binding protein dispensable for the intracellular growth, but indispensable for invasion, and active egress from cells (Plattner et al., 2008). Furthermore, TLR-11 recognizes flagellin, a main flagellar protein of flagellate organisms, causing dimerization of the receptor, activation of NF- $\kappa$ B and production of inflammatory cytokines, in mouse intestine (Hatai, Lepelley, Zeng, Hayden, & Ghosh, 2016). Indeed, TLR-11 is essential for the development of the

protective immune response in infected mice, through the induction of massive IL-12 production by dendritic cells (DCs) (Ishii, Uematsu, & Akira, 2006; Sanecka & Frickel, 2012). In humans, the *TLR-11* gene is expressed but leads to a non-functional protein (Salazar Gonzalez et al., 2014). Phylogenetic analysis between human and mouse species showed that human TLR-5 seemed to be the evolutionarily closest member to the mouse TLR-11 (Salazar Gonzalez et al., 2014; Yarovinsky et al., 2005).

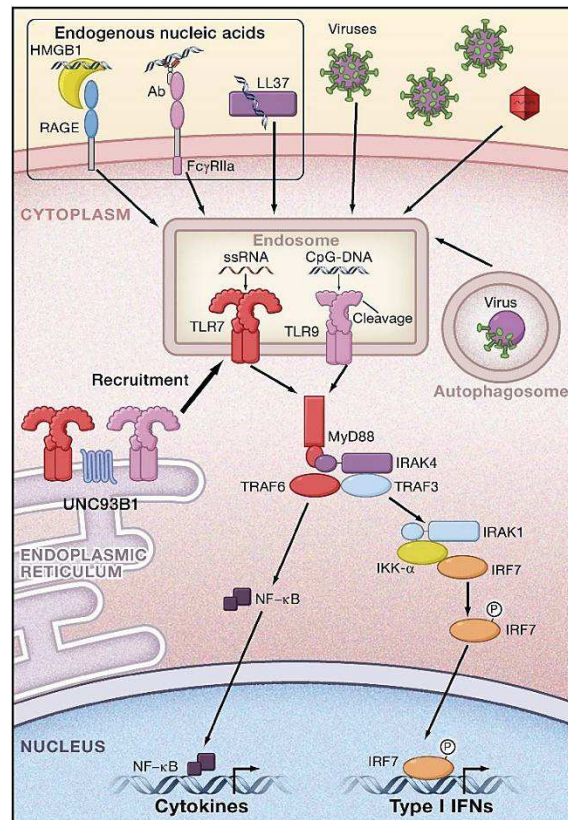
Similar to TLR-11, TLR-12 is a second receptor for *T. gondii* profilin, that colocalizes with TLR-11 and forms a heterodimer complex that interacts with the nucleic acid-sensing TLR trafficking protein UNC93B1 (W. A. Andrade et al., 2013; Pifer, Benson, Sturge, & Yarovinsky, 2011). UNC93B1 is a chaperone required to bring TLRs from the endoplasmic reticulum (ER) to the endocytic system (Y. M. Kim, Brinkmann, Paquet, & Ploegh, 2008). Mice carrying a single point mutation in UNC93B1, retain the protein in the endoplasmic reticulum thus preventing intracellular TLR trafficking. These mice were shown to be highly susceptible to *Toxoplasma* and to produce less IL-12 upon intraperitoneal infection (Melo et al., 2010).

## **6.5. TLR-7**

TLR-7 is continuously discussed with TLR-8 because they are often referred to together (TLR-7/8), and present a high degree of homology and similarity in function. These are intracellular endosomal TLRs. To encounter these endosomal TLRs, pathogens are internalized into the endocytic pathway, a hostile environment capable of detecting infection and reporting it, first to the phagocytic cell and then to other cells of the body. TLR-7/8 respond both to purine rich single stranded ribonucleic acid (ssRNA) (Figure 10) (Gantier et al., 2008). They exist as preformed dimers regardless of ligand assembly. Although both TLRs are phylogenetically and structurally related, their expression varies in different cell types and thus their agonists differ in target cell selectivity and cytokine induction profile (Gorden et al., 2005). Constitutive expression of TLR-7 is predominant in plasmacytoid dendritic cells (pDCs) and B cells compared to other circulating immune cells. TLR-8 is highly expressed in myeloid cells and to a lesser extend in pDCs (Gorden et al., 2005). TLR-7 activation induces the production of IFN



and IFN-regulated chemokines whereas TLR-8 is more effective in inducing pro-inflammatory cytokines and chemokines production (Gorden et al., 2005). Due to the importance of these two TLRs in nucleic acid pathogen recognition, some nucleoside analogs were synthesized as agonists, to enhance their activation. One example is Imiquimod, a synthetic tricyclic imidazoquinoline that was generated as a TLR-7 agonist (Schon & Schon, 2007) (see chapter VIII/section 8.1).



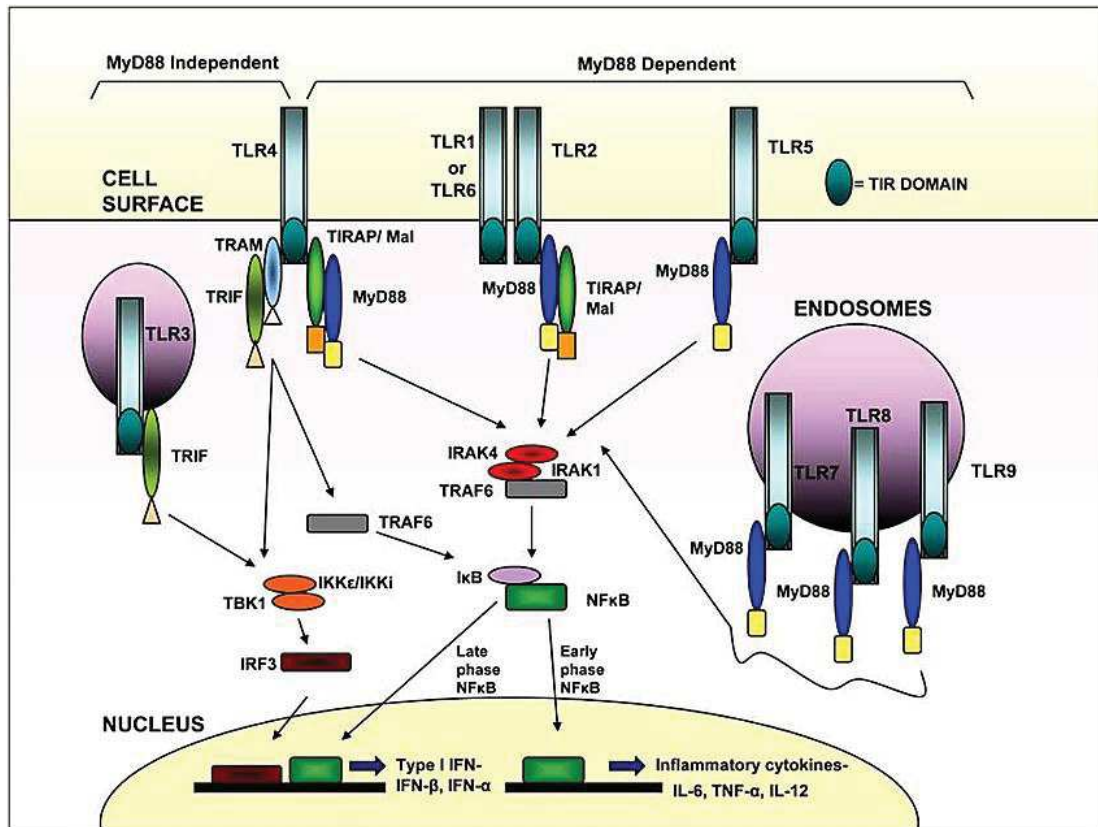
**Figure 10. Downstream signaling of endosomal Toll-like receptors (Takeuchi & Akira, 2010).** Stimulation with ligands or infection by viruses induces trafficking of endosomal TLRs from the ER to the endolysosome via UNC93B1. A complex of MyD88, IRAK-4, TRAF6, TRAF3, IRAK-1, IKK- $\alpha$ , and IRF7 is recruited to the TLR. Phosphorylated IRF7 translocates into the nucleus and upregulates the expression of type I IFN genes.

## **6.6. TLRs downstream signaling pathways**

The ligand induced TLR dimerization results in the recruitment of Toll-interleukin 1 receptor (TIR) domain-containing adaptors, to connect the receptor to

downstream effector proteins. Five adaptors have been identified to play a role in TLR signaling: MyD88, MAL (Myelin and lymphocyte), TIRAP (TIR Domain Containing Adaptor Protein), TRIF (TIR-domain-containing adapter-inducing interferon- $\beta$ ), TRAM (Translocation Associated Membrane Protein) and SARM (Sterile Alpha And TIR Motif Containing) (O'Neill & Bowie, 2007). Broadly, these adaptors trigger two main pathways, MyD88 dependent and independent pathways (TRIF dependent pathway) (Mason et al., 2004). The former pathway is required for the production of inflammatory cytokines and for the upregulation of co-stimulatory molecules and major histocompatibility complex (MHC) class II, in antigen presenting cells (APCs) (Brutkiewicz, 2016; Pasare & Medzhitov, 2003). The latter is required for the upregulation of costimulatory molecules and MHC class II in dendritic cells (DCs) (Pasare & Medzhitov, 2003; ten Broeke, Wubbolts, & Stoorvogel, 2013). MyD88 dependent pathway is essential for the downstream signaling of all TLRs, except TLR-3 that signals through TRIF dependent pathway (Figure 11). TLR-7 and TLR-8 signal in a MyD88-dependent pathway. This leads to the activation of the IRF family of transcription factors responsible for the expression of IFNs (Negishi et al., 2006). A common signaling event downstream both pathways is the activation of the canonical NF- $\kappa$ B pathway, which is responsible for transcriptional induction of pro-inflammatory cytokines, chemokines and additional inflammatory mediators in different types of innate immune cells.





**Figure 11. Myd88 dependent and independent pathways (O'Brien et al., 2008).** Overview of TLR signaling pathways. MyD88 dependent pathway: Activated TLR interacts through its TIR domain with MyD88 TIR domain. IRAKs are recruited to the receptor once ligand is bound. TRAF6 is then activated by IRAKs which in turn activates IB kinase complex. This complex phosphorylates IB and results in the nuclear translocation of NF-B and induces the expression of inflammatory cytokines. TIRAP/Mal adaptor protein is also required for TLRs 2 & 4. MyD88 independent pathway: Activated TLR (3 or 4) interact through its TIR domain with TRIF TIR domain. TLR-4 (but not TLR-3) requires TRAM also. TRIF interacts with IKK, IKKi and TBK1. This complex phosphorylates IRF3 and results in the nuclear translocation of IRF3 and induces the expression of Type I interferons. TRIF also interacts with TRAF6 and induces late phase NF-B.

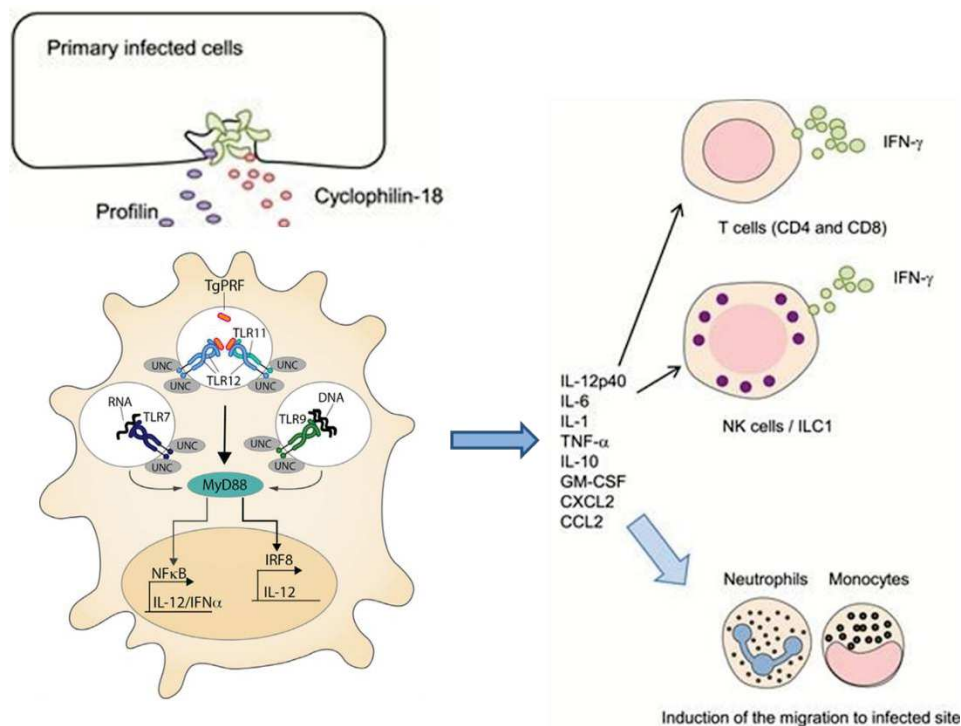
## **6.7. Toll-like receptors in the context of toxoplasmosis**

Following infection with *T. gondii*, innate immune cells migrate to the site of infection where they detect the parasite, mainly via TLRs (Yarovinsky, 2014). For instance, DCs recognize PAMPs via TLRs that mainly signal *via* the adaptor protein MyD88 (Arnold-Schrauf, Berod, & Sparwasser, 2015). This signaling pathway is required for the immune protection during many infections, which are lethal in the absence of MyD88 (Torres et

al., 2013). TLRs play a major role in *T.gondii* recognition, via two well-identified parasitic PAMPs, the above mentioned profilin and the cyclophilin-18 (Figure 12).

The actin binding protein profilin is indispensable for invasion, and active egress from cells (Egan, Sukhumavasi, Butcher, & Denkers, 2009; Kucera et al., 2010; Plattner et al., 2008). Parasites lacking profilin are unable to induce TLR-11-dependent production of IL-12 both *in vitro* and *in vivo* (Plattner et al., 2008). Indeed, profilin is essential in the context of *T. gondii* infection through two main aspects: it binds TLR-11 (Yarovinsky et al., 2005) and TLR-12 (Koblansky et al., 2013; Sanecka & Frickel, 2012), and enhances the production of IL-12 via MyD88 dependent pathway (Figure 12).

Cyclophilin-18 is recognized by both mouse and human C-C chemokine receptor type 5 (CCR5) (Yarovinsky et al., 2004) and enhances the proliferation and migration of macrophages and spleen cells (mainly T lymphocytes), to the site of infection for maintenance of the interaction between the parasite and host (Ibrahim, Xuan, & Nishikawa, 2010) (Figure 12).



**Figure 12. Recognition of *T.gondii* by innate immune cells leads to activation of acquired immunity.** Macrophages and DCs produce various inflammatory cytokines and chemokines to promote IFN- $\gamma$  production from T cells, NK cells or ILC1 and recruitment of

neutrophils and inflammatory monocytes to the infected sites (Sasai, Pradipta, & Yamamoto, 2018).

Although humans do not express either TLR-11 or TLR-12, human monocytes produce pro-inflammatory cytokines in response to *T. gondii* infection, suggesting that other TLRs in humans recognize different compartments of *T. gondii* to produce IL-12 in antigen-presenting cells (W. A. Andrade et al., 2013). The difference of relevant TLR expression in humans and mice towards *Toxoplasma* infection, prompted us to give a separate overview of these TLRs between the two species.

### **a) In mouse models:**

As discussed earlier, MyD88 protein plays a key role in the activation of several signaling pathways including NF- $\kappa$ B, and MAPK, which subsequently leads to the production of IL-12, and IFN- $\gamma$  among others (Figure 11). The role of MyD88 in resistance to bacterial, viral and protozoan parasitic infections (Scanga et al., 2002) is widely demonstrated. MyD88-deficient mice are highly susceptible to *T. gondii* infection whereas mice lacking IL-1 exhibit normal resistance to *T. gondii* infection suggesting that the absence of MyD88 alters the function of *T. gondii* TLR signaling (Scanga et al., 2002; Yarovinsky, Hieny, & Sher, 2008).

*In vitro* studies on Chinese hamsters (CHO) showed that TLR-2 and TLR-4 synthesis depend on the secretion of TNF- $\alpha$ , in the presence of glycosylphosphatidylinositol (GPI) extracted from tachyzoites (Debierre-Grockieo et al., 2007). A KO mouse for TLR-2 produces IL-12, but succumbs due to the high parasite burden of virulent strains of *T. gondii* (Scanga et al., 2002). Similarly, TLR-4 is not involved in the production of IL-12 (Scanga et al., 2002), but it may be a co-activator the transmission of cytokine signaling in the murine model (Debierre-Grockieo et al., 2007).

TLR-9 was also shown to be required for the activation of a Th1 inflammatory response, following oral infection with *T. gondii* and the chaperone protein UNC93B1 would promote the translocation of TLR-7 and 9 necessary for resistance mechanisms (Minns et al., 2006).

In addition to the previously described TLRs, TLR-11 receptor signaling mediated by MyD88, is essential for activation of the innate immune response in mice

(Pifer et al., 2011; Yarovinsky et al., 2005). In fact, inhibition of the expression of UNC93B, a chaperone protein involved in intracellular trafficking of TLRs, leads to a loss of the mechanism of resistance to toxoplasmic infection in mice (Melo et al., 2011; Pifer et al., 2011). This protein interacts directly with TLR-11 and induces regulation of the response of dendritic cells against *T. gondii* (Pifer et al., 2011; Sanecka & Frickel, 2012). Moreover, TLR-11 is important for the secretion of IL-12 in mice, as the KO mouse model for TLR-11 displayed low levels of IL-12 production (Sher, Tosh, & Jankovic, 2017; Yarovinsky et al., 2005). TLR-11 also works by forming a heterodimeric complex with TLR-12, only in the mouse. The formation of this complex is also important for dendritic cell response and IL-12 production (W. A. Andrade et al., 2013; Koblansky et al., 2013). TLR-12 has the same role as TLR-11, but is expressed specifically in hematopoietic cells, unlike TLR-11, which is expressed in these cells as well as in endothelial cells (Koblansky et al., 2013). A deficiency of the gene coding for TLR-12 results in a loss of resistance to toxoplasmic infection as well as a deficiency of the gene encoding MyD88 (Koblansky et al., 2013; Sher et al., 2017).

### **b) In humans:**

Unlike the mouse model, the TLRs responsible for the activation of the immune reaction are poorly defined in humans, during *T. gondii* infection. It has been described that parasite recognition by intracellular TLRs (TLR-3, 7 and 9) in humans facilitates resistance to toxoplasmic infection and activation of monocytes and human dendritic cells (W. A. Andrade et al., 2013; Sher et al., 2017). In humans, TLR-11 exists, but is non-functional and TLR-12 does not exist. Thus, the mechanisms of activation of the immune reaction and the production of IL-12 and IFN- $\gamma$  in humans, appears to be different from that activated in mice. It was reported that the human TLR-5 might have a similar role to the murine TLR-11 in activating cytokine production (Salazar Gonzalez et al., 2014). Other studies based on genetic association analysis for congenital toxoplasmosis in humans revealed several genes that may be involved in modulating immune responses to *T. gondii*. These include the TLR-9, the P2X7 purinergic receptor and a member of the NALP1 NOD-like receptor family (Lees et al., 2010; Peixoto-Rangel et al., 2009; Witola et al., 2011). The P2X7 purinergic receptor is associated with

congenital and ocular toxoplasmosis (Jamieson et al., 2010). It was found on the surface of macrophages, which are activated by extracellular ATP and can be upregulated synergistically by IFN $\gamma$  and TNF $\alpha$  (Krishnamurthy, Konstantinou, Young, Gold, & Saeij, 2017). A polymorphism of P2RX7 (a gene encoding the P2X7 receptor) influences the susceptibility of *T. gondii* infection (Jamieson et al., 2010). Blocking NALP1 gene expression enhances proliferation of *T. gondii* in human cells (Witola et al., 2011) and NALP1 could be involved in the process of eliminating the intracellular parasite. To date, the molecular events responsible of NALP1 activation in the cytosol of infected cells are not yet elucidated. Identification of the involvement of NALP1 in the *T. gondii* elimination process could be one of the keys to the intracellular recognition system of *T. gondii* in humans (Krishnamurthy et al., 2017).

**In summary, in murine models, *T. gondii* itself has ligands for endosomal TLR-7, TLR-9, TLR-11, and TLR-12 and for surface-expressed TLR-2 and intestinal bacteria can pass the mucosal barrier during *T. gondii*-mediated inflammation and contain ligands for TLR-2, TLR-4, and TLR-9 (Sturge & Yarovinsky, 2014). Activation of TLRs leads to downstream signaling pathways playing a key role in first dissemination and then control of the infection.**

## **Chapter VII: Immune response against toxoplasmosis**

Immune responses vary from one *Toxoplasma gondii* strain to another, and each strain has unique combinations of effectors and inducers of different pathways to promote or inhibit inflammation and other cellular responses. Some of the parasite virulence factors are secretory rhoptry and granule dense proteins, belonging to the apical complex and playing a key role during invasion and host response modulation. These proteins can be different between types of strains of *T. gondii*, hence making the modulation of host cell signaling pathways variable between these strains (Melo et al., 2011). In fact, at early time points, type I parasites do not activate pro-inflammatory responses while Type II parasites are very effective in activating an early response (Melo et al., 2011).

**In this thesis work, our main focus is the molecular and cellular characterization along with the therapeutic targeting of mostly the chronic phase of the infection. Since Type I strains fail to establish a successful chronic phase, we will focus in this chapter, on type II strains and their effect on modulating the host immune responses.**

### **7.1. Innate immune response**

When the parasite crosses the intestinal epithelial barrier, tachyzoites infect first monocytes and neutrophils (Coombes et al., 2013), then macrophages, and innate lymphoid cells which represent an identified lymphocyte subset related to innate immunity (Gregg et al., 2013). These innate immune cells produce IL-12, which participates in the differentiation of naive T cells into Th1-type T cells. T cells will then secrete IFN- $\gamma$  to activate other immune cells such as natural killer (NK) cells (Pepper et al., 2008). IFN- $\gamma$  is a key mediator of resistance against *T. gondii* in mice. This cytokine initiates multiple intracellular mechanisms to eliminate the parasite and inhibit its replication (Sasai et al., 2018).

### **7.1.1. Dendritic Cells**

Dendritic cells (DCs) are a heterogeneous population with distinct surface markers, transcription factor requirements, and functions (Belz & Nutt, 2012). All DC originate from a common bone marrow progenitor, but they subsequently differentiate into distinct subsets, including monocyte-derived DC, conventional DC, and plasmacytoid DC according to cytokines and transcription factors implicated (Miller et al., 2012; Pierog et al., 2018). Conventional DC contains three populations: CD8<sup>+</sup> DC, CD4<sup>+</sup> DC and CD8<sup>-</sup>CD4<sup>-</sup> DC (Schnorrer et al., 2006). The CD8<sup>+</sup> DC are unique since they can present exogenous antigens on their MHC class I molecules, and represent the most relevant subset of DC for IL-12 production (Schnorrer et al., 2006; Tosh et al., 2016). DC secreted IL-12, along with other IL-12 innate cell producers, play a critical role in orchestrating the innate immune functions to trigger adaptive immune response, promoting IFN- $\gamma$  production and controlling the parasite (Lakhrif et al., 2018; LaRosa et al., 2008). Indeed, infection with *T. gondii* induces increased numbers of DCs at the site of infection, then in the spleen, and lymph nodes (Mashayekhi et al., 2011). More importantly, *T. gondii* binds to TLR-11 and 12, expressed in DCs (see section 6.1), to activate MyD88 signaling among others, leading to the expression of Interferon Regulatory Factor 8 (IRF8) which in turn, induces IL-12 and subsequent production of IFN- $\gamma$  by natural killer (NK) cells and T cells (Sasai et al., 2018). Moreover, the atypical I $\kappa$ -B family member, Bcl-3, associates with p50/NF- $\kappa$ B1 or p52/NF- $\kappa$ B2 homodimers in nuclei, thereby either positively or negatively modulating transcription in a context-dependent manner. Bcl-3 is required in dendritic cells to prime protective T-cell-mediated immunity to *T. gondii* (Tassi et al., 2015).

### **7.1.2. Natural killer cells**

Natural killer (NK) cells are also involved in immunity against *T. gondii*. NK cell activity peaks early during acute infection. Although their activity is elevated during chronic toxoplasmosis, they do not appear to be significant contributors to immunity during the chronic stage of infection (Denkers, Gazzinelli, Martin, & Sher, 1993; Dupont, Christian, & Hunter, 2012). IL-12 produced by macrophages, monocytes and dendritic



cells stimulates IFN- $\gamma$  secretion by NKs to fight against the parasite. In addition to IFN- $\gamma$ , NK cells produce the cytokine IL-10 that represent a negative feedback loop when IL-12 is highly expressed (Perona-Wright et al., 2009). The rapid transfer of *T. gondii* from infected DCs to effector NK may contribute to the parasite's sequestration and shielding from immune recognition shortly after infection (Sultana et al., 2017). NK cells can also act to promote adaptive immune responses. Mice that lack T cells exhibit a limited resistance against the infection, through the ability of their NK to produce IFN- $\gamma$  to help mounting a cytotoxic response by the CD8<sup>+</sup> T cells (Combe, Curiel, Moretto, & Khan, 2005). Finally, NK cells can be directly invaded by the parasite and this invasion alters NK cell migration, producing a hyper-motile phenotype (Petit-Jentreau, Glover, & Coombes, 2018).

### **7.1.3. Monocytes**

Monocytes play a key role in toxoplasmosis and are largely manipulated by the parasite. Indeed, the parasite highjacks monocytes and changes their motility *via* it secreted rhoptry protein, ROP17, to promotes its dissemination (Drewry et al., 2019). *In vitro*, monocytes are able to inhibit *T. gondii* replication (Mordue & Sibley, 2003). Oral infection of mice with *T. gondii* induces IFN- $\beta$  production by inflammatory monocytes (IM) in mesenteric lymph nodes (Han et al., 2014). Ly6C<sup>+</sup> IMs are essential to host defense against *T. gondii*, are recruited after stimulation of TLR-11 by the parasite profilin in a C-C chemokine receptor type 2 (CCR2) - dependent manner and confer resistance in mice (Neal & Knoll, 2014). Furthermore, IMs are capable of producing IL-12 *in vitro* and *in vivo*, when stimulated by *T. gondii* (Aldebert et al., 2007; Robben, LaRegina, Kuziel, & Sibley, 2005). They can also produce IL-1 in response to soluble antigens of *T. gondii* to enhance anti-toxoplasmic effector mechanisms in macrophages and astrocytes *in vitro* (Hammouda, Rashwan, Hussien, Abo el-Naga, & Fathy, 1995). In addition, IL-1 reacts synergistically with IL-12 to promote the production of IFN- $\gamma$  from the innate and adaptive immune cells (Borthwick, 2016). IMs also contribute to the direct control of *T. gondii* *via* expression of the nitric oxide inducible synthase (iNOS), and the production of NO, which inhibits parasite replication (Dunay, Fuchs, & Sibley, 2010). Finally, monocytes are able to differentiate into dendritic cells, to induce adaptive



immune responses (Dominguez & Ardavin, 2010), or into macrophages that can then control infection by GTPase-dependent immune mechanisms (Dominguez & Ardavin, 2010).

In humans, distinct monocyte subpopulations are described based on their expression of CD14 and CD16 (Heimbeck et al., 2010). The so-called classical monocytes are CD14<sup>+</sup> and CD16<sup>-</sup> and account for ~85% of the circulating monocyte pool under steady state conditions. These appear to be the human counterparts of Ly6C<sup>+</sup> CD43<sup>low</sup> CCR2<sup>high</sup> murine monocytes used during *Toxoplasma* infection (Ehmen & Luder, 2019).

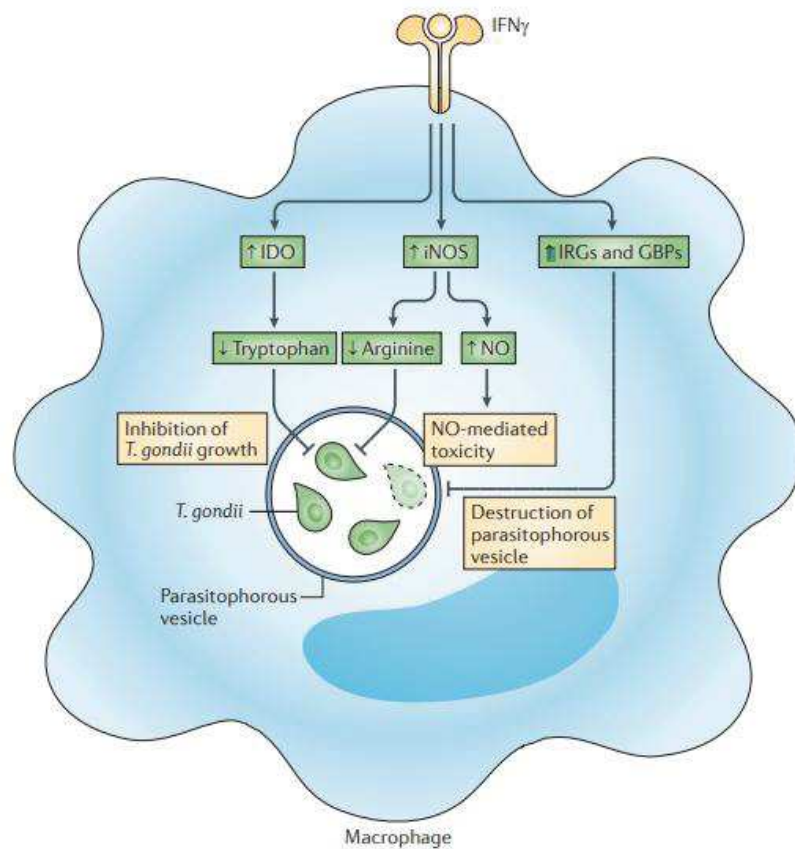
#### **7.1.4. Macrophages**

Macrophages are antigen-presenting cells that strongly activate adaptive immunity (Sasai et al., 2018). After recognition of parasite components by macrophages, they induce the expression of genes encoding chemokines such as the chemokine (C-C motif) ligand 2 (CCL2) (referred to as monocyte chemoattractant protein 1 MCP1) which induce the migration of Ly6C<sup>high</sup>CCR2<sup>+</sup> monocytes and neutrophils to the infection site (McGovern & Wilson, 2013b). Activated macrophages by *T.gondii*, produce IL-1 $\beta$  and TNF- $\alpha$  (Philip & Epstein, 1986). Importantly, IL-12 production from macrophages triggers the proliferation of NK cells, CD8 T cells and CD4 T cells, which mediate cytotoxicity and high amounts of IFN- $\gamma$  (Sasai et al., 2018), a dominant factor that enhances the ability of macrophages to destroy *T. gondii* (Yarovinsky, 2014). Furthermore, inactivation of the Nuclear factor erythroid 2-related factor 2 (Nrf2), a pathway necessary for maintaining redox homeostasis in activated macrophages, impairs parasite growth (Pang et al., 2019).

NLRP1 and 3 (nucleotide-binding domain leucine-rich repeats protein family) sense *T. gondii* infection in monocytes and macrophages as part of an inflammasome complex which mediates the secretion of IL-1 $\beta$  (Gov, Schneider, Lima, Pandori, & Lodoen, 2017) to limit parasite load and dissemination (Chu et al., 2016).

Macrophages have also the ability to induce inhibition of tachyzoite multiplication *via* iNOS, arginase-1 as well as the expression of the inhibitory proteins indoleamine 2,3- dioxygenase (IDO) and the expression of the effector proteins

immunity-related GTPases (IRGs) (Yarovinsky, 2014). IDO causes the depletion of tryptophan, an essential amino acid required for *T. gondii* survival while being unable to synthesize it. NO is generated downstream of IFN- $\gamma$  and interacts with the parasite directly in the parasitophorous vacuole (Cabral, Wang, Sibley, & DaMatta, 2018) and the IRGs contribute to the parasitophorous vacuole destruction (Yarovinsky, 2014) (Figure 13). Finally, the parasite SRS protein SAG2A antigen was shown to induce phenotypic and classical activation of macrophages in mice during the acute phase of the disease (Leal-Sena et al., 2018).



**Figure 13. Effector mechanisms of IFN $\gamma$ -mediated parasite elimination in infected macrophages:** induction of the expression of indoleamine 2,3-dioxygenase (IDO) and inducible nitric oxide synthase (iNOS) as well as immunity-related GTPases (IRGs) and guanylate-binding proteins (GBPs) (Yarovinsky, 2014).

### **7.1.5. Neutrophils**

Neutrophils control infectious pathogens by phagocytosing, degrading microbes, and producing IL-12 and reactive oxygen species (ROS) (Mantovani, Cassatella, Costantini, & Jaillon, 2011). They can also contain and eliminate extracellular microbes by releasing neutrophil extracellular traps (NETs) (Brinkmann et al., 2004). Through the release of chemokines and cytokines, neutrophils cross-talk with other immune cell types, such as macrophages, dendritic cells, and lymphocytes (Leliefeld, Koenderman, & Pillay, 2015). In the context of *T. gondii* infection, an increase in extracellular DNA is obtained at the site of the infection along with a recruitment of neutrophils. Indeed, neutrophils release DNA to form extracellular traps for *Toxoplasma* (Abi Abdallah et al., 2012). These traps decreased the parasite viability *in vitro* and may be used *in vivo* to fight against the infection (Abi Abdallah et al., 2012). Furthermore, *T. gondii* is able to inhibit IL-1 $\beta$  production in human neutrophils by impairing the activation of the NF- $\kappa$ B signaling pathway and by inhibiting the inflammasome (Lima, Gov, & Lodoen, 2018). This targeting the IL-1 $\beta$  pathway may facilitate the survival and spread of *T. gondii* during acute infection.

**To conclude this section, it has been proposed that following a *T. gondii* infection in a mouse, the parasite actin binding protein, profilin, binds to the endosomal TLR-11/TLR-12 complex, and initiates MyD88 downstream signaling, leading to activation of the transcription factor IRF8. This results in IL-12 production by DCs, eliciting an IFN- $\gamma$  production by NK cells. Infected macrophages and monocytes may also respond to *T. gondii* infection through NLRP1 and NLRP3 inflammasomes. IL-1 $\beta$  and TNF- $\alpha$  are involved in inducing IFN- $\gamma$  production from neutrophils. IL-1 $\beta$  production can also enhance the response of NK cells (Figure 14). It is worth noting that following sensing *T.gondii* by innate immune cells, IFN- $\gamma$  is produced by NK, neutrophils and subsequently by adaptive immune cells, primarily by T cells. This cytokine plays a pleiotropic role at multiple levels during toxoplasmosis.**

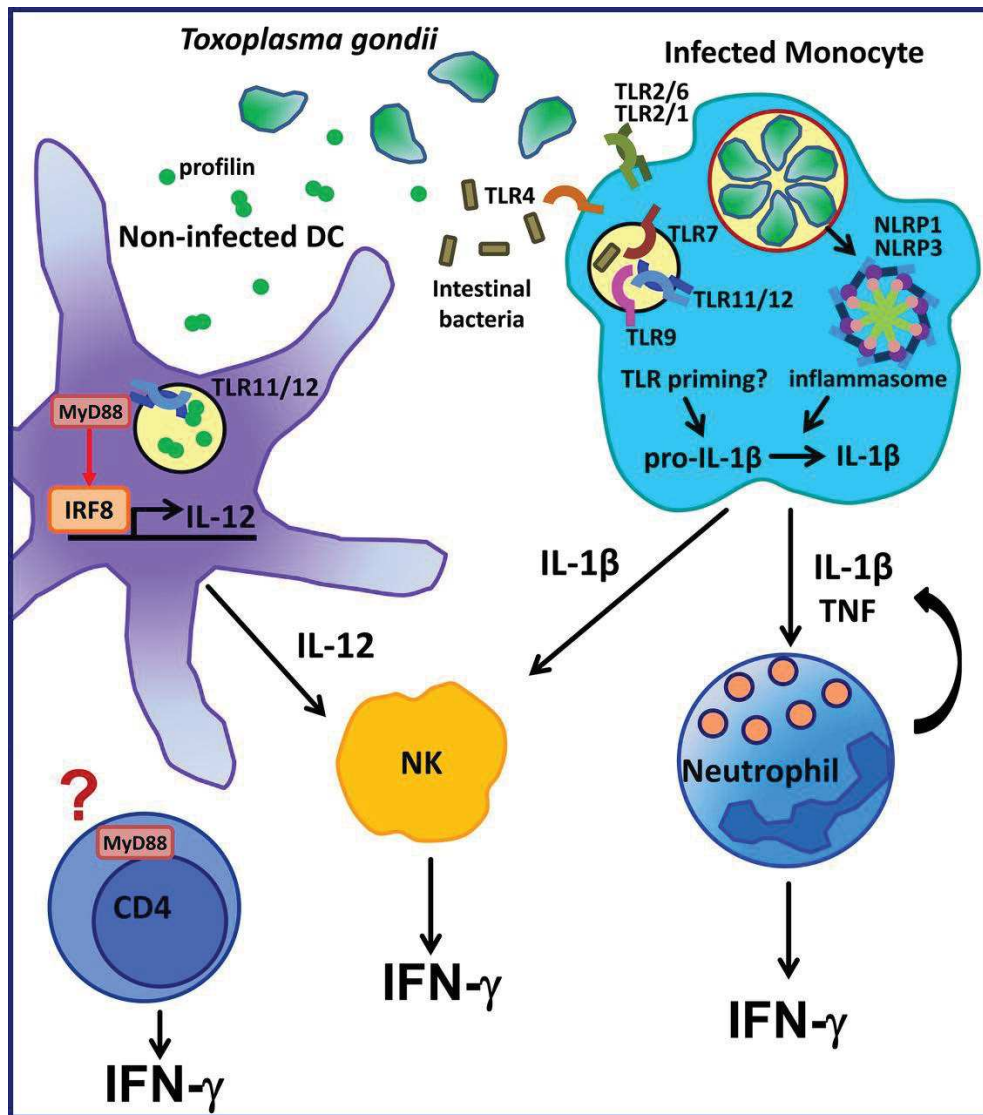


Figure 14. Current model for *T. gondii* infection and IFN- $\gamma$  production by different innate immune cells (Sturge & Yarovinsky, 2014).

## 7.2. Adaptive immune response

The importance of adaptive immune responses against *T. gondii* is demonstrated by the increased susceptibility of patients to toxoplasmosis, when primary or acquired defects in T cell functions occur. Both CD4<sup>+</sup> and CD8<sup>+</sup> T cells are important in the context of the infection and they do not play redundant or overlapping functions since individual depletion of these subsets of T cells, do not produce same phenotype, and do not recapitulate same phenotype as upon the simultaneous depletion of both subsets (R.

Gazzinelli, Xu, Hieny, Cheever, & Sher, 1992). In addition, mice lacking all T cells succumb during the acute phase of the infection (Hunter & Remington, 1994). Depletion of these cells in the chronic phase, leads to reactivation of the disease (R. Gazzinelli et al., 1992). Although CD8 T cells play an important effector role in controlling the chronic infection, their maintenance is dependent on the critical help provided by CD4 T cells. A recent study showed that reactivation of the infection in chronically infected hosts is a consequence CD4 T cell exhaustion yielding a CD8 T dysfunction. Treatment of chronically infected hosts with antigen-specific non-exhausted CD4 T cells restored CD8 T cell function and prevented reactivation of the disease (I. A. Khan, Hwang, & Moretto, 2019).

Adaptive immunity largely depends on antigen-presenting cells (APCs), such as DCs and macrophages, and their ability to present *T. gondii* antigen and activate CD4<sup>+</sup> and CD8<sup>+</sup> T and B cells, in secondary lymphoid organs. Once activated, B cells serve as APCs to stimulate CD4<sup>+</sup> T cells (Gigley, Fox, & Bzik, 2009). Activation of T cells elicits immunity to *T. gondii*, and a transfer of activated T cells can confer resistance to naive hosts (Gigley et al., 2009). CD4<sup>+</sup> and CD8<sup>+</sup> T cells are essential for long-term survival of the host, acting synergistically to prevent reactivation of cysts, and are important for the development of protective immunity against reinfection (S. Hwang & Khan, 2015) (Figure 15).

### **7.2.1. CD4<sup>+</sup> T cells**

The importance of CD4<sup>+</sup> T cells in toxoplasmosis was defined in HIV-positive immunosuppressed patients and in murine models. Initiation of T cell responses requires that CD4<sup>+</sup> T cells and naive CD8<sup>+</sup> T cells recognize APCs, carrying their corresponding antigen on MHC I or MHC II. B cells, macrophages and dendritic cells are all capable of presenting the antigen to CD4<sup>+</sup> T cells (Goldszmid et al., 2009). CD4<sup>+</sup> T cells mediate the resistance to toxoplasmosis *via* their complementary activity to CD8<sup>+</sup> cells and B cells (I. A. Khan et al., 2019; Lutjen, Soltek, Virna, Deckert, & Schluter, 2006). CD4<sup>+</sup> T cells control the chronic infection through their ability to produce IFN- $\gamma$  and to express CD40L (CD154) which a ligand necessary for the recognition of the antigen presented by APCs (R. Gazzinelli et al., 1992; Portillo et al., 2010).

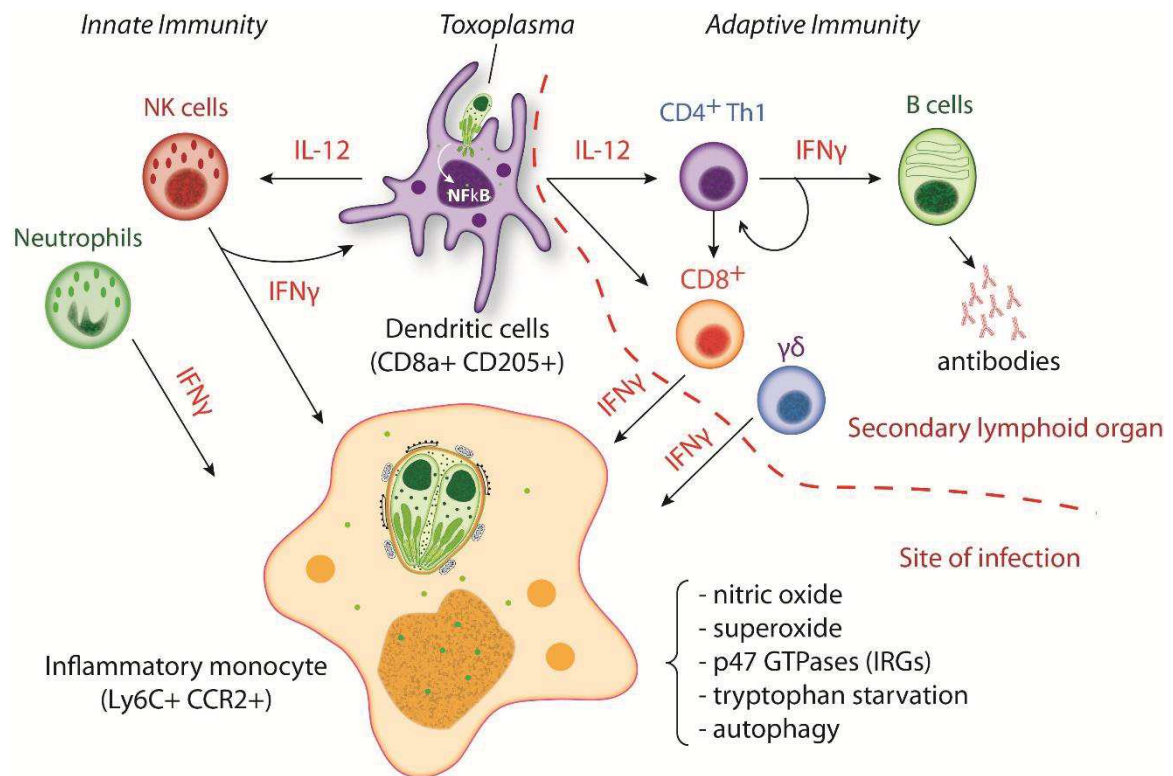
### 7.2.2. CD8<sup>+</sup> T cells

CD8<sup>+</sup> T cells can control infection through the production of inflammatory cytokines such as IFN- $\gamma$ , through CD40/CD40Ligand interactions, and through the perforin-mediated cytolysis of infected host cells (R. M. Andrade, Portillo, Wessendarp, & Subauste, 2005). Indeed, mice deficient in CD8<sup>+</sup> T cells show increased susceptibility to toxoplasmosis (Subauste & Wessendarp, 2006). *T. gondii* inactivates host innate and adaptive immune responses by targeting different host immunity related molecules such as the host endoplasmic reticulum-localizing transcription factor, ATF6 $\beta$ , to downregulate CD8<sup>+</sup> T cell-mediated type I adaptive immune responses (M. Yamamoto & Takeda, 2012). The response of CD8<sup>+</sup> T cells depends on the virulence of the strain (Tait et al., 2010). Type II strain infected mice developed a robust DC response at the site of infection and the draining lymph node and generated a population of endogenous CD8<sup>+</sup> T cells (Tait et al., 2010). CD8<sup>+</sup> T cells specific for parasite antigens can directly kill infected cells. This can be illustrated with CD8<sup>+</sup>T cells specific for an immunodominant *T. gondii* epitope, P30, which were found to kill both extracellular parasites and infected macrophages *in vitro* (Kasper, Khan, Ely, Buelow, & Boothroyd, 1992; I. A. Khan, Smith, & Kasper, 1988). CD8<sup>+</sup> T cells generated from mice vaccinated with a temperature-sensitive mutant of *T. gondii* (ts-4) are cytotoxic *in vitro* for parasite-infected or antigen-pulsed cells in a major histocompatibility complex class I (MHC-I)-restricted manner (Hakim et al., 1991). CD8<sup>+</sup> T cells are the dominant T cell responders in BALB/c models, which have a corresponding TCR restriction, V $\beta$ 8 CD8<sup>+</sup> cells in their brain (X. Wang, Claflin, Kang, & Suzuki, 2005). Adoptive transfer experiments showed that V $\beta$ 8 CD8<sup>+</sup> cells are large producer of IFN- $\gamma$  and were more protective than V $\beta$ 8 CD4<sup>+</sup> cells in response to parasite infection (X. Wang et al., 2005). CD8<sup>+</sup> T cell responses to epitopes of several identified antigenic *T. gondii* proteins, including P30, GRA4, GRA6, GRA1, GRA7, ROP2 and ROP7 in mice (Duquesne et al., 1991; Frickel et al., 2008; Hunter & Remington, 1994; Jacobs, Vercammen, & Saman, 1999; Kasper et al., 1992).

