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Characterization of the *Toxoplasma gondii* infection in SH-SY5Y cells

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1. Summary

Toxoplasmosis is a disease that affects approximately 1/3 of the population worldwide, although in the majority of cases it expresses as asymptomatic. *Toxoplasma gondii* can infect many organs but it establishes a latent infection in the form of cysts in the brain that current treatments are unable to eradicate. The majority of *in vitro* studies use fibroblasts to characterize *T. gondii* infection, but a model of neuronal cells like SH-SY5Y cells could be interesting to study the infection in the brain. For this reason this study tries to identify if SH-SY5Y cells are a good model to study *T. gondii* comparing it with human foreskin fibroblasts (HFF) cells. In this work it has been seen that *T. gondii* in SH-SY5Y cells seemed to have a fastest life cycle than in HFF but the expansion of the infection and the infection rates of the cells was similar in both cell cultures. The transcription of neuron-specific genes has been started but is yet to be evaluated and the production of cytokines by SH-SY5Y cells seemed that decreased with time with *T. gondii* infection. More studies need to be done in order to ensure that SH-SY5Y cells are a good model for study of *T. gondii* infection in neuronal cells, but as the infection is comparable to that in HFF cells it seems likely that it will be.

2. Introduction

2.1. Toxoplasma gondii

Toxoplasma gondii is an ubiquitous unicellular obligate intracellular protozoan parasite. This means that is an eukaryote that needs to live inside a host cell to survive. *T. gondii* is part of the phylum *Apicomplexa*, a group of parasites that include the *Plasmodium* species, which cause malaria (Verhoef et al., 2021), or *Cryptosporidium spp*, that causes diarrheal illnesses (Chalmers et al., 2019). The members of the Apicomplexa family have a similar morphology and most of them also contain a characteristic organelle, the apicoplast, that is essential for the parasite and its origin comes from a secondary endosymbiosis (as its 4 membranes suggest), first a cyanobacterium was endosymbiosed by an eukaryote cell and then the last was endosymbiosed by the ancestor of the phylum *Apicomplexa* (Can et al., 2020; McFadden, 2014). Some of the organisms of this family like *T. gondii* and the *Plasmodium* species also use the apicoplast in specific metabolic functions like fatty acid synthesis (Kochanowsky & Koshy, 2018). The apicoplast has been suggested as a target for new drugs given that it has been demonstrated that it is an essential organelle, but its four membranes make it a difficult target (Zhang et al., 2019). *T. gondii,* like other members of the apicomplexan family, also has a conoid and other organelles such as the micronemes and rhoptries (ROPs and RONs), that store proteins involved in the pathogenesis of the parasites (figure 1). Furthermore, some of these microorganisms also secrete a variety of proteins that are located in these organelles of the apical end during the pathogenesis (Kochanowsky & Koshy, 2018; Mendez et al., 2021; Y. Yang et al., 2023; Zhang et al., 2019).