While CD8<sup>+</sup> T cells play an important role in BALB/c mice during the chronic stage of the infection, there is a considerable amount of conflicting literature as to whether this protection is mediated by secreted IFN- $\gamma$  or by their direct cytolytic ability.



For example, perforin-deficient mice lacking cytolytic ability survive acute *T. gondii* infection and have an unimpaired level of IFN- $\gamma$ , but they have a higher cyst burden and slightly increased susceptibility at later time points of the infection. These results indicate that the cytolytic abilities of CD8<sup>+</sup> T cells contribute to control of encysted parasites (Denkers et al., 1997). In contrast, adoptive transfer of perforin-deficient CD8<sup>+</sup> T cells was still effective at preventing toxoplasmic encephalitis in a chronic reactivation model utilizing sulfadiazine-treated athymic nude mice (X. Wang et al., 2005). Although adoptive transfer of perforin-deficient CD8<sup>+</sup> T cells is effective at preventing reactivation, the model of cytolytic cyst control is more likely to be correct, as transferred IFN- $\gamma$ -deficient CD8<sup>+</sup> T cells are able to greatly reduce cyst burden in a chronic reactivation model (Y. Suzuki et al., 2010). The multifaceted effects of IFN- $\gamma$  on the immune system make it difficult to separate the contribution of CD8<sup>+</sup> T cell IFN- $\gamma$  from that of other sources of this cytokine. IFN- $\gamma$  itself increases expression of endothelial vascular cell adhesion molecule 1 (VCAM-1) to aid in recruitment of CD8<sup>+</sup> T cells to the brain of chronically infected mice and thus enhances any effects of CD8<sup>+</sup> T cells on immunity to *T. gondii* (X. Wang, Michie, Xu, & Suzuki, 2007). It seems likely that both IFN- $\gamma$  and cytolytic functions of CD8<sup>+</sup> T cells are contributing to host resistance to pathogenesis (Y. Suzuki, Sa, Gehman, & Ochiai, 2011).

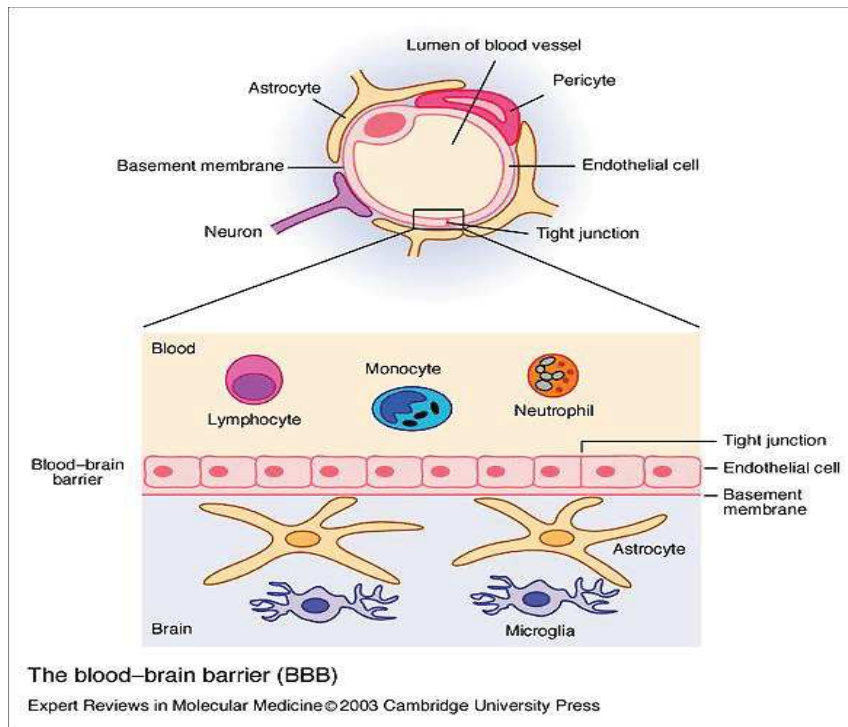


**Figure 15. Cellular immune response to initial *Toxoplasma* infection:** Cells of the innate and adaptive immune system as well as arrows indicating migration and communication between cells. IFN- $\gamma$  is central to host resistance to *Toxoplasma* as it activates many host mechanisms that kill the parasite (Jensen Lab).

### **7.3. Central Nervous System (CNS) immune response**

The central nervous system is closely linked to the immune system in several levels. The brain parenchyma is separated from the periphery by the blood brain barrier (BBB), whose integrity is maintained by the tight endothelial junctions. This barrier in normal conditions prevents the entry of mediators such as activated leukocytes, antibodies, complement factors and cytokines. Myeloid cells play a crucial role in the development of immune response in the central level, it has two major subtypes: the microglial cells which are spread in the brain parenchyma; and the perivascular macrophages located in the capillaries of the basal lamina and the choroid plexus of the brain (Elsheikha & Khan, 2010) (Figure 16).





**Figure 16: Structure of the Brain Blood Barrier (Francis, van Beek, Canova, Neal, & Gasque, 2003).**

*Toxoplasma* can be classified as a primarily neurotropic pathogen having a selective higher affinity for the CNS over other organs (Schluter & Barragan, 2019). In order to reach the brain parenchyma from the cerebral blood circulation, *Toxoplasma* has to cross the brain endothelium, primarily to the capillary bedding through two strategies by hijacking leukocytes and, as free parasites, with significant differences between *Toxoplasma* genotypes (Ginhoux & Jung, 2014; Schaeffer et al., 2009; Schluter, Deckert, Hof, & Frei, 2001; Selleck et al., 2013; Serbina, Hohl, Cherny, & Pamer, 2009).

A brain host immune response is thus triggered and *T. gondii* switches to the chronic phase of the infection. Indeed, the parasites persist as intraneuronal cysts that are controlled, but not eliminated by the immune system (Blanchard, Dunay, & Schluter, 2015). Brain-resident cells including astrocytes, microglia and neurons contribute to the intracerebral immune response by the production of cytokines, chemokines and expression of immune-regulatory cell surface molecules, such as major histocompatibility (MHC) antigens (Blanchard et al., 2015). Wandering immune cells are also recruited to

the site of infection in the CNS and contribute to the response against the infection (Blanchard et al., 2015).

IFN- $\gamma$  is key driver of this process. In the early phase of toxoplasmosis, the IFN- $\gamma$  production by NK cells and type I innate lymphoid cells is important for parasite control (Klose et al., 2014). Notably, brain recruited DCs are the main producers of IL-12, which is crucial for the maintenance of IFN- $\gamma$  during the latent phase (Figure 17) (Fischer, Bonifas, & Reichmann, 2000). *Toxoplasma* induces a hyper-migratory phenotype in human and mouse DCs. This hyper-migration of infected DCs potentiates systemic parasite dissemination in mice, including the CNS, and may cooperate with chemotactic responses of DCs (Schluter & Barragan, 2019). Although NK produce IFN- $\gamma$ , the main source of this cytokine remains the recruited T cells, which infiltrate into the brain following infection (Blanchard et al., 2015; X. Wang et al., 2005).

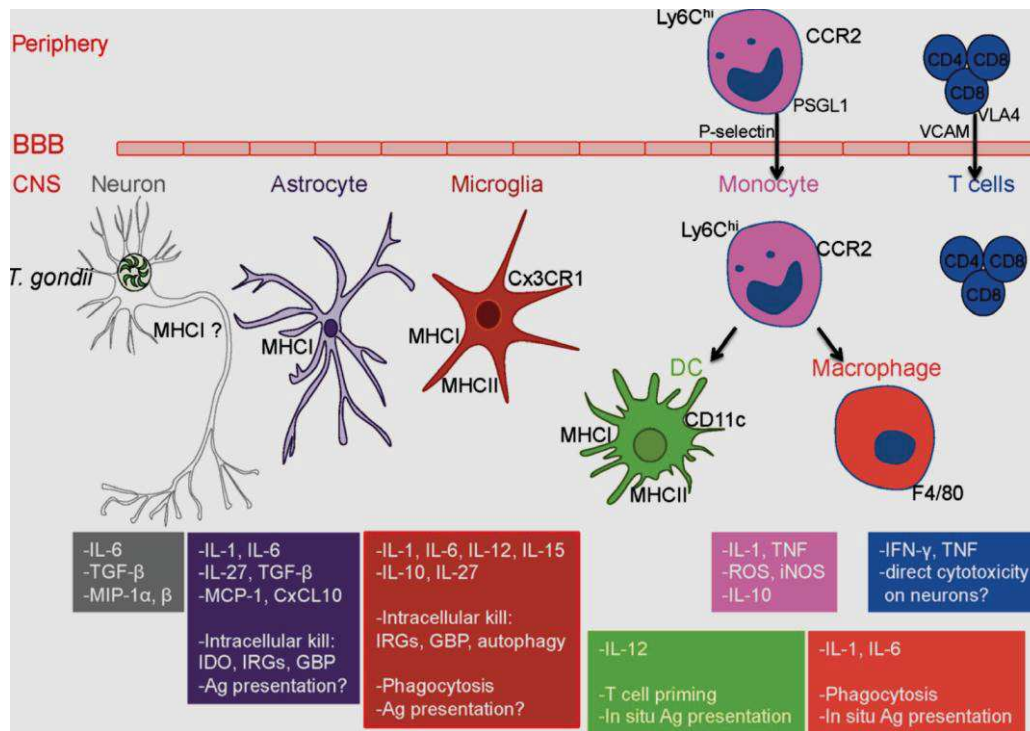
In parallel to the parasite spread to the brain, inflammatory leukocytes are recruited to the CNS. These inflammatory infiltrates are mainly composed of CD4<sup>+</sup> and CD8<sup>+</sup> T cells along with the F4/80<sup>+</sup> macrophages, CD11<sup>c+</sup> DCs, and Ly6C<sup>high</sup> inflammatory monocytes (Kwok et al., 2003). The number of infiltrated CD11<sup>c+</sup> brain DCs strongly increases in cerebral toxoplasmosis, and these cells home to the inflammatory foci in the infected brain. The inflammatory monocytes are actively recruited to sites of infection serving an immediate precursor for antigen-presenting DCs and macrophages (Serbina et al., 2009).

IFN- $\gamma$  can be also produced by the brain-resident microglia, leading to their activation and the production of NO to control the chronic cerebral infection (Blanchard et al., 2015; Chao et al., 1993; Sa et al., 2015). In murine toxoplasmosis, microglia cells are strongly activated throughout the entire brain as evidenced by upregulation of MHC class I and II antigens (Schluter, Lohler, Deckert, Hof, & Schwendemann, 1991). Microglia cells suppress the proliferation of intracerebral T cells, most probably to prevent excessive T cell proliferation and immunopathology due to the continuous T cell stimulation with the persisting parasite antigens (Schluter et al., 2001).

Neurons express MHC class I under certain circumstances, including activity dependent, long-term structural and synaptic modifications (Corriveau, Huh, & Shatz, 1998), and in functionally inactive IFN- $\gamma$ -stimulated neurons. Some cyst-harboring

neurons remain MHC class I negative and, thus, escape elimination by CD8<sup>+</sup> T cells, which in principle effectively remove cysts from the brain in a perforin-dependent manner (Neumann, Cavalie, Jenne, & Wekerle, 1995; Schluter & Barragan, 2019). Interestingly, expression of the common IL6-cytokine family receptor in neurons is required to prevent hyper-inflammation, neuronal loss, parasite replication and, death from toxoplasmic encephalitis (Handel et al., 2012). The gp130 receptor mediates survival of neurons under inflammatory conditions and is important for the production of immunosuppressive induction of TGF- $\beta$ 1 and IL-27 by neurons. During the chronic stage of toxoplasmosis the chemokines (CXCL9/ MIG, CXCL10/IP-10, and CCL5/RANTES), in addition to adhesion molecules play important roles in recruiting immune T cells and macrophages into the brain to maintain the latency of infection and to prevent toxoplasmic encephalitis (Y. Suzuki et al., 2010).

Finally, immunosuppressive cytokines are also expressed in the brain and play an important role to prevent immunopathology in toxoplasmosis. The inflammatory monocytes produce IL-10, which alleviates the immune-pathological response to the *T. gondii*-inflamed brain (Biswas et al., 2015). In addition, microglia, macrophages, regulatory B cells, and some CD8<sup>+</sup> T cells produce IL-10. IL-10 protects from lethal immunopathology in acute systemic and down-regulates the immune response in chronic cerebral toxoplasmosis (R. T. Gazzinelli et al., 1996). Astrocytes produce IL-27, which also inhibits immunopathological Th17 responses in TE (Stumhofer et al., 2006).



**Figure 17: Immune response to *T. gondii* in the infected brain by resident and recruited immune cells (Blanchard et al., 2015).**

A summary of CNS produced cytokines in the context of toxoplasmosis is provided in the table below (Table 9). The source of each cytokine, from resident and wandering brain immune cells is also presented.

Cytokines/Chemokines	Source of production	Type	References
Interferon- $\gamma$	<b>Wandering cell:</b> Phagocytic cells T cells	Pro-inflammatory	(Filisetti & Candolfi, 2004)
Interleukin-12	<b>Wandering cell:</b> Dendritic cell <b>Resident cell:</b> Microglia	Pro-inflammatory	(Robben et al., 2004)
Interleukin-1 $\beta$	<b>Wandering cell:</b> Monocytes/Macrophages <b>Resident cell:</b> Astrocytes	Pro-inflammatory	(Blanchard et al., 2015)
Interleukin-2	<b>Wandering cell:</b> LT(CD4+)	Pro-inflammatory	(Filisetti & Candolfi, 2004)

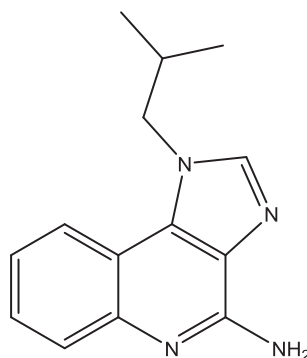
Interleukin-6	<b>Wandering cell:</b> Macrophages <b>Resident cell:</b> Neuron/Astrocyte/Microglia	Pro-inflammatory	(Filisetti & Candolfi, 2004)
Interleukin-15	<b>Wandering cell:</b> Macrophages <b>Resident cell:</b> Microglia	Pro-inflammatory	(Blanchard et al., 2015)
RANTES/CCL2	<b>Wandering cell:</b> Leucocytes/Dendritic cell <b>Resident cell:</b> Microglia	Pro-inflammatory	(Blanchard et al., 2015)
MIP	<b>Wandering cell:</b> Leucocytes/Dendritic cell <b>Resident cell:</b> Neuron	Pro-inflammatory	(Blanchard et al., 2015)
iNOS	<b>Wandering cell:</b> Monocytes	Pro-inflammatory	(Blanchard et al., 2015)
TNF- $\alpha$	<b>Wandering cell:</b> Monocytes/T cells	Pro-inflammatory	(Blanchard et al., 2015)
Interleukin-10	<b>Wandering cell:</b> Monocytes <b>Resident cell:</b> Microglia	Anti-inflammatory	(Blanchard et al., 2015)

Table 9. Summary of cytokines secreted by resident and wandering immune cells.

## Chapter VIII: Imiquimod

### 8.1. Imiquimod

Imiquimod (Figure 18), (S-26308, R-837) (1-(2-methylpropyl)-1*H*-imidazo[4,5-*c*]quinolin-4-amine), is the first member of the imidazoquinoline family, and belongs to the class of medications called immune response modifiers.



**Figure 18: chemical structure of Imiquimod**

This non-osidic nucleoside analogue of the imidazoquinoline family was the first immune response modifier used for the treatment of infectious skin conditions and shown great anti-viral and anti-tumor activities *in vivo* (reviewed in Kamath, Darwin, Arora, & Nouri, 2018). This agent was approved in 1997 by the United States Food and Drugs Administration (FDA) for topical use against some viral infections such as perianal and genital human papilloma virus (HPV) disease/genital warts by increasing the activity of the body's immune system (Miller, Gerster, Owens, Slade, & Tomai, 1999; Smith, Hamza, & Skelton, 2003). This drug is also efficacious as a topical therapy for certain types of skin cancers: basal cell carcinoma, Bowen's disease, superficial squamous cell carcinoma, some superficial malignant melanomas, and actinic keratosis (Oumata et al., 2013). Imiquimod inhibits melanogenesis and proliferation of human melanocytes. Its therapeutic spectrum was also extended to cutaneous B-cell lymphomas (Oumata et al., 2013). The exact mechanism of action in which Imiquimod activate the immune system is not yet known. Nevertheless, it is known that Imiquimod activates immune cells by ligating the Toll-like receptor 7 (TLR-7), commonly involved in pathogen recognition, on the endosomal surface of cells (Arevalo et al., 2001). Cells activated by Imiquimod *via* TLR-7 secrete cytokines (primarily IFN- $\alpha$ , IL-6, and TNF- $\alpha$ ). There is evidence that

Imiquimod, when applied to skin, can lead to the activation of Langerhans cells, which subsequently migrate to local lymph nodes to activate the adaptive immune system (Arevalo et al., 2001). Other cell types activated by Imiquimod include natural killer cells, macrophages, and B lymphocytes. There are case reports and preliminary studies suggesting Imiquimod effectiveness in the treatment of CL (Raman, Duthie, Fox, Matlashewski, & Reed, 2012). However, the effects of Imiquimod on innate immune responses, *via* toll-like receptors, suggest a potential anti leishmanial activity that was demonstrated by inducing the release of nitric oxide (El Hajj et al., 2018). Less than 1% of the drug is recovered in urine after topical application. Topical Imiquimod (5% cream) is only mildly irritating and does not lead to systemic toxic effects. Imiquimod was used in combination with a systemic antimonial administration in the treatment of CL and presented a cure rate of 90% in patients with refractory CL to pentavalent antimonial treatment (Arevalo et al., 2001). It was also shown that it is also more effective in the initial treatment of CL (Arevalo et al., 2007). Another clinical trial in Peru, Miranda-Verastegui *et al.* showed that this combination was better than placebo plus pentavalent antimony (Miranda-Verastegui et al., 2009).

# RESULTS



# **RESULTS part 1:**

Imiquimod as a novel therapeutic modality against toxoplasmosis: host immune response modulation through MyD88-dependent Toll Like receptors

**Imiquimod as a novel therapeutic modality against toxoplasmosis: host immune response modulation through MyD88-dependent Toll Like receptors**

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## Abstract

*Toxoplasma gondii* is a prevalent protozoan parasite of medical and veterinary importance. In intermediate hosts, tachyzoites and bradyzoites are responsible for acute and chronic toxoplasmosis (AT and CT), respectively. Following AT, the disease evolves into a persistent CT, in part due to the host immune system. In immunocompetent hosts, CT manifests in the brain and skeletal muscles as latent tissue cysts, which correlates with several neuro-pathologies and cancers. In immunocompromised patients, CT may reactivate and poses a life threatening condition. Current treatments primarily target AT, and present with adverse side effects.

Imiquimod is an approved immunomodulatory drug, with documented efficiency against some viral infections and cutaneous leishmaniasis. In this study, we have explored the potential efficacy of Imiquimod against AT and CT. During AT, Imiquimod led to recruitment of T cells to the peritoneum and spleen of treated mice, and significantly decreased the number of brain cysts upon establishment of CT. Remarkably, gavage of mice with the remaining brain cysts from Imiquimod treated mice, failed to induce CT.

CT remains with no effective treatment, although it is the most common form of toxoplasmosis in humans. Post-establishment of CT, we demonstrated that Imiquimod sharply reduces the number of brain cysts, concomitant with increased Toll-Like Receptors 11 and 12 expression. These TLRs are expressed by dendritic cells (DCs) and monocytes, and bind the tachyzoite actin-binding protein, Profilin. TLR-7 was also upregulated, likely due to Imiquimod reported agonistic activity. Furthermore, Imiquimod mediated interconversion as documented by the decreased protein levels of P21, and increased protein levels of P30, exclusively expressed in bradyzoites and tachyzoites, respectively. Pathways downstream from TLR-11/12 were activated, through MyD88 dependent TLR signaling, which resulted in the induction of the innate immune response and the upregulation of specific chemokines, potentially recruiting T cells to reactivated *Toxoplasma* foci, to clear the infection. Imiquimod presumably enhances the interaction of Profilin with the heterodimerized TLR-11/12 since *in vitro*, *Toxoplasma* strain lacking Profilin, which fails to bind to TLR-11/12, does not respond to Imiquimod.

Collectively, we showed that Imiquimod targets AT and more prominently CT, via conversion of bradyzoites to tachyzoites, leading to the upregulation of TLR-7, 11 and 12, subsequently activating MyD88 downstream signaling, to induce immune response and clear the infection. This study paves the way to tackle the disease at several levels, by eliminating neuro-pathologies and cancer associated with toxoplasmosis, and abrogating the transmission of the parasite in its hosts, which is at the root for human infection. This will result in lessening the consequent economic burden associated with the disease and in improved animal health.

Keywords: cerebral toxoplasmosis, dendritic cells, Toll-like receptors, cytokines, T cells, pathogen-host interaction.

## Introduction

*Toxoplasma gondii* (*T. gondii*) is the etiologic agent of toxoplasmosis, a common human and zoonotic disease. *T. gondii* infects approximately 30% of the world's human population [1], with a sero-prevalence ranging from 7 to 80% according to regions [2]. More than 40 million people in the United States are infected, which prompted the Centers for Disease Control and Prevention (CDC) to classify toxoplasmosis as a neglected parasitic infection, requiring public health action [3]. Toxoplasmosis manifests as acute and chronic forms (AT and CT respectively). AT is caused by the presence of fast-replicating tachyzoites, which deploy innate immune cells, namely dendritic cells (DCs), and monocytes, as vehicles to reach the brain and the skeletal muscles, where they convert into persistent bradyzoite cysts [4, 5].

While toxoplasmosis is considered asymptomatic in more than 80% of immunocompetent patients, more associations are made between various medical conditions and *T. gondii* infections [6]. Elevated levels of immunoglobulin G in pregnant 'toxo-positive' women were linked to prenatal anxiety and depression [7, 8]. Depression and suicide attempts also correlate with seropositivity to parasite antigens [9, 10]. CT also promotes the progression of several behavioral and neuropathies such as schizophrenia and Parkinson disease among others [11-18]. Higher anti-*T. gondii* antibodies are also reported in different types of cancer [19-21], particularly in brain cancers due to the ability of the parasite to interfere with the brain cells miRNAome [22]. In immunocompromised patients, CT may reactivate and associates with severe morbidity that might lead to death [23-26]. Reactivation usually occurs in HIV patients or in those who receive immunosuppressive therapies in the context of hematopoietic stem cells, solid organ transplant, or chemotherapy against cancer [26-30]. With the growing number of these individuals, scientists are aware of the potential occurrence of *Toxoplasma* encephalitis, not only during reactivation of latent infection, but also presenting as a primary infection [31].

Common treatments for toxoplasmosis remain limited to general anti-parasitic/anti-bacterial drugs. These include spiramycin, azithromycin, atovaquone, pyrimethamine-sulfadiazine, pyrimethamine-clindamycin and trimethoprim-sulfamethoxazole (reviewed in [32]). The recommended first-line therapy remains the

synergistic combination of pyrimethamine, an inhibitor of the dihydrofolate reductase (DHFR) enzyme, with sulfadiazine, an inhibitor of the dihydropteroate synthase reviewed in [25, 33]. However, this combination associates with several limitations, including, hematological side effects [34], elevation in serum creatinine and serum liver enzymes, hypersensitivity or allergic reactions [35] and emergence of resistant parasites [36, 37]. In addition, these drugs, whether given as prophylactic or therapeutic agents, target only AT and remain ineffective against CT [38-41].

Imiquimod is an FDA approved immune-modulatory drug, used against some viral infections [42, 43]. Imiquimod binds Toll-like receptor-7 (TLR-7) [44, 46, 47] [44, 45], which activates the innate immune response [44] through MyD88 signaling pathway. This drug also proved potent against a parasitic infection causing cutaneous leishmaniasis [47-49].

TLR family of proteins are transmembrane receptors localized on the cytoplasmic membrane and endosomal/lysosomal cellular compartments, which recognize distinct pathogenic constituents [50], to couple innate to adaptive immunity [51]. Thirteen different TLRs (TLR1-13) are described in mammals reviewed in [52]. TLRs 11, 12 and 13 are exclusively expressed in mice [53]. Following infection with *T. gondii*, TLRs expressed on innate immune cells detect the parasite [54]. TLR-11 and 12 of DCs, recognize Profilin, a *Toxoplasma* Pathogen-associated molecular pattern (PAMP) [55-57], to primarily signal through the adaptor protein MyD88 [58], leading to the activation of several signaling pathways including nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B), and mitogen-activated protein kinase (MAPK). This subsequently mounts the protective immune response through the production of interleukin-12 (IL-12), Interferon-gamma (IFN- $\gamma$ ) among others [56, 59]. Indeed, TLR-11 heterodimerizes with TLR-12, and is important for DC response and IL-12 production [55, 60]. TLR-11<sup>-/-</sup> mice display low levels of IL-12 production [56, 61], and TLR-12<sup>-/-</sup> results in a loss of resistance to toxoplasmic infection as well as a deficiency of the gene encoding MyD88 [55, 61]. While Profilin is indispensable for invasion, and active egress from cells [62-64], parasites lacking Profilin are unable to induce TLR-11-dependent production of IL-12 both *in vitro* and *in vivo* [64].

In this study, we explored the potency of Imiquimod against AT and CT. Treatment of mice during AT resulted in recruitment of T cells to the peritoneum and spleen, and significantly decreased the number of brain cysts upon establishment of CT. Remarkably, the remaining brain cysts from Imiquimod treated mice, failed to establish a new CT. In light of the absence of an efficient treatment against CT, we investigated the efficacy of Imiquimod on chronically infected mice. Interestingly, Imiquimod sharply reduced the number of brain cysts in an established CT, and significantly increased TLR-11, 12 and 7. Since these TLRs are expressed by innate immune cells upon contact with tachyzoites, we showed that Imiquimod induced interconversion. TLR-11/12 upregulation resulted in the activation of the MAPK pathway and induced its subsequent immune response, through MyD88 dependent TLR signaling. *In vitro*, a *Toxoplasma* strain depleted for Profilin, does not respond to Imiquimod treatment, suggesting that this drug promotes the interaction between this PAMP and TLR-11/12. Collectively, we showed that Imiquimod targets AT and more prominently CT, *via* conversion of bradyzoites to tachyzoites. This led to the upregulation of TLR-7, 11 and 12, subsequently activating MyD88 signaling to induce immune response and clear the infection. This study provides insights towards eradicating the disease through interfering with its persistence and transmission and paves the path towards better treatment modalities against toxoplasmosis and its associated diseases.

## **Materials and methods**

### **Parasite lines and mammalian cell cultures**

76K strain was provided by Dr. Mathieux Gissot. Profilin knock-out strain  $\Delta TgPRFe/TgPRFi$  and its control *RHTATi-1* were provided by Dr. Dominique-Favre Soldati. Tachyzoites were serially passaged in human foreskin fibroblasts (HFFs) (American Type Culture Collection-CRL 1634) cultured in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, Invitrogen) and supplemented with 10% of fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% glutamine.

Human THP-1 cells (American Type Culture Collection (ATCC TIB-202), Manassas, VA) were maintained in RPMI medium with L-Glutamine, supplemented with

10% FBS, 1% penicillin-streptomycin, and 1% glutamine (Invitrogen). The differentiation of THP-1 cells was performed as described [48]. Briefly, one million of THP-1 monocytes were seeded in a 6-well-plate, and incubated with 50 ng/mL of phorbol 12-myristate 13-acetate (Sigma) overnight. Following their adherence, differentiated macrophages were activated with 1 µg/mL of lipopolysaccharide for 4h, infected with the 76K strain, at 1:3 parasite to macrophage ratio for 24h, then treated with 1 µm of Imiquimod (Molekula, CAS N°99011-02-6) for 24h.

Peritoneal macrophages were harvested from BALB/c mice, following their induced recruitment by thioglycollate (38.5 g/L, Sigma). After peritoneal lavage, cells were collected by centrifugation. One million murine macrophages were seeded in 6-well-plates and cultured in RPMI medium supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% glutamine (Invitrogen). Infection with RHTATi-1 and  $\Delta$ TgPRFe/TgPRFi at 1:3 parasite to macrophage ratio for 24h, prior to their treatment with 1 µm of Imiquimod for 48h.

### ***In vitro* interconversion from tachyzoites to bradyzoites**

Confluent HFF cells were cultured in a 6-well plate and infected with 1000 tachyzoites of the 76K strain/well. After 24h of incubation in complete DMEM medium under 5% CO<sub>2</sub>, cells were maintained in induction medium (RPMI 1640 without NaHCO<sub>3</sub>, HEPES 50mM, 3% FBS, pH 8.2) and in absence of CO<sub>2</sub>. The basic medium was changed every other day to maintain the pH at 8.2. After 10 days, cells infected with bradyzoites were treated every other day with 1 µm of Imiquimod. On day 14, infected cells with bradyzoites were harvested for western blot and immuno-fluorescence assays.

### **Immunofluorescence and confocal microscopy**

Bradyzoite conversion was confirmed by staining the cyst wall with Biotinylated Dolichos biflorus lectin (DBA) [65]. Following *in vitro* switch, coverslips of cells infected with cysts of 76K, were fixed with 4% paraformaldehyde in PBS for 20 minutes, permeabilized in 0.2% Triton for 10 minutes, blocked with 10% FBS in PBS for 30 min. T<sub>8</sub>3B<sub>1</sub> or T<sub>8</sub>2C<sub>2</sub> primary monoclonal antibodies directed against P18 and P34 respectively



[66] were used at the dilution of 1:500. Biotinylated DBA (Sigma, Cat. NoB-1035) was used at the dilution of 1:100. Anti-mouse secondary antibody (Abcam, ab150116) was used at the concentration of 1:500. Streptavidin (Sigma) was used at the dilution of 1:100. Coverslips were mounted on slides using a Prolong anti-fade kit (Invitrogen, P36930). Z-section images were acquired by confocal microscopy using confocal microscopy (Zeiss LSM 710) and all images were analyzed using Zeiss Zen software.

### **Western blot analysis**

Proteins from various experimental procedures were separated on polyacrylamide gels with different percentages according to the molecular weight of desired proteins and transferred to nitrocellulose membranes (BIO RAD Cat# 162-0112). Membranes were probed with different primary antibodies followed by anti-mouse (m-IgGk BP-HRP, sc-516102) and anti-Rabbit (Mouse anti-rabbit IgG-HRP, sc-2357) (Santa Cruz, 1:5000) secondary antibodies conjugated to Horseradish peroxidase (HRP). Bands were visualized using luminol chemiluminescent substrate (Bio-Rad, Cat# 170-5061).

Primary antibodies used in our study are: T<sub>8</sub> 3B<sub>1</sub> primary monoclonal antibody directed against p18 (1:1000) (Gift from Jean-Francois Dubremetz)[66, 67], T<sub>4</sub> 1E<sub>5</sub> monoclonal antibody directed against P30 (SAG-1/1:1000) (Gift from Jean-Francois Dubremetz)[66], TLR-11, 12, and 7 TLR-12 polyclonal antibodies Thermofisher (Cat# PA1-41080; 1:1000; Cat# PA1-41037; 1:500), Cat#PA5-11605; 1:500, respectively), MYD88 monoclonal antibody Abcam (Cat#ab135693; 1:1000), total ERK1/2 (*I37F5*) Rabbit polyclonal cell signaling (Cat#4695; 1:1000), Phospho-P44/42 MAPK ERK1/2 (*Thr202/Tyr204*) Rabbit polyclonal antibody cell signaling (Cat#4397; 1:1000),  $\beta$ -Actin Mouse cell signaling (Cat#8H10D10; 1:1000), GAPDH antibody conjugated to HRP from Abnova (Cat#MAB5476; 1:20000).

### **Quantitative Real Time PCR (qRT PCR)**

qRT PCR was performed using CFX96 (Biorad). Primers to detect different transcripts in the brains of BALB/c infected mice (Timeline described in Figure 3A), are listed in (Table 1). Glyceraldehyde-3-Phosphate dehydrogenase (GAPDH) was used as

housekeeping gene (Table 1). In qRT-PCR, individual reactions were prepared with 0.25  $\mu$ M of each primer, 150 ng of cDNA and SYBR Green PCR Master Mix to a final volume of 10  $\mu$ l. PCR reaction consisted of a DNA denaturation step at 95°C for 3min, followed by 40 cycles (denaturation at 95°C for 15 sec, annealing at the appropriate temperature of the used primers for 60 sec, extension at 72°C for 30 sec). For each experiment, reactions were performed in duplicates and the expression of individual genes was normalized to GAPDH Ct values. The Threshold cycle (Ct) corresponds to the cycle at which there is a significant detectable increase in fluorescence. Data were plotted by calculating  $\Delta$ Ct ( $C_{t_{\text{target gene}}} - C_{t_{\text{GAPDH}}}$ ). Thereafter,  $\Delta\Delta$ Ct is calculated according to the Livak method:  $2^{-\Delta\Delta\text{Ct}}$  to obtain the percentage of expression [68].

Table 1. Summary of primers used for Real-time quantitative PCR

Primer	Sequence 5'→3'	Annealing T <sup>0</sup> C
GAPDH Forward Primer	5'-CATggCCTTCCgTgTTCCTA-3'	59.4
GAPDH Reverse Primer	5'-CCTgCTTCACCACCTTCTTgAT-3'	60.3
SAG-1 Forward primer	5'-ACT CAC CCA ACA ggC AAA TC 3'	56.5
SAG-1 Reverse primer	5'- gAg ACT AgC AgA ATC CCC Cg-3'	56.6
BAG-1 Forward primer	5'-gCggAgAAAgTggACgATgATgg-3'	62
BAG-1 Reverse primer	5'-gTCgggCTTgTAATTAATCggg-3'	62
TLR-11 Forward primer	TCCTTCCTCTGATTAGCTGTCCTAA	57

TLR-11 Reverse primer	TCCACATAATTTCCACCAACAAGT	57
TLR-12 Forward primer	GCCGCCATTCCAAGCTATC	57
TLR-12 Reverse primer	CTCCACAGTCCGAGGTACAACCTT	57
TLR-7 Forward primer	TTCCTTCCGTAGGCTGAACC	57
TLR-7 Reverse primer	GTAAGCTGGATGGCAGATCC	57
CXCL9 Forward Primer	5'-TgT ggA gTT CgA ggA ACC CT-3'	60.5
CXCL9 Reverse Primer	5'-TgC CTT ggC Tgg TgC Tg-3'	57.2
CXCL10 Forward Primer	5'-AgA ACg gTg CgC TgC AC-3'	57.2
CXCL10 Reverse Primer	5'-CCT ATg gCC CTg ggT CTC A-3'	61.7

### Enzyme-linked immunosorbent assay (ELISA)

Brains from infected BALB/c mice with 76K were harvested after three weeks of treatment with 50 $\mu$ g of Imiquimod/mouse. Following brain homogenization, supernatants were collected, and ELISA was performed using Multi-Analyte ELISArray Kit (Qiagen) according to the manufacturer's instructions. Briefly, supernatants were spun for 10 min at 1000g and transferred to new Eppendorf tubes, and diluted using a specific cocktail of antigens (IL-12, IL-1 $\beta$ , and IFN- $\gamma$ ) provided by the kit. Samples were then transferred to ELISA plate, and were incubated for 2 hours. After three washes, the detection antibody was added and incubated for 2 hours, followed by Avidin-HRP addition for 30 min.

Wells were washed and developed in the dark for 15 min, before addition of the stop solution. Optical density was recorded at 450 and 570 nm.

### ***In vivo studies***

Eight to ten weeks old female BALB/c mice (5 mice per condition) were intraperitoneally injected with 250 parasites of 76K and with 50 and 100 µg of Imiquimod per mouse to determine dose efficacy, or Sulfadiazine (200mg/L administered in drinking water) (Timeline described in Figure 1A). Imiquimod treatment was performed every other day from day 2 until day 8 and mice were sacrificed on day 10 to assess tachyzoite burden in the spleen.

To assess the effect of Imiquimod on immune cell recruitment during AT, eight to ten weeks old female BALB/c mice (5 mice per condition) were intraperitoneally injected with 1000 parasites of 76K. Treatment with 50 µg/mouse was performed on days 2 and 3 and mice were sacrificed on day 4 p.i. (Timeline described in Figure 1C).

To test the potency of Imiquimod on brain cyst formation, eight to ten weeks old female BALB/c mice (10 mice per condition) were intraperitoneally injected with 250 parasites of 76K, and treated during AT, with 50µg of Imiquimod from day 4 until day 32 (Timeline described in Figure 2B). Mice were then sacrificed, brains were harvested, and cysts were extracted using an optimized Percoll method (GE Healthcare Percoll Bio-Sciences AB Lot 10221921) [69, 70]. To test the viability of the remaining cysts found in the brains of treated mice, 20 cysts were orally injected into new BALB/c mice (10 mice per condition) (Timeline described Figure 2B).

To explore the effect of Imiquimod on CT, eight to ten weeks old female BALB/c mice (20 mice per condition) were intraperitoneally injected with 250 parasites of 76K. Chronically infected mice were treated with 50 µg of Imiquimod, every other day, and starting day 21 and until day 49 p.i. (Timeline described in 2C). On day 49 p.i., mice were sacrificed and brain cysts were counted following an optimized percoll extraction method [69, 70].

To assess the upregulation and the time point at which TLR-11, 12, and 7 are upregulated, eight to ten weeks old female BALB/c mice (5 mice per condition) were intraperitoneally injected with 250 parasites of 76K. Chronically infected mice were treated with 50 µg of Imiquimod, every other day, starting day 21 and until day 49 p.i. (Timeline described in 3A). Mice were sacrificed on a weekly basis (days 35, 42 and 49) p.i. Total brains were harvested and TLR-11, 12 and 7 transcript levels were assessed by qRT PCR. All remaining molecular studies on *in vivo* treated mice, were performed on brains of mice sacrificed at day 49 p.i.

### **Ethics statement**

All mice protocols were approved by the Institutional Animal Care and Utilization Committee (IACUC) of the American University of Beirut (AUB) (Permit Number: #18-02-461). All animals were housed in specific pathogen free facility with a 12h ON/OFF light cycle. Humane endpoints were fully respected as per AUB IACUC following Association for Assessment and Accreditation of Laboratory Animal Care International guidelines and guide of animal care use book (Guide, NRC 2011). Mice were monitored on a daily basis. To verify the acute phase of the infection, blood was withdrawn following deep anesthesia with isoflurane by inhalation. Mice were sacrificed if any abnormal ethical features are noticed.

### **Statistics**

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

## Results

### **Imiquimod exhibits a toxoplasmicidal effect *in vitro***

We investigated the potency of Imiquimod on infected macrophages with tachyzoite stage. Macrophages were infected at 1:3 parasite to cell ratio. Twenty-four hours p.i., cells were treated with 1  $\mu$ M of Imiquimod for 24h (Timeline described in Figure 1A). Cells were harvested 24h post-treatment, and the effect of Imiquimod on tachyzoites was assessed using a monoclonal antibody directed against SAG-1. Imiquimod significantly decreased the protein expression of SAG-1 by more than 70% (Figure 1A), proving the anti-parasitic efficacy of this drug on tachyzoites *in vitro*.

### **Imiquimod potently decreased parasite burden during acute toxoplasmosis *in vivo***

BALB/c mice were infected intraperitoneally with 250 tachyzoites of the 76K strain. Initially, we tested the dose of 50 and 100  $\mu$ g of Imiquimod/mouse [71], every other day, from day 2 until day 8 (Timeline described in Figure 1B). Mice were sacrificed on day 10 p.i., and spleens were harvested to evaluate parasite burden. SAG-1 transcripts were quantified by real time PCR. Consistent with the *in vitro* potency of Imiquimod on the tachyzoites stages, we observed a significant decrease of SAG-1 transcripts in the spleen of treated mice, presumably reflecting a diminished number of disseminated tachyzoites to this organ (Figure 1B). Testing at 100  $\mu$ g concentration did not result in significant difference as compared to the dose of 50  $\mu$ g (Figure 1B). Hence, the dose of 50  $\mu$ g was adopted throughout the study.

### **Imiquimod recruits T cells to the peritoneum and spleen of mice during AT**

Upon intraperitoneal infection with tachyzoites of *T. gondii*, innate immune cells, namely DCs and monocytes are recruited to the site of infection [61, 72-74]. These cells produce IL12, and present *T. gondii* antigens to prime T cells, leading to high systemic levels of IFN- $\gamma$  required to control AT (reviewed in [61, 75, 76]). Type I strains recruit monocytes, macrophages and DCs to peritoneum as early as day 2 p.i., while T cell migration to this site peaks at Day 6 p.i. [77-79]. Type II strains are more studied in the

brains of infected mice. We assessed the effect of Imiquimod on the recruitment of DCs, macrophages and T cells to the peritoneum and spleen of infected mice during AT. BALB/c mice were intraperitoneally injected with 1000 tachyzoites of the 76K strain (Timeline described in Figure 1C). Mice were treated with Imiquimod or sulfadiazine, on days 2 and 3 p.i., and sacrificed on day 4 p.i. Immune cell recruitment was assessed by Flow cytometry of specific surface markers. Treatment with Imiquimod or sulfadiazine led to non-significant effect on peritoneal macrophages, while no DC were detected (Figure 1C, and data not shown). However, both treatments led to a significant and higher recruitment of macrophages to the spleen of infected mice (Figure 1C). Strikingly, our results demonstrate that Imiquimod led to a significant recruitment of T cells to the peritoneum and the spleen of infected mice at day 4 p.i., while sulfadiazine has no effect on this cell population in the tested infection sites (Figure 1C). This was further asserted by the transcriptional upregulation of IFN- $\gamma$ , predominantly produced by the recruited T cells (Figure 1D). These results support a role for Imiquimod on mounting a faster adaptive immune response to clear the infection, potentially explaining the lower tachyzoite burden observed during AT.

### **Imiquimod reduces the number of bradyzoite cysts *in vitro* and *in vivo***

*In vitro* interconversion from tachyzoites to bradyzoites was performed in HFF. On day 10, Imiquimod was added to cells infected with bradyzoites, at the dose of 1 $\mu$ M and maintained for 4 days (Timeline described in Figure 2A). On day 14 p.i., treatment with Imiquimod significantly decreased protein levels of the bradyzoite surface marker P18 [66, 67], as compared to the untreated controls (Figure 2A). Similar results were obtained by immunofluorescence assay, upon quantification of bradyzoite cysts using a DBA specifically binding to a selectin on the cyst wall [65], and another bradyzoite marker P34 using the T<sub>8</sub>2C<sub>2</sub> monoclonal antibody [66, 67].