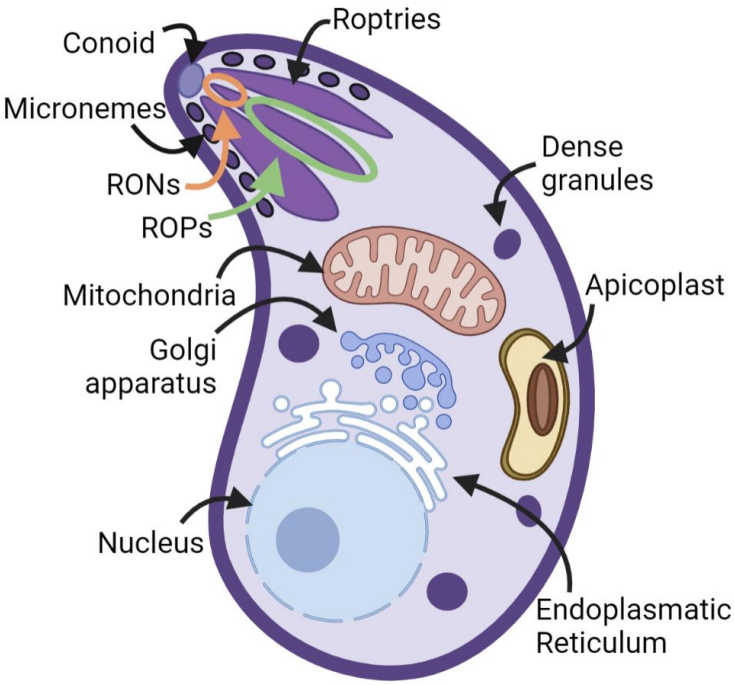

Figure 1: *DRAWN IMAGE OF THE STRUCTURE OF T.GONDII. Highlighted are the organelles of the protozoan parasite. Rhoptry proteins, RONs (neck in orange) and ROPs (body in green) are contained within the rhoptries and along with the micronemes are important for invasion. Created with BioRender.com from the information extracted from the work of Kochanowsky and Koshy (2018), Zhang et al. (2019), and Mendez et al. (2021).*

T. gondii has infected approximately one third of the human population globally (Blanchard et al., 2019). While it generally presents as a mild or asymptomatic infection, *T. gondii* can cause severe life-threatening infections in immunocompromised individuals and the developing foetus

(Blanchard et al., 2019; Mendez et al., 2021). Once inside the host, *T. gondii* can infect all nucleated cells like white blood cells or neuronal cells. In the brain, it has been seen that in mice, *T. gondii* infects predominantly neurons but not glia, although there is some debate on whether or not glia cells are infected (Mendez et al., 2021).

There are different treatments against *T. gondii*, but none are able to eradicate the disease completely as the current treatments are not able to eliminate the parasites that are in cyst form, what allows the parasites to reside in a latent form inside the host cells (Halonen & Weiss, 2013). Furthermore, most of the current treatments against active *T. gondii* have side effects such as hypersensitivity, kidney stones and bone marrow suppression, what limits their use. More studies of *T. gondii* and its infection mechanisms and cyst formation are needed in order to be able to propose new treatments for the infection (M. McPhillie et al., 2016; M. J. McPhillie et al., 2020).

2.1.1. Life cycle

The life cycle of *T. gondii* has a sexual and an asexual phase (figure 2). The sexual cycle only occurs in the *Felidae* family's gut epithelium, and for this, cats are referred to as "definitive" hosts for *T. gondii*. When *T. gondii* is in the cat gut epithelium it differentiates from a bradyzoite (the most common form of the *T. gondii* when it enters the cat organism, although it can also enter in an oocyst form (Halonen & Weiss, 2013)) to a merozoite, and from this form it can differentiate into male and female gametocytes, what allows sexual reproduction (Warschkau & Seeber, 2023). This means that in a cat infected with two different strains of *T. gondii* there can be a genetic recombination (Kochanowsky & Koshy, 2018). With this mechanism millions of oocytes with four haploid sporozoites each can be produced and ultimately, shed. At the moment it is not known yet what makes the cat gut epithelium special, but this is currently being studied (Attias et al., 2020; Kochanowsky & Koshy, 2018; Zhang et al., 2019).

T. gondii can also infect any warm-blooded hosts, like birds, humans or rodents, that are called intermediate hosts. In these intermediate hosts, *T. gondii* goes through asexual replication, which means that the parasite only replicates its haploid genome and divides itself into two daughter cells, a process known as endodyogeny (Kochanowsky & Koshy, 2018). This process is different from a typical eukaryotic cell division, as the two daughter cells form within the mother cell which is recycled as the daughter cells form and separate from each other. The haploid form of *T. gondii* is called a tachyzoite and it disseminates and fast replicates in the host. The tachyzoite is considered the main target for the immune system (Kochanowsky & Koshy, 2018). This form is characteristically found in acute infections and is the one that composes the asexual cycle, that represents the fast reproduction and egression from the host cells (Zhang et al., 2019). Tachyzoites can convert into bradyzoites in some tissues and cell types when in situations of stress, as *T. gondii* is able to sense the host cell's stress signals like the eukaryotic translation of inhibition factor 2 (eIF2) kinases, although the stress factors *in vivo* are not well known (Warschkau & Seeber, 2023). The bradyzoite is a form that is mostly found in the chronic infection (Warschkau & Seeber, 2023). The bradyzoites are the slow replicating forms of tachyzoites that make cysts that contain many bradyzoites and evade the immune response. When in this form *T. gondii* enters in a dormant stage as it has a low replicating ratio and it does not lyse the cells (Kochanowsky & Koshy, 2018). This allows *T. gondii* to establish a persistent infection in the host that can evade drugs and the immune system. In humans, this encystment and persistence is carried out specially in the brain (the major organ of encysment) and in cardiac and skeletal muscle (Kochanowsky & Koshy, 2018; Smith et al., 2021; Zhang et al., 2019).

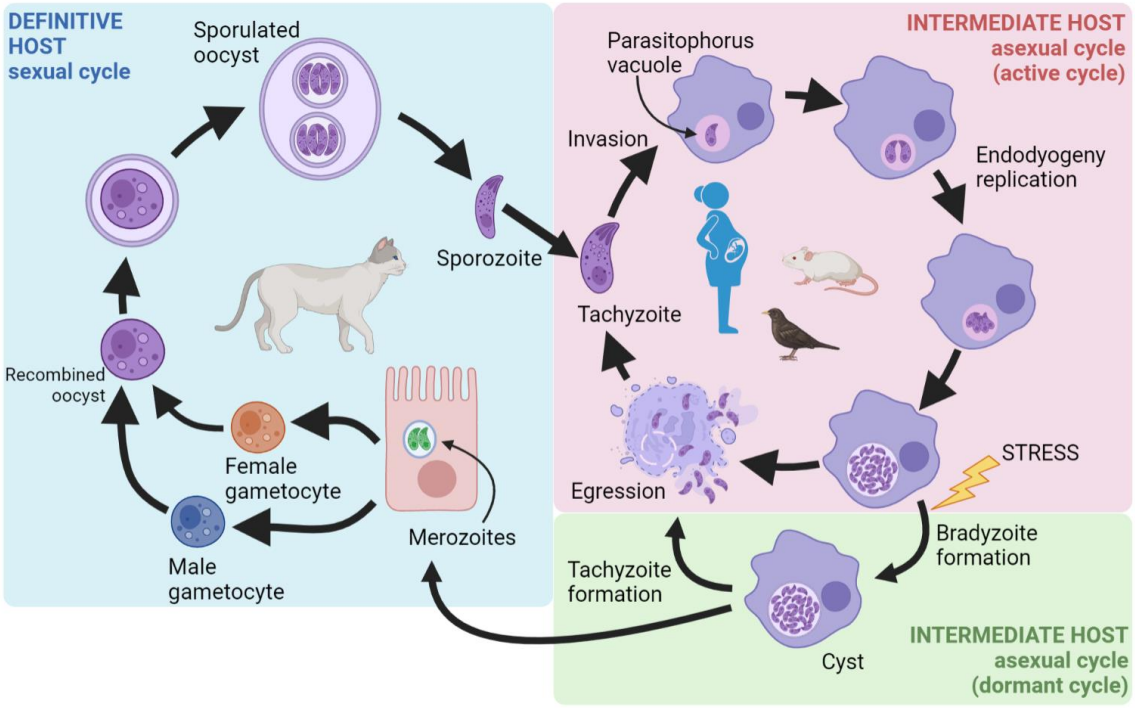

Figure 2: *TOXOPLASMA GONDII'S LIFE CYCLE. Toxoplasma gondii's sexual life cycle (in blue) and asexual life cycle, that can be seen in its two parts, active cycle (in pink) and dormant cycle (in green). Created with BioRender.com adapting the information from the work of Kochanowsky and Koshy (2018), Zhang et al. (2019), Attias et al. (2020) and Warschkau and Seeber (2023).*

2.1.2. Infection

The infection of cats, the definitive host is carried out by carnivorism or ingestion of sporulated oocysts (Kochanowsky & Koshy, 2018). On the other hand, the infection of intermediate hosts happens by ingesting food or water that is contaminated with oocysts or tissue cysts (Madireddy et al., 2022). In humans the infection can also be passed from mother to foetus, via infected organs during transplants or via blood transfusions (Madireddy et al., 2022).