We then tested the *in vivo* potency of Imiquimod on the establishment of CT, following treatment of mice during AT. BALB/c mice were intraperitoneally infected with 250 tachyzoites of the 76K. Treatment with 50  $\mu$ g of Imiquimod was performed every other day, from day 4 until day 32 (Timeline described in Figure 3B). AT was

verified seven days p.i. by immune reactivity of infected mice on tachyzoite extracts [80]. Thirty-two days p.i., brains were harvested, and bradyzoite cysts were extracted [69, 70] before counting. Interestingly, treatment of mice with Imiquimod during AT, led to a significant decrease of cyst number by around three folds (Figure 2B, left panel). We then assessed the viability and the capacity of the remaining cysts to establish a successful CT infection. Therefore, we followed the same timeline described in Figure 2B. After extracting and counting brain cysts, new BALB/c mice were orally infected with 20 cysts harvested from brains of either untreated or treated mice with Imiquimod. Similarly, on day 7 post-oral infection, the acute phase was verified by immune reactivity of infected mice on tachyzoite extracts [80]. Surprisingly, reactivity with tachyzoites was less prominent in mice infected with cysts derived from brains of Imiquimod treated mice (data not shown), suggesting a potential effect of Imiquimod on either the viability or the conversion of bradyzoites to establish a successful AT. We further expanded our results to assess CT establishment, and harvested brains of orally infected mice at day 32 post-oral infection. Brains of infected mice with cysts derived from Imiquimod treated animals were free of cysts (Figure 2B, middle panel). To check whether it is a problem of low amount of detected cyst, we used the sensitive qRT PCR method and checked for bradyzoite expression using BAG-1 specific primers. Transcript data was very consistent with the lack of presence of cysts (Figure 2B, right panel). These results demonstrate the potency of Imiquimod on bradyzoite cysts *in vitro* and *in vivo* and support a role for this drug either on bradyzoite viability or on its capacity to convert back to tachyzoite to establish a new infection.

### **Imiquimod reduces the number of bradyzoite cysts in chronically infected mice**

CT is the most common form of toxoplasmosis [25] and correlates with several neuro-pathologies and cancers [11-18] [19-21]. Furthermore, CT reactivation may become life threatening in immunocompromised patients [23-25]. In light of the lack of effective treatment options against CT, we assessed the effect of Imiquimod on chronically infected mice. BALB/c mice were infected with 250 tachyzoites of the 76K strain on day 0. The acute phase was verified seven days p.i. by immune reactivity of infected mice on tachyzoite extracts [80]. Following establishment of CT, Imiquimod



treatment was administered at the dose of 50 µg, every other day, from day 21 until day 49 p.i. (Timeline described in Figure 2C). At this time point, brain cysts were harvested and counted [69, 70]. Imiquimod treatment resulted in 50% reduction of the number of brain cysts in chronically infected mice (Figure 2C). Together with our *in vitro* data, Imiquimod presents as a potent drug of CT.

### **Imiquimod induces the expression of TLR-11, TLR-12 and TLR-7 in the brains of chronically infected mice**

Toll-Like Receptor (TLR) signaling is one of the first defense systems against infections in mammalian innate immune protection. Profilin, a well-characterized *T.gondii* PAMP, plays a role in the recognition of parasite antigens by TLR-11 and 12 of DCs and monocytes during murine AT, resulting the production of IL-12 via MyD88 [54-56, 64, 81]. Due to the importance of TLR-11 and 12, in *Toxoplasma* recognition, and due to the potency of Imiquimod on established CT, we investigated the expression of these TLRs. On the other hand, Imiquimod is an immunomodulatory agonist of TLR-7 which is also known to signal through MyD88 [42, 46, 47]. This prompted us to study TLR-7. Chronically infected BALB/c mice were treated with 50µg of Imiquimod every other day, from day 21 until day 49 p.i. (Timeline described in figure 3A). Mice were sacrificed at days 35, 42 and 49, brains were harvested and TLR-11, TLR-12 and TLR-7 transcript levels were quantified by real time PCR. At day 35, treatment with Imiquimod induced a non-statistically significant increase of transcript levels of the three tested TLRs, as compared to untreated or sulfadiazine treated groups (Figure 3A). The transcriptional levels of TLR-11, 12 and 7 progressively increased and attained high significance at day 42 (Figure 3A) to reach the highest level at day 49 p.i. (Figure 3A). Hence, we assessed protein levels of these TLRs at day 49. Predictably, protein levels of TLR-11, 12 and 7 proteins were significantly increased upon treatment of chronically infected mice with Imiquimod (Figure 3B). These results reinforce a role for Imiquimod in signaling through TLR-11, TLR-12 and TLR-7 in chronic murine toxoplasmosis.

### **Imiquimod induces the interconversion of bradyzoites to tachyzoites in the brains of chronically infected mice**

Signaling through TLRs is well documented in the tachyzoite stages of *T.gondii*, while CT is characterized by the presence of intra-neuronal bradyzoite cysts. TLR-11, 12 and 7, known to be expressed in murine DCs, macrophages and monocytes, the presumed vehicles for dissemination, are also triggered by the tachyzoite stage [60]. We studied the potential effect of Imiquimod on interconversion from bradyzoites to tachyzoites. Remarkably, the bradyzoite specific marker P21 [66, 67], was sharply decreased in brains of chronically infected mice, after four weeks of treatment with Imiquimod (Figure 4A, left panel). This decrease was concomitant with a significant upregulation of the exclusively tachyzoite-expressed surface marker SAG-1 [66](Figure 4A, right panel). These results implicate Imiquimod treatment in the interconversion from bradyzoites and tachyzoites in chronically infected mice.

### **Imiquimod activates MyD88 pathway**

MyD88 signaling pathway is activated as a result of TLR-11/12 binding to the parasite [58]. This activation promotes the secretion of IL-12, which in turn, induces natural killer (NK) cells and T cells to produce IFN- $\gamma$ , to fight the infection [54, 56, 59, 82]. Furthermore, mice deficient in the adapter molecule MyD88 are acutely susceptible to toxoplasmosis [56, 83, 84]. We evaluated, following four weeks of treatment of chronically infected mice with Imiquimod, MyD-88 expression levels. Consistent with TLR-11 and 12 upregulation, MyD88 protein levels were upregulated (Figure 3B).

MyD88 a key role in the activation of signaling pathways including MAPK [85]. A hallmark of the activation of this pathway is the phosphorylation of ERK1/2 (P-ERK1/2) [85]. We observed a significant and sharp upregulation in P-ERK1/2 in chronically infected mice after four weeks of treatment with Imiquimod, indicating the activation of MyD88 pathway (Figure 4C). This activation was concurrent with the production of IL-12, IL-1 $\beta$  and IFN- $\gamma$  (Figure 4D), known to be among the cytokines necessary to mount a protective immune response against *T. gondii* infection [56, 60, 85]. Altogether, our results demonstrate that Imiquimod induces the conversion to tachyzoites,

presumably recruiting innate immune cells to the brain. These express TLR-11, 12 and 7 and activate the MyD88-TLR dependent signaling, which subsequently leads to mount the protective immune response, presumably clearing the infection.

### **Imiquimod upregulates CXCL-9 and CXCL10 in the brains of chronically infected mice**

During murine CT, CXCL9, and CXCL10, are predominantly expressed in the brains of infected BALB/c mice [86, 87]. Furthermore, CXCL9 is crucial for the recruitment of T cells to control reactivation [88]. Interestingly, Imiquimod led to a significant upregulation of both CXCL9 and 10 to control the treatment-induced reactivation in the brains of chronically infected mice (Figure 5).

### **Profilin/TLR-11 and 12 complex *in vitro* influences Imiquimod activity**

Profilin is a parasitic PAMP, playing a major role in TLR binding and hence parasite invasion. It binds TLR-11 [56] and TLR-12 [55], and enhances the production of IL-12 via MyD88 dependent pathway [62-64]. Parasites lacking Profilin are unable to induce TLR-11-dependent production of IL-12 both *in vitro* and *in vivo* [64]. To assert that Imiquimod signals through TLR-11 and TLR-12, we used a *T. gondii* line depleted for Profilin ( $\Delta$ TgPRFe/TgPRFi) and its control strain (RHTATi-1). Since TLR-11 and TLR-12 are only expressed in mice [53], we conducted our experiments in murine macrophages. Primary elicited macrophages were extracted from BALB/c mice by peritoneal lavage. Differentiated macrophages were infected with  $\Delta$ TgPRFe/TgPRFi or RHTATi-1 at 1:3 parasite to cell ratio. Infected cells were treated, 24h p.i., with 1  $\mu$ M of Imiquimod for 24h. Our results showed that Imiquimod does not exhibit any effect on murine macrophages infected with  $\Delta$ TgPRFe/TgPRFi line as SAG-1 protein levels remained unchanged when compared with the significant decrease of this protein marker in the treated control strain (Figure 5A). Furthermore, the protein level of TLR-11 and 12 remained unchanged in the macrophages infected with the knock out Profilin line (Figure 5B). These results confirm our earlier data on the effect of Imiquimod on TLR-11/12 signaling, presumably through enhancing the binding of Profilin to its hetero-dimerized receptors.

## Discussion

This study unravels the potency of Imiquimod, an immunomodulatory drug, against AT and more importantly against CT. During the last decade, eighty clinically available drugs, including a large number of new compounds were used against *T.gondii* *in vitro* and *in vivo*, with more than forty mechanisms of action (reviewed in [40]). Several target based drug screens were also identified [40]. However, most of these drugs are effective against tachyzoites, and only very little trigger bradyzoites or the back and forth switch between both stages [40]. It is worth noting that an ideal drug against toxoplasmosis, should not only be effective against the proliferative tachyzoite stage of the parasite, but it should also exert an activity against the tissue cyst stage, especially that the chronic form is the most common form of the disease in humans and other intermediate hosts. In addition, these drugs should be capable to cross the blood brain barrier and to penetrate the cysts targeting bradyzoites [89]. We showed that Imiquimod induces interconversion of bradyzoites to tachyzoites in the brains of chronically infected mice, leading to overexpression of TLR-7, 11 and 12 and their downstream signaling (Figure 6C). Activation of these TLRs upon treatment with Imiquimod indicates that this drug could successfully cross the blood brain barrier to exert its effect.

Innate immunity represents the first line of defense against *T. gondii*. DCs and monocytes represent a major forefront exploited by the parasite, due to the capacity of both cell types to secrete defense molecules, and to the capacity of DCs to present antigens mediating crosstalk to T cells. But the most important feature triggered by the parasite is their shuttling role to various organs, hence escaping the induced host inflammatory response [90-94]. DCs recognize PAMPs via MyD88 TLRs signaling [58]. This signaling pathway is required for the immune protection during many infections, which are lethal in the absence of MyD88 [95]. Two parasitic PAMPs, Profilin and cyclophilin-18, play a role in TLR recognition. Profilin binds TLR-11 [56] and TLR-12 [55, 57], and enhances the production of IL-12 via MyD88 dependent pathway. TLR-11 also works by forming a heterodimeric complex with TLR-12, only in the mouse. The formation of this complex is important for DC response and IL-12 production [55, 60]. Our results demonstrated that Imiquimod led to the upregulation of TLR-11 and 12, most

likely by recruited DCs and monocytes, triggering our observed MyD88-downstream signaling, the activation of MAPK, and the subsequent secretion of immune mediators including IL-1 $\beta$ , IL-12 and IFN- $\gamma$  (Figure 6C). The production of IL-12 and IL-1 $\beta$  may be essential for the recruitment of neutrophils and natural killer cells, which will produce IFN- $\gamma$  until recruited T cells produce this cytokine and control the infection (reviewed in [54, 75, 76, 96-99]). Indeed, infected DCs and monocytes with *T. gondii*, participate, with astrocytes and microglia to present antigens to activated CD4<sup>+</sup> and CD8<sup>+</sup>, following their recruitment to the brain [100, 101], to control the infection [102]. We demonstrated that Imiquimod treatment yielded higher expression of CXCL9 which is crucial to recruit T cells into tachyzoite reactivated foci in the brain [88], supporting the recruitment and activation of these T cells, hence controlling the Imiquimod-induced conversion to tachyzoites.

A strain depleted for Profilin is not affected upon treatment with Imiquimod (Figure 6A) and TLR-11 and 12 expression levels in cells infected with this parasite line remained unaffected (Figure 6B). These results suggest that Imiquimod may enhance the binding of this parasite PAMP, to induce TLR-11 and 12 mediated MyD88 signaling (Figure 6C).

Since TLR-11 and TLR-12 are functional in mice but not in humans [53], our data can be extrapolated to target the transmission of the parasite between intermediate and definitive hosts, thus interfering with the transmission and the life cycle of the parasite. Although humans do not express either TLR-11 or TLR-12, human monocytes produce pro-inflammatory cytokines in response to *T. gondii* infection, suggesting that other TLRs in humans recognize different compartments of *T. gondii* to produce IL-12 in antigen-presenting cells [60]. It has been described that parasite recognition by intracellular TLRs (TLR3, 7 and 9) in humans facilitates resistance to toxoplasmic infection and activation of monocytes and human DCs [60, 61]. A study of human innate receptors showed that the human TLR5 may have a similar role to the mouse TLR-11, in activating cytokine production [103]. The effect of Imiquimod on TLR-5 is thus worth investigating. Our results showed that Imiquimod, that was initially generated as a TLR-7 agonist [104], leads to the upregulation of TLR-7. TLR-7 is an endosomal receptor,

known to recognize ribonucleic acid [99], and can signal through MyD88 to induce inflammatory cytokines [106-109]. The upregulation of TLR-7, after treatment of CT with imiquimod, can also contribute to the observed MyD88 signaling and the induced immune response. This plausible mechanism can be extrapolated to CT associated diseases in humans.

Noteworthy, the efficient recruitment of T cells to both peritoneum and spleen during AT, may imply the other parasite PAMP, cyclophilin-18. Indeed, cyclophilin-18 is recognized by both mouse and human C-C chemokine receptor type 5 (CCR5) [110] and enhances the proliferation and migration of macrophages and spleen cells (mainly T lymphocytes), to the site of infection for maintenance of the interaction between the parasite and host [111]. We showed that Imiquimod leads to recruitment of T cells during AT. This effect might involve cyclophilin-18, and requires further investigation. We also demonstrated that treatment with Imiquimod during AT, reduced the number of cysts upon establishment of CT, and the remaining bradyzoites were either dead or failed to convert to tachyzoites following oral infection. This result may be extrapolated to treat AT infection in both humans and animals.

Our study has implications in immunocompetent hosts where CT correlates with several neuro-pathologies and cancers [1, 11, 22, 23, 25, 31, 34, 35, 47, 53, 55, 70, 81, 84, 88]. Furthermore, targeting bradyzoite cysts, which hide in bays and skeletal muscles, interferes with parasite survival, and persistence in intermediate hosts, as well as with the transmission between intermediate hosts and/or definitive hosts. However, since Imiquimod induces interconversion between brain stages, it is less likely to be potentially administered to immunocompromised patients. Imiquimod, as supported by our data, stands to become a major player in *T.gondii* related diseases, since it at least equaled or exceeded the results obtained from current gold standard treatment.

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## **Acknowledgments**

We thank the American University of Beirut Core Facilities for providing access to their imaging, Animal Care, and core culture facilities. We also thank the Office of Grants and Contracts at the American University of Beirut. This work was made possible through core support from the Medical Practice Plan (Faculty of Medicine, American University of Beirut) and the American University of Beirut and the Centre National de Recherche Scientifique Libanais (AUB- CNRS- L GRP) funds.



## Figure legends

### Figure 1: Imiquimod exhibits a potent effect against acute toxoplasmosis. (A)

Timeline for the *in vitro* treatment of infected monocytes derived macrophages with 1 $\mu$ m of Imiquimod. Western Blot analysis for SAG-1 (P30) expression (left panel) and corresponding densitometry (right panel) in the treated macrophages as compared to the untreated one. (B) Timeline schedule for assessment of tachyzoite expression following treatment in BALB/c mice. Briefly, on day 0, BALB/c mice were injected with 1000 tachyzoites/mouse of 76K, on day 2 post injection mice were treated every other day with Imiquimod (50 $\mu$ g or 100 $\mu$ g/mouse) or the vehicle (Lipofendin+DMSO). At day 10, spleens were harvested and Quantitative Real-Time PCR for SAG-1 (# 5 mice per condition). SAG-1 expression was normalized to GAPDH. The results are expressed as percentage of untreated control ( $\pm$ ) SD. The t-test was performed to validate significance. \*, \*\* and \*\*\* indicate p values  $\leq$  0.05; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant. (C, D) Timeline schedule for immune cell recruitment using CD11b, and CD3 markers by flow cytometry, and cytokine expression in the peritoneum and the Spleen. Briefly, on day 0, BALB/c mice were injected with 1000 tachyzoites/mouse of 76K; on day 2 and 3 mice were treated either with imiquimod (50 $\mu$ g/mouse) or with sulfadiazine (200mg/L in drinking water). At day 4, spleens and peritoneal lavage were performed. Flow cytometry showing the percentage of CD11b and CD3 are shown as indicated (C) and quantitative Real-Time PCR for SAG-1, (# 5 mice per condition), IL-12 (# 5 mice per condition) and IFN- $\gamma$  (# 5 mice per condition) (D). The t-test was performed to validate significance. \*, \*\* and \*\*\* indicate p values  $\leq$  0.05; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant.

### Figure 2: Imiquimod reduces cyst number and bradyzoites protein *in vitro* and *in vivo*. (A)

Timeline schedule for assessment of bradyzoite formation/number *in vitro* following treatment with 1 $\mu$ m of Imiquimod. Western Blot analysis for p18 expression and corresponding densitometry (left panel) in the treated bradyzoites as compared to the untreated one following *in vitro* switch from tachyzoites to bradyzoites. The

quantification of cysts was confirmed by confocal microscopy following IF assay (right panel), using a biotinylated lectin (green), with specific binding to a selectin on the cyst wall and the bradyzoite marker P34 (red). The results depict one representative experiment among at least three independent ones. Number of cysts was determined in 50 independent fields per condition. **(B)** Timeline schedule for assessment the effect of Imiquimod on the conversion from acute to chronic toxoplasmosis (CT) in Balb/c mice. Briefly, on day 0, BALB/c mice were injected with 250 tachyzoites/mouse of 76K. On day 4, the treatment starts every other day using 50µm/mouse of Imiquimod during 4 weeks. At day 32, brains were harvested for cysts quantification or for gavage into new Balb/c mice. Cyst count following percoll extraction for the treated Balb/c mice (Left panel, 10 mice per condition). Bradyzoite cyst count for BALB/c orally injected with 20 cysts from brains of treated mice with Imiquimod (Middle panel, 10 mice per condition), and Quantitative Real-Time PCR for BAG-1 (right panel, 10 mice per condition). BAG-1 expression was normalized to GAPDH. **(C)** Timeline schedule for assessment the effect of Imiquimod on on a developed CT by treating chronically infected mice at day 21 for 4 weeks. After treatment, brains of BALB/c mice were harvested. Cyst count following percoll extraction for the treated BALB/c mice (10 mice per condition). The results are expressed as percentage of untreated control ( $\pm$ ) SD. The t-test was performed to validate significance. \*, \*\* and \*\*\* indicate p values  $\leq$  0.05; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant.

**Figure 3: Imiquimod increases the expression levels of TLR-11, TLR-12 and TLR-7 over weeks.** **(A)** Timeline schedule for assessment of TLR(s) profile in BALB/c mice. Briefly, Balb/c mice were injected with 250 tachyzoites/mouse of 76K. After treatment with 50µg/mouse of Imiquimod or Sulfadiazine (200mg/L in drinking water), mice were sacrificed at days 35, 42, 49 respectively. Quantitative Real-Time PCR for TLR-11, TLR-12 and TLR-7 (5 mice per condition) from brains of these mice. TLRs expression was normalized to GAPDH. The results are expressed as percentage of untreated control ( $\pm$ ) SD. The t-test was performed to validate significance. \*, \*\* and \*\*\* indicate p values  $\leq$  0.05; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant. **(B)** Western Blot analysis (3 representative mice per condition) for TLR-11 (left panel),

TLR-12 (middle panel) and TLR-7 (right panel) and their corresponding densitometry in the brains of Balb/c mice at day 49 following treatment with Imiquimod.

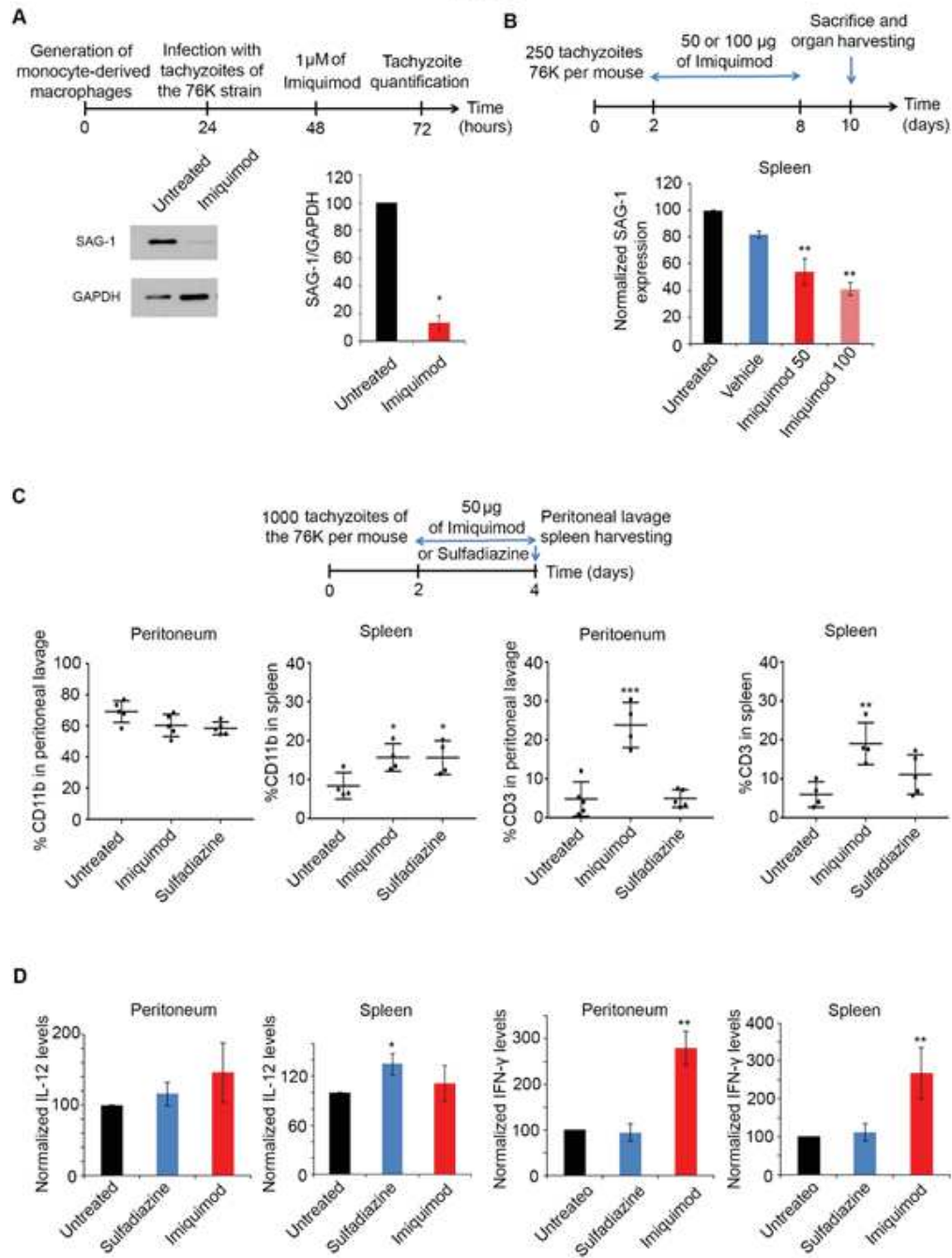
**Figure 4: Imiquimod induces interconversion and activates the TLR-MyD88 signaling pathway.** (A) Western Blot analysis for P21 (left panel), and SAG-1 (right panel) (3 representative mice per condition) and their corresponding densitometry in the brains of Balb/c mice at day 49 following treatment with Imiquimod. (B) Western Blot analysis for MYD88 (2 representative mice per condition) and their corresponding densitometry in the brains of BALB/c mice at day 49 following treatment with Imiquimod. (C) Western Blot analysis for P-ERK1/2 (upper gel), Total ERK1/2 (down gel) (3 representative mice per condition), and their corresponding densitometry in the brains of Balb/c mice at day 49 following treatment with Imiquimod. (D) ELISA showing the percentage of secretion levels of IL-1 $\beta$ , IL-12 and IFN- $\gamma$  in the brains of BALB/c mice chronically infected with 76K and treated with 50 $\mu$ g/mouse of Imiquimod (one representative experiment out of 2 #2 mice per condition). \*, \*\* and \*\*\* indicate p values  $\leq$  0.05; 0.01 and 0.001, respectively.

**Figure 5: Imiquimod Induces the upregulation of T cell markers during CT** (A) Quantitative Real-Time PCR for CXCL-10 (left panel/ 5 mice per condition) and CXCL-9 (right panel/ 5 mice per condition) from brains of mice injected with 250 parasites of 76K and treated with Imiquimod or Sulfadiazine (200mg/L in drinking water). CXCL-9 and CXCL-10 expressions were normalized to GAPDH. The results are expressed as percentage of untreated control ( $\pm$ ) SD. The t-test was performed to validate significance. \*, \*\* and \*\*\* indicate p values  $\leq$  0.05; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant.

**Figure 6: Imiquimod signals through Profilin/TLR-11/12 complex.** Western Blot analysis for (A) SAG-1 and the corresponding densitometry, (B) TLR-12 and the corresponding densitometry in the murine macrophages treated with 1 $\mu$ m of Imiquimod following infection either with the wild type strain (left panel), or with the KO Profilin strain (right panel). The results depict one representative experiment among at least three independent ones. \*, \*\* and \*\*\* indicate p values  $\leq$  0.05; 0.01 and 0.001, respectively. P-

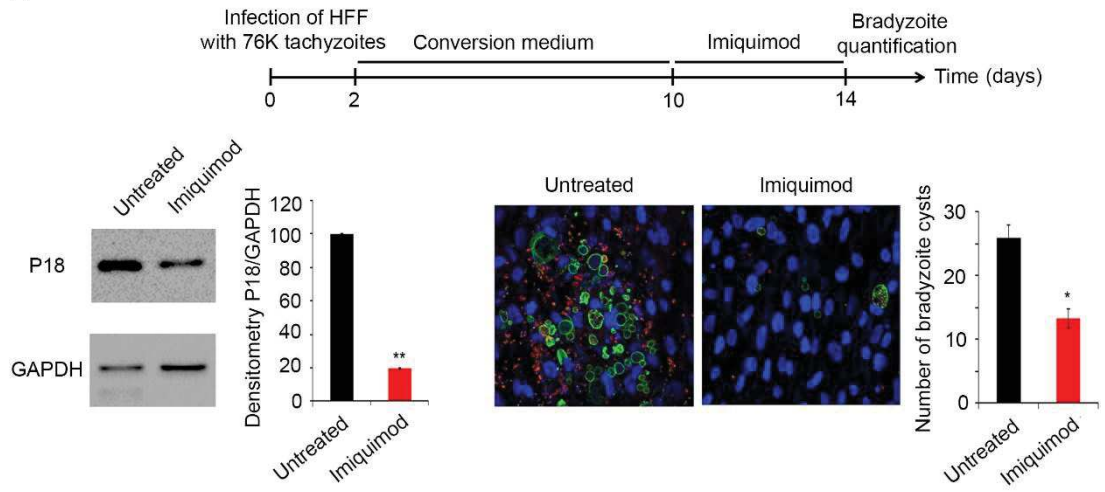
values less than 0.05 were considered significant. (C) Proposed model for the mechanism of action of Imiquimod during CT.

**Figure 1**

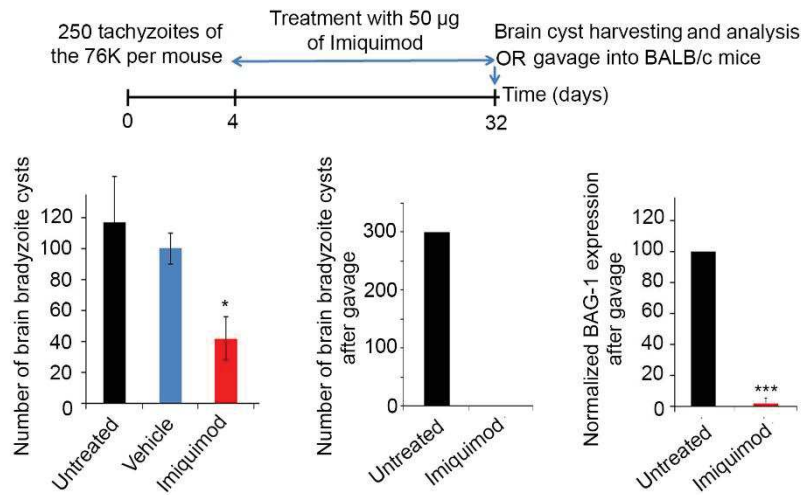


**Figure 2**

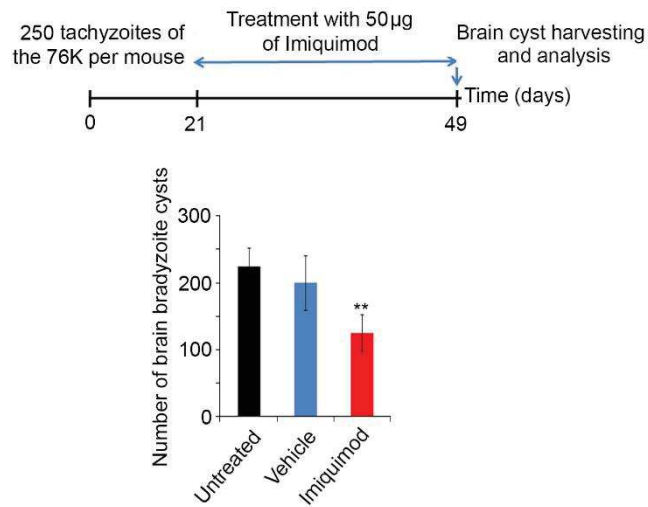
**A**



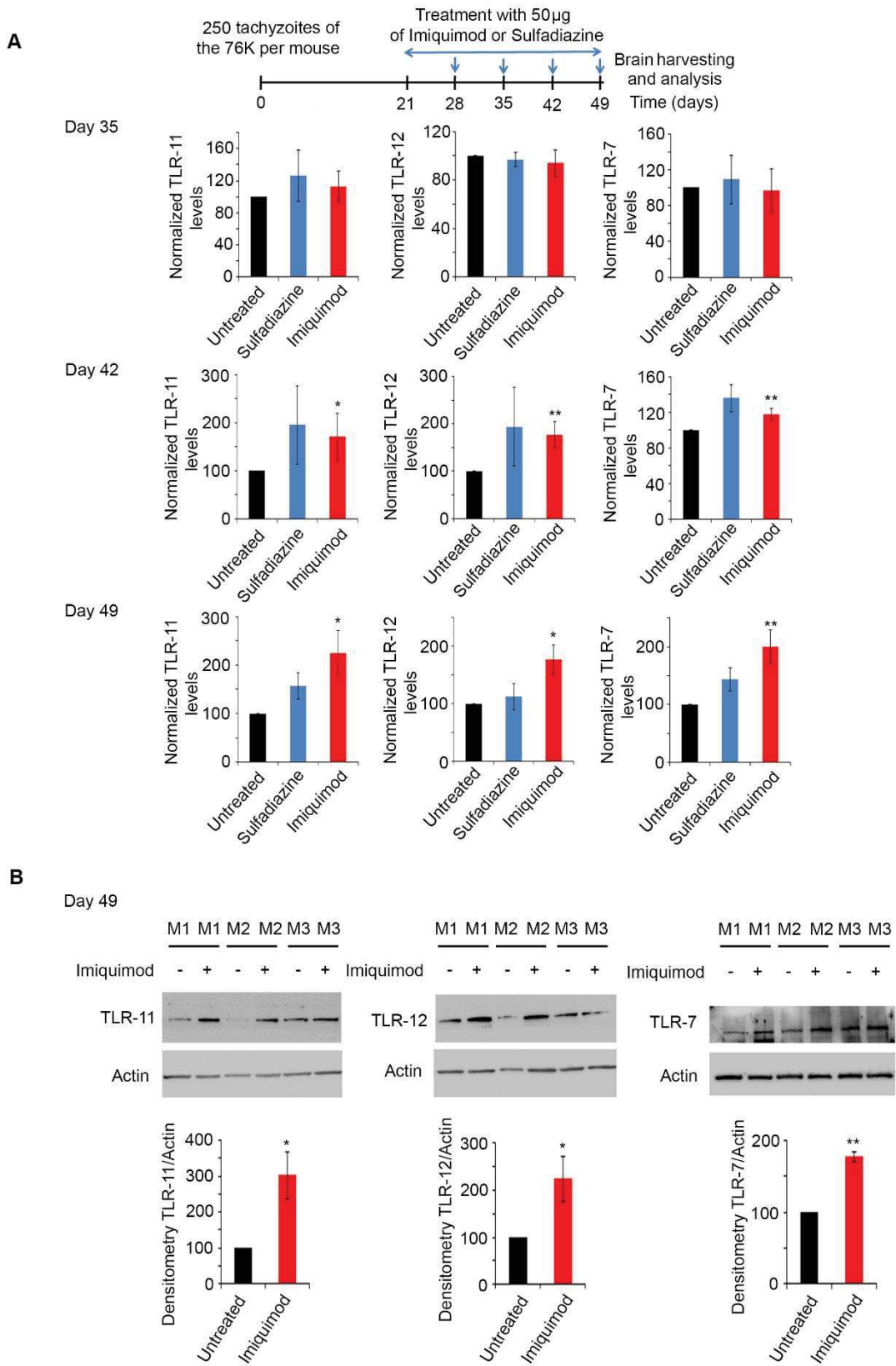
**B**



**C**



**Figure 3**





**Figure 4**

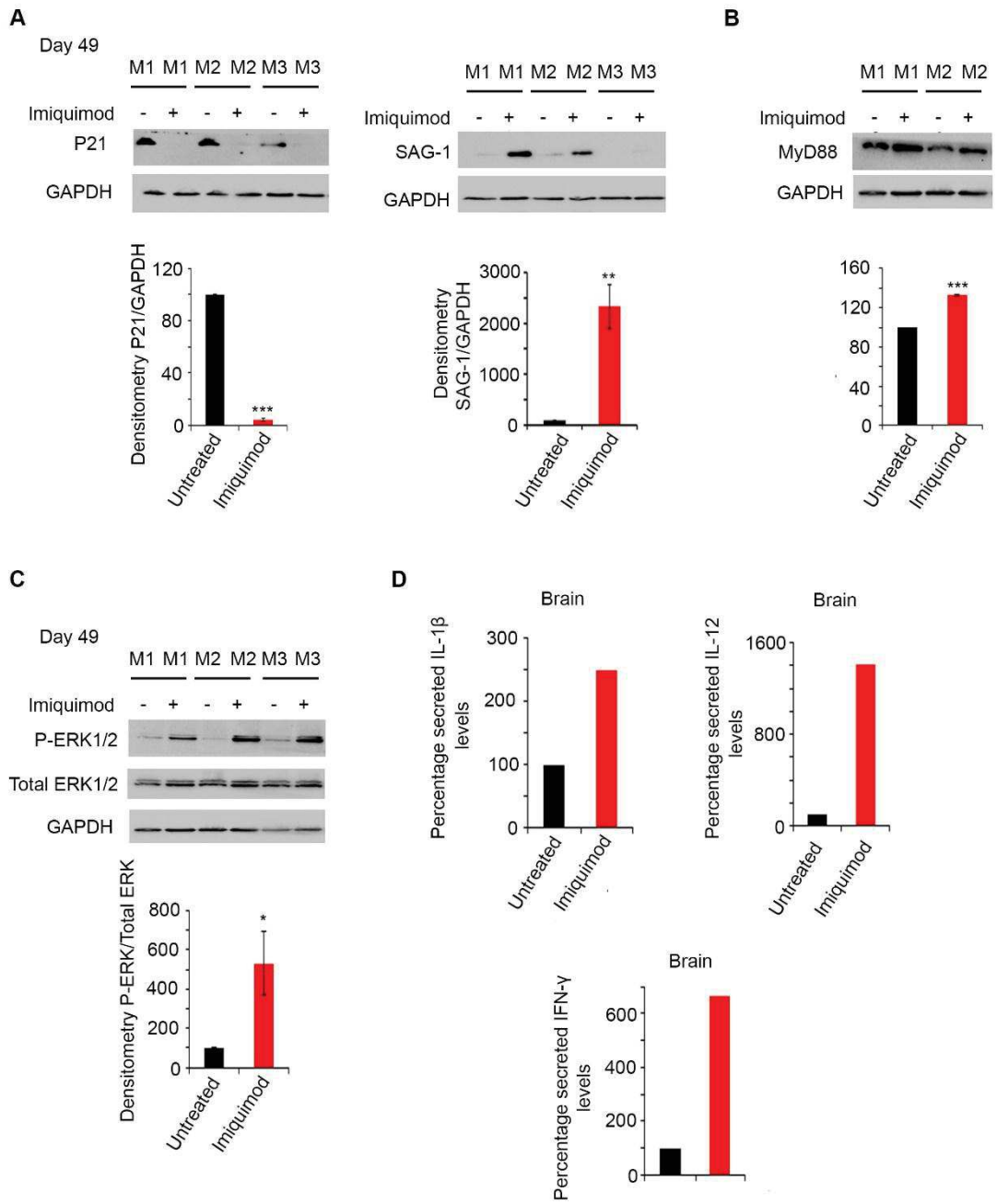
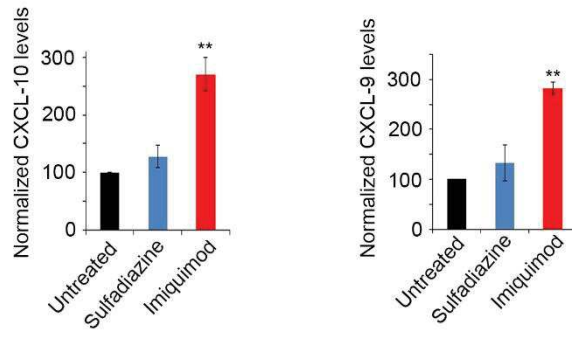




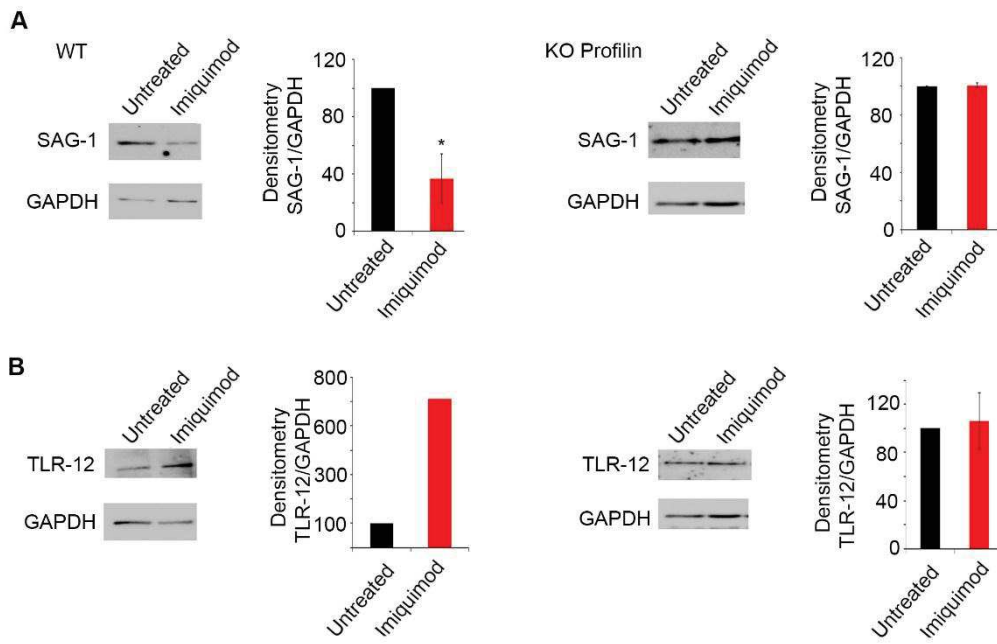
Figure 5

A

Day 49

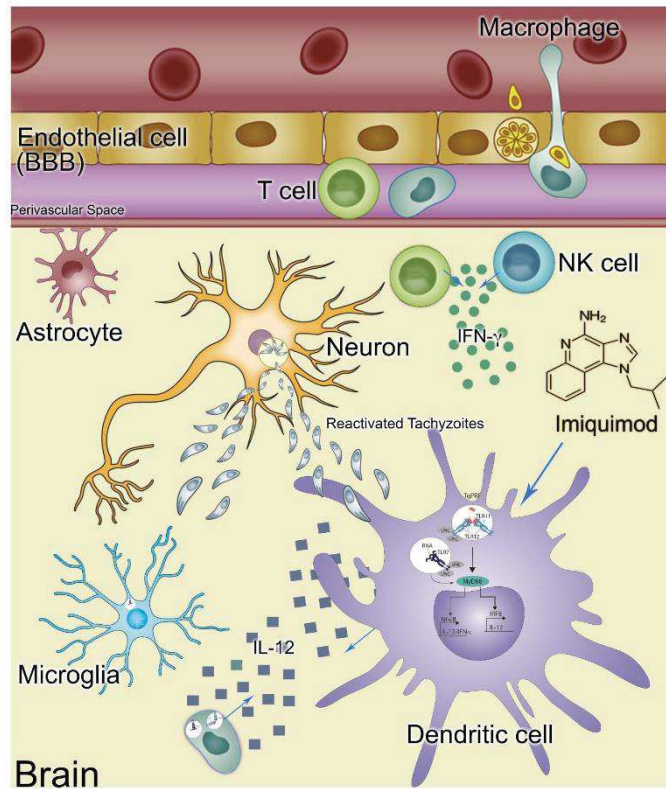


**Figure 6**



**C**

....Model still lacks some info some question marks related to microglia, NK



## **RESULTS part 2:**

P18 (SRS35/TgSAG4) is involved in the interconversion between acute and chronic toxoplasmosis and delays reactivation through modulation of host immunity in murine models.

**P18 (SRS35/TgSAG4) is involved in the interconversion between acute and chronic toxoplasmosis and delays reactivation through modulation of host immunity in murine models**

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## Abstract

Chronic toxoplasmosis (CT) is a prevalent disease caused by *Toxoplasma gondii*. Under the control of the host immune system, *T. gondii* persists as latent brain neuronal bradyzoite cysts in infected patients. Host immunosuppression leads to the reactivation of cerebral CT, a potentially life-threatening disease, but the parasite factors underlying interconversion between chronic and acute phases are poorly understood. Here, we investigated the role of the parasite surface antigen P18, belonging to the Surface-Antigen 1 (SAG-1) Related Sequence (SRS) family. We showed that P18 is an important modulator of the reactivation of CT following immunosuppression. Depletion of P18 in a cyst-forming strain of *T. gondii*, results in a decrease of virulence in BALB/c mice, during the acute phase. We observed that P18 depletion led to a faster clearance of the parasites from the peritoneum of these mice, paralleled by a substantial recruitment of dendritic cells, presumably a vehicle for tachyzoite dissemination. This result emphasizes a potential role of P18 in the virulence of the parasite through delaying the host immune response during the acute phase of the infection. Concomitantly, a lower number of tachyzoites was detected in the spleens while a higher number of parasites reached the brains of infected mice, resulting in a higher number of bradyzoite cysts. We also detected in the brain an increase of expression of immunomodulatory cytokines/chemokines, including Chemokine (C-X-C motif) ligand 9 (CXCL9) and 10 (CXCL10), known to control reactivation. Significantly, a delayed reactivation was observed upon immunosuppression of KO *P18*-BALB/c infected mice. Furthermore, upon oral infection of Severe Combined Immunodeficiency (SCID) (with IFN- $\gamma$  secreting innate immune cells), and NOG (NOD/Shi-scid/IL-2R $\gamma$ null) (NSG) mice (lacking IFN- $\gamma$  production), a significant prolonged survival of infected SCID but not NSG mice was observed. This suggests a role for IFN- $\gamma$  in the P18-mediated conversion from bradyzoites to tachyzoites. All together, these data support a potential role for P18 surface antigen, in the virulence of the parasite and in orchestrating the host immune response, during the acute and more importantly the chronic phase of infection. P18 plays also a central role in controlling parasite reactivation and dissemination in an IFN- $\gamma$  dependent fashion. Understanding mechanisms of switch between parasite stages, cysts formation and persistence, has far reaching implications in light of the documented

association of *T. gondii* brain cysts and neurological diseases, as well as the reactivation of the disease in immunocompromised patients.