2.1.2.1. Secretory proteins

T. gondii secretes different proteins during the infection process to be able to infiltrate the host cells and regulate the expression of host proteins. Some of these proteins are excreted by the microneme proteins (MICs), rhoptry proteins (ROPs) and dense granules (GRAs). These proteins vary in their localization within the cell and their time of release (Zhang et al., 2019). To be precise, ROPs and MICs are located in the apical end and GRAs are dispersed throughout the parasite (Gubbels & Duraisingh, 2012). ROPs can be divided into two subclasses depending on their localization in the cell, thus the two categories are named Rhoptry neck proteins (RONs) and Rhoptry proteins (ROPs) that are located in the rhoptry neck and bulb respectively (figure 1) (Zhang et al., 2019).

MICs play a role in motility and adhesion to the host cell. MICs are also responsible for the attachment to the host cell by binding to a wide spectrum of targets, like ICAM-1 and heparin (Zhang et al., 2019). The most studied MIC proteins are MIC2 and AMA1, specifically, MIC2 is thought to have a role in motility as parasites deficient in *mic2* have deficient surface attachment properties (Gras et al., 2017), while AMA1 is involved in host cell invasion and forms the moving or tight junction that bridges the interface between the host and parasite during penetration. Although these moving junctions can be formed differently in the absence of AMA1 (Zhang et al., 2019).

ROPs and GRAs are implicated in the fine-tuning of immunological pathways. An example of this is the action of ROP 18, an active threonine kinase whose expression plays an important role in phosphorylation of the immunity related GTPases, blocking their recruitment and preventing the macrophage mediated removal of intracellular parasites (Zhang et al., 2019). Some of the mentioned proteins are also responsible for the differentiation of tachyzoites into bradyzoites due to stress factors like the exposure to alkaline stress (Warschkau & Seeber, 2023; Zhang et al., 2019).

In the motility mechanism model of *T. gondii* named the 'linear motor' model, it is thought that MIC2 proteins and other proteins like Sag1 or AMA1 are secreted at the apical end of the parasite and have the role of transmitting the force of movement by interacting with the hosts substrate surface and the parasites internal actomyosin system (Meissner et al., 2013). This model suggests that the motility force is mainly given by the myosins (Whitelaw et al., 2017). On the other hand, a more accurate model can be explained in the 'retrograde flow motor' that comes from the secretion of the micronemes at the apical end, generating tension along the membrane as the 'flow' moves towards the rear and finally sheds of the membrane bound proteins or recycles them via endocytosis (figure 3) (Whitelaw et al., 2017). This new motility model describes that the majority of the surface proteins are shed in the motility process at the rear end of the parasite, while proteins like MIC2 are endocytosed and degraded in the cell forming a cycle (Whitelaw et al., 2017).