### **Author Summary**

Chronic toxoplasmosis (CT) is a prevalent disease caused by *Toxoplasma gondii*. Under the control of the host immune system, *T. gondii* persists as latent brain neuronal bradyzoite cysts in infected patients. An increasing number of reports associate seropositivity for *T. gondii* with host behavior, several neurological disorders (e.g. schizophrenia, and Parkinson diseases), and brain cancer incidence. Furthermore, host immunosuppression leads to the reactivation of cerebral CT, a potentially life-threatening disease in immunocompromised patients. In this study, we demonstrated that the surface antigen P18, plays a role in the virulence of the parasite and is modulating the host immune response. P18 is also implicated in brain cyst formation, reactivation of CT following immunosuppression, as well as in interconversion and dissemination of the parasite in immunocompromised hosts. Studying the parasite's factors underlying interconversion between stages, cyst formation and persistence in the host, has far-reaching implications on toxoplasmosis and its documented association with different diseases, especially in light of the recent flagging on considering toxoplasmosis as a neglected parasitic infection, requiring public health action.

## Introduction

*Toxoplasma gondii* is an obligate intracellular parasite that infects all warm-blooded animals. Approximately 30% of the human population is infected worldwide (1). According to regions, the sero-prevalence of *T. gondii* in humans varies between 10 and 70%, and significantly increases with age (2-4). Tachyzoites, the rapidly multiplying forms of *T. gondii*, lead to tissue damage and are responsible for acute toxoplasmosis. Tachyzoites exploit dendritic cells, and monocytes to spread into various organs, and subsequently form bradyzoite cysts in the brain and in skeletal muscles (5). These slow-growing bradyzoites are responsible for a persistent disease known as chronic toxoplasmosis (CT).

Until recently, parasite persistence in healthy individuals was regarded as clinically asymptomatic. However, an increasing number of reports associate seropositivity for *T. gondii* with host behavior (6), several neurological disorders (e.g. schizophrenia, and Parkinson diseases) (7, 8), and brain cancer incidence (9). In immunocompromised patients, despite the availability of prophylactic and treatment options, reactivation of CT can still occur, imposing a life threatening situation (10-15). These include Human Immunodeficiency Virus (HIV)-infected patients, cancer patients after chemotherapy, or following bone marrow or organ transplantation (11-15).

The interconversion between acute and CT is exquisitely controlled by the host immune system (16). During the acute infection, the host's innate immunity mounts a robust anti-*Toxoplasma* cytokine response, characterized by high interferon-gamma (IFN- $\gamma$ ) production by natural killer (NK) and T cells (17-19), following IL-12 production by dendritic cells, neutrophils and macrophages (20-25). IFN- $\gamma$ -mediated immune response provokes intracellular elimination of tachyzoites (19, 22-25). In the brain, IFN- $\gamma$  production by brain resident and recruited cells, including microglia and primarily T-cells, is also crucial for the maintenance of cerebral CT latency (26-28). Importantly, IFN- $\gamma$  regulates the recruitment of immune T cells into the brain of BALB/c mice during the acute and chronic phases of the infection (29, 30). During murine CT, CXCL9 and 10 CXCL10, are predominantly expressed in the brains of infected BALB/c mice (31, 32). Furthermore, CXCL9 is crucial to recruit T cells into the brain and to

induce their accumulation in zones of tachyzoite proliferation to prevent reactivation of CT (33).

*Toxoplasma* expresses 144 proteins belonging to the SRS family (34). These SRS are differentially expressed in a life cycle stage specific manner, and are potentially involved through mediating attachment to host cells, and regulating the immune response, in the successful initiation of infection. In addition, some SRS proteins play important functions in the context of parasite reactivation. For instance, p36 (SRS9/SRS16B), one of the most abundant bradyzoite-specific proteins plays a major role in both persistence in the brain and reactivation in the intestine (35). Four monoclonal antibodies recognizing four selective pellicular antigens (P36, P34, P21 and P18) were generated against the bradyzoite stage (36, 37). Yet, Expressed Sequence Tag (EST) data reveal the expression of a very low number of transcripts (7 ESTs) of P18 in the tachyzoite stage and a very high number of transcripts (187 ESTs) in the bradyzoite stage, making P18 transcripts amongst the most abundant expressed EST, between the SRS family members (34). The function of P18, encoded by *SAG4/SRS35* (38), remains to be elucidated.

In this study, we investigated the role of P18 in the phase of acute infection, in brain cyst formation, and along the path towards the reactivation of CT following immunosuppression. We showed that P18 deletion impacts the virulence of the parasites, by prolonging survival of acute infected mice in a dose dependent manner. P18 depletion led to a faster clearance of the parasites from the peritoneum of BALB/c mice, concurrent with a higher recruitment of dendritic cells, presumably indicating a role in modulating the host immune response. A lower number of tachyzoites was detected in the spleens of infected mice with the knock-out strain and a higher number of parasites reached the brain at the same time point. Consistent with the higher tachyzoite number reaching the brain, P18 depletion induced a higher number of brain bradyzoite cysts in BALB/c infected mice. This phenotype is concurrent with the induction of immunomodulatory cytokines/chemokines, and the upregulation of CXCL9 and CXCL10, which are predominantly expressed on activated T-cells and natural killer cells, and known to play a role in the control of reactivation of CT. Furthermore, *P18* knock out parasites (KO *P18*)



significantly delayed reactivation, in immunosuppressed BALB/c mice. Notably, SCID mice, having normal production of IFN- $\gamma$  by innate immune cells, survived oral infection with the KO *P18* but not WT strain cysts. In contrast, NSG mice lacking IFN- $\gamma$  production, succumbed from the infection, clearly indicating a role for IFN- $\gamma$  in the interconversion from bradyzoites to tachyzoites in *P18*-depleted parasites. Altogether, these results implicate P18 in the virulence of the parasite, in the modulation of the host immune response, during the acute and chronic phases of the infection from acute to chronic toxoplasmosis, in the control of reactivation of cerebral toxoplasmosis, and the conversion and dissemination of the infection in immunocompromised hosts.

## Results

### Generation of the *P18* knock out and the complemented lines

To gain insights into P18 function, we generated KO *P18* parasites by replacing the corresponding *SRS35/TgSAG4* gene by the selectable marker *hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT)*, in the WT Pru $\Delta$ *Aku80* type II strain (39) (Fig 1A). The successful genetic modification was verified by PCR (Fig 1B) and the P18 expression level was assessed in tachyzoites and upon *in vitro* switch from tachyzoites to bradyzoites, by immunoblot using specific anti-P18 antibodies (36). Consistent with the published EST data (34), P18 protein levels were abundantly expressed in bradyzoites of the WT strain. Trace levels of P18 were detected in tachyzoites of this strain as they required a higher exposure of the nitrocellulose membrane with Luminol (Fig 1C, left panel). Importantly, we could confirm P18 abrogation in the KO *P18* strain in both tachyzoites and bradyzoites, upon deletion of its encoding gene (Fig 1C). We next generated the Cpt *P18* complemented strain by adding an extra copy of *P18*, under its own promoter (Fig 1A). Stable transgenic clone was isolated (Fig 1B) and showed expression of P18 by western blot, in both tachyzoites and bradyzoites of Cpt *P18 in vitro* (Fig 1C). Consistent with the western blot data, a total extinction of *P18* expression was observed in cysts of the mutant. Differentiating vacuoles was distinguished using a fluorescent *Dolichos biflorus* lectin (DBL) which recognizes *N*-acetylgalactosamine on the bradyzoite-specific cyst-wall protein CST1 (40). After 14 days under alkaline stress,

the vacuoles of both control and KO *P18* parasites were positive for DBL, but vacuoles of the mutant were negative for P18 stained with anti-P18 antibodies (Fig 1D), confirming the loss of expression of P18 in the KO *P18*. P18 expression was restored in the Cpt *P18* strain by IFA (Fig 1D). Collectively, these results showed that *P18* depletion and complementation were successful and allow a functional characterization of the P18 protein.

### **Deletion of P18 dramatically affects the parasite virulence in mice**

The expression of P18 in the tachyzoite stage, even if low, prompted us to investigate the phenotype of KO *P18* during the acute phase of infection. A clear impact of P18 removal was observed on survival of infected mice (Fig 2A). While all BALB/c mice survived intraperitoneal infection with  $10^5$  tachyzoites of the KO *P18* strain, mice infected with the WT strain died between 10 and 17 days post-infection. Inoculation of mice with a higher dose of parasites ( $10^6$ ), does not show any significant difference (Fig 2A), reflecting an attenuated virulence *in vivo*.

The difference in the survival phenotype prompted us to investigate the dissemination of the parasites to different organs, during the acute phase of the infection. BALB/c mice were infected with 250 tachyzoites of WT, KO *P18* or Cpt *P18*. Four days post-infection, we assessed parasite number and expression in the peritoneum and in relevant organs to the infection, namely spleen and brain. Interestingly, numbers of tachyzoites in the peritoneum of the KO *P18* infected mice were significantly lower than those of the WT infected control mice (Fig 2B, left panel). This was paralleled with a significantly higher number of dendritic cells, as shown in the percentage of CD11c<sup>+</sup> using flow cytometry (Fig 2B, middle panel). This result presumably indicates the ability of P18 to contribute to the parasite virulence in delaying the host immune response, and implies the capacity of the KO *P18* to use dendritic cells, as a vehicle (41), for a faster dissemination to brain. This hypothesis was further supported by the significant lower transcript levels of SAG-1 in the spleen, and their significant higher levels in the brains of KO *P18* infected mice (Fig 2C). Kinetic studies demonstrated that the spleen is among the targeted organs upon infection with type II strains, before reaching the brain to establish CT (42, 43). This further supports our hypothesis that the KO *P18* highjacks

DCs to reach the brain faster and establish CT. To ascertain that these observed *in vivo* phenotypes are due to P18, we tested Cpt *P18* parasites and showed similar tachyzoite numbers to those of WT strain in the peritoneum of infected mice (Fig 2B, left panel). SAG-1 expression patterns were also very similar between Cpt *P18* and WT strains, in both the spleens and brains of infected mice with the complemented strain (Fig 2B, right panel and Fig 2C). Altogether, these results show that the observed phenotypes implicate P18 in modulating the virulence of the WT strain *in vivo*, and point a role for P18 in modulating the host immune system to induce a faster escape to the brain, for the establishment of the latent chronic phase of the infection.

### **P18 depletion results in increased number of bradyzoite cysts *in vitro* and *in vivo***

It is well documented that P18 is expressed in the bradyzoite stage, at both the transcript and protein levels (34, 38). We assessed the effect of P18 depletion on bradyzoite cyst formation and number. After 14 days of *in vitro* conversion from tachyzoites of WT, KO *P18* or Cpt *P18* strains, increased number of cysts was observed in the P18 depleted strain, suggesting a higher number of bradyzoites (Fig 3A). This result was obtained by assessing several fields, by immunofluorescence microscopy. Not only the number of cysts formed by the KO *P18* strain was significantly higher, but also a significant difference in the cyst size was observed (Fig 3A).

We then investigated the number of brain cysts *in vivo* using intraperitoneal injection of BALB/c mice with 250 parasites of WT, KO *P18* or Cpt *P18* strains. The acute phase was verified seven days post infection by immune reactivity of infected mice on tachyzoite extracts (44). Twenty-eight days post infection, brains of infected mice with the three different strains were harvested for cyst quantification (Timeline described in Fig 3B). P18 depletion significantly increased the number of cysts in the brains of infected mice (Fig 3B). Complementation of *P18* in the Cpt *P18* strain reverted the observed phenotype (Fig 3B). Altogether, these results demonstrate a function for P18 in the control of bradyzoite cyst number *in vitro* and *in vivo*.

### **P18-deficient bradyzoites elicit a stronger brain immune response**

Nitric-oxide (NO), produced by microglial cells and macrophages that infiltrate the CNS, inhibits *T. gondii* replication and plays a vital role in the progression of the infection (45-50). Inducible nitric oxide synthase (iNOS) expression is also up-regulated in the brains of mice infected with type II strains (51). Consistent with the higher number of cysts obtained upon *P18* depletion (Fig 3C), iNOS transcriptional levels were elevated in the knock out strain (Fig 4A). This increase of expression was restored in the complemented strain, suggesting a potential role for NO *via* iNOS up-regulation in mediating stress responses, leading to the conversion of the higher number of tachyzoites reaching the brain upon *P18* depletion, into bradyzoite cysts.

Another elicited key immune response upon establishment of CT, is the IFN- $\gamma$  produced by microglia, intracerebral T cells, and NK to control cerebral *T. gondii* growth (27). IFN- $\gamma$  elicits intracerebral immune response by the production of cytokines and chemokines (5, 16, 20, 26, 27, 32). We showed that, upon depletion of *P18*, the secreted levels of IL-12, IL-1 $\beta$ , IFN- $\gamma$ , IL-6, TNF- $\alpha$ , IL-10, MCP-1, MIP-1 $\alpha$  and MIP-1 $\beta$  were specifically up-regulated in the brains of chronically infected mice (Fig 4B). Restoring *P18* expression in the complemented cell line led to cytokine/chemokine levels similar to those induced by the parental cell line (Fig 4B). These results indicate that the KO *P18* strain induces a stronger immune response in the brains of infected BALB/c mice.

During murine CT in BALB/c mice, CXCL9, and CXCL10, are predominantly expressed in the brains of infected mice (31, 32). Furthermore, CXCL9 is crucial to recruit T cells into the brain and to prevent reactivation of CT (33). Interestingly, *P18* depletion led to a significant upregulation of both CXCL9 and 10, suggesting a role of *P18* in controlling reactivation (Fig 4C).

Collectively, the elicited immune response may correlates with the higher parasitic burden, manifesting with a high number of tachyzoites reaching the brain, and explaining the higher conversion rate from tachyzoites to bradyzoites, thus a greater number of bradyzoites in the *P18* knock-out strain.

### **KO *P18* parasites induce a delayed reactivation upon immunosuppression**

The higher expression of chemokines involved in T cell recruitment to the brain, and their documented role in the prevention of CT reactivation (33), prompted us to address the potential role of *P18* in reactivation. Following establishment of CT, BALB/c mice were immunosuppressed using dexamethasone (52) (Timeline described in Fig 5A). Reactivation of CT in mice infected with the wild type WT strain led to 100% lethality between 29 and 33 days post-dexamethasone administration (Fig 5B, left panel). Interestingly, reactivation of CT in mice infected with KO *P18* parasites was significantly delayed and death was recorded between days 80 and 81 after initial administration of dexamethasone (Fig 5B, left panel). Upon complementation with *P18*, the delay of reactivation was reverted, and animals succumbed between days 28 and 33 post-dexamethasone administration (Fig 5B, left panel), indicating a role for *P18* in the observed phenotype. To confirm that the death of mice is a result of CT reactivation, we quantified the tachyzoite marker SAG-1 transcript levels in the brains of infected mice. Since the average time of death of mice infected with control or complemented strains was day 30 post-dexamethasone administration, we assessed SAG-1 expression at this specific time point. Consistent with the survival results, SAG-1 expression was significantly lower in the brains of infected mice with the KO *P18* parasites (Fig 5B, right panel). Altogether, these data support a role for *P18* in the reactivation of CT in BALB/c mice.

### **IFN- $\gamma$ plays a major role in *P18* bradyzoite to tachyzoite interconversion**

Due to the delayed reactivation of the KO *P18* strain, we explored the capacity of bradyzoites to reconvert into tachyzoites and establish a successful acute infection in immunocompromised animals. We used SCID and NSG mice. SCID mice lack adaptive B and T cells related immunity, but yet retain a normal innate immunity with intact macrophages, antigen-presenting cells, and natural killer (NK) cells, thus a normal IFN- $\gamma$  production (53). The NSG model present deletion or truncation of the gamma chain of interleukin 2 (IL-2) receptor (54). Thus, NSG mice possess a defective production of a

big number of interleukins and a severe impairment of the dendritic cells and mostly their capacity in producing IFN- $\gamma$  upon stimulation (54).

Harvested cysts (20 cysts) from the brains of BALB-c mice infected with WT, KO *P18* or Cpt *P18* were administered by oral gavage to SCID or NSG mice. While SCID mice infected with the wild-type or complemented strains succumbed between 15 and 18 days post-oral infection, SCIDs infected with *P18* depleted parasites, showed a significant prolonged survival (26-122 days) in 60% of tested animals, while 40% of the mice remained disease free (Fig 5C, left panel). Conversely, oral gavage of NSG mice resulted in 100% death of mice after 15 days post-infection (Fig 5C, right panel). These results indicate that the *P18* bradyzoite conversion to tachyzoites is impaired in SCID but not in NSG mice, suggesting a role of IFN- $\gamma$  in the *P18*-mediated conversion from the chronic to the acute phase of the infection.

## Discussion

This study demonstrates a role for *P18*, not only in the virulence and dissemination of the parasite during the acute phase of the infection, but also in the persistence of CT and in the reactivation of *Toxoplasma* bradyzoites. This contributes to the general understanding of toxoplasmosis, a disease that poses significant health and economical burdens. Our study has implications in both immunocompetent hosts where CT correlates with several neuro-pathologies and in immunocompromised patients where recurrence of CT is severely morbid and potentially lethal (10-15). Hence, understanding the mechanisms of cyst formation and reactivation of CT is essential for our ability to interfere with parasite survival and persistence in the host.

*T. gondii* exploits the immune system of intermediate hosts, transforming challenges into opportunities. Dendritic cells represent a major forefront exploited by the parasite, due to their capacity to secrete defense molecules, present antigens mediating crosstalk to T cells, but most importantly due to their shuttling role to various organs. Indeed, infection with type II *Toxoplasma* strains leads to both higher migration and hypermotility of DCs (55-59). Notably, type II *Toxoplasma* use DCs more effectively as a shuttle, to travel through various tissues, escaping the host inflammatory response (57). Infected DCs and

monocytes with *T. gondii* cross the blood brain barrier, participating, with astrocytes and microglia to present antigens to activated CD4<sup>+</sup> and CD8<sup>+</sup>, following their recruitment to the brain (29, 30), to control the infection (60). These activated T cells, in cooperation with natural killer cells among others, are the main source of IFN- $\gamma$ , which plays a role in intracellular elimination of tachyzoites (19, 22-25). Moreover, IFN- $\gamma$  can activate microglia (61) and astrocytes (48, 62) to prevent tachyzoite proliferation. IFN- $\gamma$  is also crucial for the maintenance of cerebral CT latency (26-28). Our results showed a higher number of recruited DCs upon P18 depletion during the acute phase of infection. Recognizing that some of the parasites will be eliminated by dendritic cells, the remaining surviving parasites presented with a lower number of tachyzoites in spleens of infected mice while a higher number of tachyzoites reaching the brain (42, 43). It is worth noting that the recruitment of more innate immune cells upon depletion of P18, suggests a role of this marker in attenuating the immune response during the acute phase of the infection, contributing to the virulence of the parasite. Moreover, P18 depletion yielded higher expression of CXCL9 which is crucial to recruit T cells into the brain and to prevent reactivation of CT (33), along with a higher number of cysts and a deficit in the reactivation capacity of these parasites. These results may suggest a role for P18 in modulating and activating DCs to travel from the infection site to the brain. This role might implicate production of IL-12 by DCs, and to present antigens to induce the recruitment and activation of T cells, hence controlling both the persistence and reactivation of cerebral toxoplasmosis. Furthermore, the IFN- $\gamma$  mediated P18-conversion obtained in SCID mice suggests, not only a role of T cells in producing this cytokine as dominant and necessary immune response for maintenance and persistence of the bradyzoite stage, and prevention of recrudescence to tachyzoites (28), but also involves innate immune cells in its production. These are potentially involved in controlling the dissemination of the parasite, following conversion to tachyzoites.

A critical step in *T. gondii* pathology is the interconversion between tachyzoite and bradyzoite stages, that are crucial for persistence and transmission of the parasite. The superfamily of SRS genes comprises 144 members, of which 35 are pseudo genes (34). Differential expression of SRS during life cycle stages of the parasite is essential for the initiation of infection, modulation of host immunity and establishment of



transmissible infections (63-65). Of all the recognized proteins, the tachyzoite-specific SAG1/SRS29B functions as an adhesin, influences virulence and induces lethal ileitis in mice (64, 66-70). In addition, SAG1/SRS29B, along with other tachyzoite-specific members like SRS34A, and SRS29C proteins, elicit high antibody titers during the acute phase of the infection (71). This immune response is essential to drive the switch to latent bradyzoites expressing specific members of this family. For instance, CST1/SRS44, a mucin-like domain glycoprotein, is essential to construct an intact and rigid cyst wall (40). This protein presents a heavily glycosylated domain to maintain the cyst wall structural integrity (72). Another protein, SRS13 was identified and localized to the cyst wall and matrix. However, unlike CST1/SRS44, SRS13 is not necessary for the assembly of the cyst wall (73). Recently, Tu et al. expanded their functional analysis to two other cyst wall proteins, CST2 and CST3. The generation of the respective knock out strains revealed a normal phenotype with respect to growth or cyst formation *in vitro*, yet, CST2-KO parasites were markedly less virulent during the acute infection in mice (74).

SRS9/SRS16B encodes an abundant bradyzoite-specific protein, p36 (35). SRS9 plays an important role in both persistence in the brain and reactivation in the intestine (35). Added evidence of p36 role in CT persistence, its deletion decreases the cyst number in the brains of infected mice (35). In this study, we identified a role for P18 (*SAG4/SRS35*) in cyst formation and reactivation of CT. In contrast to the reported function of p36, P18-depleted parasites resulted in more cysts *in vitro* and *in vivo*. Despite this higher number of cysts, a delayed reactivation of CT was observed upon dexamethasone-induced immunosuppression. While p36 was implicated in the reactivation in the intestine (35), P18 seems to exert its function in the cerebral reactivation, while not excluding also a role in the intestine. Whether p36 may influence P18 expression, and affect the number and the persistence of bradyzoite cysts during CT is yet to be explored. Our results highlight a non-redundant function of another member of the SRS superfamily in the reactivation process.

Our data support a role for P18 in both the virulence of the parasites and in modulating the host immune system to faster escape and reach the brain, where it actively converts to CT. It also shows a role of this protein in the persistence and maintenance of



CT, as well as a control of reactivation of CT. Moreover, P18 seems to implicate IFN- $\gamma$  in the process of conversion from bradyzoites to tachyzoites, highlighting a modulation of the immune system, by this SRS protein at multiple levels within the intermediate host. The identified role in conversion and reactivation will set the stage for further studies that may lead to novel targets for preventional therapy. This has far reaching implications in light of the documented association of *T. gondii* brain cysts and neurological diseases, brain tumors as well as the reactivation of the disease in immunocompromised patients, and the recent flagging on considering toxoplasmosis as a neglected parasitic infection, requiring public health action (CDC 2019).

## **Materials and methods**

### **Ethics statement**

All mice protocols were approved by the Institutional Animal Care and Utilization Committee (IACUC) of the American University of Beirut (AUB) (Permit Number: #1312273). All animals were housed in specific pathogen free facility with a 12h ON/OFF light cycle. Humane endpoints were fully respected as per AUB IACUC following AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) guidelines and guide of animal care use book (Guide, NRC 2011). Mice were monitored on a daily basis. To verify the acute phase of the infection, blood was withdrawn following deep anesthesia with isoflurane by inhalation. Mice were sacrificed if any abnormal ethical features are noticed as described previously [63]. Animals were deeply anesthetized before cervical dislocation.

### **Mammalian cells and parasite cultures**

Tachyzoites from Pru $\Delta ku80$  (WT) (deleted for the *ku80* gene [64]), Pru $\Delta ku80\Delta P18$  (KO *P18*) or the complemented Pru $\Delta ku80\Delta P18+P18$  (Cpt *P18*) were used throughout this study. Parasites were maintained by serial passage in human foreskin fibroblasts (HFFs) (American Type Culture Collection-CRL 1634), grown in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, Invitrogen) supplemented with

10% of fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% kanamycin and 1% glutamine.

### **Molecular cloning**

Genomic DNA purification from a pellet of  $3 \times 10^8$  parasites of WT strain, was performed using wizard Genomic DNA purification system Promega (Ref A 2361 0000035819). PCR amplifications were performed on genomic DNA using PrimeSTAR HS DNA/Phusion® High-Fidelity DNA Polymerases (New England Biolabs).

Plasmid pKO-*P18* was designed to replace by double- homologous recombination, the genomic sequence of *P18* by the open reading frame of *HXGPRT* gene to produce KO *P18* line. A genomic fragment of 2274 bp corresponding to the 5' non-coding sequence of *P18* was amplified using ML1514 and ML1515 primers and subcloned into the HindIII and ApaI sites of plasmid 2854.HX (75). Then a genomic fragment of 2719 pb corresponding to the 3' non-coding sequence of *P18* was amplified using ML1516 and ML1517 primers and subcloned at Not I and Spe I sites of plasmid 2854.HX. Forty micrograms of pKO*P18* was digested by HindIII and SpeI prior to transfection in the WT strain and was subjected to Mycophenolic Acid and xanthine selection.

To complement the KO *P18* line, we amplified the open reading frame of *P18* flanked by 1024 pb of 5' non-coding sequence and 502bp of 3'UTR and cloned into pLIC-HA vector (76) , containing a DHFR selectable marker.

Table 1. Summary of PCR primers used to generate 5' *P18*-P2854 HXPRT- 3' *P18*. Restriction site specific for each enzyme is underlined and highlighted in yellow and the rest of the primer complementary to the 3' or 5' regions is highlighted in green.

Primers' names	Primers sequences (5'-3')	Restriction Enzymes
ML1514	gcgcGGGCCC CGATCCGCAGACATCT GGGGGTCTCTTGGCGTTCGTCCCCG CCAACAAAGCG	Apal
ML1515	cccAAGCTTGGTTGAAGACAGACGA AAGCAGTTGCAGTATGCTGCGACGC GTCTTCCGAG	HindIII
ML1516	ggACTAGTGAGTTCATTGCCAGTGA AGAAGGTGACTGGTAGTGTCACATT TGGCAACTGG	SpeI
ML1517	ataagaatGCGGCCGC TTGTTACCTGGC ACACGTCACCTGCAACATTGTAAAC TTGTTTGTGTCTGG	NotI

### Generation of transgenic parasites

For transfection,  $20 \times 10^6$  parasites were re-suspended in 800  $\mu$ l of cytomix (10 mM  $KPO_4$ , 120 mM KCl, 0.15 mM  $CaCl_2$ , 5mM  $MgCl_2$ , 25 mM Hepes, 2 mM EDTA) complemented with 3 mM ATP and 3 mM of reduced glutathione and electroporated with 30–50  $\mu$ g DNA as described previously [65]. Selections of the transgenic parasites were performed with mycophenolic acid (20  $\mu$ g  $ml^{-1}$ ) and xanthine (50  $\mu$ g  $ml^{-1}$ ) for HXGPRT selection or pyrimethamine (1  $\mu$ M) for DHFR-TS selection and chloramphenicol (20  $\mu$ M) for chloramphenicol acetyltransferase selection. The isolation of clonal transgenic populations was performed using limiting dilution in 96-well plates.

### *In vitro* switch from tachyzoites to bradyzoites

Confluent HFF cells were cultured in a 6-well plate and on coverslips and infected at a concentration of 1000 parasites from WT, KO *P18* or Cpt *P18*/well. After a 24h incubation in complete DMEM medium under 5%  $CO_2$ , the medium was changed to

induction medium (RPMI 1640 without NaHCO<sub>3</sub>, HEPES 50mM, 3% FBS, pH 8.2). Cells were then maintained in absence of CO<sub>2</sub>. The basic medium was changed every other day to maintain the pH at 8.2. After 2 weeks, infected cells with bradyzoites were harvested for immuno-fluorescence assay.

### **Immunofluorescence and confocal microscopy**

Bradyzoite conversion was confirmed by staining the cyst wall with Biotinylated *Dolichos biflorus* lectin (DBA) (77). Following *in vitro* switch, coverslips of cells infected with cysts of WT, KO *P18* or Cpt *P18* were fixed with 4% paraformaldehyde in PBS for 20 minutes, permeabilized in Triton (0.2%) for 10 minutes, blocked for 30 min with 10% FBS in PBS. T<sub>8</sub>3B<sub>1</sub> or T<sub>8</sub>2C<sub>2</sub> primary monoclonal antibodies directed against P18 and P34 respectively (37) were used at the dilution of 1:500. Biotinylated DBA (Sigma, Cat. NoB-1035) was used at the dilution of 1:100. Anti-mouse secondary antibody (Abcam, ab150116) were used at the concentration of 1:500. Streptavidin (Sigma) was used at the dilution of 1:100. Coverslips were mounted on slides using a Prolong Anti-fade kit (Invitrogen, P36930). Z-section images were acquired by confocal microscopy using a Zeiss LSM 710 confocal microscope (Zeiss, Oberkochen, Germany) and all images were analyzed using Zeiss Zen software.

### **Western blot analysis**

Following *in vitro* switch, HFF cells infected with cysts of WT, KO *P18* or Cpt *P18* were scrapped, washed with PBS and collected by centrifugation. Pellets were re-suspended in 1x Laemmli buffer and proteins were separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes (BIO RAD Cat# 162-0112). Membranes were probed with T<sub>8</sub> 3B<sub>1</sub> primary monoclonal antibody directed against P18 (37), followed by anti-mouse secondary antibody conjugated to Horseradish peroxidase (HRP) (m-IgGk BP-HRP, Santa Cruz, sc-516102, 1:5000). Bands were visualized using luminol chemi-luminescent substrate (Bio-Rad, Cat# 170-5061).

## Quantitative Real Time PCR (qRT PCR)

SYBR green qRT PCR was performed using CFX96 (Biorad). SAG-1 primers were used to quantify mRNA representing tachyzoites in wild type WT, KO *P18* or Cpt *P18* strains (Table 2). Glyceraldehyde-3-Phosphate dehydrogenase (GAPDH) was used as housekeeping gene (Table 2). In qRT-PCR, individual reactions were prepared with 0.25  $\mu$ M of each primer, 150 ng of cDNA and SYBR Green PCR Master 53 Mix to a final volume of 10  $\mu$ l. PCR reaction consisted of a DNA denaturation step at 95°C for 3min, followed by 40 cycles (denaturation at 95°C for 15 sec, annealing at the appropriate annealing temperature for each couple of primers (Table 2) for 60 sec, extension at 72°C for 30 sec). For each experiment, reactions were performed in duplicates and the expression of individual genes was normalized to GAPDH Ct values. The Threshold cycle (Ct) corresponds to the cycle at which there is a significant detectable increase in fluorescence. Data were plotted by calculating  $\Delta$ Ct ( $Ct_{\text{target gene}} - Ct_{\text{GAPDH}}$ ). Thereafter,  $\Delta\Delta$ Ct is calculated according to the Livak method:  $2^{-\Delta\Delta Ct}$  to obtain the percentage of expression (78).

Table 2. Summary of primers used for Real-time quantitative PCR

Primer	Sequence 5'→3'	Annealing T <sup>0</sup> C
Mouse GAPDH Forward Primer	5'-CATggCCTTCCgTgTTCCTA-3'	59.4
Mouse GAPDH Reverse Primer	5'-CCTgCTTCACCACCTTCTTgAT-3'	60.3
SAG-1- Forward primer	5'-ACT CAC CCA ACA ggC AAA TC 3'	56.5
SAG-1 Reverse primer	5'- gAg ACT AgC AgA ATC CCC Cg-3'	56.6
Mouse CXCL9 Forward Primer	5'-TgT ggA gTT CgA ggA ACC CT-3'	60.5
Mouse CXCL9 Reverse Primer	5'-TgC CTT ggC Tgg TgC Tg-3'	57.2
Mouse CXCL10 Forward Primer	5'-AgA ACg gTg CgC TgC AC-3'	57.2
Mouse CXCL10 Reverse Primer	5'-CCT ATg gCC CTg ggT CTC A-3'	61.7

## **Enzyme-linked immunosorbent assay (ELISA)**

Brains from chronically infected BALB/c mice with WT, KO *P18* or Cpt *P18* were harvested at week 4 post-infection (p.i.) with either parasite strain. Following brain homogenization, supernatants were collected, and ELISA was performed using Multi-Analyte ELISArray Kit (Qiagen) according to the manufacturer's instructions. Briefly, supernatants were spun for 10 min at 1000g and transferred to new Eppendorf tubes, and diluted using a specific cocktail of antigens (IL-12, IL-1 $\beta$ , IFN- $\gamma$ , IL-6, Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-10, Monocyte chemoattractant protein 1 (MCP-1), Macrophage Inflammatory Proteins MIP-1 $\alpha$  and MIP-1 $\beta$ ) provided by the kit (Qiagen). Samples were then transferred to ELISA plate, and were incubated for 2 hours. After three washes the detection antibody was added and incubated for 2 hours, followed by Avidin-HRP addition for 30 min. Wells were 4 washed and development solution was added in the dark and kept for 15 min, before addition of the stop solution according to manufacturer's instructions. The optical density (O.D) was determined at 450 and 570 nm and calculated according to the standard values of a positive control according to manufacturer's instructions.

## ***In vivo* study**

Eight to ten weeks old immunocompetent female BALB/c mice were intraperitoneally injected with WT, KO *P18* or Cpt *P18* parasites. Mice experimental protocols are indicated in timelines (Figs 2A, 2B, 3B, 4A and 5A).

To assess the KO *P18* virulence *in vivo*, freshly harvested tachyzoites ( $10^6$  or  $10^5$ ) of KO *P18* or WT strains were i.p. injected into ten mice per condition. Invasiveness of the parasites was evaluated by simultaneous plaque assay of a similar dose of parasites on HFFs. Mouse survival was monitored daily until their death, end-point of all experiments. The immune response of surviving animals was tested day 7 post infection and sera of infected mice were verified by Western blotting against tachyzoite lysates.

To assess the pattern of dissemination of the KO *P18* parasites during the acute phase of infection, 250 freshly harvested tachyzoites of WT, KO *P18* or Cpt *P18* strains

were i.p. injected into ten females per condition. On day 4 post-infection, mice were sacrificed, peritoneal lavage was performed to screen for tachyzoites number and dendritic cells recruitment. Spleens and brains were harvested to assess tachyzoite burden in these organs.

For cyst quantification, and iNOS and cytokine production, brains of infected BALB/c mice were harvested at d28 post-infection. To assess the reactivation capacity of the KO *P18*, ten mice per condition were allowed to establish chronic infection for 28 days, then mice were immunosuppressed by administrating dexamethasone (Medochemie), at the dose of 5mg/L in drinking water, and were assessed for survival.

To elaborate on the role of IFN- $\gamma$ , Immunocompromised SCID and NSG mice were infected by oral gavage with 20 cysts from WT, KO *P18* or Cpt *P18*, to assess their survival (ten mice per condition).

Survival data were represented in Kaplan Meier plot. Histogram and dot plot analysis were performed using GraphPad Prism 7 or Excel software.

## **Statistics**

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

## **Acknowledgments**

We thank the American University of Beirut Core Facilities for providing access to their imaging, Animal Care, and core culture facilities. We also thank the Office of Grants and Contracts at the American University of Beirut. This work was made possible through core support from the Medical Practice Plan (Faculty of Medicine, American University of Beirut) and the American University of Beirut and the Centre National de Recherche Scientifique Libanais (AUB- CNRS- L GRP) funds.

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## Figure legends

**Fig 1: Generation of *P18* knock-out and complemented transgenic lines.** (A) Schematic representation of the knock-out generation. *5'P18-P2854-3'P18* construct is replaced by *P18* by the selectable marker *hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT)*. *5'P18-P2854-3'P18* plasmid was then introduced by electroporation to the WT type II strain to generate KO *P18* parasites. Selection of stably depleted parasites was then performed under Xanthine and Mycophenolic acid. *P18* gene was re-introduced to the KO *P18* for confirmation of its role. The LIC-HA3 vector containing the dihydrofolate reductase (DHFR) selection was used and *P18* was introduced under its own promoter. Following electroporation of the KO *P18* with the LIC-*P18* promoter-*P18*-HA3 generated vector, stable transgenic clones were isolated following pyrimethamine and successful generation of the Cpt *P18* complemented with the *P18* gene was obtained. (B) Gel electrophoresis following PCR amplification for the verification of the successful integration of the *5'P18-P2854-3'P18* and LIC-*P18* promoter-*P18*-HA3 in the generated KO *P18* and Cpt *P18* transgenic strains respectively. (C) Western Blot analysis for the verification of the stable generation of KO *P18* and Cpt *P18* strains, in both tachyzoites and bradyzoites following *in vitro* switch. Two exposures are presented reflecting the abundance of P18 in the bradyzoites as compared to its low expression in tachyzoites of the WT strain. The results depict one representative experiment among three independent ones. (D) Confocal microscopy following IFA assay on *T.gondii* cysts after *in vitro* switch. P18 abrogation is confirmed in the KO *P18* strain (middle panel) as compared to the WT strain (upper panel). *P18* gene is restored in the complemented Cpt *P18* strain (lower panel). P18 protein expression was used using T<sub>8</sub>B<sub>1</sub> (green), cysts were stained using a biotinylated lectin (red), with specific binding to a selectin on the cyst wall. . Scale bar = 10 μM. The results depict one representative experiment among at least three independent ones.

**Fig 2: P18 depletion impacts the acute phase of the infection by dramatically affecting the parasite virulence, dissemination to organs and recruitment of dendritic cells in mice.** (A) Timeline schedule for the assessment of lethal parasitic dose.

Survival of BALB/c mice infected with  $10^6$  (left panel, 10 mice per condition), or  $10^5$  (right panel, 10 mice per condition) of WT or KO *P18*. **(B)** Timeline schedule for assessment of parasite number, dissemination to organs and recruitment of dendritic cells. On day 0, mice were injected with 250 tachyzoites/mouse of either WT, KO *P18*, or Cpt *P18* strains. Tachyzoites count in the peritoneal lavage (left panel, 15 mice per condition), percentage of CD11c<sup>+</sup> (right panel, 8 mice per condition) were assessed at day 4 post infection as indicated. Quantitative Real-Time PCR for SAG-1 transcripts in spleens of mice injected with WT, KO *P18*, or Cpt *P18* strains and sacrificed at day 4 post-infection (right panel, 15 mice per condition). **(C)** Quantitative Real-Time PCR for SAG-1 transcripts in brains of mice injected with WT, KO *P18*, or Cpt *P18* strains and sacrificed at day 4 post-infection (15 mice per condition). SAG-1 expression was normalized to GAPDH. The results are expressed as percentage of untreated control ( $\pm$ ) SD and depict one representative experiment among two independent ones. The t-test was performed to validate significance. \*, \*\* and \*\*\* indicate p values  $\leq 0.05$ ; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant.

**Fig 3: KO *P18* parasites form more bradyzoite cysts *in vitro* and *in vivo*.** **(A)** Quantification and size of cysts by confocal microscopy following IF assay, after *in vitro* switch using a biotinylated lectin (green), with specific binding to a selectin on the cyst wall and the bradyzoite marker P34 (red). The results depict one representative experiment among at least three independent ones. Number of cysts was determined in 50 independent fields per condition. Scale bar = 2  $\mu$ M. **(B)** Timeline schedule for assessment of bradyzoite formation/number following *P18* depletion in BALB/c mice. Briefly, on day 0, BALB/c mice were injected with 250 tachyzoites/mouse of the WT, KO *P18*, or Cpt *P18* strains. Acute toxoplasmosis was verified on day 7 p.i. On day 28 p.i., Brains of *Toxoplasma* positive mice were harvested. Cyst count following percoll extraction (10 mice per condition). The results are expressed as percentage of untreated control ( $\pm$ ) SD and depict one representative experiment among at least three independent ones. The t-test was performed to validate significance. \*, \*\* and \*\*\* indicate p values  $\leq 0.05$ ; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant.

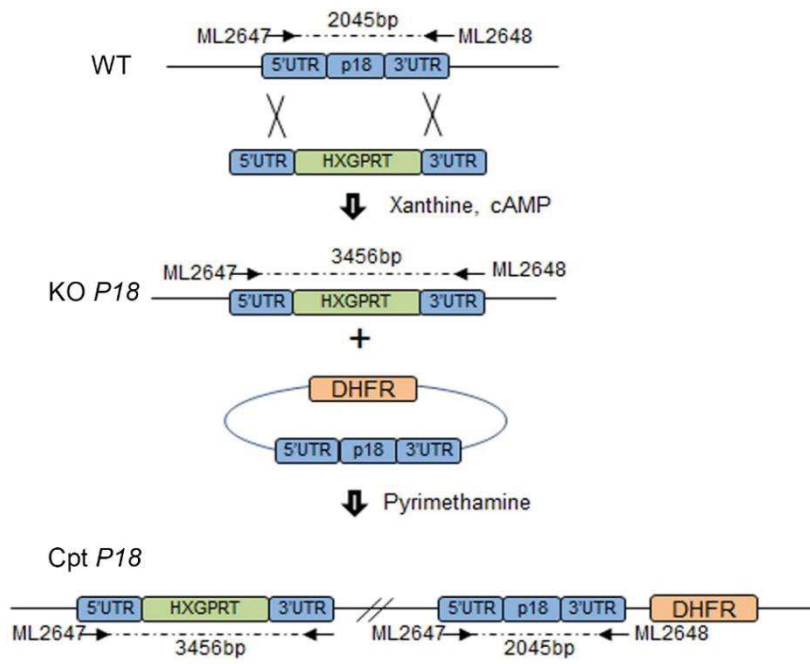
**Fig 4: KO  $\Delta P18$  elicits a higher immune response favoring the higher number of bradyzoites cysts upon establishment of chronic infection in BALB/c mice. (A)** Timeline schedule for assessment of immune response following *P18* depletion in BALB/c mice. Briefly, on day 0, BALB/c mice were injected with 250 tachyzoites/mouse of the WT, KO *P18*, or Cpt *P18* strains. Acute toxoplasmosis was verified on day 7 post-infection. On day 28, brains of *Toxoplasma* sero-positive mice were harvested. Quantitative Real-Time PCR for iNOS (right panel, 10 mice per condition) from brains of mice injected with WT, KO *P18*, or Cpt *P18* strains. iNOS expression was normalized to GAPDH. **(B)** ELISA showing the secretion levels of different cytokines/chemokines (IL-12, IL-1 $\beta$ , IFN- $\gamma$ , IL-6, TNF- $\alpha$ , IL-10, MCP-1, MIP-1 $\alpha$  and MIP-1 $\beta$ ) in BALB/c mice chronically infected with the WT, KO *P18*, or Cpt *P18* strains. **(C)** Quantitative Real-Time PCR for CXCL-9 and CXCL-10 (12 mice per condition) from brains of mice injected with WT, KO *P18*, or Cpt *P18* strains. CXCL-9 and CXCL-10 expressions were normalized to GAPDH. The results are expressed as percentage of untreated control ( $\pm$ ) SD and depict one representative experiment among at least three independent ones for A and B, and of two independent experiments for C. The t-test was performed to validate significance. \*, \*\* and \*\*\* indicate p values  $\leq$  0.05; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant.

**Fig 5: KO *P18* parasites induce a delayed reactivation in BALB/c and affects the interconversion from bradyzoites to tachyzoites in an IFN- $\gamma$  dependent fashion. (A)** Timeline schedule for establishing chronic infection in BALB/c mice and stimulating immunosuppression. On day 0, mice were injected with 250 tachyzoites/mouse of either WT, KO *P18*, or Cpt *P18* strains. Acute toxoplasmosis was verified on day 7 post-infection. On day 28, mice were treated with the immunosuppressive dexamethasone drug until death. **(B)** Survival of immunosuppressed BALB/c mice infected with WT, KO *P18*, or Cpt *P18* strains following reactivation of chronic toxoplasmosis (left panel, 15 mice per condition). Quantitative Real-Time PCR for SAG-1 (right panel, 15 mice per

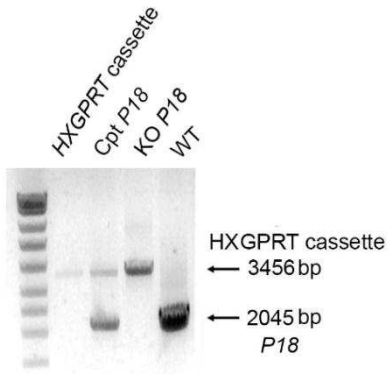
condition) from mice injected with WT, KO *P18*, or Cpt *P18* strains. SAG-1 expression was normalized to GAPDH. The results are expressed as percentage of untreated control ( $\pm$ ) SD and depict one representative experiment among at least three independent ones. The t-test was performed to validate significance. \*, \*\* and \*\*\* indicate p values  $\leq$  0.05; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant. **(C)** Survival of SCID (left panel, 10 mice per condition) versus NSG mice (right panel, 10 mice per condition) following oral gavage with 20 cysts of the WT, KO *P18*, or Cpt *P18* strains.

Figure 1

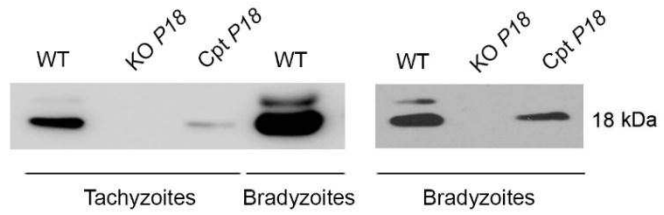
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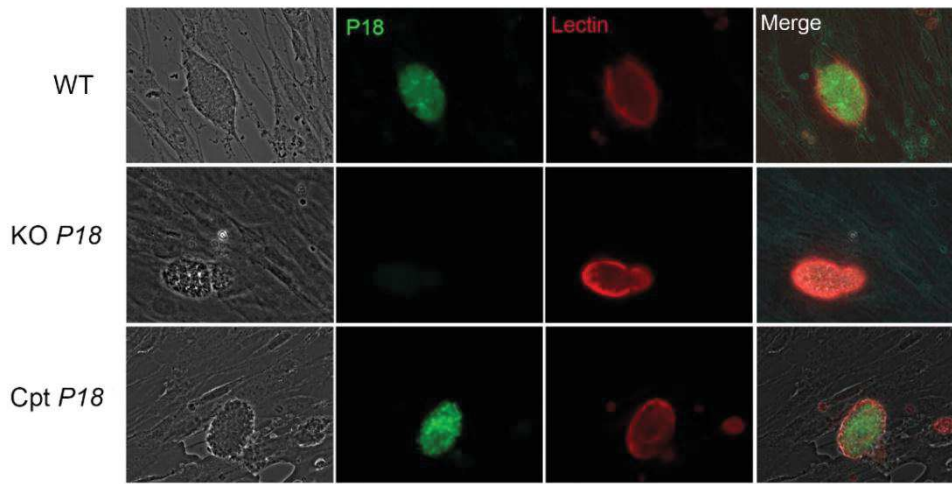
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**Figure 2**

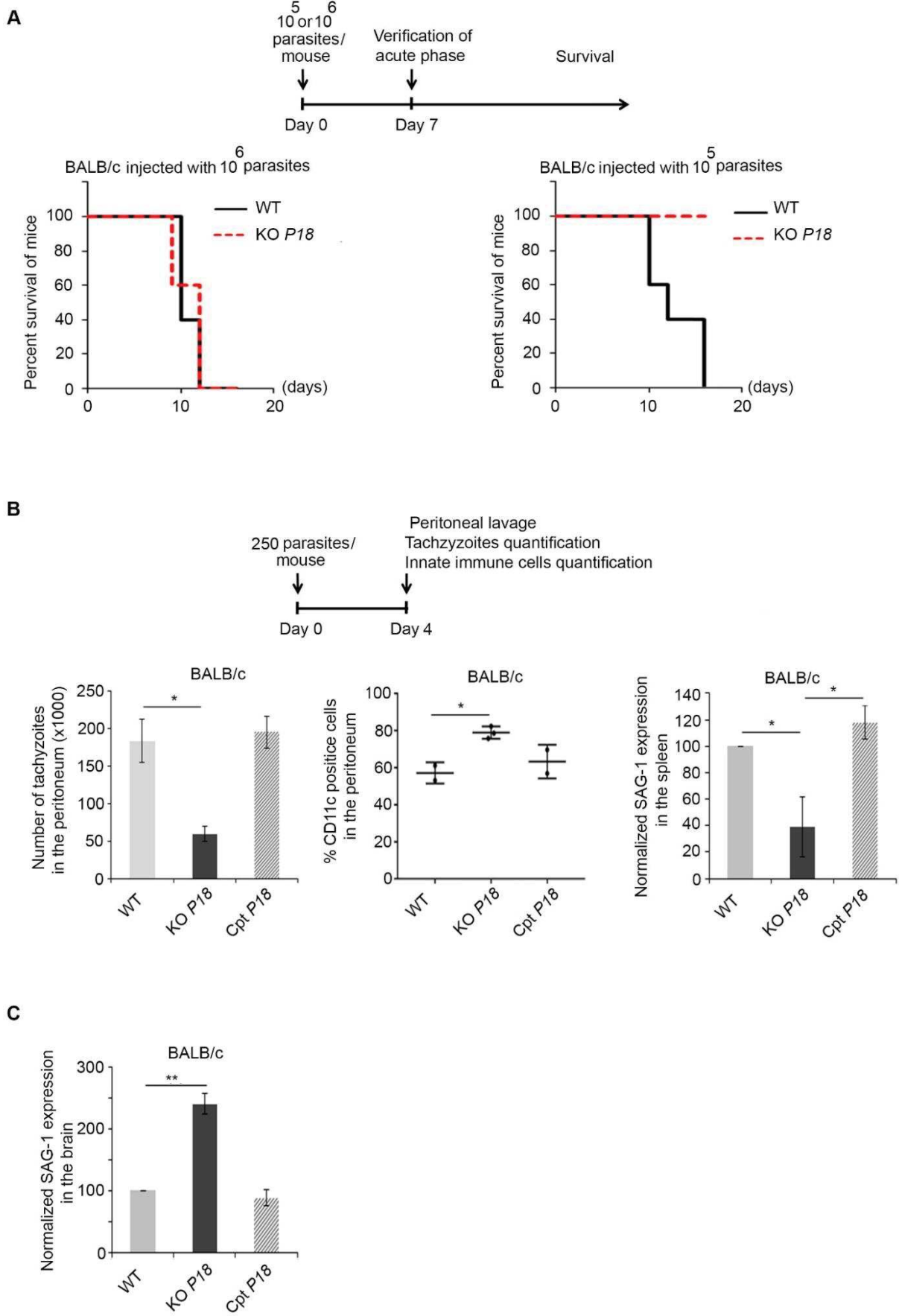
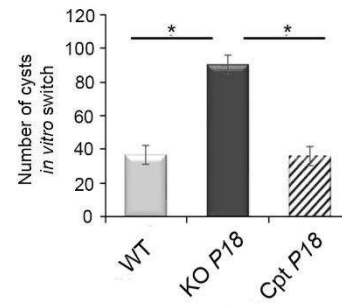
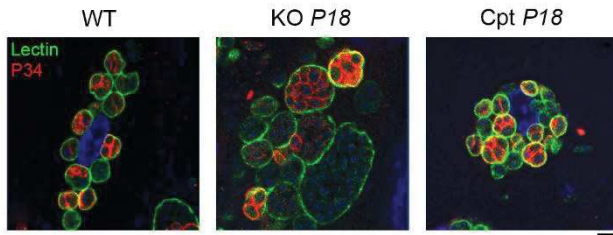
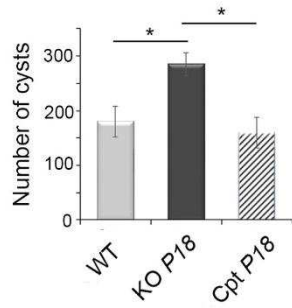
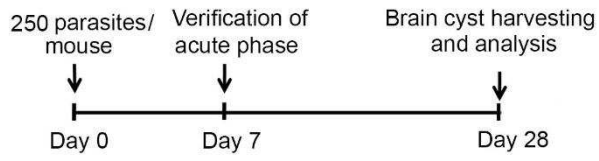


Figure 3

A

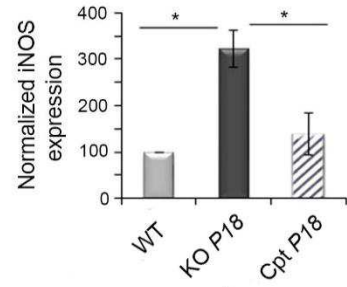
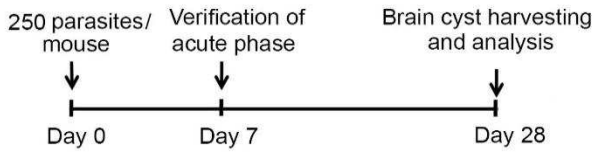


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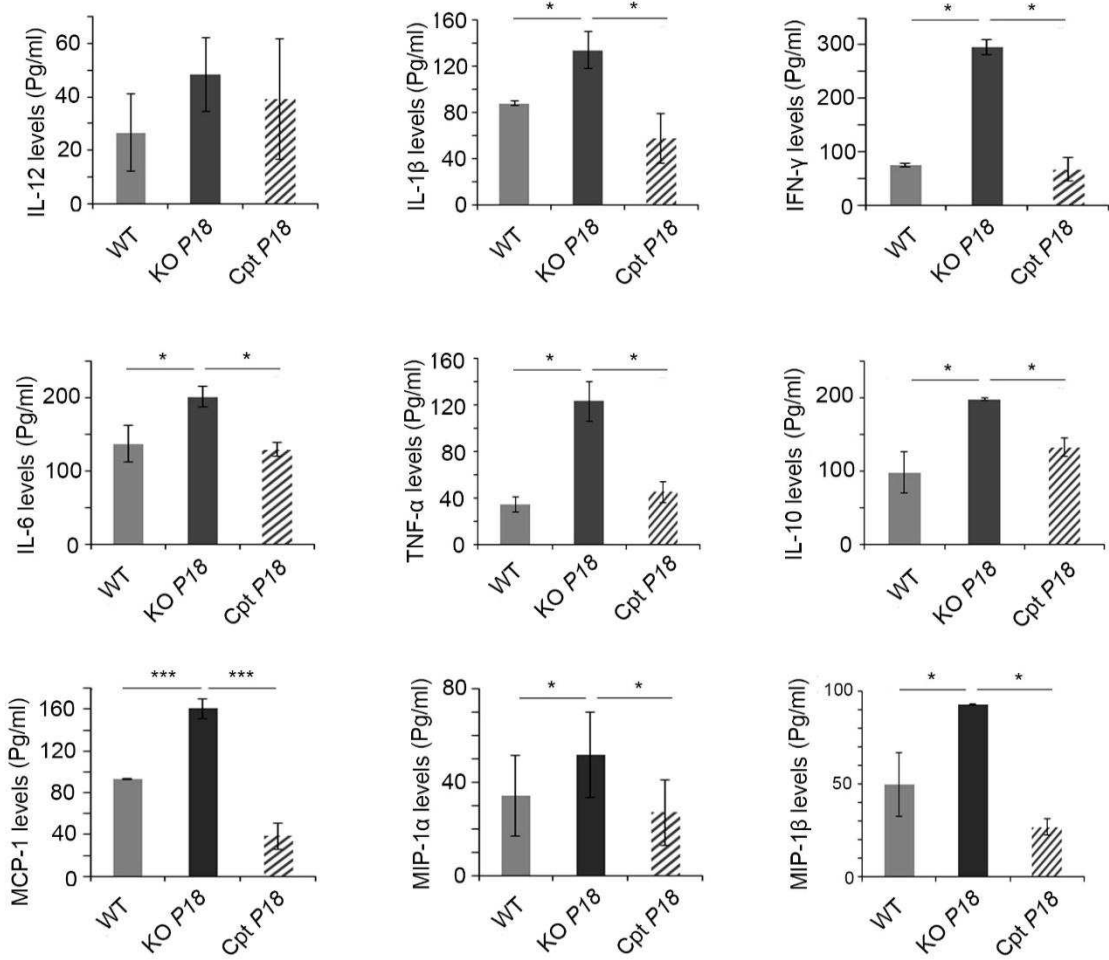


**Figure 4**

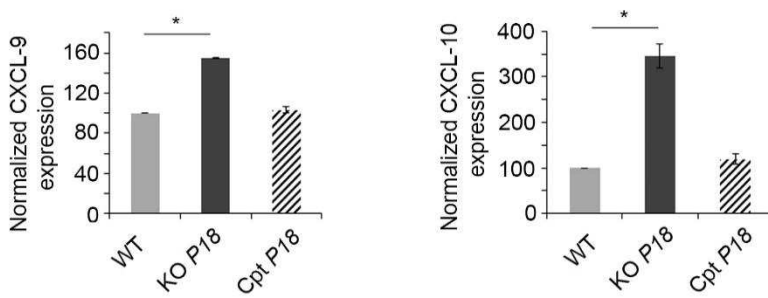
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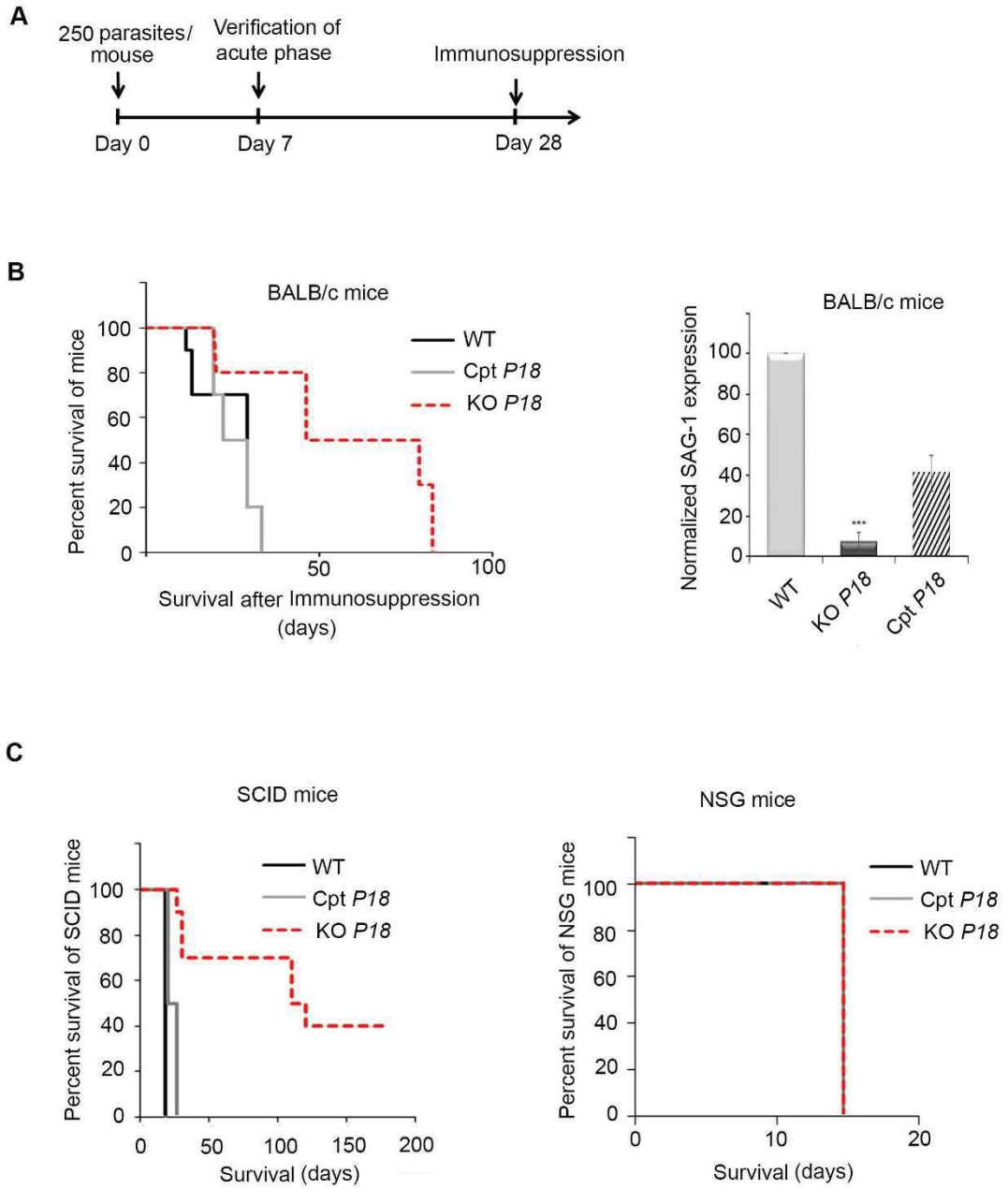
**B**



**C**



**Figure 5**



# DISCUSSION

*Toxoplasma gondii* is one of the most successful and widespread parasitic pathogen that infects, not only one third of the population worldwide, but also all warm blooded animals including mammals, reptiles, and birds (J. P. Dubey, 1998; J. P. Dubey et al., 2010) reviewed in (Schluter & Barragan, 2019) . This parasite is responsible for health problems and contributes to high economic losses due to its burden in chicken, sheep, fish, ovine, and bovine among others (Dong et al., 2018; Innes et al., 2009) In mammals including humans, *Toxoplasma* causes acute and chronic toxoplasmosis (AT and CT) (Blader & Saeij, 2009; J. P. Dubey, 1998). In certain countries including Lebanon, the prevalence of this parasitic infection reaches an alarming percentage of almost 70% of the population (Bouhamdan et al., 2010).

In France, we estimated seroprevalence to be 41 to 53% (Fromont et al., 2009). In the USA, more than 40 million people are infected and the burden of the parasite is leading to high economic losses. This prompted the Centers for Disease Control and Prevention (CDC) to consider toxoplasmosis as a neglected parasitic infection, which requires public health action (Ben-Harari & Connolly, 2019).

Toxoplasmosis is largely controlled by the host immune system. Indeed, the infection leads, at the first place, to the recruitment of innate immune cells, primarily DCs and monocytes, to the site of infection (Dupont et al., 2012). The interplay between the parasite and the host immunity was well documented. Indeed the parasite has multiple virulence factors known to modulate the host immunity. These include micronemes, rhoptry proteins and dense granules among several other factors (Hunter & Sibley, 2012; Poncet, Blanchard, & Marion, 2019). Yet, members of the SRS family differentially expressed during life cycle stages of the parasite are important for the initiation of infection, modulation of host immunity and establishment of transmissible infections (Boothroyd et al., 1998; Manger, Hehl, Parmley, et al., 1998; Manger, Hehl, & Boothroyd, 1998). For instance, the tachyzoite-specific SAG1/SRS29B functions as an adhesin, and influences virulence in mice (Boulanger et al., 2010; He et al., 2002; Manger, Hehl, Parmley, et al., 1998; Mineo et al., 1993; Pollard et al., 2008; Rachinel et al., 2004). In addition, SAG1/SRS29B, along with other tachyzoite-specific members like SRS34A, and SRS29C proteins, elicit high antibody titers during the acute phase of the infection (Lekutis et al., 2001). We showed that another member of the SRS family,

P18/SRS35 plays a role in modulating the host immunity at multiple levels. During AT, this protein, albeit not abundantly expressed in the tachyzoites stage (Odberg-Ferragut et al., 1996; Wasmuth et al., 2012), seems to lower the recruitment of DCs to the site of infection, as its depletion induced higher numbers of DCs to the peritoneum of infected mice. This result can suggest that P18/SRS35 may counterbalance the high induced immune response by SAG-1/SRS29B to ensure parasite survival and escape from the host immune response, thus ensuring its transmission to the brain and skeletal muscles to sustain its life cycle.

Recruited immune cells, mainly DCs, monocytes and macrophages are known to be used by the tachyzoites as “Trojan Horses”, to present antigens, elicit adaptive immune responses, and to spread to various tissues (Hunter & Sibley, 2012; Melo et al., 2011). Under this immune pressure, tachyzoites will transform into bradyzoite cysts in the brain and in skeletal muscles, ensuring the parasite sustainability for further propagation between intermediate hosts, or between intermediate and definitive hosts. At the molecular level, we showed that P18 depleted parasites presented with a lower number of tachyzoites in spleens of infected mice while a higher number of tachyzoites reached the brain (Sumyuen et al., 1995; Zenner et al., 1999). This higher number in the brain corroborated with a higher number of bradyzoite cysts. On the therapeutic level, we showed that Imiquimod affects parasite replication in human macrophages proving the anti-parasitic efficacy of this drug on the replication of the parasites *in vitro*. In addition, Imiquimod led to a decrease in the number of tachyzoites in the spleen and the peritoneum of infected mice during AT, by eliciting an adaptive immune response as early as two days post-treatment. Our results show, not only a protein marker that plays a role in the virulence and the dissemination of the parasite during AT, but also an efficacious treatment that increases mounting of a fast adaptive immune response to clear the infection during AT. These results can be extrapolated to the human patients who present with AT, as well as to animals dying from AT, especially following congenital toxoplasmosis, which leads to high economic losses (Wallon & Peyron, 2018).

The high burden of disseminated tachyzoites to the brains of infected mice upon depletion of P18, elicited a stress response translated by higher iNOS levels. This was concurrent with a high inflammatory response, which led to the formation of more

bradyzoite cysts in the brain of infected mice when P18 was depleted. On the other hand, treatment with imiquimod during AT, sharply reduced the number of bradyzoite cysts upon establishment of CT. Our results offer a better understanding on a surface antigen marker, which implicates the immune and stress responses, upon establishment of CT, and a therapeutic modality which, when used during AT, negatively impacts the establishment of CT in the brain of intermediate hosts, and highly affects the interconversion to acute phase. This was demonstrated by the failure of establishment of a new AT, upon oral gavage of new mice with cysts from Imiquimod treated mice. These results offer a better understanding on the conversion between AT and CT and show the efficacy of an immunomodulatory drug, if used during AT, on the establishment of CT, and thus affecting the persistence of the parasite.

On an established CT in murine models, we showed that, albeit the higher number of obtained cysts upon depletion of P18, CT failed to reactivate upon immunosuppressing the mice with dexamethasone. This offers a better understanding of such an occurring condition in immunocompromised patients. Moreover, treatment with Imiquimod significantly reduced the number of bradyzoite cysts in chronically infected mice. This result is to our sense, one of the most important findings of this work, in light of the absence of a current treatment that targets CT, which is the most common and widespread form of the infection (Montazeri et al., 2016; Montazeri et al., 2017). Imiquimod decreased p21, exclusively expressed in bradyzoites, and increased P30, exclusively expressed in tachyzoites, indicating the induction of interconversion. While this interconversion was important to elicit immune cell responses via TLR expression by innate immune cells, Imiquimod is less likely to be potentially administered to immunocompromised patients. Indeed, the Imiquimod-induced interconversion of bradyzoites to tachyzoites in the brains of chronically infected mice, led to the overexpression of TLR-7, 11 and 12 and their downstream signaling. Activation of these TLRs upon treatment with Imiquimod indicates that this drug could successfully cross the blood brain barrier to exert its effect. TLR-11 and 12 are known to be expressed in monocytes, macrophages and DCs, which represent a major forefront exploited by the parasite, due to their capacity to secrete defense molecules, and to the capacity of DCs to present antigens mediating crosstalk to T cells (Sanecka & Frickel, 2012). TLR-7, is



expressed by microglia, in addition to these mentioned innate immune cells (Butchi, Du, & Peterson, 2010). DCs recognize two parasitic PAMPs, Profilin and cyclophilin-18. Profilin binds TLR-11 (Yarovinsky et al., 2005) and TLR-12 (Koblansky et al., 2013; Sanecka & Frickel, 2012), and enhances the production of IL-12 via MyD88 dependent pathway. Our results demonstrated that Imiquimod upregulates TLR-11 and 12, most likely by recruited DCs and monocytes, triggering our observed MyD88-downstream signaling, the activation of MAPK, and the subsequent secretion of immune mediators including IL-1 $\beta$ , IL-12 and IFN- $\gamma$ . The production of IL-12 and IL-1 $\beta$  may be essential for the recruitment of neutrophils and natural killer cells, which will produce IFN- $\gamma$  until recruited T cells produce this cytokine and control the infection (reviewed in (T. Scharon-Kersten, Nakajima, Yap, Sher, & Leonard, 1998; Sher et al., 1993; Sturge & Yarovinsky, 2014; Y. Suzuki et al., 1988; Yarovinsky, 2014). A strain depleted for Profilin is not affected upon treatment with Imiquimod and TLR-11 and 12 expression levels in cells infected with this parasite line. These results suggest that Imiquimod may enhance the binding of this parasite PAMP, to induce TLR-11 and 12 mediated MyD88 signaling, to activate downstream signaling pathways and the corresponding immune response (LaRosa et al., 2008; Yarovinsky et al., 2005). This result coincides with the activation of phosphorylated ERK1/2 in the treated mice as compared to the untreated one, and the triggered induced immune response upon treatment with Imiquimod. TLR-11 and TLR-12 are functional in mice but not in humans (D. De Nardo, 2015). Our obtained results are still very promising and can be extrapolated to target the transmission of the parasite between intermediate and definitive hosts, thus interfering with the transmission and the life cycle of the parasite. Although humans do not express either TLR-11 or TLR-12, human monocytes produce pro-inflammatory cytokines in response to *T. gondii* infection, suggesting that other TLRs in humans recognize different compartments of *T. gondii* to produce IL-12 in antigen-presenting cells (W. A. Andrade et al., 2013). It has been described that parasite recognition by intracellular TLRs (TLR3, 7 and 9) in humans facilitates resistance to toxoplasmic infection and activation of monocytes and human DCs (W. A. Andrade et al., 2013; Sher et al., 2017). A study of human innate receptors showed that the human TLR5 may have a similar role to the mouse TLR-11, in activating cytokine production (Salazar Gonzalez et al., 2014). The

effect of Imiquimod on TLR-5, and probably on TLR3 and 9, is thus worth investigating. Interestingly, we showed that Imiquimod, that was initially generated as a TLR-7 agonist (Schon & Schon, 2007) leads to the upregulation of TLR-7. TLR-7 is an endosomal receptor, known to recognize ribonucleic acid (Sher et al., 1993), and can signal through MyD88 to induce inflammatory cytokines (Goff et al., 2017; Nickerson et al., 2010). This TLR is common between human and mouse species, thus can offer a broader understanding of the mechanism of action through which Imiquimod exhibits its efficacy among different species. This endosomal TLR can be expressed in monocytes, macrophages, DCs, microglia among other resident or wandering brain cells (Kielian, 2006). Previous studies demonstrated that Imiquimod acts in the context of leishmaniasis, another parasitic infection, *via* binding to TLR-7, leading to the activation of the NF- $\kappa$ B signaling pathway (Arevalo et al., 2001; El Hajj et al., 2018). In our study, Imiquimod proved a high efficiency against another parasitic infection and signaled through TLR-7. The observed upregulation of TLR-7, after treatment of CT with imiquimod, can also contribute to the observed MyD88 signaling and the induced immune response. This result shows first the importance of repositioning medicine, and second reflects a broader anti-parasitic activity of this immuno-modulatory drug proving its promising potency.

Adaptive immune response is a key mechanism by which the host fights against *Toxoplasma* (Dupont et al., 2012). Noteworthy, the efficient recruitment of T cells to both peritoneum and spleen during AT, may imply the other parasite PAMP, cyclophilin-18, which is recognized by both mouse and human C-C chemokine receptor type 5 (CCR5) (Yarovinsky et al., 2004). During AT, we showed that Imiquimod leads to a relatively early recruitment of T cells to the sites of infection. This effect demonstrates that Imiquimod enhances mounting the adaptive immune response and might involve cyclophilin-18, which requires further investigation.

At the level of the brain, P18 depletion treatment yielded higher expression of CXCL9, which is crucial to recruit T cells into the brain and to prevent reactivation of CT (Ochiai et al., 2015), along with a higher number of cysts and a deficit in the reactivation capacity of these parasites. On the other hand, Imiquimod treatment induced the interconversion from bradyzoites to tachyzoites and led to the activation of innate immune response, which thereafter was accompanied by the increase of CXCL9 and 10,

presumably indicating the recruitment of T cells to clear the infection at the reactivated foci. Indeed, these results may suggest a role for P18 in modulating and activating DCs to travel from the infection site to the brain. Similarly, Imiquimod treatment may exhibit a similar role on DCs. This might implicate production of IL-12 by DCs, and to present antigens to induce the recruitment and activation of T cells, hence controlling both the persistence and reactivation of cerebral toxoplasmosis. Within the brain, CT correlates with several neuro-pathologies and cancers (Bannoura et al., 2018; Ben-Harari et al., 2017; Fekadu et al., 2010; Gharamti et al., 2018; Katlama, De Wit, et al., 1996; Koblansky et al., 2013; Miranda-Verastegui et al., 2009; Montoya & Liesenfeld, 2004; Ochiai et al., 2015; Skariah et al., 2010; Thirugnanam et al., 2013; Watts et al., 2017). Thus, understanding the molecular mechanisms of CT and reducing the cyst number within the established CT may strongly help affecting parasite survival, to eradicate its persistence and transmission, hence targeting its related diseases.

During the last decade, eighty clinically available drugs, including a large number of new compounds were used against *T.gondii* *in vitro* and *in vivo* (reviewed in (Montazeri et al., 2017)). However, most of these drugs are effective against tachyzoites, and only very little trigger bradyzoites or the back and forth switch between both stages. An ideal drug against toxoplasmosis, should not only be effective against the proliferative tachyzoite stage of the parasite, but it should also exert an activity against the tissue cyst stage, especially that the chronic form is the most common form of the disease in humans and other intermediate hosts. Throughout our work, we proved the potency of Imiquimod against bradyzoite cysts. We also proved that deleting P18 led to higher number of brain cysts, yet a delay in the reactivation. Hence, our study offers a better molecular characterization of persistence and reactivation of CT, and more importantly an efficient therapeutic modality targeting it. This study can address diseases associated with CT and has a far-reaching impact on animal health, since it is important to keep in mind that rodents play a crucial role in the predator-bait life cycle of the parasite, whereas humans are accidental intermediates host of *T. gondii* (R. T. Gazzinelli, Mendonca-Neto, Lilue, Howard, & Sher, 2014). Imiquimod, as supported by our data, stands to become a major player in *T.gondii* related diseases, since it at least equaled or exceeded the results obtained from current gold standard treatment.

Together, our approach should result in a better understanding of Host/Parasite interaction in the context of *Toxoplasma* infection and paves the path towards a comprehensive solution to this endemic disease.

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# **RÉSUMÉ FRANÇAIS DE LA THÈSE**

## **1. Généralités**

La toxoplasmose est une parasitose infectieuse causée par le protozoaire intracellulaire obligatoire *Toxoplasma gondii*, appartenant au phylum des Apicomplexes. Ce parasite est capable d'infecter tous les animaux à sang chaud, y compris l'homme. Le cycle de vie du toxoplasme nécessite un hôte définitif, qui peut être n'importe quel membre des félinés, le plus souvent un chat, chez lequel se déroule la partie sexuée du cycle. Ceci conduit à la formation des oocystes excrétés dans les crottes de l'animal. Dans l'environnement, ces oocystes deviennent sporulés, et contiennent deux sporocystes contenant quatre sporozoïtes chacun. L'ingestion de ces oocystes par un hôte intermédiaire, déclenche la voie asexuée du cycle parasitaire. Suite à l'acidité de l'appareil digestif, les sporozoïtes sont libérés dans l'intestin de l'hôte intermédiaire, et se transforment rapidement en tachyzoïtes, formes répliquatives rapides responsables de la phase aiguë de la maladie. Ces stades sont capables d'infecter toutes les cellules nucléées, et se transforment, suite à la pression de la réponse immunitaire de l'hôte intermédiaire, en formes répliquatives plus lentes, dites bradyzoïtes. Ces derniers stades s'enkystent dans le cerveau et les muscles squelettiques, causant la phase chronique de toxoplasmose.

Environ un tiers de la population mondiale est infecté par ce parasite. De plus, la prévalence augmente avec l'âge et varie en fonction des régions, de l'environnement, du régime alimentaire et de l'hygiène. En France, comme au Liban, la prévalence de ce parasite peut aboutir à 70% de la population. Aux Etats Unis, plus de quarante millions de personnes sont infectées par ce parasite. Récemment, le Centre de contrôle et de prévention des maladies infectieuses (CDC) a considéré la toxoplasmose comme une infection parasitaire tropicale négligée, nécessitant des mesures de santé publique. L'homme contracte ce parasite suite à la consommation de viande crue ou mal cuite, contaminée par des kystes de bradyzoïtes, des crudités mal lavées ou de l'eau souillée par des oocystes libérés dans les crottes du chat.

Bien que la toxoplasmose soit considérée comme asymptomatique chez plus de 80% de patients immunocompétents, des associations assez récentes sont établies entre cette infection et un nombre de maladies. En effet, certains affirment que les niveaux élevés d'immunoglobuline G chez les femmes enceintes, séropositives pour le parasite, sont liées à l'anxiété et à la dépression prénatale. D'autres établissent un lien entre la

séropositivité au Toxoplasme, la dépression et les tentatives de suicide. De plus, la phase chronique de la maladie corrèle positivement avec la progression de plusieurs troubles comportementaux et neurologiques tels que la schizophrénie et la maladie de Parkinson, parmi d'autres. Les anticorps anti-Toxoplasme ont également été signalés plus élevés chez des patients ayant différents types de cancers, en particulier ceux atteints d'un cancer du cerveau ; cela pourrait être dû à la capacité du parasite à interférer avec le micro-ARNome cérébral.

Chez les patients immunodéprimés, la maladie peut devenir grave, voire même fatale. C'est le cas dans le contexte d'une toxoplasmose congénitale ou d'une neurotoxoplasmose. La toxoplasmose congénitale est une embryofœtopathie caractérisée par des lésions oculaires, viscérales ou intracrâniennes secondaires à une primo-infection maternelle par le parasite durant la grossesse. Quant à la neurotoxoplasmose, elle se manifeste chez les patients immunodéprimés, la réactivation des bradyzoïtes en tachyzoïtes est responsable de la nécrose des tissus cérébraux. Cette réactivation peut également se produire chez les patients sidéens, ou chez ceux qui reçoivent des traitements immunosuppresseurs, dans le contexte de greffe de cellules souches hématopoïétiques, de greffe d'organe ou de chimiothérapie contre le cancer.

## **2. Traitements de la toxoplasmose**

Les traitements actuels de la toxoplasmose demeurent limités aux drogues antiparasitaires et antibactériennes classiques. Il s'agit de la spiramycine, l'azithromycine, l'atovaquone, la pyriméthamine-sulfadiazine, la pyriméthamine-clindamycine et le triméthoprime-sulfaméthoxazole. En outre, le traitement recommandé reste l'association de la pyriméthamine, un inhibiteur de l'enzyme dihydrofolate réductase (DHFR), et de la sulfadiazine, un inhibiteur de dihydroptéroate synthase. Cette association est habituellement administrée avec de l'acide folinique, ce qui bloque la biosynthèse du folate, donc la synthèse des acides nucléiques du parasite inhibant sa réplication. Cependant, ce régime de traitement s'avère être associé à plusieurs effets secondaires hématologiques tels que la neutropénie, une baisse importante du nombre de plaquettes, la thrombocytopénie, la leucopénie, une élévation du taux de créatinine et des enzymes

hépatiques, des réactions allergiques d'hypersensibilité et surtout l'émergence de parasites résistants, en particulier chez les patients immunodéprimés.

De plus, ces médicaments, administrés comme agents prophylactiques ou thérapeutiques, ne ciblent que la phase aiguë de l'infection et demeurent inefficaces contre les kystes tissulaires, caractérisant la forme chronique de la toxoplasmose. A l'heure actuelle, aucun traitement utilisé n'élimine les kystes tissulaires et cérébraux responsables de la toxoplasmose chronique.

### **3. SAG-4 ou p18 : découverte et clonage**

Pendant de longues années, la recherche sur le toxoplasme fut focalisée sur le stade tachyzoïte, qui est facile à cultiver. Cependant, l'importance de l'interconversion de la phase aiguë à la phase chronique, pour le maintien du cycle parasitaire et le progrès de la maladie, suscita l'intérêt pour l'étude du bradyzoïte. Des anticorps contre les marqueurs spécifiques des bradyzoïtes furent donc développés. Ces anticorps monoclonaux incluent T<sub>8</sub> 4A<sub>12</sub> reconnaissant une protéine de 36 kDa nommée P36, T<sub>8</sub> 2C<sub>2</sub> reconnaissant une protéine de 34 kDa appelée P34, T<sub>8</sub> 4G<sub>10</sub> reconnaissant une protéine de 21 kDa appelée P21 et T<sub>8</sub> 3B<sub>1</sub> reconnaissant une protéine de 18 kDa appelée P18. Ces protéines représentent quatre antigènes pelliculaires, dont trois sont exposés à la surface du parasite. Parmi ces trois marqueurs de surfaces de bradyzoïtes (P34, P21 et P18), seul le gène codant pour P18 a été séquencé et publié et ne présentait aucune homologie substantielle avec aucun des gènes connus. Conformément à la nomenclature proposée par Sibley, le gène *P18* a été nommé *SAG4*. Plus récemment, par analogie de séquence, SAG4 ou P18 a été attribué à la famille de protéines de surface (Surface-Antigen 1 (SAG-1) Related Sequence) (SRS), et le gène codant pour cette protéine était nommé SRS35. Bien que des anticorps dirigés contre P18 aient été générés contre le stade du bradyzoïte, des études plus récentes de transcriptome (Express Sequence Tag ou EST) ont révélé l'expression des transcrits de P18, très faible, dans les tachyzoïtes. Chez les bradyzoïtes, les EST (Express Sequence Tag) de P18 sont très abondants, faisant de cette protéine l'une des SRS les plus abondamment exprimée chez ce stade. La fonction de P18 demeure énigmatique.

#### **4. Les récepteurs Toll-Like dans le contexte de la toxoplasmose**

Suite à l'infection par *T. gondii*, les cellules immunitaires de type inné migrent vers le site de l'infection où elles détectent le parasite, principalement *via* les récepteurs de type Toll-Like (TLR). Les TLR jouent un rôle majeur dans la reconnaissance de *T.gondii*, *via* deux « Pathogen-associated molecular pattern » (PAMP) parasitaires bien identifiées, la profiline et la cyclophiline-18. La profiline est une protéine parasitaire, qui ressemble à l'actine, et qui s'avère indispensable à l'invasion et à la sortie active du parasite à partir des cellules infectées. La profiline se lie au TLR-11, en formant un hétérodimère avec TLR-12, induisant la production de l'interleukine-12 (IL-12) par les cellules dendritiques, suite à l'activation de la voie de signalisation MyD88, et par conséquent, la réponse immunitaire correspondante. Des parasites dépourvus de profiline sont incapables d'induire la production d'IL-12 dépendante de TLR-11, à la fois *in vitro* et *in vivo*. La cyclophiline-18 est reconnue par le récepteur de chimiokine CC de type 5 (CCR5) aussi bien chez les souris que les êtres humains. La cyclophiline-18 favorise la prolifération et la migration des macrophages et des cellules de la rate (principalement des lymphocytes T), vers le site de l'infection, afin de maintenir l'interaction entre le parasite et les cellules immunitaires de l'hôte, notamment les neutrophiles et les monocytes inflammatoires, au niveau du site de l'infection.

Bien que ni les TLR-11 ni TLR-12 ne soient pas exprimés chez l'homme, les monocytes humains produisent des cytokines pro-inflammatoires en réponse à l'infection par *T. gondii* ; ceci suggère que d'autres TLR chez l'homme reconnaissent différents compartiments du parasite, pour produire l'IL-12 par les cellules présentatrices d'antigène.

#### **5. Imiquimod**

L'Imiquimod, (S-26308, R-837) (1- (2-méthylpropyl) -1H-imidazo [4,5-c] quinoléine-4-amine), est le premier membre de la famille des imidazoquinoléines, et appartient à la classe de médicaments appelés modificateurs de la réponse immunitaire. Cet analogue nucléosidique non osidique de la famille des imidazoquinoléines est le premier modificateur de la réponse immunitaire, et est utilisé pour le traitement des maladies cutanées infectieuses, notamment les maladies cutanées virales. En effet,

l'Imiquimod présente une activité antivirale et anti tumorale considérable *in vivo*. Cet agent a été approuvé en 1997, comme traitement topique activant le système immunitaire contre certaines infections virales, telles que le virus du papillome humain (VPH) causant des verrues génitales et périanales. Ce médicament s'avère également efficace contre certains types de cancers de la peau tels que le carcinome baso-cellulaire, la maladie de Bowen, certains mélanomes ... En effet, L'Imiquimod inhibe la mélanogénèse et la prolifération des mélanocytes humains. Son spectre thérapeutique est également étendu aux lymphomes cutanés à cellules B. Le mécanisme d'action exact par lequel l'Imiquimod active le système immunitaire n'est pas encore connu. Néanmoins, il est connu que l'Imiquimod active les cellules immunitaires, en se liant au récepteur endosomal TLR-7, couramment impliqué dans la reconnaissance des agents pathogènes. Les cellules activées par l'Imiquimod *via* TLR-7, sécrètent des cytokines, principalement IFN- $\alpha$ , IL-6 et TNF- $\alpha$ . L'Imiquimod peut aussi entraîner l'activation des cellules de Langerhans, qui migrent ensuite vers les ganglions lymphatiques locaux pour activer le système immunitaire adaptatif. Récemment, l'Imiquimod a montré son efficacité contre une parasitose causée par certaines espèces de *Leishmania*, la leishmaniose cutanée. Cette activité antiparasitaire est principalement due à l'interaction de l'Imiquimod avec le TLR-7, et à l'activation de la voie NF- $\kappa$ B et par conséquent, la réponse immunitaire pro-inflammatoire permet de lutter contre cette infection parasitaire.

## **6. Objectifs et résultats**

Dans ce travail de thèse, nous avons axé nos travaux sur l'identification potentielle d'une nouvelle approche thérapeutique de la toxoplasmose et sur l'obtention d'une meilleure compréhension des mécanismes moléculaires de de cette maladie. Notre intérêt s'est plus particulièrement focalisé sur l'étude de la phase chronique. En effet, cette phase est la plus commune de la maladie, elle s'associe à des neuropathologies et des cancers, elle peut devenir fatale lorsqu'elle est réactivée chez les patients immunodéprimés, et surtout elle demeure sans traitement efficace jusqu'à l'heure.

Dans la première partie de ce travail, nous avons exploré l'activité potentielle de l'Imiquimod contre la toxoplasmose aiguë et chronique. En premier lieu, nous avons testé l'effet de l'Imiquimod sur des macrophages humains infectés par la souche 76K. Nos



résultats ont montré que l'Imiquimod affecte plus de 70% de la réplication parasitaire, prouvant son efficacité antiparasitaire *in vitro*. De plus, l'Imiquimod entraîne une réduction significative du nombre des tachyzoïtes disséminés dans la rate chez des souris BALB/c infectées durant la phase aiguë de l'infection, et une diminution considérable du nombre de kystes cérébraux lors de l'établissement de la phase chronique de la maladie. D'une manière importante, les kystes de bradyzoïtes qui restent dans le cerveau de souris, suite au traitement à l'Imiquimod, sont incapables d'induire une nouvelle infection aiguë ainsi qu'une nouvelle infection chronique. Ceci souligne l'efficacité de l'Imiquimod contre la phase aiguë, affectant l'établissement de la phase chronique, ce qui suggère soit la mort des bradyzoïtes soit leur échec de se reconvertir en tachyzoïtes.