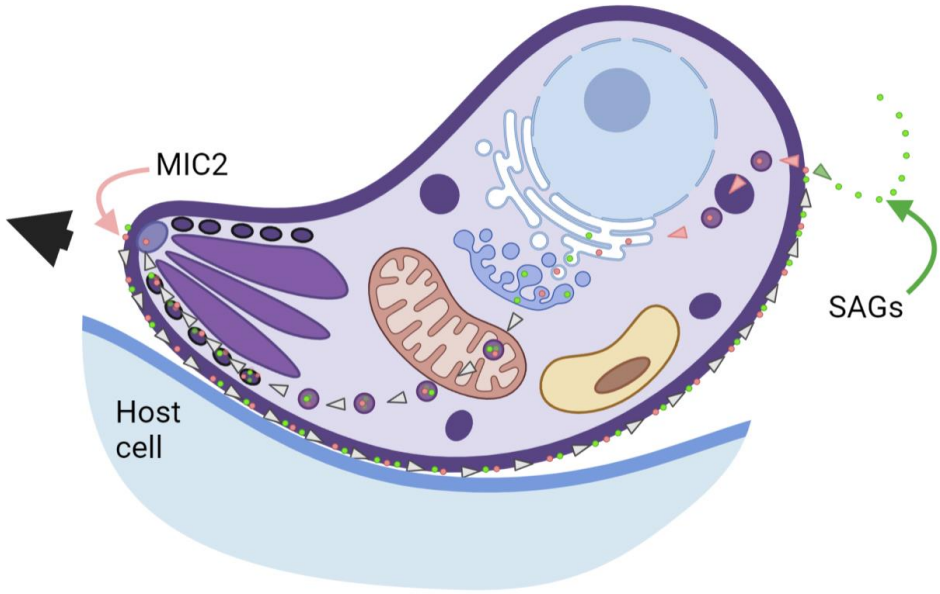

Figure 3: *MEMBRANE PROTEIN MOVEMENT AND SHEDDING/REPURPOSING EXPLAINED IN THE RETROGRADE FLOW MOTOR MODEL. The MIC2 and SAG proteins are exposed in the outer membrane of the parasite and MIC2 attaches to the membrane of the host cell. To be able to glide over the surface of the host cell MICs and SAGs move along the membrane of the parasite. MIC2 is repurposed and enters the parasite in the rear end and SAGs are shed. In green shed proteins, in pink MIC2 that is endocytosed and repurposed, black arrow determines the movement of the parasite. Image made using BioRender adapting the work of Whitelaw* et al. *(2017).*

2.1.2.2. Infection mechanisms

In intermediate hosts like humans, the tissue cysts and oocysts walls are removed by digestive enzymes liberating bradyzoites and sporozoites respectively. When the bradyzoites and sporozoites are inside the new host, they move by a unique gliding mechanism (Attias et al., 2020). First, MIC proteins are secreted, these are essential for gliding and the initial adhesion to the hosts cell surface. The gliding mechanism comprises a complex assembly of proteins

anchored to the plasma membrane of the tachyzoite and to an actin-myosin motor located between the plasma membrane and the inner pellicle. This is known as the glideosome in which take part proteins like AMA1 and MIC2 (both anchored to the plasma membrane) that recognize and attach to receptors on the plasma membrane of the host cell (Attias et al., 2020; Kochanowsky & Koshy, 2018).

The process of infection usually occurs in any nucleated cell, but mostly happens in macrophages, epithelial cells, muscle cells and neurons (Attias et al., 2020). For internalization, the tachyzoite binds to the host cells' surface and then it reorientates the apical end towards it by the secretion of proteins like MICs and RONs (Attias et al., 2020). During invasion, the rhoptry secretes toxofilin, a ROP that binds to actin and can change the host cells' cytoskeleton and help with the formation of the membrane of the parasitophorus vacuole (Delorme-Walker et al., 2012; Li et al., 2018). Then, the tachyzoite assembles the moving junction with the cell membrane of the host cell. This junction forms a ring around the tachyzoite at the point of entry into the host cell that results from the attachment of AMA1 (in the tachyzoite surface) to RON2 (that is secreted into the host and exposed in the membrane of the host cell) (figure 4). The tachyzoite squeezes itself through the moving junction and changes its shape into an hourglass shape that is constricted in the point of contact. Once the internalization is complete, the parasitophorus vacuole closes and the parasite is able to reside safely avoiding the immune system (Attias et al., 2020; Kochanowsky & Koshy, 2018).