Nous nous sommes par la suite intéressés à étudier l'effet de l'Imiquimod contre la phase chronique de la toxoplasmose. Nos données ont montré une diminution du nombre de kystes suite au traitement à l'Imiquimod *in vitro* et *in vivo*. En effet, après établissement de la phase chronique dans un modèle murin, le traitement des souris chroniquement infectées par l'Imiquimod, réduit significativement le nombre de kystes parasitaires cérébraux. Ce résultat qui, à notre sens, est parmi les résultats les plus importants de notre travail en vue de l'absence de traitements actuels ciblant cette phase, prouve l'efficacité de l'Imiquimod contre la toxoplasmose chronique dans des hôtes immunocompétents. Ceci nous a mené à notre second objectif qui était le mode d'action potentiel de cette molécule. Des études antérieures ont montré que l'Imiquimod, un agoniste du TL-7, est efficace contre une autre infection parasitaire, la leishmaniose cutanée causée par certaines espèces. L'Imiquimod agit dans le contexte de la leishmaniose *via* la liaison à TLR-7, conduisant à l'activation de la voie de signalisation NF- $\kappa$ B, qui par conséquent entraîne une réponse immunitaire de l'hôte. Dans nos travaux, nous avons d'abord étudié le TLR-7, vue son activation par l'Imiquimod, et les TLR-11 et TLR-12, vue leurs importances dans la reconnaissance du parasite par les cellules dendritiques et les monocytes. Nos données ont montré que les niveaux de transcrits des trois TLR augmentent progressivement depuis la deuxième semaine de traitement des souris porteuses de toxoplasmose chronique et traitées par l'Imiquimod, pour atteindre un maximum à la quatrième semaine après traitement. Nous avons donc choisi cette quatrième semaine pour mesurer le taux protéique de ces TLRs. En accord avec les

niveaux de transcrits, les niveaux protéiques sont significativement augmentés dans les cerveaux des souris infectées par la toxoplasmose chronique et traitées par l'Imiquimod. L'activation des TLRs se fait normalement par les tachyzoïtes, par liaison de la profiline ou la cyclophiline 18 parasite aux TLRs dans les monocytes et les cellules dendritiques. Nous avons donc testé l'effet de l'imiquimod sur l'interconversion de bradyzoïtes en tachyzoïtes. Nos résultats montrent que l'Imiquimod a diminué la P21, exclusivement exprimée dans les bradyzoïtes, et a augmenté la P30, exclusivement exprimée dans les tachyzoïtes, indiquant l'induction de l'interconversion. Nous avons par la suite examiné l'activation des TLRs, en étudiant la voie de signalisation MyD88 qui est en aval, et qui est connue être activée dans le contexte de la toxoplasmose pour induire la réponse immunitaire. Le traitement par l'Imiquimod stimule MyD88, active la voie de signalisation de la protéine kinase activée par un mitogène (MAPK), se traduisant par la phosphorylation d'ERK1/2 et conduisant à améliorer la réponse immunitaire IL-1 $\beta$ , IL-12 et IFN- $\gamma$  dans le cerveau des souris traitées. Afin de confirmer que l'Imiquimod induit une activation des TLR-11/12, nous avons utilisé une souche de *T.gondii* dépourvue de la profiline. Nos données ont démontré que cette souche qui est incapable de se lier aux TLR 11/12 et ne répond pas au traitement par l'Imiquimod. Ceci suggère que cette drogue augmente l'interaction de la profiline à aux deux TLRs pour stimuler la réponse immunitaire anti-parasitaire. En outre, une augmentation significative des niveaux de transcription des 2 chimiokines, le ligand 9 (motif C-X-C) (CXCL9) et le ligand (motif C-X-C) 10 (CXCL10), a également été remarquée suite au traitement par l'imiquimod, indiquant probablement le recrutement des lymphocytes T pour éliminer les foyers réactivés de *Toxoplasma* et éliminer l'infection.

Globalement, la première partie de cette thèse démontre une activité importante de l'Imiquimod contre la phase aigüe et plus particulièrement contre la phase chronique de la toxoplasmose. Elle a permis d'identifier le mode d'action potentiel qui est l'activation des TLR, conduisant à l'activation de la voie de signalisation MyD88 et déclenchant la réponse immunitaire appropriée pour éliminer l'infection. Cette première partie de l'étude peut être extrapolée pour proposer une nouvelle modalité de traitement des maladies associées à la toxoplasmose chronique et pourrait avoir un impact considérable sur la santé animale. En effet, il est important de signaler que les rongeurs

jouent un rôle crucial dans le maintien du cycle de vie du parasite après prédation par les hôtes définitifs.

Dans la deuxième partie de ce travail, nous nous sommes concentrés sur les mécanismes moléculaires impliqués dans la toxoplasmose aiguë et surtout dans la toxoplasmose chronique. Nous nous sommes intéressés à caractériser P18, un membre de la superfamille de la séquence liée à l'antigène de surface 1 (SAG-1), en supprimant le gène codant pour cette protéine. Nos résultats ont montré que l'élimination de cette protéine atténue la virulence du parasite pendant la phase aiguë, suite à un recrutement plus élevé de cellules dendritiques dans le péritoine des souris, une charge parasitaire moins abondante dans la rate, et un plus grand nombre de tachyzoïtes arrivant aux cerveaux des souris infectées par la souche KO P18. De plus, l'élimination de P18 entraîne une augmentation significative du nombre de kystes de bradyzoïtes *in vitro* et *in vivo* dans les cerveaux de souris infectées. Ce nombre plus élevé de kystes cérébraux est très cohérent avec le nombre plus élevé des tachyzoïtes qui s'infiltrent aux cerveaux en s'échappant du système immunitaire pendant la phase aiguë de la maladie. Il est accompagné d'une expression importante des transcrits de iNOS, de sécrétion des cytokines et de chimiokines immuno-modulatrices, et notamment les transcrits CXCL9 et CXCL10, connues pour être impliquées dans le recrutement de cellules T dans les cerveaux infectés par *T.gondii* pour empêcher la réactivation. Ceci nous a mené à étudier la capacité du KO P18 à se réactiver. Les souris infectées par la souche sauvage ou le KO P18, ont été immunodéprimées par la dexaméthasone. A la vue du grand nombre de kystes obtenu suite à l'élimination de P18, nous avons noté une réactivation retardée de ces souris par rapport aux souris injectées par la souche contrôle. Ces données démontrent l'implication de la protéine P18 dans le contrôle de la réactivation. Finalement, nous avons exploré la capacité des bradyzoïtes à se reconvertir en tachyzoïtes et à établir une infection aiguë. Nous avons utilisé des souris immunodéprimées, SCID et NSGn qui diffèrent surtout dans la capacité de leurs cellules immunitaires innées, notamment les macrophages, à sécréter l'interféron gamma. Après gavage des SCIDs, dont les macrophages sont capables de sécréter de l'interféron-gamma, par des kystes cérébraux dérivant de souris infectées par le KO P18, nous avons obtenu un prolongement significatif de survie chez 60% des animaux testés et un échec

total des parasites à établir une phase aiguë dans 40% des souris. Cependant, chez les souris NSG, dont les macrophages sont incapables de produire de l'interféron gamma, toutes les souris succombent après 15 jours de gavage par le KO *P18* ou la souche sauvage. Nos résultats soulignent un rôle de P18, dépendant de l'interféron gamma, dans la conversion de la phase chronique en phase aiguë de l'infection.

La deuxième partie de cette thèse montre le rôle de P18 dans la phase aiguë, et surtout la phase chronique de la toxoplasmose. Cette protéine s'avère jouer un rôle de modulation de la réponse immunitaire à plusieurs niveaux de l'infection. Pendant la phase aiguë, la P18 semble atténuer le recrutement des cellules dendritiques, probablement pour moduler la réponse immune induite par la P30. Cette protéine semble également être importante pour moduler à la fois la réponse immunitaire et le stress oxydatif pendant l'établissement de la phase chronique de la toxoplasmose. Finalement, P18 semble moduler la réactivation, la conversion de la phase chronique vers la phase aiguë et la dissémination du parasite. Cette régulation de conversion semble être dépendante de l'interféron gamma.

Dans l'ensemble, ce travail de thèse a démontré le potentiel thérapeutique prometteur de l'imiquimod contre la toxoplasmose et caractérisé le rôle de P18 dans l'immunomodulation afin de contrôler la dissémination et l'inter-conversion. Notre étude ouvre la voie à de nouvelles approches thérapeutiques contre la toxoplasmose, sa persistance et sa réactivation. Notre approche devrait ouvrir des horizons pour trouver une solution globale à cette maladie endémique.

# ANNEXE

# **ANNEXE 1**

# Imidazoquinoxaline Derivative EAPB0503: A Promising Drug Targeting Mutant Nucleophosmin 1 in Acute Myeloid Leukemia

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**BACKGROUND:** Nucleophosmin 1 (NPM1) is a nucleocytoplasmic shuttling protein mainly localized in the nucleolus. *NPM1* is frequently mutated in acute myeloid leukemia (AML). NPM1c oligomerizes with wild-type nucleophosmin 1 (wt-NPM1), and this leads to its continuous cytoplasmic delocalization and contributes to leukemogenesis. Recent studies have shown that Cytoplasmic NPM1 (NPM1c) degradation leads to growth arrest and apoptosis of *NPM1c* AML cells and corrects wt-NPM1 normal nucleolar localization. **METHODS:** AML cells expressing wt-NPM1 or NPM1c or transfected with *wt-NPM1* or *NPM1c* as well as *wt-NPM1* and *NPM1c* AML xenograft mice were used. Cell growth was assessed with trypan blue or a CellTiter 96 proliferation kit. The cell cycle was studied with a propidium iodide (PI) assay. Caspase-mediated intrinsic apoptosis was assessed with annexin V/PI, the mitochondrial membrane potential, and poly(adenosine diphosphate ribose) polymerase cleavage. The expression of NPM1, p53, phosphorylated p53, and p21 was analyzed via immunoblotting. Localization was performed with confocal microscopy. The leukemia burden was evaluated by flow cytometry with an anti-human CD45 antibody. **RESULTS:** The imidazoquinoxaline 1-(3-methoxyphenyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine (EAPB0503) induced selective proteasome-mediated degradation of NPM1c, restored wt-NPM1 nucleolar localization in *NPM1c* AML cells, and thus yielded selective growth arrest and apoptosis. Introducing *NPM1c* to cells normally harboring *wt-NPM1* sensitized them to EAPB0503 and led to their growth arrest. Moreover, EAPB0503 selectively reduced the leukemia burden in *NPM1c* AML xenograft mice. **CONCLUSIONS:** These findings further reinforce the idea of targeting the NPM1c oncoprotein to eradicate leukemic cells and warrant a broader preclinical evaluation and then a clinical evaluation of this promising drug. *Cancer* 2017;123:1662-73. © 2017 American Cancer Society.

**KEYWORDS:** acute myeloid leukemia, apoptosis, 1-(3-methoxyphenyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine (EAPB0503), nucleophosmin 1, xenograft mice.

## INTRODUCTION

Acute myeloid leukemia (AML) is a complex, heterogeneous blood malignancy in which a failure to differentiate and an overproliferation of undifferentiated myeloid precursors result in impaired hematopoiesis and bone marrow (BM) failure. AML is associated with a highly variable prognosis and a high mortality rate, with overall survival exceeding 2 years for only 20% of elderly patients and 5 years for less than 50% of adult patients.<sup>1</sup>

The prognosis of AML is mostly dependent on somatic genetic alterations used to classify the risk as favorable, intermediate, or unfavorable.<sup>2</sup> In AML patients with a normal karyotype, the most important genetic mutations influencing both the prognosis and the treatment strategies are mutations in nucleophosmin 1 (*NPM1*) and FMS-like tyrosine kinase 3 (*FLT-3*) internal tandem duplication.<sup>3</sup> Recently, more heterogeneous genomic categories for AML have been reported.<sup>4</sup>

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We thank Dr. Ali Bazarbachi for a critical reading of the manuscript; Dr. Tala Kansoun, Miss Jamal Al Saghir, and Dr. Marwan El Sabban for their help with the CD34<sup>+</sup> extraction and flow cytometry analysis; and Mr. Abdel Rahman Itani for his help in training Rita S. Hleihel, Martin M. Karam, and Maguy H. Hamie in intravenous injection and bone marrow flushing for human CD45-positive cell analysis.

Additional supporting information may be found in the online version of this article.

**DOI:** 10.1002/cncr.30515, **Received:** August 24, 2016; **Revised:** November 10, 2016; **Accepted:** November 17, 2016, **Published online** January 5, 2017 in Wiley Online Library (wileyonlinelibrary.com)

*NPM1* is an essential gene<sup>5</sup> encoding a phosphoprotein<sup>6</sup> continuously shuttling between the nucleus, nucleolus, and cytoplasm but mainly residing in the nucleolus.<sup>7,8</sup> *NPM1* has many functions, including p14<sup>Arf</sup> stabilization, ribosomal biogenesis regulation, centrosomal duplication control, and p53 activation in response to stress stimuli.<sup>5,6,9</sup> In AML, *NPM1* mutations account for approximately one-third of patients, and this makes it one of the most frequently mutated genes.<sup>6,10</sup> These mutations lead to the creation of a *de novo* nuclear export signal,<sup>6,10,11</sup> which results in cytoplasmic accumulation of *NPM1c*, along with wild-type nucleophosmin 1 (wt-*NPM1*) and thus leukemogenesis in these AML patients.<sup>10</sup>

Despite all the advances in genetic and epigenetic changes in AML, there is still little progress in the treatment of the disease. Although complete remission is reached by almost 70% of patients with standard induction chemotherapy, refractory disease is common, and relapse represents the major cause of treatment failure.<sup>12</sup> Stem cell transplantation remains the best chance for long-term survival but is associated with several complications.<sup>13</sup> Therefore, new therapeutic approaches, specifically ones directly targeting the products of AML genetic alterations, are needed.

In *NPM1c* AML, degradation of the *NPM1c* oncoprotein leads to leukemic cell growth arrest and apoptosis.<sup>14-16</sup> We and others have recently shown that arsenic trioxide and retinoic acid selectively induce *NPM1c* proteasomal degradation and thus lead to apoptosis in *NPM1c* AML cells.<sup>15,16</sup> This combined treatment restores *NPM1* nucleolar localization *ex vivo* and *in vivo*. However, although the clearance of AML blasts was observed in a few treated patients, no cure was achieved, likely because of the complexity and status of the disease burden. This underlies the need for novel therapies to improve treatment outcomes.

Imiquimod is a toll-like receptor 7 immunomodulator<sup>17,18</sup> used to treat certain skin cancers<sup>19</sup> and genital warts.<sup>20</sup> Imiquimod analogues, called imidazoquinoxalines, have been synthesized<sup>21</sup>; among them, 1-(2-phenylethyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine (EAPB0203) and 1-(3-methoxyphenyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine (EAPB0503) have been reported with promising antitumor activity.<sup>22,23</sup> Indeed, EAPB0203 displayed pronouncedly higher *in vitro* potency against melanoma and adult T-cell leukemia cells in comparison with imiquimod.<sup>23,24</sup> Later, EAPB0503 showed 10-fold higher cytotoxicity than EAPB0203 against melanoma cells.<sup>25</sup> More recently, EAPB0503

showed a potent apoptotic effect in chronic myeloid leukemia cells through BCR-ABL degradation.<sup>26</sup>

Here we demonstrate that EAPB0503 induces *NPM1c* proteasomal degradation selectively in *NPM1c* AML cells and leads to their apoptosis. Importantly, introducing *NPM1c* to wt-*NPM1*-harboring cells sensitizes them to EAPB0503. Moreover, EAPB0503 treatment restores wt-*NPM1* nucleolar localization *in vitro* and also *in ex vivo* treated blasts and selectively reduces the leukemia burden in *NPM1c* AML xenograft mice. These findings expand the antileukemic use of EAPB0503, reinforce the idea of targeting oncoprotein degradation to kill leukemic cells, and warrant a broader preclinical evaluation and then a clinical evaluation of this promising drug.

## MATERIALS AND METHODS

### Cell Lines

KG-1 $\alpha$ , ML-2, and THP-1 cell lines (from F. Mazurier) and IMS-M2 (from H. de Thé) were grown in Roswell Park Memorial Institute 1640 medium. OCI-AML3 cells (from D. Bouscary) were grown in minimum essential medium  $\alpha$ . Cells were seeded at a concentration of  $2 \times 10^5$ /mL. EAPB0203 or EAPB0503 was used at 0.1 to 5  $\mu$ M, the caspase inhibitor Z-Val-Ala-DL-Asp(OMe)-fluoromethylketone (zVAD) (Bachem Bioscience) was used at 50  $\mu$ M, and the proteasome inhibitor PS-341 was used at 10 nM.<sup>15</sup> Cell growth was assessed with trypan blue or a CellTiter 96 proliferation kit (Promega).

Primary AML cells from patients' BM were extracted as described by El Hajj et al<sup>15</sup> after approval by the institutional review board at the American University of Beirut and after the patients had consented according to the Declaration of Helsinki.

### Drugs

The synthesis of EAPB0203 and EAPB0503 was performed as described by Deleuze-Masquefa et al.<sup>21,22</sup> Further optimization of EAPB0503 synthesis was achieved with microwave-assisted chemistry.<sup>27</sup>

### Generation of Cells Expressing wt-*NPM1* or *NPM1c*

Green fluorescent protein (GFP) wt-*NPM1* or *NPM1c* inserts were amplified and ligated into a pBye lentiviral vector by the EcoRI site. Stable OCI-AML2 expressing wt-*NPM1* or *NPM1c* was generated by lentiviral transduction followed by blasticidin selection. GFP-positive cells were sorted with the FACSria Special Order Research Product (Becton Dickinson) and grown in minimum essential medium  $\alpha$  before the cell growth assessment.



HeLa cells were transfected with pcDNA hemagglutinin (HA) expressing *wt-NPM1* or *NPM1c* (from G. Tell)<sup>28</sup> with Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations and were grown in Dulbecco's modified Eagle's medium.

### **Xenograft Animal Studies**

NOD/Shi-*scid* *IL2r $\gamma$* <sup>-/-</sup> (NSG) mice were obtained from Jackson Laboratories (United States). Mouse protocols were approved by the institutional animal care and utilization committee of the American University of Beirut. OCI-AML3 or THP-1 cells ( $1 \times 10^6$ ) were injected into the tail vein of 8-week-old females (5 mice per group). On day 5 after the AML injection, the mice were treated with EAPB0503 (15 mg/kg) for 5 days a week over a period of 2 weeks. EAPB0503 was dissolved in dimethyl sulfoxide and diluted in an equal volume of lipofundin (vehicle) before its intraperitoneal administration to the mice.<sup>24,29</sup>

### **Flow Cytometry**

#### **Cell cycle analysis**

Propidium iodide (PI) staining was used to assess the cell cycle as described by El Hajj et al.<sup>15</sup>

#### **Annexin V staining**

An annexin V–fluorescein isothiocyanate kit (BD Pharmingen) was used to assess phosphatidylserine exposure. Cells were treated with 1  $\mu$ M EAPB0503 for 24 hours before annexin V/PI labeling and flow cytometry analysis.

#### **Mitochondrial membrane potential (MMP)**

The MMP was assessed by a cell's ability to retain rhodamine 123 (Sigma-Aldrich), as described by Saliba et al.<sup>26</sup>

A Becton Dickinson FACS instrument was used; 10,000 events per condition were acquired, and FlowJo software (FlowJo LLC) was used for the analysis of the results.

#### **Human CD45 staining**

BM from the femurs and tibias of euthanized animals was flushed at the end of week 3 after AML inoculation. Cell surface staining was performed on 100  $\mu$ L of a sample with 20  $\mu$ L of an anti-human CD45 Peridinin Chlorophyll Protein (PerCP) conjugated antibody (345809; Becton Dickinson). After incubation for 15 minutes in the dark, erythrocytes were lysed with 1 mL of an FACS lysis solution (Becton Dickinson). Labeled samples were washed twice and analyzed on a Guava flow cytometer.

### **Immunoblot Analysis**

After 48 hours of treatment with EAPB0203 or EAPB0503, proteins were probed with poly(adenosine diphosphate ribose) polymerase (PARP), p53, p21, HA (Santa Cruz), phosphorylated p53 (Biolabs), or NPM1 (Abcam) before incubation with the monoclonal horseradish peroxidase–conjugated secondary antibodies. The loading control was performed via probing with the mouse horseradish peroxidase–conjugated glyceraldehyde 3-phosphate dehydrogenase antibody (Abnova) or  $\beta$ -actin (Abcam). Immunoblots were detected with a luminol detection kit (Santa Cruz), and images were captured with the X-OMAT or BioRad ChemiDoc MP system.

### **Immunofluorescence Microscopy**

AML cells or patients' blasts were spun down onto glass slides, fixed, and permeabilized with ice-cold methanol for 30 minutes. Immunostaining was performed with a monoclonal antibody against anti-B23 NPM1 (Santa Cruz) and a polyclonal antibody against the nucleolar marker fibrillarin (Abcam). Primary antibodies were revealed by Alexa Fluor 488– or Fluor 594–labeled secondary antibodies (Santa Cruz). Images were acquired with a Zeiss LSM 710 laser scanning microscope operated with Zen 2009 software (Carl Zeiss).

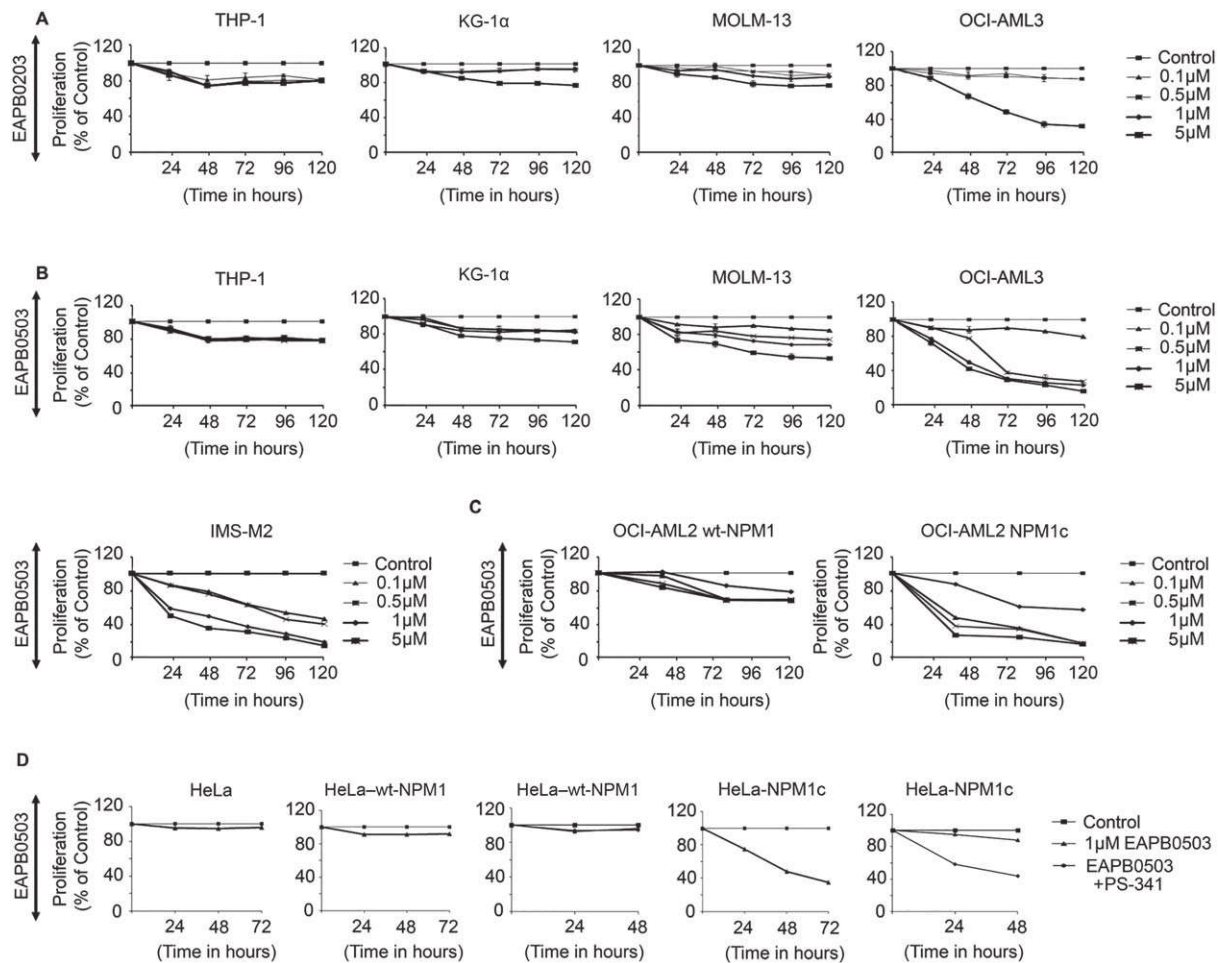
### **Statistical Analysis**

Data are reported as averages and standard deviations. Statistical analyses were performed with the Student *t* test; a *P*-value less than .05 was considered significant.

## **RESULTS**

### **EAPB0203 and EAPB0503 Induce Growth Arrest in NPM1c AML Cells**

We used 3 *wt-NPM1* cell lines (THP-1, KG-1 $\alpha$ , and MOLM-13) and the 2 available *NPM1c* AML cell lines (OCI-AML3 and IMS-M2) to test for EAPB0203 and EAPB0503 effects on cell growth and viability. We tested a range of drug concentrations (0.1–5  $\mu$ M) and assessed cell growth for 5 days after treatment. Both treatments resulted in pronounced time-dependent growth inhibition of OCI-AML3 cells (Fig. 1A,B). EAPB0203 at 5  $\mu$ M resulted in significant OCI-AML3 growth inhibition ( $P < .05$ ), which started 72 hours after treatment. Strikingly, EAPB0503 was more potent and at 0.1  $\mu$ M resulted in significant growth inhibition, which started 96 hours after treatment ( $P < .001$ ). Similarly significant results were obtained for both OCI-AML3 and IMS-M2: a concentration of 0.5  $\mu$ M induced growth inhibition starting 72 hours after treatment ( $P < .001$ ), and concentrations



**Figure 1.** EAPB0503 induces selective growth inhibition in *NPM1c* AML cells. AML cell lines with normal *NPM1* (THP-1, KG-1α, and MOLM-13) and *NPM1c* (OCI-AML3 and IMS-M2) were treated with increasing concentrations (0.1–5 μM) of (A) EAPB0203 and (B) EAPB0503 for 24, 48, 72, 96, and 120 hours. (C) Stably transfected OCI-AML2 with green fluorescent protein *wt-NPM1* or *NPM1c* was treated with increasing concentrations (0.1–5 μM) of EAPB0503 for 24, 48, 72, 96, and 120 hours. (D) HeLa cells transfected with hemagglutinin-tagged *wt-NPM1* or *NPM1c* were treated with 1 μM EAPB0503 alone or in combination with 10 nM PS-341 for 24, 48, and 72 hours as indicated. Cell growth (percentage of the control) was assayed in triplicate. The results represent the average of at least 3 independent experiments. AML indicates acute myeloid leukemia; EAPB0203, 1-(2-phenylethyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine; EAPB0503, 1-(3-methoxyphenyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine; NPM1, nucleophosmin 1; wt-NPM1, wild-type nucleophosmin 1; Cytoplasmic NPM1 (NPM1c) Hemagglutinin (HA)-tagged confirmed green fluorescent protein (GFP)-tagged wt-NPM-1 or NPM-1c.

of 1 and 5 μM induced the same inhibitory effect 24 hours after treatment ( $P < .05$  and  $P < .001$ , respectively; Fig. 1B). Importantly, a median inhibitory concentration of 1 μM in OCI-AML3 and IMS-M2 cells was achieved 2 days after treatment with EAPB0503 ( $P < .05$  and  $P < .001$ , respectively), whereas a concentration of 5 μM was achieved after treatment with EAPB0203 in OCI-AML3 (Fig. 1A,B). This more potent effect of EAPB0503 versus EAPB0203 is in line with previously reported results.<sup>26</sup> THP-1 and KG-1α cells were minimally sensitive to the compounds, with only approximately 20% growth inhibition even 5 days after treatment (Fig. 1A,B). MOLM-13 cells were also minimally sensitive to

EAPB0203 but displayed approximately 50% growth inhibition 72 hours after treatment with EAPB0503 (Fig. 1A,B). This percentage did not become more pronounced even 5 days after treatment, and the only significant result was obtained with concentrations of 1 and 5 μM, 120 and 72 hours after treatment, respectively ( $P < .05$ ; Fig. 1B).

#### Introduction of *NPM1c* Into *wt-NPM1*-Expressing Cells Sensitizes Them to EAPB0503

To examine whether the growth inhibition solely observed in *NPM1c* cell lines was due to *NPM1* mutations, we introduced *NPM1c* to *wt-NPM1*-expressing cells and checked for their sensitivity to EAPB0503. We used the

wt-NPM1-expressing AML cell line (OCI-AML2) and generated by lentiviral transduction and then blasticidin selection cells stably expressing either GFP-tagged wt-NPM1 or NPM1c. GFP-positive cells were sorted, and a range of EAPB0503 concentrations (0.1–5  $\mu$ M) were tested to assess cell growth more than 5 days after treatment. Interestingly, stable expression of NPM1c in OCI-AML2 resulted in significantly pronounced growth inhibition at 0.1  $\mu$ M that started 72 hours after treatment and at 0.5, 1, and 5  $\mu$ M that started 48 hours after treatment ( $P < .05$ ; Fig. 1C). A minimal effect was observed in *wt-NPM1* OCI-AML2: maximum growth inhibition of 30% (nonsignificant) was obtained 48 hours after treatment with concentrations of 0.5, 1, and 5  $\mu$ M (Fig. 1C). Similar results were obtained with HeLa cells: a concentration of 1  $\mu$ M induced growth arrest starting 48 hours after treatment in HA *NPM1c*-transfected cells ( $P < .001$ ) but not *wt-NPM1*-transfected cells (Fig. 1D). This growth inhibition was reversed upon the addition of PS-341 only in NPM1c-expressing cells both 24 and 48 hours after treatment ( $P < .05$ ; Fig. 1D). Our results strongly suggest that introducing *NPM1c* into cells harboring *wt-NPM1* sensitizes them to EAPB0503. Because of its potency, especially in *NPM1c* AML cells, only EAPB0503 was adopted at its median inhibitory concentration of 1  $\mu$ M for the remainder of the study.

### **EAPB0503 Induces Massive Apoptosis in NPM1c AML Cells**

To examine the mechanisms dictating growth inhibition and cell death, a cell cycle analysis was performed 48 hours after treatment with 1  $\mu$ M EAPB0503. A sharp increase in the pre-G<sub>0</sub> cell percentage, which reached more than 80%, was obtained upon the treatment of OCI-AML3 with EAPB0503. Minimal effect was observed in the *wt-NPM1* cells (THP-1, KG-1 $\alpha$ , and MOLM-13; Fig. 2A and Supporting Fig. 1A [see online supporting information]). The cell cycle distribution showed no major variation in all the tested AML cells untreated or treated with EAPB0503 (Fig. 2B and Supporting Fig. 1A [see online supporting information]), and this shows that the drug is mostly inducing pre-G<sub>0</sub> accumulation in *NPM1c* AML without affecting the other cell cycle phases.

To confirm the apoptosis, annexin V/PI labeling was performed, and a significant increase of 40% in annexin V positivity was observed only in OCI-AML3 cells treated with 1  $\mu$ M EAPB0503 for 24 hours ( $P < .005$ ; Fig. 2C and Supporting Fig. 1B [see online supporting information]). In contrast, all *wt-NPM1* cells remained virtually annexin V-negative upon treatment with the drug (Fig. 2C and Supporting Fig. 1B [see online supporting information]).

### **EAPB0503-Induced Apoptosis in NPM1c AML Cells Involves the Dissipation of MMP and Caspase Activation**

The intrinsic apoptotic cascade is characterized by many steps, the earliest of which is the disruption of the MMP.<sup>30</sup> Because EAPB0503 induces apoptosis in *NPM1c* AML cells, we measured MMP in untreated cells or 2 days after treatment with EAPB0503. Treated OCI-AML3 cells failed to retain the rhodamine 123 dye inside their mitochondria (Fig. 2D and Supporting Fig. 1C [see online supporting information]). Conversely, all *wt-NPM1* AML cells showed no loss of MMP up to 48 hours after treatment (Fig. 2D and Supporting Fig. 1C [see online supporting information]).

To study the effect of MMP dissipation in EAPB0503-treated AML cells on the caspase cascade, we examined PARP cleavage. The treatment of OCI-AML3 for 48 hours with EAPB0503 but not with EAPB0203 led to PARP cleavage into its death-associated fragment (Fig. 2E); this occurred to a much lesser extent in the *wt-NPM1* AML cells treated with either drug (Fig. 2E). Interestingly, the cotreatment of cells with the general caspase inhibitor zVAD and EAPB0503 reversed EAPB0503 growth-induced inhibition in OCI-AML3 (Fig. 2F), whereas no effect was observed in *wt-NPM1* cells (THP-1 and MOLM-13; Fig. 2F). Altogether, our results indicate that the selective growth arrest obtained in *NPM1c* AML with EAP0503 involves caspase activation.

### **EAPB0503 Treatment Activates p53 Signaling in NPM1c AML Cells**

To determine whether the EAPB0503-associated growth inhibition and apoptosis were p53-mediated, p53 protein levels were monitored 48 hours after treatment with 1  $\mu$ M EAPB0203 or EAPB0503, and the results were compared with untreated controls. EAPB0503 induced substantial upregulation of total p53 protein levels and the p53 phosphorylated form exclusively in the *NPM1c* OCI-AML3 cell line (Fig. 2G), whereas no effect was observed upon the treatment of these cells with EAPB0203 (Fig. 2G). Accordingly, p21 protein levels were upregulated only in EAPB0503-treated OCI-AML3 (Fig. 2G). Because p53 is mutated in both THP-1 and KG-1 $\alpha$  cell lines,<sup>31</sup> we tested p53 only in the *wt-NPM1* MOLM-13 cell line and found that p53, phosphorylated p53, and p21 protein levels remained unchanged upon treatment with either drug (Fig. 2G). Altogether, these results show that EAPB0503 is a potent inducer of apoptosis exclusively in *NPM1c* AML cells.

**EAPB0503 Induces *NPM1c* Proteasomal Degradation and Restores wt-*NPM1* Nucleolar Localization in *NPM1c* AML Cells**

Given the selective activity of EAPB0503 in *NPM1c* AML cells, we examined its effect on *NPM1c* oncoprotein

degradation. Although no effect of EAPB0203 or EAPB0503 on *NPM1* expression was obtained in THP-1, MOLM-13, or KG-1 $\alpha$  cells (Fig. 3A), EAPB0503 but not EAPB0203 triggered *NPM1* downregulation in OCI-AML3 cells (Fig. 3B), and this suggests that *NPM1c* is the

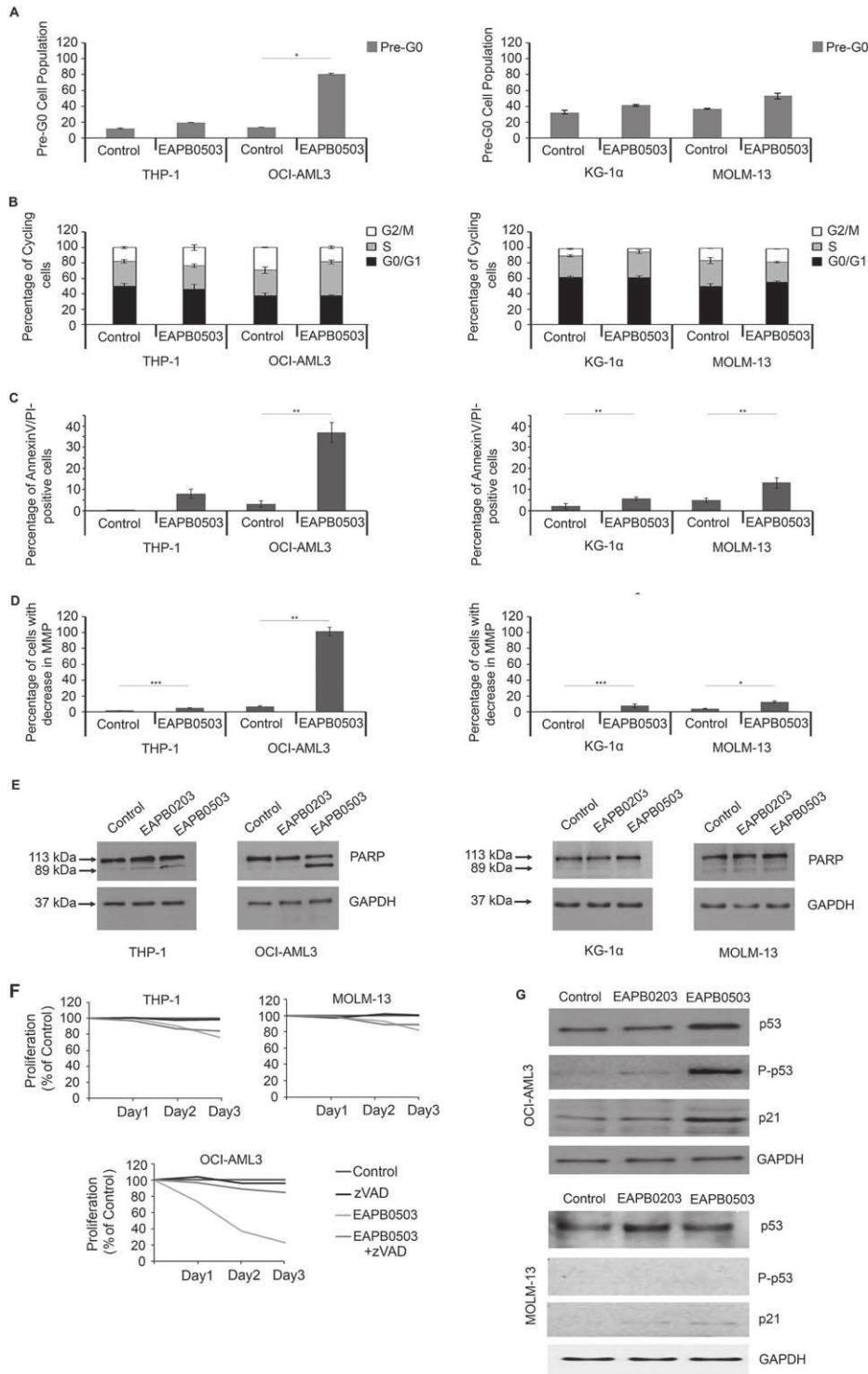


Figure 2.



primary target of EAPB0503. Critically, adding the proteasome inhibitor PS-341 reversed both NPM1 downregulation and growth arrest (Fig. 3C) specifically in OCI-AML3 (Supporting Fig. 2 [see online supporting information]). To eliminate any potential off-target effect of the treatment, we treated HA-tagged, *wt-NPM1*- or *NPM1c*-transfected HeLa cells with EAP0503 alone or in combination with PS-341. With an anti-HA antibody, our results showed that EAPB0503 proteasome-mediated degradation was selective for NPM1c and was reversed upon the addition of PS-341 (Fig. 3D). Using primers specific for either *wt-NPM1* or *NPM1c* messenger RNA, we found that neither transcript level was affected in EAPB0503-treated cells (Supporting Fig. 3 [see online supporting information]), and this shows that NPM1 downregulation occurs at the protein level. Collectively, these results strongly suggest that EAPB0503-treated *NPM1c* AML cells are secondary to oncoprotein degradation.

In *NPM1c* AML, *wt-NPM1* oligomerized with NPM1c and was delocalized to the cytoplasm (Fig. 3E),<sup>6,10,11</sup> whereas the treatment of THP-1 cells with EAPB0503 did not affect NPM1 nucleolar localization (Fig. 3E), EAPB0503 treatment of OCI-AML3 restored the nucleolar localization of the remaining NPM1 protein (Fig. 3E). This suggests that EAPB0503-triggered degradation of NPM1c releases *wt-NPM1* and thus corrects the nucleolar organization defect.

#### **EAPB0503 Selectively Inhibits Proliferation, Induces NPM1c Degradation, and Restores wt-NPM1 Nucleolar Localization in Ex Vivo Treated NPM1c AML Blasts**

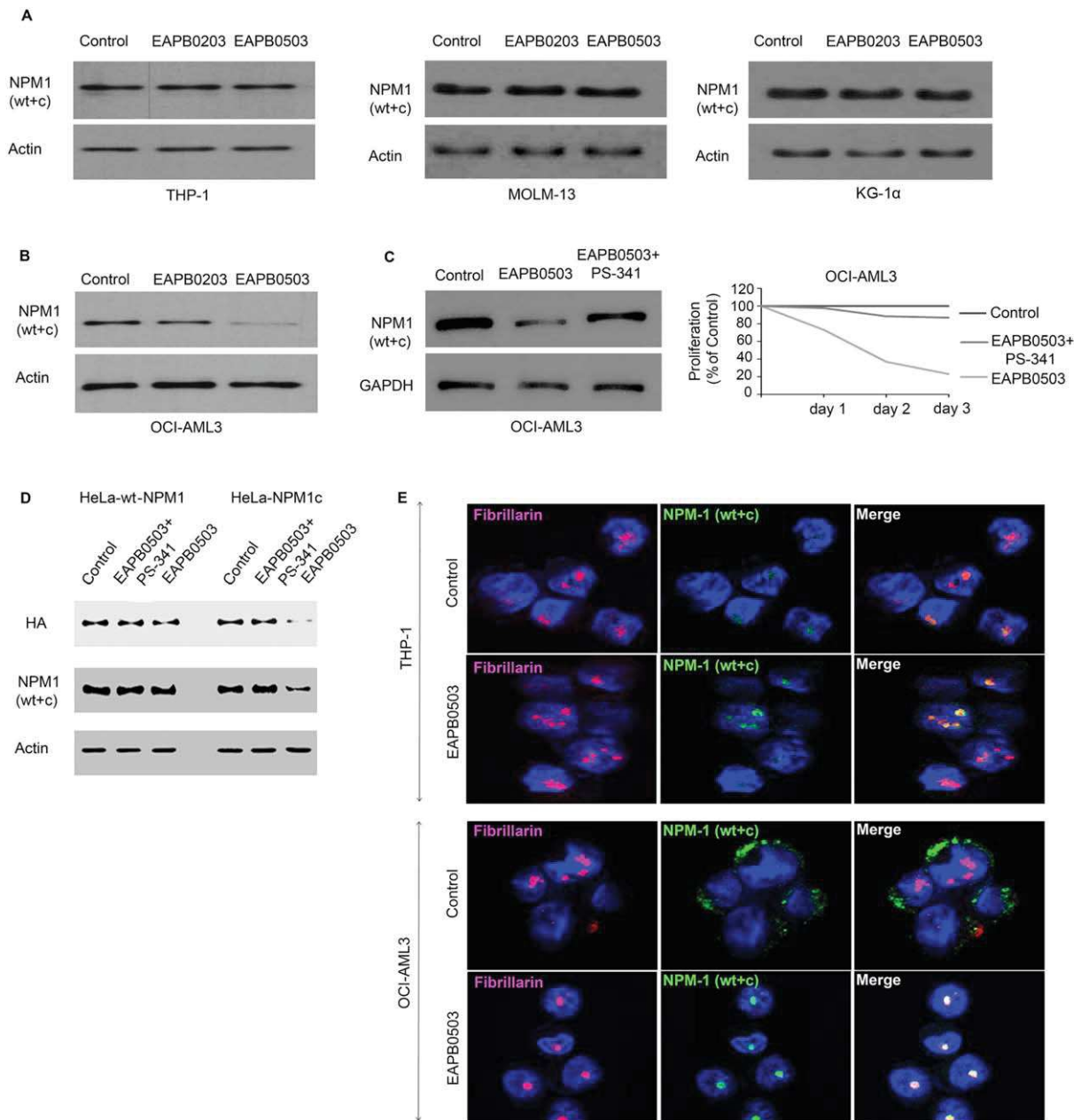
Primary blasts derived from the BM of 6 AML patients were treated with EAPB0503. Patient 1 had acute promyelocytic leukemia with PML/RARA rearrangement, patients

2 and 6 were AML patients with *wt-NPM1*, and patients 3 to 5 harbored an *NPM1* mutation without *FLT-3* internal tandem duplication. Although leukemic cells derived from patients 1, 2, and 6 were not sensitive to EAPB0503 treatment, those derived from patients 3 to 5 were highly sensitive, and almost all died within the first 48 hours after treatment (Fig. 4A). Moreover, EAPB0503 induced NPM1c selective degradation in patients 3 to 5 (Fig. 4B) and restored the *wt-NPM1* nucleolar localization only in those patients (Fig. 4C). Collectively, EAPB0503 exerts its growth-inhibition effect, induces NPM1c degradation, and corrects the *wt-NPM1* nucleolar localization selectively in treated *NPM1c* AML blasts ex vivo.

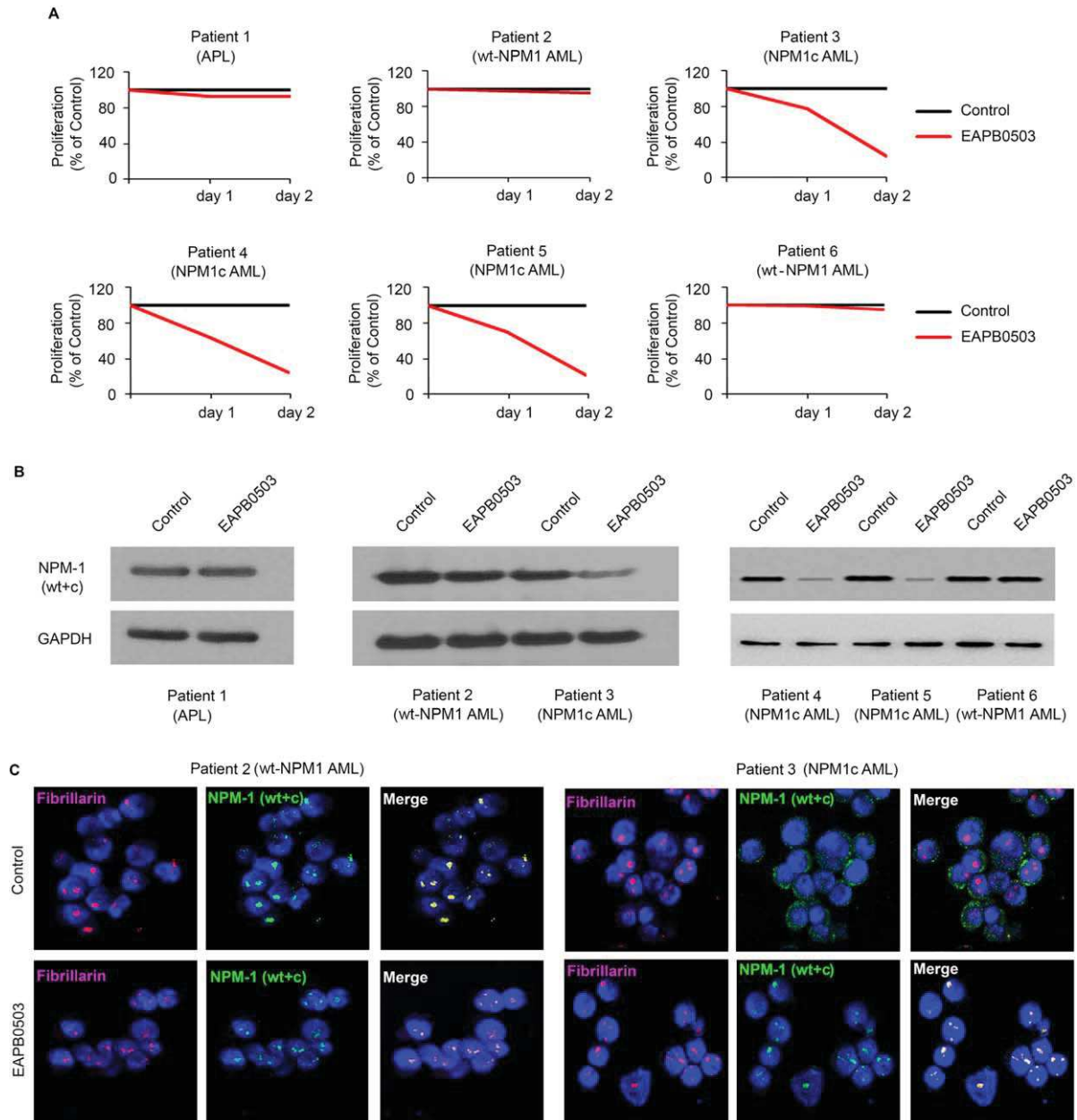
#### **EAPB0503 Selectively Reduces the Leukemia BM Burden in OCI-AML3 Xenograft Mice**

Several xenograft mouse models have been generated.<sup>32,33</sup> Furthermore, OCI-AML3 and THP-1 cells are known to express the hCD45 marker.<sup>33,34</sup> To assess the in vivo efficacy of EAPB0503, we injected NSG mice with OCI-AML3 or THP-1 cells. Five days after the AML cell injection, xenograft mice were treated intraperitoneally with EAPB0503 or its respective vehicle (dimethyl sulfoxide/lipofundin) once daily for 5 consecutive days a week over a period of 2 weeks. At the end of week 3 after the AML cell inoculation, BM was flushed from the femurs and tibias of untreated mice and vehicle- or EAPB0503-treated mice. Human AML xenograft cells were stained with the human-specific hCD45<sup>+</sup> antibody and analyzed with flow cytometry. Our results show that the OCI-AML3 BM burden was markedly reduced from 34% to 10% upon EAPB0503 treatment ( $P < .05$ ; Fig. 5A,B), whereas the THP-1 burden was not affected (22% for untreated mice vs 23% for EAPB0503-treated mice; Fig. 5B,C). These results indicate that EAPB0503 is a promising drug that selectively

**Figure 2.** EAPB0503 induces caspase-mediated apoptosis in *NPM1c* AML cells. (A) Pre-G<sub>0</sub> cell population after PI staining upon the treatment of AML cell lines with the median inhibitory concentration dose (1 μM) of EAPB0503 for 48 hours. (B) Percentage of cycling cell populations after PI staining upon the treatment of AML cells for 48 hours as described previously. Histograms represent the relative distributions of nonapoptotic cells between the G<sub>0</sub>/G<sub>1</sub>, S, and G<sub>2</sub>/M phases. (C) Annexin V staining of AML cells treated for 48 hours as described previously. (D) MMP assay. After the treatment with AML cells as described previously and rhodamine 123 staining, rhodamine 123 was excited at 488 nm, and the fluorescence emission at 525 nm was assessed with flow cytometry. (E) Western blot analysis for PARP upon the 48-hour treatment of AML cells with EAPB0203 and EAPB0503. (F) Proliferation assay after the treatment of AML cells (THP-1, MOLM-13, and OCI-AML3) with 1 μM EAPB0503 alone or in combination with 50 μM zVAD (general caspase inhibitor) for 24, 48, and 72 hours. Cell growth is represented as the percentage of the control as indicated. (G) Western blot analysis for p53, P-p53, p21, and GAPDH in OCI-AML3 and MOLM-13 cells treated for 48 hours as described. In all flow cytometry assays, histograms represent 1 of 3 independent experiments. *P* values less than .05 were considered significant (\* $P \leq .05$ , \*\* $P \leq .01$ , \*\*\* $P \leq .001$ ). AML indicates acute myeloid leukemia; EAPB0203, 1-(2-phenylethyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine; EAPB0503, 1-(3-methoxyphenyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MMP, mitochondrial membrane potential; PARP, poly(adenosine diphosphate ribose) polymerase; PI, propidium iodide; P-p53, phosphorylated p53; zVAD: z-Val-Ala-DL-Asp(Ome)-fluoromethylketone Rhodamine 123 phosphorylated-p53.



**Figure 3.** EAPB0503 induces proteasomal degradation of the NPM1c protein and restores the correct wt-NPM1 nucleolar localization in the *NPM1c* OCI-AML3 cell line. Western blot analysis of NPM1 recognizing both NPM1 (wt+c) and actin in (A) AML cell lines with *wt-NPM1* (THP-1, MOLM-13, and KG-1 $\alpha$ ) and (B) *NPM1c* OCI-AML3 cell lines treated with 1  $\mu$ M EAPB0203 or EAPB0503 for 48 hours as indicated. (C) NPM1 (wt+c) and GAPDH in OCI-AML3 treated with 1  $\mu$ M EAPB0503 alone or in combination with 10 nM PS-341 (proteasome inhibitor) for 48 hours as indicated and proliferation assay after the treatment of OCI-AML3 with 1  $\mu$ M EAPB0503 alone or in combination with 10 nM PS-341 for 24, 48, and 72 hours. Cell growth is presented as the percentage of the control as indicated. (D) Western blot analysis for HA, NPM1 (wt+c), and actin in HeLa cells transfected with HA-tagged *wt-NPM1* or *NPM1c* and treated with 1  $\mu$ M EAPB0503 alone or in combination with 10 nM PS-341 for 48 hours as indicated. (E) Confocal microscopy analysis of NPM1 localization in THP-1 or OCI-AML3 cells after treatment with EAPB0503 for 48 hours. NPM1 was stained with an antibody recognizing NPM1 (wt+c) (green), nucleoli were stained with anti-fibrillarin (red), and nuclei were stained with 4',6-diamidino-2-phenylindole (blue). Images represent z-sections. AML indicates acute myeloid leukemia; EAPB0203, 1-(2-phenylethyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine; EAPB0503, 1-(3-methoxyphenyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HA, hemagglutinin; NPM1, nucleophosmin 1; wt-NPM1, wild-type nucleophosmin 1; NPM1c, cytoplasmic NPM1; NPM-1 (wt+c): wild type and cytoplasmic NPM1.

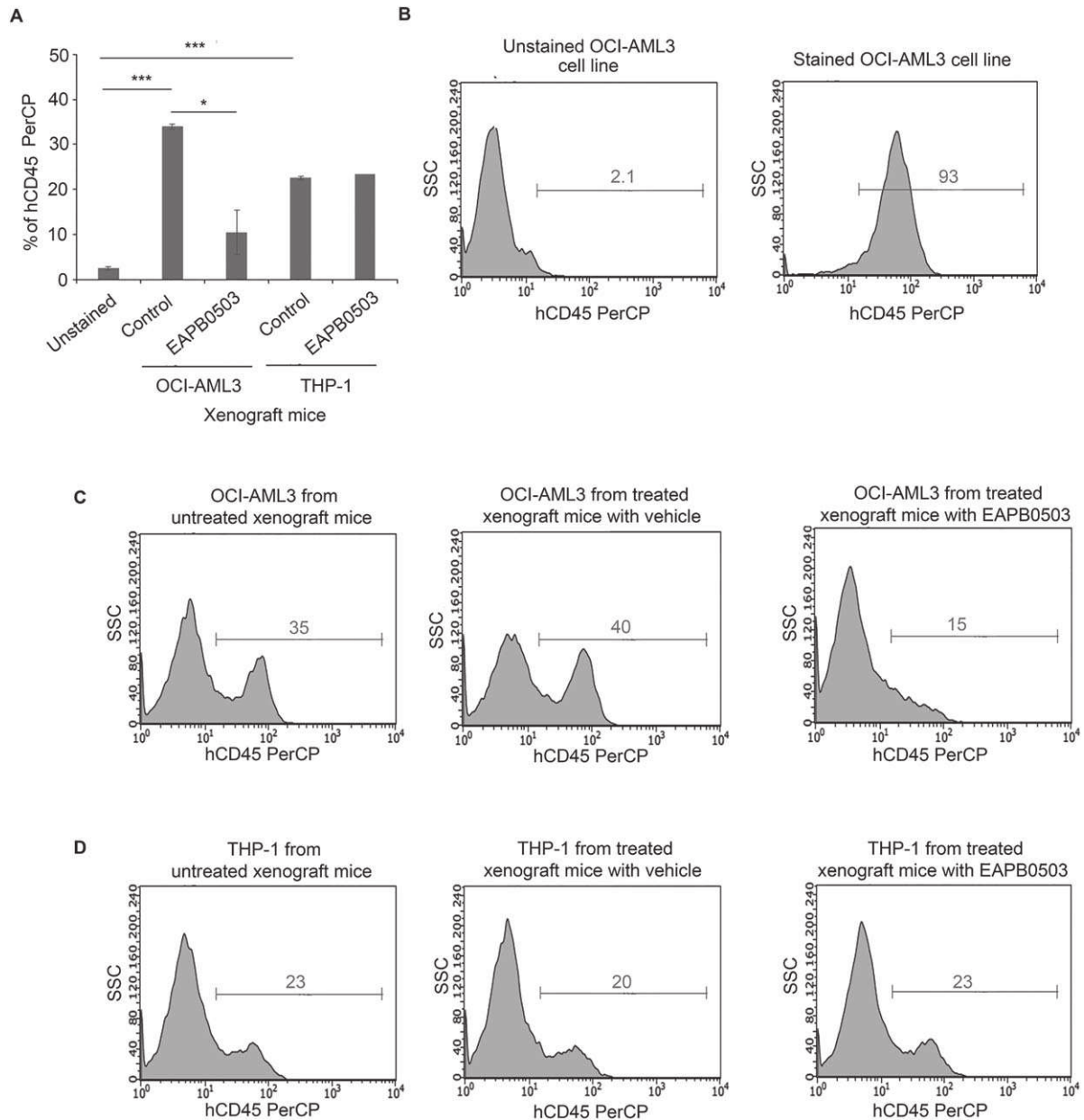


**Figure 4.** EAPB0503 inhibits proliferation, induces the degradation of NPM1c, and restores the nucleolar localization of *wt-NPM1* selectively in ex vivo treated blasts derived from *NPM1c* AML patients. Primary leukemic blasts were harvested from 3 patients and treated with 1  $\mu$ M EAPB0503. Patient 1 had APL with PML/RARA rearrangement, patients 2 and 6 were AML patients with *wt-NPM1*, and patients 3 to 5 were AML patients harboring an *NPM1* mutation without *FLT-3* internal tandem duplication. (A) Proliferation of AML blasts after treatment for 24, 48, and 72 hours. Cell growth is represented as the percentage of the control. (B) Western blot analysis for NPM1 (wt+c) and GAPDH in treated AML blasts as indicated previously. (C) Confocal microscopy of derived blasts from patients 2 and 3. NPM1 (wt+c) was stained with an anti-NPM1 (wt+c) antibody (green), nucleoli were stained with anti-fibrillarlin (red), and nuclei were stained with 4',6-diamidino-2-phenylindole (blue). Images represent z-sections. AML indicates acute myeloid leukemia; APL, acute promyelocytic leukemia; EAPB0503, 1-(3-methoxyphenyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine; FLT-3, FMS-like tyrosine kinase 3; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NPM1, nucleophosmin 1; wt-NPM1, wild-type nucleophosmin 1; NPM1c, cytoplasmic NPM1; NPM1 (wt+c), wild-type and cytoplasmic NPM1.

reduces the *NPM1c* AML BM burden in xenograft animals and warrants more preclinical investigation and then a clinical investigation.

## DISCUSSION

In this report, we examine the effects of EAPB0503 and EAPB0203, 2 imidazoquinoxaline agents, on AML cell



**Figure 5.** EAPB0503 selectively reduces the leukemia bone marrow burden in OCI-AML3 xenograft NSG mice. Eight-week-old female NSG mice were injected with  $1 \times 10^6$  OCI-AML3 or THP-1 cells intravenously. EAPB0503 or its vehicle was administered for 5 days per week over a period of 2 weeks intraperitoneally. At the end of week 3, bone marrow was harvested from femurs and tibias of xenograft mice and then stained with the anti-hCD45 antibody. (A) Histograms showing the hCD45 PerCP percentage in xenograft animals. (B) Unstained and stained OCI-AML3 cell lines with the hCD45 antibody. (C) Representative histograms of stained and untreated OCI-AML3 xenograft mice, OCI-AML3 xenograft mice treated with the vehicle, and OCI-AML3 xenograft mice treated with EAPB0503. (D) Representative histograms of stained and untreated THP-1 xenograft mice, THP-1 xenograft mice treated with the vehicle, and THP-1 xenograft mice treated with EAPB0503. EAPB0503 indicates 1-(3-methoxyphenyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine; NSG, NOD/Shi-*scid* *IL2 $\gamma$* <sup>-/-</sup>; PerCP: peridinin chlorophyll protein (\* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ ); SSC, side scatter.

lines. Imidazoquinoxalines have arisen as promising anti-cancer drugs on the basis of their *in vitro* activity in T-cell leukemia and chronic myeloid leukemia and their *in vivo* activity in melanoma.<sup>22,23,26</sup> We show that EAPB0503

has a specific growth-inhibition effect on *NPM1c* OCI-AML3 and IMS-M2 cells in a dose- and time-dependent manner. EAPB0503 activity in OCI-AML3 cells is considerably more pronounced than EAPB0203



activity, and this in line with its higher antitumor potency in other cancer types.<sup>24,25</sup> Introducing *NPM1c* into cells harboring *wt-NPM1* sensitizes them to EAPB0503. The phenyl group is directly linked to the core imidazoquinoline heterocycle in EAPB0503, whereas an ethyl link exists in EAPB0203 between the 2 parts. This ethyl linker in EAPB0203 appears to abolish the antileukemic activity in most of the tested leukemia models in comparison with the direct linkage in the EAPB0503 compound.<sup>26</sup> Indeed, this change in the EAPB0503 structure enhanced its *in vitro* activity and led to better bioavailability in rats.<sup>29</sup>

We have shown that EAPB0503 induces growth arrest and apoptosis in *NPM1c* AML cells. Apoptosis is accompanied by the dissipation of MMP and PARP cleavage, and this strongly suggests the involvement of the intrinsic apoptotic pathway. Our results are consistent with previous studies showing antitumor activity of EAPB0503 in melanoma and chronic myeloid leukemia with a mode of action similar to the mode of this compound.<sup>23,26</sup>

*NPM1c* characterizes one-third of AML patients,<sup>6,10</sup> and when it alone is present in the case of a normal karyotype, it confers a better prognosis.<sup>35</sup> *NPM1* mutations mediate malignancies as observed in transgenic and knock-in mice.<sup>36</sup> Mutated *NPM1* is the key hallmark of OCI-AML3 and IMS-M2 cells for maintaining their malignant proliferation. In *NPM1c* AML, emerging studies have shown that therapies targeting NPM1c oncoprotein degradation lead to inhibition of proliferation and the cell death of leukemic cells.<sup>14-16</sup> In line with these findings, we have demonstrated that EAPB0503 degrades the NPM1c oncoprotein in a proteasome-dependent manner. This results in correcting the *wt-NPM1* nucleolar localization in both *NPM1c* AML cells and *ex vivo* treated blasts derived from *NPM1c* AML patients. Furthermore, in *in vivo* *NPM1c* AML xenograft animals, EAPB0503 showed a selective reduction of the BM leukemia burden.

Recently, EAPB0503 was shown to exert potent inhibition of tubulin polymerization that correlated with its antiproliferative activity.<sup>27</sup> Therefore, the corrective effect of *wt-NPM1* nucleolar localization after NPM1c degradation warrants testing the disruption of the microtubule network in *NPM1c* AML cells to further explain the mechanism of cell death.

Nowadays, most AML patients are still dying, especially because the basic therapies have remained unchanged or have only slightly changed over the last 2 decades. Nonetheless, before novel clinical therapies are introduced, a deep understanding of the therapeutic approach is required. The evolutionary changes emerging in

AML classification based on the morphology and cytogenetic/genetic changes reflect the importance of identifying the subtype-specific biology to determine the appropriate targeted therapy triggering degradation of the byproducts of these genetic modifications.<sup>13</sup> Our results suggest that EAPB0503 holds promise for the treatment of *NPM1c* AML, especially in those patients with mutation A,<sup>37</sup> which represents 80% of *NPM1* mutations in AML<sup>38</sup> and is the hallmark mutation present in OCI-AML3 and IMS-M2.<sup>39</sup> These promising results were translated *in vivo*: among treated mice, EAPB0503 decreased the BM leukemia burden only in *NPM1c* xenograft mice. Further *in vivo* studies (survival and organ infiltration) and *ex vivo* studies (treated blasts) are required for us to have a complete idea of EAPB0503's mechanism of action.

## FUNDING SUPPORT

This work was supported by the For Women in Science Levant and Egypt Regional Fellowship (L'Oréal/United Nations Educational, Scientific, and Cultural Organization) and by the International For Women in Science Rising Talents 2016 Fellowship (L'Oréal/United Nations Educational, Scientific, and Cultural Organization; to Hiba A. El Hajj).

## CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

## AUTHOR CONTRIBUTIONS

**Ali I. Nabbouh:** Performance of experiments and reporting to Hiba A. El Hajj. **Rita S. Hleihel:** Performance of experiments and reporting to Hiba A. El Hajj. **Jessica L. Saliba:** Performance of experiments and reporting to Hiba A. El Hajj. **Martin M. Karam:** Performance of experiments and reporting to Hiba A. El Hajj. **Maguy H. Hamie:** Performance of experiments and reporting to Hiba A. El Hajj. **Hsin-Chieh J.M. Wu:** Performance of experiments and reporting to Hiba A. El Hajj. **Caroline P. Berthier:** Performance of experiments and reporting to Hiba A. El Hajj. **Nadim M. Tawil:** Performance of experiments and reporting to Hiba A. El Hajj. **Pierre-Antoine A. Bonnet:** Planning of study. **Carine Deleuze-Masquefa:** Planning of study. **Hiba A. El Hajj:** Planning of study and writing of manuscript.

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# **ANNEXE 2**

Review

# A Critical Review of Animal Models Used in Acute Myeloid Leukemia Pathophysiology

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Received: 5 July 2019; Accepted: 1 August 2019; Published: 13 August 2019



**Abstract:** Acute myeloid leukemia (AML) is one of the most frequent, complex, and heterogeneous hematological malignancies. AML prognosis largely depends on acquired cytogenetic, epigenetic, and molecular abnormalities. Despite the improvement in understanding the biology of AML, survival rates remain quite low. Animal models offer a valuable tool to recapitulate different AML subtypes, and to assess the potential role of novel and known mutations in disease progression. This review provides a comprehensive and critical overview of select available AML animal models. These include the non-mammalian *Zebrafish* and *Drosophila* models as well as the mammalian rodent systems, comprising rats and mice. The suitability of each animal model, its contribution to the advancement of knowledge in AML pathophysiology and treatment, as well as its advantages and limitations are discussed. Despite some limitations, animal models represent a powerful approach to assess toxicity, and permit the design of new therapeutic strategies.

**Keywords:** Zebrafish; *Drosophila*; rats; mice; NPM-1; FLT3 ITD; ETO-1; IDH1/2

## 1. Introduction

Acute myeloid leukemia (AML) is an aggressive and heterogeneous hematological group of neoplasms characterized by increased proliferation of myeloid progenitor cells and a reduced capacity to differentiate. This results in the accumulation of myeloblasts in the bone marrow (BM), which negatively impacts hematopoiesis and leads to BM failure [1]. AML is one of the most common acute leukemia in adults [2]. Its incidence rate is 2.5 per 100,000 cases/year and the median overall survival (OS) is approximately nine months [3]. AML treatment and prognosis largely depend on the patients' age [4–6]. AML was historically divided into eight major groups according to cell morphology and immune phenotype (M0 to M7) [7]. This classification has been revised several iterations since then [8–12]. Exome sequencing in AML patients led to the current classification through identification of more than 20 driver recurrent mutations [13]. These mainly include *Nucleophosmin-1* (*NPM1*), *DNA methyltransferase 3A* (*DNMT3A*), *Fms-like tyrosine kinase-3* (*FLT3*), *isocitrate dehydrogenase* (*IDH*), *Ten–Eleven Translocation 2* (*TET-2*), *Runt-related transcription factor* (*RUNX-1*), *CCAAT enhancer binding protein  $\alpha$*  (*CEBPA*), *additional sex comb-like 1* (*ASXL1*), *mixed lineage leukemia* (*MLL*), tumor protein p53

(*TP53*), *c-KIT* [14]. These mutations dictate the response to treatment, rates of complete remission, disease-free survival, overall survival, and classify AML into three prognostic risk factors (favorable, intermediate, and adverse) (Table 1).

Animal models provide an excellent tool to understand the biology of pathological mechanisms involved in human diseases. Diverse animal species were used to answer pivotal questions related to disease progression, genetic mutations, immunity, and response to treatment. Among these models, Zebrafish was exploited to generate different mutations mimicking several subtypes of human AML.

**Table 1.** 2017 European LeukemiaNet (ELN) prognostic groups according to genetic abnormalities of acute myeloid leukemia (AML) [12].

Prognostic Group	Genetic Mutations and Abnormalities
Favorable	<ul style="list-style-type: none"> <li>t(8;21)/RUNX1-RUNX1T1</li> <li>inv(16) or t(16;16)/CBFB-MYH11</li> <li>Mutated <i>NPM1</i> without <i>FLT3-ITD</i></li> <li>or with <i>FLT3-ITD</i><sup>low</sup> *</li> <li>Biallelic mutated <i>CEBPA</i></li> </ul>
Intermediate	<ul style="list-style-type: none"> <li>Mutated <i>NPM1</i> and <i>FLT3-ITD</i><sup>high</sup> *</li> <li>Wild-type <i>NPM1</i> without <i>FLT3-ITD</i> or with <i>FLT3-ITD</i><sup>low</sup> *</li> <li>t(9;11)/MLLT3-KMT2A</li> <li>Cytogenetic abnormalities not classified as favorable or adverse</li> </ul>
Adverse	<ul style="list-style-type: none"> <li>t(6;9)/ DEK-NUP214</li> <li>t(v;11q23.3)/<i>KMT2A</i> rearranged</li> <li>t(9;22)/<i>BCR-ABL1</i></li> <li>inv(3) or t(3;3)/<i>GATA2,MECOM(EV11)</i></li> <li>Complex karyotype</li> <li>Monosomal karyotype</li> <li>Wild-type <i>NPM1</i> and <i>FLT3-ITD</i><sup>high</sup> *</li> <li>Mutated <i>RUNX1</i> †</li> <li>Mutated <i>ASXL1</i> †</li> <li>Mutated <i>TP53</i></li> </ul>

\* Low, low allelic ratio (<0.5); high, high allelic ratio (>0.5); † these mutations should not be used as an adverse prognostic marker if they co-occur with favorable-risk AML subtypes.

## 2. Zebrafish: Characteristics and Relevance to Human Blood Malignancies

*Danio rerio*, commonly known as Zebrafish, shares genetic and molecular mechanisms of hematopoiesis with humans [15]. This model offers many advantages, including low-cost, optically transparent embryos, high fecundity, rapid embryogenesis, and short gestation time. The genome editing in zebrafish was known since 1970s, when the first transgenic zebrafish was generated by inserting naked linear DNA [16]. Since then, the genetic manipulation of this model evolved to include clustered regularly interspaced short palindromic repeats (CRISPR) technology [17], which renders zebrafish an attractive model for studying specific gene involvement and for drug screening in blood malignancies [18–20].

During normal zebrafish hematopoiesis, both the primitive and definitive waves arise from the mesoderm germ layer under the control of the Transforming Growth Factor beta (TGF- $\beta$ ) superfamily proteins, known as bone morphogenic proteins (BMP such as *bmp2b* and *bmp7*) [21–23]. The generated transient primitive erythroid and myeloid cells are essential for the embryonic development, while the hematopoietic stem cells (HSCs) and progenitor cells (HSPCs) produce blood lineages in the adult fish [24]. In the below section, we will provide an overview of AML models of Zebrafish (summarized in Table 2).



## 2.1. AML Models of Zebrafish

### 2.1.1. Spi-1: MYST3/NCOA2-EGFP

MYST3 (MOZ) is a member of the MOZ, YBF2, SAS2, TIP60 (MYST) family of histone acetyl-transferases (HAT), while NCOA2 (TIF2) is a member of the p160 HAT family [25–28]. The first AML model in Zebrafish was created by expressing the fusion protein, MYST3/NCOA2 (MOZ/TIF2). This fusion targets hematopoietic cells under the control of *spi-1* (*pu.1*), an early myeloid promoter [29]. *pu.1* is an ETS-domain transcription factor expressed in both immature lymphoid/hematopoietic cells and myeloid cells during zebrafish hematopoiesis [30]. Cells expressing *pu.1* differentiate into myeloid progeny, whereas cells with low *pu.1* expression shift to the erythroid fate [31]. After an extended latent period, a small percentage of transgenic fish developed AML [29]. These animals presented with an extensive invasion of kidneys by myeloid blast cells, proving the oncogenic potency of MYST3/NCOA2 fusion gene [29]. Although this model is useful as a chemical library screen, especially for compounds that target epigenetic regulation of gene expression [29], the long latency and low incidence waned the enthusiasm for its use.

### 2.1.2. hsp70: AML1-ETO

A chromosomal translocation between chromosomes 8 and 21 (t(8;21)(q22;q22)) occurs in 12–15% of AML patients [32]. This chromosomal rearrangement yields a fusion transcription factor encoding AML1 (RUNX1) linked to ETO, forming the AML1-ETO fusion product [33–35]. This translocation was introduced under the control of the heat shock promoter *hsp70* in zebrafish embryos (*hsp70: AML1-ETO*). Transgenic Zebrafish recapitulated the human AML features, at both the cytological and transcriptional levels [36]. The expression of this fusion protein led to the accumulation of non-circulating hematopoietic cells, whereby the intermediate cell mass was enriched with myeloperoxidase positive neutrophils and morphologically immature hematopoietic blasts [36]. The disruption of definitive hematopoiesis led to switching the cells fate from the erythroid to the myeloid lineage [36]. Overexpression of the transcription factor reversed the observed phenotypes, implicating *scl*, as major player downstream of AML1-ETO [36]. This model enabled the screening of a small molecule library and discovery of compounds that antagonize the activity of AML1-ETO in the hematopoietic progenitor cells (HPCs) [36]. Inhibition of COX-2 and  $\beta$ -catenin signaling antagonized AML1-ETOs effects on HPCs differentiation and may have implications in human AML [37].

### 2.1.3. MYCN: HSE: EGFP

MYCN (N-myc) proto-oncogene is upregulated in many types of hematological malignancies [38,39] including 20 to 40% of pediatric AML patients [40]. To unravel the molecular and transcriptional networks by which MYCN induces malignancy, Shen et al. established a transgenic embryonic zebrafish model, Tg (*MYCN: HSE: EGFP*), expressing the murine MYCN under a heat shock promoter [41]. MYCN overexpression induced immature myeloid blast cell expansion and reprogrammed the hematopoietic cell fate through MYCN downstream-regulated gene 1b (*ndrg1b*) and other lineage-specific hematopoietic transcription factors regulation [41]. The primitive hematopoiesis was enhanced through *scl* and *lmo2* upregulation. Furthermore, erythroid differentiation was blocked through downregulation of *gata1*, while myelopoiesis was promoted by *pu.1* overexpression [41]. This model presents a high AML incidence (~75% of transgenic zebrafish) and a rapid onset occurrence, providing a platform for whole-organism chemical suppressor screens, to identify compounds that can reverse MYCN function in vivo [41].

### 2.1.4. FLT3-ITD and NPM1c+ Models in Zebrafish

FLT3-ITD and NPM1 are two major players in defining the prognosis and response to treatment in AML patients. FLT3 is a tyrosine kinase receptor that plays a major role in hematopoiesis through the regulation of proliferation, differentiation, and apoptosis of HPCs [42]. It is highly expressed on

leukemic blasts of 70–100% of AML patients [43,44]. Several mutations occur in the FLT3 receptor, the most common of which leads to an internal tandem duplication (ITD) [45]. FLT3-ITD occurs in 20% of AML patients and is strongly associated with poor prognosis [46,47]. NPM1, a shuttling protein between the nucleoplasm and the cytoplasm, plays several roles, notably ribosomal biogenesis [48,49]. NPM1 is mutated (NPM1c+) in around 30% of AML patients with normal karyotype [50]. NPM1c+ is continuously translocated to the cytoplasm contributing to leukemogenesis [50].

FLT3-ITD plays a role in embryonic primitive and definitive hematopoiesis in zebrafish. Transgenic zebrafish embryos with human FLT3-ITD showed expansion and clustering of myeloid cells [51]. Thus far, the impact of FLT3-ITD on adult zebrafish remains underexplored.

Bolli et al. generated a transgenic zebrafish model expressing NPM1c+, which perturbed primitive hematopoiesis by promoting the early expansion of pu.1+ myeloid cells [52]. This phenotype was even more pronounced in a p53-deficient background [52]. An increase in the number of gata1+/lmo2 indicating expansion of erythro-myeloid progenitors (EMPs) was also observed. These EMPs highly expressed both c-myb and CD41 but not RUNX1, suggesting a disruption of definitive hematopoiesis where these cells could be the main target of NPM1c+. This model provides a tractable in vivo system for the study of the mechanisms through which hematopoietic development is perturbed in the presence of NPM1c+ [52].

Transgenic zebrafish models expressing either human FLT3-ITD or NPM1 proteins under the control of *pu.1* promoter were also generated [53]. For that purpose, *spi-1*: FLT3-ITD-2A-EGFP/CG2 expressing mutant FLT3-ITD and *spi-1*: NPM1-Mut-PA/CG2 expressing mutant NPM1 constructs were designed. This double mutant transgenic fish (FLT3-ITD/NPM1.Mut) exhibited an accelerated rate of myeloid leukemogenesis [53]. By the age of six months, around 66% of the transgenic fish produced significantly increased precursor cells in the kidney marrow along with dedifferentiated myeloid blasts [53].

#### 2.1.5. Spi-1: CREB-EGFP

The cAMP response element binding protein (CREB) plays a major role in hematopoiesis through the regulation of proliferation and differentiation of myeloid progenitor cells [54]. Overexpression of CREB is associated with immortalization, growth factor-independent proliferation and blast-like phenotype in BM progenitor cells [55]. CREB is highly expressed in BM samples of both adult and pediatric AML patients [56]. Tregnago et al. generated a transgenic zebrafish model (*spi-1*: CREB-EGFP) expressing the CREB gene downstream *pu.1* promoter in the myeloid cell lineage. CREB overexpression resulted in upregulation of erythroid and myeloid genes, altering primitive hematopoiesis. Among adult transgenic zebrafish, 80% of the fish developed AML after 9–14 months through the blockage of myeloid differentiation [57]. These fish showed aberrant expression of a set of 20 genes in common with pediatric AML. The most intriguing is the CCAAT-enhancer-binding-protein- $\delta$  (C/EBP $\delta$ ) that acts downstream CREB. It resulted in impaired myeloid differentiation that could be reversed through inhibition of the CREB-C/EBP $\delta$  axis. These findings are complementary with the data obtained by screening for CREB and C/EBP $\delta$  in pediatric AML patients, offering an opportunity to test for novel therapeutics through this model [57].

#### 2.1.6. Spi-1: SOX4-EGFP

SOX4 is a transcription factor belonging to the SOX (Sry-related high-mobility groupbox) family [58]. In AML patients, SOX4 overexpression results in poor prognosis and short overall survival [59]. SOX4 was reported to contribute to the leukemic phenotype of C/EBP $\alpha$  mutant AML in murine models as well as in human AML. C/EBP $\alpha$  protein typically inhibits the self-renewal of leukemic cells and restores cellular differentiation. SOX4 overexpression results in C/EBP $\alpha$  inactivation, enabling leukemic cells proliferation and AML development [60,61].

Lu et al. generated a transgenic zebrafish model Tg (*spi-1*:SOX4-EGFP) expressing SOX4 protein downstream the *spi-1* myeloid promoter. Early developmental stages of transgenic zebrafish did not



reveal a difference of expression of SOX4. However, by the age of five months, Tg (spi-1:SOX4-EGFP) zebrafish kidneys started showing mild vacuoles in the renal tubule which evolved into effacement, distorted structure, and increased infiltration of myeloid cells by the ages of 9 and 12 months. A higher number of myeloid progenitor cells and excess blast cells with focal aggregation were observed in the kidney marrow blood cells of 9-, 12-, and 15-months old fish but not younger ones, highlighting that myeloid transformation is age-dependent [59].

### 2.1.7. IDH 1/2 Mutation

Mutations identified in a family of enzymes involved in the citric acid cycle, isocitrate dehydrogenases 1/2 (IDH1/2), account for 16% of AML patients [62]. These mutations substitute arginine residue almost exclusively at codon 132 in IDH1 (IDH1-R132H) and codons 140 and 172 in IDH2 [62]. To study the involvement of IDH in AML, *zidh1* was either suppressed or deleted and resulted in the blockage of differentiation and accumulation of early myeloid progenitor cells, while decreasing macrophage and natural killer progenitor cells [63]. The importance of IDH1 mutation was asserted when plasmids of IDH1-R132H were injected into zebrafish embryos [63]. An increase in 2-hydroxyglutarate (2-HG) level, a reduction of 5-Hydroxymethylcytosine (5-hmC), and an expansion of myelopoiesis were obtained in these embryos. A human IDH1-R132H-specific inhibitor significantly ameliorated both hematopoietic and 2-HG responses in human but not zebrafish IDH1 mutant expression [63]. This result is not surprising and highlights some of the drawbacks using Zebrafish as a model for human diseases. On the other hand, studies on *zidh2* were restricted to the regulation of embryonic hematopoiesis in zebrafish but with no relevance to the human AML [63].

Even with the drawbacks of not possessing many mammalian-like organs, zebrafish still provides an excellent, affordable, and rapid platform for evaluating several aspects of AML. The variations in the biological microenvironment might impede drug delivery and performance in humans. Additionally, zebrafish are ectothermic (cold-blooded), so their physiology is not identical to humans, which might affect enzyme kinetics and metabolism. The genetic diversity detected between individual zebrafish belonging to the same strain confounds data and could be misleading [64]. The sparsity of reagents to study zebrafish at the molecular level is contrasted by the abundance of mouse-specific reagents.

## 3. Rodent Models

Due to the complexity and heterogeneity of AML in humans, rodent models have been instrumental in providing a platform for answering pivotal questions related to AML pathogenesis, disease progression, and developing new effective therapeutic approaches. Among these models, rats and mice represent the closest accepted mammalian models to AML.

### 3.1. Rats

Several transplantable leukemia rat models were established using carcinogens, radiations, and pollutants [65–67].

#### Transplantable Rat Models

Acute Myeloid Leukemia/ Chronic Myelogenous Leukemia (AML/CML) leukemia: Repeated intravenous injections of 7, 12-dimethylbenz (a) anthracene (DMBA) into WOP/H-Onc strain or Wistar/H-Onc strain, induced leukemia in 10% of the rats in 5–9 months. This leukemia has myeloid characteristics as revealed by hematological and histological examination, as well as infiltration of myeloid blasts into several organs (BM, liver, spleen, and lymph nodes). This myeloid nature showed similarities with both human CML (as demonstrated by high peroxidase and Sudan black B positive cells and reduction in alkaline phosphatase positivity) and human AML (non-specific esterase activity, highly reduced in the peripheral blood but slightly reduced in BM). These findings do not support the use of these rats as an exclusive AML model [68].

Brown Norwegian Myelogenous Leukemia (BNML): The transplantable promyelocytic leukemia in BN rat (BNML) was first described in 1971. This slow growing leukemia shares many common characteristics with AML, including the disappearance of normal hematopoiesis [69]. Similarities in in vitro colony forming assays between AML patients and BNML rats validated it as a model for AML [70,71]. Several therapeutic modalities were optimized using this model; these include the combination of anthracyclines, [72,73] Ara-C, [74,75], 4'-(9-acridinylamino) methanesulfon-m-anisidide (AMSA) [76], and other therapeutics [77–79]. One of the most significant advantages in the BNML model is its contribution to the improvement of minimal residual disease (MRD) detection by karyotyping [80] and multidimensional flow cytometry [81,82].

### 3.2. Mice

Mice offer an invaluable model due to their small size, cost-effectiveness, and easy maintenance, availability of research tools, and ease of manipulation to produce and recapitulate several human diseases, including cancer. Since hematopoiesis in mice has been well characterized, they provide a reasonably reproducible model to study AML pathogenesis and potential therapies. Murine AML models include induced, transgenic animals, and humanized mouse models (Table 3) among others.

#### 3.2.1. Chemically-Induced Model

AML models were generated using the L1210 and p388 cell lines, isolated from DBA/2 mice chemically exposed to the carcinogen 3-methylcholantrene [83]. These models were transplantable and provided a platform for testing chemotherapeutic drugs, studying their kinetics, and evaluating their anti-leukemic effectiveness [84]. The L1210 model was used to screen anthracyclines [85] and antimetabolites [86,87] including Cytarabine [88]. The p388 model was used to investigate the efficacy of natural products as topoisomerase II inhibitors [89]. These models allowed significant improvement in the treatment of AML, including the currently used Cytarabine [90]. The main limitation of using these animal models is the induction of more lymphoid than myeloid leukemia, and the needed prolonged exposure to those carcinogens to develop leukemia [91].

#### 3.2.2. Radiation-Induced Model

The correlation between radiation and leukemia was established in patients exposed to x-rays, and survivors of nuclear attacks. Among this cohort of subjects, children presented mostly with ALL, whereas adults were more prone to CML and AML [92–95]. All established radiation-induced AML models carry deletions on chromosome 2, where the hematopoietic transcription factor *Sfpi1/pu.1* is located [96].

#### RF Model

The RF strain was developed by Furth in 1933 at the Rockefeller Institute [97]. In this model, myeloid leukemia was developed following exposure to fission neutron irradiation or gamma irradiation [98]. In the RF model, a single dose of ionizing radiation-induced myeloid leukemogenesis in 4–6 months, with symptoms reminiscent to human AML [99]. Flt3-ITD mutations were identified in 10% of RF mice [100], which correlates with the occurrence of this mutation in human AML [101].

#### SJL/J Model

This model is characterized by high spontaneous frequency of reticulum cell neoplasm type B at an early age [102]. The radiation-induced AML in this model is similar to the secondary human AML occurring after irradiation of Hodgkin disease patients [103]. The efficient development of AML required the addition of promoting factors, such as corticosteroids and growth factors, colony stimulating factor CSF-1, known to be high in AML patients [104].

### C3H/He and CBA Models (CBA/Ca, CBA/Cne, and CBA/H)

These models were generated in 1920, by cross-breeding Bragg albino with DBA mice. While C3H/He was specifically selected for the high incidence of mammary tumors [105], CBA was selected for a lower incidence of mammary tumors. The C3H/He was detected 24 h after irradiation in BM cells; this indicates that chromosomal 2 alteration is responsible for the initiation of myeloid leukemogenesis [106]. CBA showed chromosome 2 and 4 aberrations [107,108]. Moreover, an 8% decrease in DNA methylation was observed after exposure to radiation. This hypomethylation played a role in leukemogenesis [109]. The CBA model is considered the most favorable model in radiation-induced AML because of low spontaneous leukemia incidence (0.1 to 1%), high incidence of AML after exposure to radiation or benzene, with lower latency, compared to other models, and more importantly, it mimics human AML at the cytological, histopathological, and molecular levels.

#### 3.2.3. Virally Induced Leukemia Models

Murine leukemia viruses (MuLV) induce non-B and non-T cell leukemia in mice [110,111] and are considered among the simplest retroviruses that shed light on the pathogenesis of leukemia [112,113]. A model was created by injecting cell-free filtrates, including replication-deficient spleen focus forming virus (SFFV) and a replication-competent Friend MuLV [114,115]. It was noticed that the same infection of MuLV induces several subtypes of AML (Table 4), resembling French–American–British (FAB) classification of human AML [116]. Furthermore, MuLV-induced AML led to the discovery of several genes with a significant role in the regulation of growth, death, lineage determination, and development of hematopoietic precursor cells [117]. MuLV induced AML is considered a critical landmark for understanding the pathogenesis of human AML, since it unraveled relevant unknown oncogenes to leukemogenesis (Table 4).

#### 3.2.4. Transposon Models

Sleeping Beauty (SB) transposon is an insertional mutagenesis system, allowing overexpression or inactivation of specific genes depending on the transposon orientation and integration site [118,119]. SB consists of a mobilized piece of DNA, transposon, and a transposase enzyme [120]. In a transgenic animal with a humanized NPM1c+ knock-in allele, this system enhanced the incidence and onset of AML in NPM1c+ mice [121]. An advantage of this model was the identification of mutations in leukemia genes [121].

#### 3.2.5. Transgenic Models: Single Mutation

##### PML-RAR $\alpha$ t(15;17)

Acute promyelocytic leukemia (APL) is a subtype of AML, characterized by t(15;17) chromosomal translocation, resulting in the promyelocytic leukemia-retinoic acid receptor  $\alpha$  (PML-RAR $\alpha$ ) fusion protein [122,123]. PML-RAR $\alpha$  was expressed in three mouse models under the myeloid regulatory promoters. Under the *CD11b* promoter, transgenic mice showed abnormal myelopoiesis and increased radiation sensitivity, however, did not develop any leukemia [124]. Mice expressing the transgene under the human cathepsin G (*HCG*) and human MRP8 (*hMRP8*) promoters [124–126] developed APL phenotypes after a long period of latency [125,126]. These two models recapitulated the remissions seen after all trans-retinoic acid (ATRA) treatment in human APL [125,126].