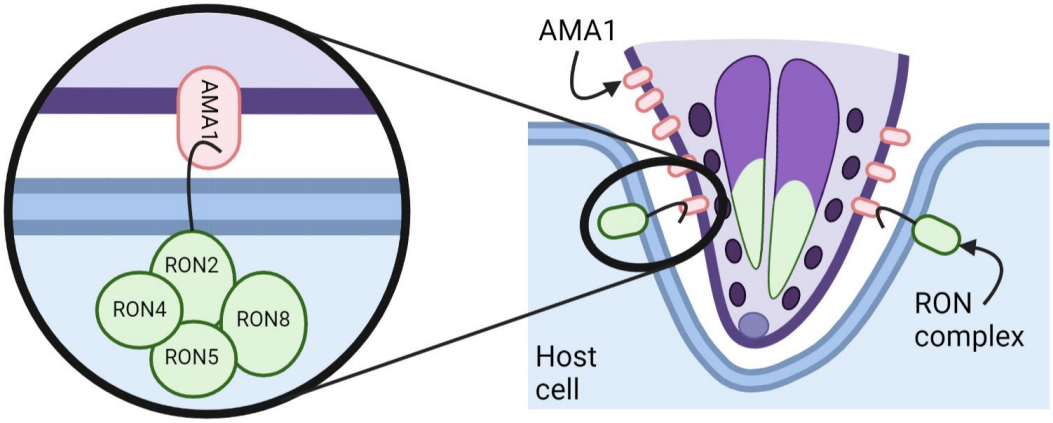

Figure 4: *FORMATION OF THE MOOVING JUNCTION DURING THE INTERNALIZATION PROCESS. The proteins of the RON complex are secreted by the parasite and enter the host cell forming the complex. Then RON2 binds to AMA1 and form the moving junction that moves upwards in the membrane of the parasite as it enters the host cell. Image made using Biorender adapting the work of Kochanowsky and Koshy (2018) and Attias et al. (2020).*

2.1.2.3. Infection of the central nervous system

During the acute stage of the infection, *T. gondii* can infect different organs of which the brain is one of the most important, as here the parasite can transform into bradyzoites and persist latently (Mendez & Koshy, 2017). This way it can evade the immune response and the drug attack as it is also protected by the blood-brain barrier. *T. gondii* enters the brain by infected white blood cells (what is known as the "trojan horse" mechanism) or crossing the blood-brain barrier, although the last has not been thoroughly studied and it is not clear what mechanisms are involved in it (Ross et al., 2022).

When the parasite enters the brain, it infects parenchymal cells that express the innate defences and recruit immune cells. Some studies state that microglial cells can be infected, in which case once the microglial cells have been infected it has been reported that they undergo drastic

morphological changes and acquire a high motility which suggest that they may contribute to the spreading of the infection in the parenchyma (Bhandage et al., 2019; Blanchard et al., 2015).

The infection in the CNS has been reported to produce changes in the neurotransmitters not in specific cell types but within the brain in general (Mendez & Koshy, 2017). The dopaminergic system is one to be believed to have a big impact, as dopamine is responsible for movement and learning. For this reason it is believed that the modulation of dopamine can be related to behavioural changes during infection (Mendez & Koshy, 2017; Vyas et al., 2007).

2.1.3. Effects of *T. gondii* infection in neuronal development

Some recent studies have shown that *T. gondii* may be an underlying cause of several neurodegenerative disorders despite remaining asymptomatic in the majority of the cases (Saftawy et al., 2020). This is because it is thought to be distributed within the brain preferable in the amygdala and the hippocampus, two medial temporal areas that act combined to allow flexible cognitive performance. Specifically, the amygdala modulates the programming process of hippocampal-dependent memories and the hippocampus stores the emotional symbols of events to affect the responses of the amygdala (Piekut et al., 2022; Saftawy et al., 2020). The infection by *T. gondii* in the brain has been linked to a microglial activation and thus inflammation. It has also been proven that *T. gondii* interfered with immunoregulation in the brain (Shinjyo & Kita, 2021).

Another form of neuronal development alteration led by *T. gondii* is the alterations suffered by the foetus in the event of congenital transmission form a seropositive mom to the descendant. The specific mechanisms of affectation of the foetus are yet to be studied thoroughly, but it is well known that the infection of a foetus can result in hydrocephaly, microcephaly or even more severe neurological underdevelopments, and in some severe cases it can result in spontaneous abortion or stillbirth (Teimouri et al., 2020).

2.1.4. Effects of *T. gondii* infection in the brain and behaviour

There have been studies conducted in immunocompetent rodents that show that *T. gondii* infection can affect the behaviour in different ranges (Kochanowsky & Koshy, 2018). For example a decrease of avoidance of cat urine. Although the exact cause of the loss of avoidance of cat urine is still not sure, the fact that there is a noticeable change in behaviour is clear (Vyas et al., 2007). Some studies say that this behaviour indicates specialized loss of olfactory neuronal junctions, while more recent ones suggest that this may be caused because of a loss of fear due to inflammation or because of the loss of dopamine secretion. Different mechanisms have been proposed to explain this change like the infection of specific neurons with cysts, changes in circuits secondary to inflammation in the brain or changes in systemic hormone levels. For now, a definitive mechanism has not been defined, although it has been demonstrated that the location of the cysts does not affect the behavioural changes (Kochanowsky & Koshy, 2018; Mendez & Koshy, 2017).