##### AML1-Eight-Twenty One Oncoprotein

AML1-Eight-Twenty One oncoprotein (ETO) chimeric product, encoded by the t(8;21), occurs in around 12–15% of AML [32]. Knock-in mice expressing AML1-ETO is embryonic lethal due to the complete absence of liver-derived definitive hematopoiesis [127,128]. Embryonic livers contained dysplastic multilineage hematopoietic progenitors that had an abnormally high self-renewal

capacity in vitro, a phenotype typical of leukemic cells [129]. To bypass the embryonic lethality, inducible transgenic models were generated. These mice expressed AML1-ETO in their BM progenitor cells [130,131]. Although abnormal maturation and proliferation of progenitor cells were observed, mice failed to develop leukemia [130,131]. Expression of AML1-ETO under the control of *hMRP8* promoter was unable to develop AML until their exposure to a robust DNA-alkylating mutagen, *N*-ethyl-*N*-nitrosourea [132]. To further enhance AML development, this mouse model was modified by either the expression of other factors or mutations in tyrosine kinases such as c-KIT, FLT3-ITD, or the TEL-platelet-derived growth factor receptor  $\beta$  (PDGFbR) [133,134].

#### CBFB-MYH11

The beta subunit of the core binding complex (CBFB) is a heterodimeric core-binding transcription factor, with a critical role in hematopoiesis [135]. CBF products, due to chromosomal translocations, account for approximately 25% of pediatric and 15% of adult AML patients [136]. The translocation Inv(16) (p13;q22) is a result of the binding of CBFB subunit to the tail region of the smooth muscle myosin heavy chain (*SMMHC*) gene, MYH11 [137]. The resulting fusion protein (CBFB-MYH11) competes with the binding of CBF to target genes, disrupting transcriptional regulation, thus contributing to leukemic transformation [137]. Similar to embryos with homozygous mutations in AML1 [128], knock-in embryonic mice (*Cbfb*<sup>+/Cbfb-MYH11</sup>) lacked definitive hematopoiesis and died during gestation [138]. Chemically or retrovirally induced mutations in heterozygous *CBFB-MYH11* adults led to AML development [138,139]. A conditional knock-in mouse model expressing *CBFB-MYH11* fusion protein in adult mice (*Cbfb*<sup>+56M</sup>) was also generated [140] and led to AML development in 90% of the mice within five months [140].

#### Mutant Nucleophosmin-1 (NPM1c+)

Mutations in the *Nucleophosmin-1* (*NPM1*) gene represent one of the most frequent genetic aberrations in AML [141] and account for 30% of AML patients [50]. Transgenic mice harboring the *NPM1c+* mutation developed myeloproliferation in BM and spleen, supporting a role of NPM1c+ in AML [142]. Chou et al. generated a knock-in transgenic mouse model by inserting the most frequent mutation, TCTG called mutation A, in the C-terminus of wt-NPM1 [143]. Mice homozygous for the transgene encountered embryonic lethality, whereas one-third of the heterozygotes (*Npm1wt/c+*) developed the fetal myeloproliferative disease but not AML [143]. Conditional expression of *NPM1c+* with further genetic manipulations resulted in two models [121,144]. In one model, one-third of the transgenic mice developed leukemia after a long period of latency associated with AML features [144]. In the other model, the expression of humanized NPM1c+ in the hematopoietic stem cells caused *HOX* overexpression, enhanced self-renewal, and expanded myelopoiesis [121].

#### Fms-Related Tyrosine Kinase 3 Internal Tandem Repeats

The second most common genetic aberrations in de novo AML patients occur in the *fms*-related tyrosine kinase 3 internal tandem repeats (*FLT3-ITD*) gene on chromosome 13. These associate with poor prognosis and short overall survival (OS) [145]. A transgenic mouse model expressing FLT3-ITD under the *vav* hematopoietic promoter was created [146]. The majority of transgenic mice developed a myeloproliferative syndrome (MPS) characterized by megakaryocytic hyperplasia and thrombocytosis but not AML [146]. In FLT3-ITD knock-in mice, loss of FLT3 wild-type allele contributed to myeloid expansion and aggressiveness of the MPS disease [147]. Several other models expressing this mutation also revealed MPS but not AML [148,149].

#### Mixed Lineage Leukemia (MLL)

The translocation t(9;11)(p22;q23) produces the fusion product MLL-AF9 [150,151]. In one model, embryonic stem cells were generated from an in-frame fusion of AF9 with exon 8 of mouse MLL [152]. Other models conditionally expressed MLL-AF9 [153]. These models developed only

AML despite the widespread activity of the MLL promoter [152,153]. Conditional expression of MLL-AF9 in long-term hematopoietic stem cells (LT-HSC) produced aggressive AML with extensive tissue infiltration, chemo-resistance, and expressed genes related to epithelial-mesenchymal transition in solid cancers [154]. MLL early introduction results in abnormalities of myeloid cell proliferation and differentiation [155]. Moreover, HOXA9 was found to be essential for the MLL-dependent leukemogenesis in vivo [156].

The translocation t(4;11)(q21;q23) produces the fusion product MLL-AF4. This translocation is associated with pro-B-ALL and rarely AML [157]. Although several models have been established for this translocation, only few models resulted in AML. MLL-AF4 models generated using both a knock-in [158] and *Cre*-inducible inverter model [159] produced large B-cell lymphoma rather than the immature acute leukemia observed in humans [158,159]. The MLL-AF4 expression in hematopoietic precursors, during mouse embryonic development, developed long latency B-cell lymphoma [159,160]. Furthermore, MLL-AF4 knock-in followed by in vitro inducible transduction generated mice with both AML and pre-B-ALL as well as a few MLLs [161].

Leukemia with the t(11;19)(q23;p13.3) translocation express MLL-ENL fusion proteins capable of malignant transformation of myeloid and/or lymphoid progenitor(s). Immortalized cells containing MLL-ENL proviral DNA or enriched primary hematopoietic stem cells transduced with MLL-ENL induced myeloid leukemia in syngeneic and SCID recipients [162]. Using an in vitro B-cell differentiation system, retroviral transduction of *MLL-ENL* generated a leukemia reminiscent of human MLL-ENL ALL [163]. Other models expressed MLL-ENL-ERTm, the ligand-binding domain of the estrogen receptor modified to specifically recognize synthetic but not endogenous estrogens, using retroviral transduction approach [164]. Several other models were generated encountering more mutation along with MLL-ENL [165,166].

## IDH 1/2

A conditional knock-in mouse model was created by inserting the mutated human IDH1 (R132H) into the endogenous murine *idh1* locus. IDH1 (R132H) was expressed in all hematopoietic cells under the *vav* promoter (*vav*-KI mice) or specifically in cells of the myeloid lineage (*LysM*-KI mice) [167]. Transgenic mice showed increased number of early hematopoietic progenitors and developed splenomegaly and anemia with extramedullary hematopoiesis, characteristics of a dysfunctional BM niche, along with partial blockage in myeloid differentiation [167]. Moreover, *LysM*-KI cells have hypermethylated histones and changes to DNA methylation similar to those observed in human *IDH1*- or *IDH2*-mutant AML, demonstrating the induction of leukemic DNA methylation signature in the mouse model [167].

### 3.2.6. Transgenic Models: Compound Transgenic Mouse Models

#### K-RAS-G12D + PML-RAR $\alpha$

4% and 10% of APL patients with PML-RAR $\alpha$  fusion had oncogenic *N-RAS* and *K-RAS* mutations, respectively [168,169]. The conditional expression of oncogenic *K-RAS* and PML-RAR $\alpha$  in mice induced a rapid-onset and highly penetrant, lethal APL-like disease [170].

These mice may be used to test for the therapeutic efficacy of inhibitors of RAS post-translational modifications and RAS downstream signaling [170].

#### N-RASD12 + BCL-2

*N-RAS*, a protein belonging to the family of RAS GTP-ases, is mutated in patients at risk of leukemic transformation after chemotherapy and/or radiotherapy [171]. *N-RAS* mutation at codon 12 is the most frequent abnormality in myelodysplastic syndromes (MDS), associated with AML transformation and poor OS [172]. B-cell lymphoma 2 (*BCL-2*) protein is an apoptosis regulatory protein. *BCL-2* is overexpressed in AML patients [173], which blocks the differentiation of myeloid



progenitors [174]. Both mutants have been previously identified as risk factors for AML in MDS patients [172].

Two murine models of initiation and progression of human MDS/AML were generated [175]. The transplantable model expressing hBCL-2 in a primitive compartment by mouse mammary tumor virus–long terminal repeat (*MMTV $\lambda$ TA /TBCL-2/NRAS $\Delta$ 12*) represents human MDS, whereas the constitutive *MRP8 [BCL-2/NRAS $\Delta$ 12]* model is closer to AML [175]. Both models showed expanded leukemic stem cell (Lin<sup>-</sup>/Sca-1<sup>+</sup>/c-Kit<sup>+</sup>) populations. hBCL-2 is observed in the increased RAS-GTP complex within the expanded Sca-1<sup>+</sup> compartment [175]. The difference of hBCL-2 oncogenic compartmentalization associates with the pro-apoptotic mechanisms in MDS and the anti-apoptotic in AML mice [175]. Downregulation of hBCL-2 in MDS mice partially reversed the phenotype and prolonged survival; however BM blasts and tissue infiltration persisted [175]. This model revealed that the two candidate oncogenes *BCL-2* and mutant *N-RAS* can cooperate to give rise to malignant disease with a penetrance of around 80% and a latency period of 3 to 6 months [175].

#### Mixed Lineage Leukemia-Partial Tandem Duplication + FLT3-ITD

Mixed lineage leukemia-partial tandem duplication (*MLL*-PTD) is expressed in 5 to 7% of cytogenetically normal (CN)-AML patients [176,177]. Approximately 25% of these patients have constitutive activation of FLT3-ITD, conferring a poor prognosis [178]. To recapitulate the *MLL<sup>PTD/WT</sup> ;flt3<sup>ITD/WT</sup>* AML found in humans, a double knock-in mouse model was generated by expressing these two mutated genes under their respective endogenous promoters [179]. After a period of latency, this model developed AML with a short life span, extensive extramedullary involvement, and increased aggressiveness [179]. Reminiscent of this subtype of AML in humans, these transgenic mice have normal chromosomal structures, reduced *MLL*-WT expression, loss of *FLT3*-WT, and increased total *FLT3* expression [179–182]. Moreover, increased *HOXA9* transcript levels were observed, rendering this model valuable for the assessment of epigenetic modifying agents combined with tyrosine kinase inhibitors [179].

#### NUP98-HOXD13 + FLT3-ITD

The chromosomal translocation t(2;11)(q31;p15) leads to the fusion of Nucleoporin (*NUP98*), a structural component of the nuclear pore complex, to the homeobox protein NHD13 (*HOXD13*), inducing leukemogenesis [183]. *NUP98-HOX* fusions are observed in human and murine MDS [184]. Clinical and experimental evidence demonstrated that high rate of FLT3-ITD mutations was observed in patients with *NUP98* translocations [185]. High-level transcriptional expression of *NUP98-HOX* correlated with higher transcript levels of *FLT3* and an increased incidence of FLT3 activating mutations [185]. A novel model combining an FLT3-ITD mutation with NHD13 (*HOXD13*) was generated using their respective endogenous promoters [186]. Initially, these transgenic mice developed leukemia with both primitive myeloid and lymphoid origin. Later, strictly myeloid leukemia with minimal differentiation were monitored [186]. Indeed, *NHD13* transgene enhanced the overexpression of the *HOX* genes, *HOXA7*, *HOXA9*, *HOXB4*, *HOXB6*, *HOXB7*, *HOXC4*, and *HOXC6* [186], shown to play an important role in HSC self-renewal and are upregulated in acute leukemia [187–189]. Nevertheless, mice encountered a spontaneous loss of heterozygosity with a high frequency, resulting in the loss of WT *FLT3* allele, [186], a characteristic of patients with FLT3-ITD mutations [180]. These transgenic mice provide a model to study the molecular pathways underlying MDS-related AML [186].

#### NPM1c+/FLT3

*NPM1c+* and FLT3-ITD double mutations are found in about 40% of AML patients [190]. A compound transgenic mouse model with a double mutation in *NPM1* and *FLT3* was generated by crossing conditional *Npm1<sup>fllox-cA/+</sup>* with constitutive *Flt3<sup>ITD/+</sup>* mice [191]. Inducing recombination of *Npm1<sup>fllox-cA</sup>* in hematopoietic stem cells was accomplished by crossing the double heterozygous mice into *Mx1-Cre* transgenic mice [191]. Double mutant mice developed AML and died by the age of 31–68 days. Peripheral blood showed increased leukocyte counts, reduced numbers of circulating

B and T lymphocytes along with a marked population of immature blasts, while BM cells exhibited increased self-renewal potential [191]. Solid organs were infiltrated with abnormal myeloid cells inducing splenomegaly and hepatomegaly by the time of death, highlighting the role of this double mutation in leukemogenesis [191].

#### N-RAS-G12D + CBFβ-MYH11

A knock-in mice (*Nras*<sup>LSL-G12D</sup>; *Cbfb*<sup>56M</sup>) with an allelic expression of oncogenic N-RAS<sup>G12D</sup> and CBFβ-MYH11 developed leukemia in a cell-autonomous manner, with a short median latency and high leukemia-initiating cell activity [192]. Mice displayed an increased survival of pre-leukemic short-term HSCs and myeloid progenitor cells with a sustained blocked differentiation induced by the fusion protein [192]. *Nras*<sup>LSL-G12D</sup>; *Cbfb*<sup>56M</sup> leukemic cells were sensitive to pharmacologic inhibition of the MEK/ERK signaling pathway [192], highlighting the importance of this pathway in AML and proposing MEK inhibitors as potential therapeutic agents in *inv16*/N-RAS<sup>G12D</sup> AML [192].

#### NPM1c + N-RAS-G12D

One of the most common mutations with NPM1c+ is the *N-RAS* mutation occurring in 20% of NPM1c+ AML patients [190]. *NPM1* and *N-RAS* double mutant transgenic mice (*Npm1*<sup>cA/+</sup>; *Nras*<sup>G12D/+</sup>) developed high penetrance, enhanced self-renewal capacity in hematopoietic progenitors, and AML-like myeloid differentiation bias [193]. At the genomic level, frequent amplification of the mutant *N-RAS-G12D* allele was observed, along with other somatic mutations in AML driver genes [193]. Within the *HOX* genes, which were overexpressed, *HOXA* genes and downstream targets were crucial for the survival of the double-mutant mice [193].

#### WT1-R394W + FLT3-ITD

Wilms tumor 1 (WT1) is a zinc finger transcriptional regulator of target genes implicated in cell differentiation and quiescence [194]. Mutations in *WT1* occur in 10–15% of CN-AML, and it is frequently associated with mutations in several genes [194,195]. *FLT3-ITD* and *WT1* mutations, when present concomitantly, identify a group of AML patients that fail to respond to the standard induction chemotherapy, which results in poor OS [195,196]. Double mutant mice *Flt3*<sup>+/ITD</sup>/*Wt1*<sup>+/R394W</sup> displayed manifestations of shortened survival, myeloid expansion in the BM, anemia, and erythroid dysplasia [197]. Although this model did not appear sufficient to consistently recapitulate human AML, it demonstrated that the combined mutations resulted in a more aggressive disease than either mutant genotype [197].

### 3.2.7. Humanized Models

Humanized mouse models, injected with AML cell lines or patient-derived AML blasts, offered a faster approach and were instrumental in studying different aspects of AML. Several models were attempted to study AML in Nude mice with little success [198,199]. This section will focus on promising models for AML studies.

#### SCID Mice

The severe combined immuno-deficient (*SCID*) mice lacking B and T cell immunity [200], represent essential humanized AML mouse models [201]. Indeed, patient-derived AML cells engraftment enabled the identification of leukemia-initiating cells (LIC), expressing CD34<sup>+</sup> CD38<sup>-</sup> surface markers, recapitulating the human HSCs signature [202]. Engraftment of AMLs from different FAB classes into *SCID* mice reflected their intrinsic biologic behavior, suggesting a clinical correlation to the growth and dissemination of these leukemic subtypes [203]. However, lack of species cross-reactivity of cytokines and the innate host immunity against human AML cells resulted in poor engraftment of the BM [204]. In an attempt to overcome these limitations, exogenous human cytokines and growth factors were



provided, which resulted in better engraftment of human cells [202,204–206]. One limitation of this model is the “leakiness” of the *SCID* mutation occurring in around 10% of the mice [207]. These mice present functional B and T cells, enhanced natural killer (NK) cell activity, and complement activation decreasing the engraftment efficiency [208]. An attempt to bypass this problem uses radiation and/or anti-asialo-GM1 antibody pretreatment. Unfortunately, it reduced the survival of the host, rendering this model unsuitable for human xenograft [209,210].

#### NOD/SCID Mice

To further improve tumor engraftment, a non-obese diabetic (NOD/*SCID*) model exhibiting further impairment of NK activity, reduced mature macrophage, and total lack of B and T cells was generated [211]. This model yielded higher engraftment rates with fewer human AML cells, yet with preserved morphological, phenotypical, and genotypical characteristics of the AML donors [212–215]. This model was used successfully in the screening for new therapeutics in AML [216]. In addition, human AML cells engraftment enabled the fractionation of LICs (CD34<sup>+</sup> CD38<sup>-</sup>) into CD34<sup>+</sup>/CD71<sup>-</sup>/HLA-DR [217], CD34 Thy1 hematopoietic stem cells [218] and CD34/CD117 (or *ckit*) [219] subpopulations. Nevertheless, the NOD/*SCID* model presents the limitation by which higher engraftment rates required the supplementation of human cytokines or transplantation of growth-factor producing cells [220,221]. Moreover, long term engraftments (more than 8.5 months) were disabled due to the development of thymic lymphomas and restoration of NK cells activity during this period [211]. A variant with NOD/*SCID* background is the NSS model (N/S-S/GM/3) expressing Steel factor (SF), granulocyte macrophage-colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) human growth factors was generated [222]. NSS displayed enhanced engraftment of pre-leukemic myeloid cell cultures, as well as primary human AML samples, suggesting that the NSS mouse is a better host for at least a subset of AML samples [223].

#### NSG Mice

NOD/*SCID* mice were further immunosuppressed to generate the NOD/*SCID* b2-microglobulin null mice with a complete abolishment of the NK cell activity [224]. Importantly, a NOD/*SCID* IL2-R $\gamma$ <sup>-/-</sup> or NSG model was generated by deletion or truncation of the gamma chain of IL-2R [225]. In addition to all the abnormalities of their predecessors, NSG mice possess a defective production of IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 as well as a severe impairment of the dendritic cell (DC) and their capacity to produce interferon  $\gamma$  (IFN- $\gamma$ ) upon stimulation [225,226]. Engraftment of newborn NSG mice with human CD34<sup>+</sup> HSCs leads to the generation of a complete hematopoietic system, including red blood cells and platelets [226]. Studies revealed a significantly higher potential of AML cells engraftment in adult NSG mice in comparison to previous immunodeficient hosts [227,228]. Attempts to create different subtypes of AML were successful in NSGs [228]. NSG mice xenotransplanted with five well-characterized AML cell lines established AML models of particular relevance and significance to drug-sensitivity studies [228]. These models were exploited to study the *in vivo* potency of an Imidazoquinoxalines immunomodulatory drug, EAPB0503, and showed its specific activity in NPM1c+ AML subtype [229]. The usability of NSG model allowed the evaluation of the effect of a synthetic retinoid ST1926, or its encapsulated form in nanoparticles (ST1926-NP). El-Houjeiri et al. demonstrated that ST1926-NP is more potent in NSG injected with THP-1 cells [230]. MOLM-13-injected NSG mice showed strong efficacy to chemotherapy (cytarabine, 50 mg/kg) and 5+3 regimen of daunorubicin (1.5 mg/kg) [231]. These models enabled the *in vivo* tracking of UCB-NK cells, demonstrating their capability to migrate to BM and inhibit progression of human leukemia cells. Administering a low dose of human IL-15 enhanced survival of these mice, emphasizing the role of innate immunity in AML outcome [232]. In that sense, utilization of NSG model enabled the assessment of the combination of HSPC-NK cell adoptive transfer with the hypomethylating agents (HMAs), azacitidine (AZA), and decitabine (DAC). Cany et al. signified that the therapeutic combination exerted a significant delay in AML progression in these mice [233].

**Table 2.** A summary of generated AML Zebrafish models and their contribution to the understanding

Zebrafish Model	Zebrafish Manipulation	Model Features and Major Findings
<i>spi-1: MYST3/NCOA2-EGFP</i>	Transgenic expression of human MYST3/NCOA2 fusion under the <i>spi-1/pu.1</i> promoter	First AML model in zebrafish 1.1% of transgenic fishes expressing the transgene showed AML-like phenotype after long latency
<i>hsp70: AML1-ETO</i>	Transgenic expression of human AML1-ETO fusion under <i>hsp70</i> promoter	A phenotype similar to human AML Disruption of definitive hematopoiesis: the switch from primitive to myeloid through <i>gata1</i> downregulation and <i>pu.1</i> upregulation AML1-ETOs effects on HPCs differentiation was mediated by <i>Cyclooxygenase-2 (COX-2)</i> and $\beta$ -catenin signaling
<i>mRNA: NPMc+</i>	mRNAs injection into 1-cell-stage embryos followed by morpholinos (MOs) targeting <i>npm1a</i> and <i>npm1b</i>	Perturbation of primitive and definitive hematopoiesis Alterations in the expression of major transcription factors ( <i>csf1r+</i> , <i>c-myb</i> , <i>CD41</i> , <i>RUNX1</i> )
<i>HSE-MYC-N-EGFP</i>	Induction of murine <i>N-myc</i> gene through heat-shock promoter	AML development with high incidence and rapid progression Enhancement of primitive hematopoiesis through upregulation of transcription factors ( <i>pu.1</i> , <i>gata1</i> , <i>scl</i> , <i>lmo2</i> , <i>p27kip</i> and <i>p21cip1</i> ) Activation of major cancer signaling pathways
IDH1/2 mutants	Knockdown of zebrafish <i>idh1</i> and <i>idh2</i> ( <i>zidh1</i> and <i>zidh2</i> ) by morpholino knockdown and Transcription activator-like effector nuclease (TALEN)-mediated mutagenesis	<i>zidh1</i> suppression/deletion is correlated with a block in primitive hematopoiesis in the myeloid lineage <i>zidh1</i> effects definitive hematopoiesis exclusively <i>zidh2</i> affects primitive hematopoiesis exclusively
	Transgenic expression of human IDH1 mutation	Embryos recapitulated the features of human AML
<i>FLT3-ITD-2A-EGFP spi-1: NPM1-Mut-PA spi-1:</i>	Transgenic expression of human FLT3-ITD or/and NPM1 mutations under the <i>spi-1</i> promoter	Myeloproliferative neoplasm (MPN) development in embryos 66.6% of double mutant transgenic fish showed myeloid leukemia in the kidney marrow along with dedifferentiated myeloid cells
<i>spi-1: CREB-EGFP</i>	Expression of <i>CREB-EGFP</i> under <i>spi-1</i> promoter in myeloid lineage	Alteration of primitive hematopoiesis in embryos AML development in 79% of adult fishes by 9–14 months Aberrant expression of 20 genes diagnosed in pediatric AML
<i>Spi-1: SOX4-EGFP</i>	Expression of <i>SOX4</i> protein downstream the <i>spi-1</i> promoter	Increase in the number of myeloid progenitor cells in the kidney marrow Distortion of the kidney structure

**Table 3.** A summary of generated AML mice models and their contribution to the understanding of

Mouse Model	Manipulation	Outcomes and M
<b>Chemically-Induced Model</b>	Transplantable AML models were generated using the L1210 and p388 cell lines, isolated from DBA/2 mice chemically exposed to the carcinogen 3-methylcholantrene.	Provide a platform for testing chemotherapeutic drugs kinetics, and evaluating effectiveness (mainly Cy
<b>Radiation- Induced Model</b>	<b>RF model</b>	Myeloid leukemia was developed following exposure to fission neutron irradiation or $\gamma$ irradiation
	<b>SJL/J model</b>	The radiation induced AML (RI-AML) in this model, is similar to the secondary human AML occurring after irradiation of Hodgkin disease patients
	<b>C3H/He and CBA models (CBA/Ca, CBA/Cne, and CBA/H)</b>	These models were generated by cross breeding Bragg albino with DBA mice
<b>Virally-induced leukemia models MuLV</b>	Murine leukemia viruses (MuLV) induce non-B and non-T cell leukemia in mice	CBA model is considered model in RI-AML. High incidence of AML by radiation or benzene with compared to other models. Mimics human AML at the histopathological, and molecular
<b>Transposon models</b>	Sleeping Beauty (SB) transposon is another insertional mutagenesis system, allowing overexpression or inactivation of specific genes depending on the transposon orientation and integration site	Same infection of MuLV subtypes of AML that res classification. Identifies unknown oncogenes to leukemogenesis.
		Identification of mutation genes, which provided new insights and potential therapeutic targets in NPM1c+ AML

Table 3. Cont.

	Mouse Model	Manipulation	Outcomes and M	
Trans-genic models	Single mutation	Promyelocytic Leukemia protein (PML)-RAR $\alpha$ t(15;17)	Expressing PML-RAR $\alpha$ under <i>CD11b</i> promoter	Abnormal myelopoiesis radiation sensitivity No AML development
			Expressing PML-RAR $\alpha$ under human cathepsin G ( <i>HCG</i> ) promoter	APL phenotype after lon Remission seen after All (ATRA) treatment in AP
			Expressing PML-RAR $\alpha$ under human MRP8 ( <i>hMRP8</i> ) promoter	APL phenotype after lon Remission seen after ATR
		AML1- Eight-Twenty One oncoprotein (ETO)	Knock-in of AML1-ETO into mouse embryos (AML1-ETO/+)	Absence of liver-derived hematopoiesis Embryonic lethality
			Expressing AML1-ETO in adult bone marrow progenitor cells	Abnormal maturation ar progenitor cells No AML development
			Expressing AML1-ETO under human MRP8 ( <i>hMRP8</i> ) promoter	AML development after <i>N</i> -ethyl- <i>N</i> -nitrosourea
		CBF $\beta$ -MYH11	Knock-in embryonic mice (Cbf $\beta$ +/ <i>Cbf</i> $\beta$ -MYH11)	Lack of definitive hemat Embryonic lethality
			Chemical/ retroviral mutagens on heterozygous CBF $\beta$ -MYH11 adults	AML development
			Conditional knock-in adult mice ( <i>Cbf<math>\beta</math>+/<i>56M</i></i> )	AML development in 90 months
		Mutant Nucleophosmin-1 (NPM1c+)	Knock-in mice expressing NPM1 with mutation A (NPM1c+)	Homozygotes encounter lethality 1/3 of the heterozygotes developed fetal myeloproliferation but not AML

Table 3. Cont.

Mouse Model	Manipulation	Outcomes and Mechanisms
	Expression of NPM1 with mutation A (NPM1c+) under the pCAG promoter	1/3 of the transgenic mice develop leukemia after a long period
	Expression of humanized NPM1c+ in the hematopoietic stem cells	HOX overexpression Enhanced self-renewal Expanded myelopoiesis
<b>Fms-related tyrosine kinase 3 internal tandem repeats (FLT3-ITD)</b>	Expressing FLT3-ITD under the vav hematopoietic promoter	Myeloproliferative syndrome Megakaryocytic hyperplasia thrombocytosis No AML development
	FLT3-ITD knock-in mice with lost FLT3 wild-type allele	Myeloid expansion and development of the MPS disease No AML development
<b>Mixed Lineage Leukemia (MLL)</b>	Embryonic stem cell formed by in-frame fusion of AF9 with exon 8 of mouse MLL	AML development
	Conditional expression of MLL-AF9 using programmed interchromosomal recombination	AML development
	Conditional expression of MLL-AF9 in LT-HSC	Aggressive AML Extensive tissue infiltration Chemoresistance Expression of genes related to epithelial-mesenchymal transition in solid cancers
	Early introduction of MLL	Abnormalities of myeloid lineage and differentiation
<b>IDH 1/2</b>	Expressing IDH1/2 under the vav promoter (Vav-KI mice) or specifically in cells of the myeloid lineage (LysM-KI mice)	Increased number of early myeloid progenitors Splenomegaly Anemia Extramedullary hematopoiesis characteristics of a dysfunctional myeloid lineage and partial blockage in myeloid differentiation Induction of leukemic DMP signature in mouse model

Table 3. Cont.

Mouse Model	Manipulation	Outcomes and Mechanisms
Compound mutations	K-RAS-G12D + PML-RAR $\alpha$	Constitutive expression of K-RAS and PML-RAR $\alpha$ Rapid-onset and highly aggressive APL-like disease
	N-RAS12D + BCL-2	<i>MMTV</i> / <i>TA</i> / <i>TBCL-2</i> / <i>NRASD12</i> Expression of hBCL2 in a primitive compartment by mouse mammary tumor virus–long terminal repeat MDS development Expanded leukemic stem cell population (Lin <sup>-</sup> /Sca-1 <sup>+</sup> /c-Kit <sup>+</sup> ) population Increased apoptosis Malignant disease with a latency around 80% and a latency of 3–6 months
		<i>MRP8</i> [ <i>BCL-2</i> / <i>NRASD12</i> ] Constitutive expression of <i>BCL-2</i> under human <i>MRP8</i> promoter AML development Expanded leukemic stem cell population (Lin <sup>-</sup> /Sca-1 <sup>+</sup> /c-Kit <sup>+</sup> ) population No apoptotic cells Malignant disease with a latency around 80% and a latency of 3–6 months
	MLL-PTD + FLT3-ITD	Expressing MLL-PTD and FLT3-ITD under their respective endogenous promoters Latent AML with a short latency extramedullary involvement aggressiveness Normal chromosomal status Reduced <i>MLL</i> -WT expression Loss of <i>FLT3</i> -WT and increased expression of <i>FLT3</i> -ITD Increased <i>HOXA9</i> transcription
	NUP98-HOXD13 + FLT3-ITD	Expressing FLT3-ITD and NHD13 (HOXD13) under their respective endogenous promoters Myeloid leukemia with a short latency differentiation Overexpression of several genes Spontaneous loss of heterozygosity of the <i>FLT3</i> allele at high frequency, resulting in a homozygous <i>FLT3</i> allele
NPM1c+ - FLT3	Crossing conditional <i>Npm1</i> <sup>fllox-cA/+</sup> with constitutive <i>Flt3</i> <sup>ITD/+</sup> mice AML development Lethality by the age of 3 weeks Modified blood cell counts Immature blasts in BM Myeloid cells infiltration Splénomegaly and hepatomegaly	

Table 3. Cont.

Mouse Model	Manipulation	Outcomes and Mechanisms	
N-RAS-G12D + CFBF-MYH11	Allelic expression of oncogenic N-RAS <sup>G12D</sup> and CFBF-MYH11	Leukemia development in a myeloid-like manner with a short median survival High leukemia-initiating capacity Increased survival of pre-leukemic short-term HSCs and myeloid progenitor cells with blocked differentiation Leukemic cells were sensitive to JAK2 inhibitors	
NPM1c + N-RAS-G12D	Conditional expression of NPM1c+ and N-RAS-G12D	AML-like myeloid differentiation Hematopoietic progenitor cell expansion, penetrance and enhanced self-renewal capacity Frequent amplification of N-RAS-G12D allele Somatic mutations in AML-associated genes Overexpression of HOX9	
WT1-R394W + FLT3-ITD	Crossing <i>Flt3</i> <sup>+ITD</sup> mice with <i>Wt1</i> <sup>+R394W</sup> mice	MDS/MPN development Shortened survival Myeloid expansion in the bone marrow Anemia Erythroid dysplasia	
Xenograft/humanized models	SCID mice	Autosomal recessive mutation Lack of B and T cells Retained innate immunity Identification of leukemia-initiating cells (LIC) Poor engraftment of human hematopoietic stem cells (HSC) in BM	
	NOD/SCID mice	NOD/SCID model: Express additional mutations	Impairment of NK activity Reduced mature macrophage numbers Total lack of B and T cells Fractionation of LIC into myeloid and lymphoid
		NSS model (N/S-S/GM/3): variant of NOD/SCID mice expressing SF, GM-CSF and IL-3	Better host for a subset of human HSCs
NSG mice	Deletion or truncation of the $\gamma$ chain of IL-2R	Defective production of IFN- $\gamma$ and IFN- $\gamma$ Impairment of dendritic cell function Complete abolishment of NK cell activity Higher engraftment capacity of human HSCs than previous models	



**Table 4.** Murine leukemia virus (MuLV) induced AML models: Major gene discoveries and their involvement in different AML subtypes.

MuLV Virus	Mouse Strain	AML Subtype	FAB Classification	Major Gene Discoveries
CasBrM-MuLV	NFS	Granulocytic	M1 or M2	<i>His-1</i>
CasBrE MuLV	NIH Swiss	Myeloid	M1 or M2	<i>Fli-1</i>
Endogenous ecotropic MuLV	AKXD-23	Granulocytic	M1 or M2	<i>Evi-1</i>
Friend-MuLV	C57BL/6	Granulocytic	M1 or M2	<i>Ccnd1</i>
Friend-MuLV	DBA/2	Myeloblastic	M1 or M2	<i>Evi-1, &amp; c-</i>
M-MuLV	BALB/c	Promonocytic	M5	<i>c-myb</i>
B ecotropic MuLV	BXH-2	Myelomonocytic	M4	<i>c-myb, HOXA7, HOXA9, Mafk, Hhex, Rarg, Sharp1, Ccnd3, Clabp, Hmgcr, Nfya</i>

#### 4. *Drosophila Melanogaster*

##### *AML1-ETO*

The chromosomal translocation t(8:21)(q22;q22) is frequent and common in AML. It represents up to 40% of AML subtype M2 of the FAB classification [256]. The fusion gene resulting in this translocation encodes for the chimeric protein AML1-ETO, which contains the N-terminus of AML1 (including its DNA binding domain) and most of the ETO protein [33,257], and inhibits the expression of AML1 target genes leading to leukemogenesis [258]. The detailed molecular mechanism governing this interference is poorly understood, which enticed the generation of several animal models to understand its mode of action. AML1-ETO alone is not sufficient to induce leukemia unless accompanied by secondary mutations [130,131,259]. The simplicity of genetics and ease of manipulation in *Drosophila* presents it as an attractive model to study this complex translocation. In addition, *Drosophila* hematopoiesis is comparable to that of mammals [260]. Two AML1-ETO models of genetically engineered *Drosophila* were generated. In the first model, AML1-ETO is a constitutive transcriptional repressor of AML1 target genes. In the second model, AML1-ETO dominantly interferes with AML1 activity by potentially competing for a common co-factor [261]. The transcription factor Lozenge (Lz) that is similar to human AML1 protein is necessary for the development of crystal cells, one of the major *Drosophila* blood cells, during hematopoiesis [262]. Using these models and by comparison with loss-of-function phenotypes of Lz, AML-1-ETO was shown to act as a constitutive transcriptional repressor [261]. Osman et al. reported that AML1-ETO inhibits the differentiation of crystal cell lineage, and induces an increase in the number of circulating LZ+ progenitors. Moreover, large scale RNA interference screen for suppressors of AML1-ETO in vivo showed that *calpainB* is required for AML1-ETO-induced leukemia in *Drosophila*. Surprisingly, calpainB inhibition in Kasumi-1 cells (AML patient cell line carrying t(8;21) translocation) leads to AML1-ETO degradation and impairs their clonogenic potential [263]. Another study identified pontin/RUVBL1 as a suppressor of AML1-ETO. Indeed, PONTIN knock-down inhibits the proliferation of t(8;21) positive cells, and that PONTIN is essential for Kasumi-1 clonogenic potential and cell cycle progression [264]. Thus, AML1-ETO can be recapitulated in *Drosophila* blood for investigating its mechanism and identifying potential targeted therapeutics for this AML subtype.

Despite advances in our understanding of many molecular mechanisms, in vitro research falls short in determining overall effect of treatment modalities or drug discovery. AML is an intricate disease where culture consisting of a single cell line system, can never recapitulate the complexity of the disease. In the difficulty of obtaining primate models of AML, small rodents, zebrafish, and *Drosophila* with well characterized genetic background and relative ease of manipulation, are the backbone of current work where leukemic cells are interfaced with the host immunity, metabolic environment and importance of the niche ation. Not one model is sufficient to address all posed questions. However, collectively, these models have expanded our knowledge and understanding of several pathways and important players in AML pathogenesis.

**Author Contributions:** All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication. H.S., B.J., R.H., M.H. writing—original draft preparation, N.D., A.B. writing—review and editing, M.E.S. and H.E.H. supervision, review and editing.

**Funding:** This research received no external funding.

**Conflicts of Interest:** The authors declare no conflict of interest.

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## **Résumé**

*Toxoplasma gondii* est un parasite répandu, ayant un impact médical et vétérinaire. Chez les hôtes intermédiaires, les tachyzoïtes et les bradyzoïtes sont responsables de la toxoplasmose aiguë (TA) et chronique (TC), respectivement. Sous la réponse immunitaire, la TA évolue en TC, se manifestant par des kystes latents dans le cerveau et les muscles squelettiques. De plus, une forte corrélation existe entre la TC et plusieurs neuropathologies et cancers. Chez les patients immunodéprimés, la TC peut être réactivée et conduire à une maladie potentiellement fatale. Les traitements actuels ciblent principalement les TA, et présentent plusieurs effets secondaires. Nous nous sommes concentrés sur la TC et la compréhension de ses mécanismes moléculaires. Nous avons d'abord étudié l'efficacité de l'imiquimod contre la TA et la TC. Au cours de la TA, l'imiquimod a entraîné le recrutement de cellules T dans le péritoine et la rate de souris traitées et a considérablement diminué le nombre de kystes cérébraux lors de l'établissement de la TC. Remarquablement, le gavage de souris avec les kystes cérébraux restants chez des souris traitées à l'imiquimod n'a pas pu induire de TC. Après l'établissement de la TC, nous avons démontré que l'imiquimod réduisait considérablement le nombre de kystes cérébraux chez les souris chroniquement infectées et augmentait les récepteurs Toll-Like 11 et 12, qui se lient à une protéine du tachyzoïte, la profiline. Parallèlement, l'expression de TLR-7 augmentait, probablement par son agoniste, l'imiquimod. L'imiquimod induit une interconversion, comme l'indiquent la diminution du taux de protéine P21 et l'augmentation du taux de protéine P30, exprimées exclusivement et respectivement chez les bradyzoïtes et les tachyzoïtes. Les voies en aval de TLR-11/12 ont été activées via la voie MyD88 de signalisation, entraînant une induction ultérieure de la réponse immunitaire. In vitro, l'imiquimod n'affecte pas la souche *Toxoplasma* dépourvue de profiline, suggérant un rôle via le complexe Profilin/TLR-11/12. Enfin, le traitement par l'imiquimod a régulé positivement les transcrits des ligands 9 (CXCL9) et 10 (CXCL10), connus pour induire le recrutement de lymphocytes T dans des foyers réactivés du *Toxoplasma* afin d'éliminer l'infection. Ensuite, nous nous sommes concentrés sur les mécanismes moléculaires impliqués dans la TA et particulièrement dans la TC. Nous avons caractérisé P18, un membre de la superfamille SRS. Lorsque nous avons supprimé P18, la virulence était atténuée au cours de la TA, dû à un échappement plus rapide des tachyzoïtes du péritoine de souris, parallèle à un recrutement significatif de cellules dendritiques. De manière concomitante, moins de tachyzoïtes étaient détectés dans la rate, tandis que plus de parasites ont atteint le cerveau de souris infectées. L'élimination de P18 a augmenté le nombre de kystes de bradyzoïtes in vitro et dans le cerveau de souris infectées. Une expression induite de cytokines, notamment CXCL9 et 10, a également été observée. L'immunosuppression de souris KO P18 infectées a retardé la réactivation. L'infection orale de souris immunodéficientes ayant des macrophages fonctionnels a montré un prolongement de survie, contrairement aux souris n'ayant pas de macrophage, soulignant un rôle de l'IFN- $\gamma$  dans l'interconversion. Collectivement, ces données confirment le rôle de P18 dans la modulation de la réponse immunitaire, facilitant le passage des tachyzoïtes dans le cerveau et favorisant la formation de kystes. P18 joue également un rôle central dans la réactivation et la dissémination de parasites de manière dépendante de l'IFN- $\gamma$ . Dans l'ensemble, nous avons montré le potentiel thérapeutique prometteur de l'imiquimod contre la toxoplasmose et caractérisé le rôle de P18 dans l'immunomodulation afin de contrôler la dissémination et l'interconversion. Notre étude ouvre la voie à de nouvelles approches thérapeutiques contre la toxoplasmose, sa persistance et sa réactivation.

Mots-clés: toxoplasmose chronique, récepteurs Toll-like 11, 12, 7, interféron- $\gamma$ , réactivation, Imiquimod, p18.

## **Abstract**

*Toxoplasma gondii* is a prevalent parasite of medical and veterinary impact. In intermediate hosts, tachyzoites and bradyzoites are responsible for acute and chronic toxoplasmosis (AT and CT), respectively. In immunocompetent patients, AT evolves, due to the host immunity, into a persistent CT, which manifests as latent tissue cysts in the brain and skeletal muscles. CT correlates with several neuro-pathologies and cancers. In immunocompromised patients, CT may reactivate and poses a life threatening condition. Current treatments primarily target AT, are limited to general anti-parasitic/anti-bacterial drugs, and associate with several limitations. Here, we focused on targeting CT and understanding its molecular mechanisms. First, we explored the efficacy of Imiquimod against AT and CT. During AT, Imiquimod led to recruitment of T cells to peritoneum and spleen of treated mice and significantly decreased the number of brain cysts upon establishment of CT. Remarkably, gavage of mice with the remaining brain cysts from Imiquimod treated mice, failed to induce CT. Post-establishment of CT, we demonstrated that Imiquimod sharply reduced the number of brain cysts in chronically infected mice, and significantly increased Toll-Like Receptors 11 and 12. These TLRs are usually expressed by dendritic cells and monocytes, and bind a tachyzoite actin-binding protein, profilin. Concomitantly, TLR-7 was upregulated, likely by its agonist Imiquimod. Imiquimod induced interconversion as documented by the decreased protein levels of P21, and increased protein levels of P30, exclusively expressed in bradyzoites and tachyzoites respectively. Pathways downstream from TLR-11/12 were activated, through MyD88 dependent TLR signaling, which resulted in subsequent immune response induction. In vitro, *Toxoplasma* strain lacking profilin, does not respond to Imiquimod, suggesting a role through Profilin/TLR-11/12. Finally, Imiquimod treatment upregulated the transcript expression levels of Chemokine (C-X-C motif) ligand 9 (CXCL9) and 10 (CXCL10), known to induce T cell recruitment to reactivated *Toxoplasma* foci to clear the infection. Then, we focused on molecular mechanisms involved in AT and notably CT. We characterized P18, a Surface-Antigen 1 (SAG-1) Related Sequence (SRS) superfamily member. When we deleted P18, the virulence was attenuated during AT. Indeed, P18 depletion led to a faster clearance of the parasites from the peritoneum of mice, paralleled by a substantial recruitment of dendritic cells, presumably a vehicle for tachyzoite dissemination. Concomitantly, a lower number of tachyzoites was detected in the spleens while a higher number of parasites reached the brains of infected mice. P18 depletion increased the number of bradyzoite cysts, in vitro and in the brains of infected mice. An induced expression of cytokines/chemokines, including CXCL9 and 10 was also observed. Immunosuppression of infected mice with KO P18, delayed reactivation. Oral infection of Severe Combined Immunodeficiency (SCID) (with IFN- $\gamma$  secreting macrophages), and NOD/Shi-scid/IL-2R $\gamma$ null (NSG) mice (lacking IFN- $\gamma$ ), showed a significant prolonged survival in infected SCID but not NSG mice. This underlines a role for IFN- $\gamma$  in the conversion from bradyzoites to tachyzoites. Collectively, these data support a role of P18 in orchestrating the immune response, which ultimately facilitates tachyzoite trafficking to the brain and favors cyst formation. P18 plays also a central role in parasite reactivation and dissemination in an IFN- $\gamma$  dependent fashion. Altogether, we showed the promising therapeutic potential of Imiquimod against toxoplasmosis and characterized P18 role in immunomodulation to control dissemination and interconversion. Our study paves the path towards new therapeutic approaches against toxoplasmosis. It tackled key questions pertaining to establishment, maintenance and reactivation of CT and should result in a comprehensive solution to this endemic disease.

Keywords: chronic toxoplasmosis, Toll-like receptors 11, 12, 7, Interferon- $\gamma$ , reactivation, Imiquimod, p18.