These behavioural changes have been demonstrated in *T. gondii* infected rodents, but data on changes in humans is inconsistent. What has been found is that *T. gondii* seropositivity is enriched in diseases like schizophrenia, Parkinson's and Alzheimer's (Fuglewicz et al., 2017; Kochanowsky & Koshy, 2018; Piekut et al., 2022). Furthermore, the persistence of the conditions linked to *T. gondii* is the same in countries with high and low *T. gondii* seropositivity. This leads to the conclusion that the link between *T. gondii* and changes in behaviour in humans is still inconsistent (Kochanowsky & Koshy, 2018).

2.2. In vitro models for *T. gondii* infection study

As the infection by *T. gondii* is important in the brain and little is known about it, a model for in vitro studies should be considered to be able to understand how the invasion is conducted in neurons and have a better understanding of the base for the disease in this type of cells. For this reason, in this project the SH-SY5Y cell line is proposed.

2.2.1. SH-SY5Y cells

The SH-SY5Y cell line is a neuroblastoma cell line derived from a metastatic bone marrow tumour biopsy of a 4-year-old female neuroblastoma patient with a sympathetic adrenergic ganglial origin (Xie et al., 2010). This specific cell line is derived from the parental cell line SK-N-SH that was subcloned 3 times: to SH-SY, SH-SY5 and finally to SH-SY5Y (Kovalevich et al., 2013). These cells are used to mock the neuronal cells found in the brain since the early 1980's because they possess the biochemical and functional characteristics of neurons. These cells are widely used, as the use of primary mammalian neurons derived from embryonic CNS tissue is limited by the fact that when these cells differentiate into mature neurons are no longer able to propagate. The main usage for these cells is the study of pathogenic molecular mechanisms of neurodegenerative diseases and pain disorders, as well as oxidative stress (Aliño et al., 2022; Şahin et al., 2021; Xie et al., 2010).

The SH-SY5Y cells have three important characteristics that have to be acknowledged. First, cultures have adherent and floating cells, both of which are viable. Although, most of the studies conducted with these cells use the adherent cells and discard the floating cells (Kovalevich et al., 2013). Second, some early studies showed that the parental differentiated SK-N-SH cells had two different phenotypes: neuroblast-like cells and epithelial-like cells (Kovalevich et al., 2013). These two phenotypes are known as N and S respectively in SH-SY5Y cells (Encinas et al., 2000). The difference between these two phenotypes is that the cells with the neuroblast-like morphology are positive for Tyrosine hydroxylase and dopamine-β-hydroxilase that are characteristic of catecholaminergic neurons, while the epithelial-like cells lack these enzymes. Lastly, the SH-SY5Y cells can also be differentiated into mature neuron-like phenotypes characterized by specific neuronal markers. These cells can be differentiated into the different phenotypes following specific methods, although the use of retinoic acid is the most common. While differentiated cells have been extensively used, undifferentiated cells remain understudied in multiple aspects like their cell surface study (Aliño et al., 2022; Kovalevich et al., 2013).

One of the most important features of SH-SY5Y cells is that these cells can be differentiated into mature neuron-like phenotypes through the manipulation of the medium, which means it is a rather easy technique that benefits the research with these cells. Also, as these are considered a cell line, the ethical concerns associated to primary human neuronal culture do not apply. Furthermore, these cells are human derived, which means that these cells express human neuronal markers and are a more accurate model than a rodent neuron cell culture. The differentiation also synchronizes the cell culture to produce homologous neuronal population, something that can fluctuate in undifferentiated cells (Kovalevich et al., 2013).

2.2.1.1. Differentiation of the cells

Both differentiated and undifferentiated cells have been used in research, but neuronal differentiation comprises a number of specific events like the formation and extension of neurotic processes, an increased electrical excitability of the plasma membrane, the formation of synaptosin-positive functional synapses and induction of neuron-specific enzymes,

neurotransmitters and neurotransmitter receptors; that help making them a more accurate research model (Kovalevich et al., 2013).

The undifferentiated SH-SY5Y cells are characterized by neuroblast-like, non-polarized cell bodies with few truncated bodies as their morphology (see figure 5). These cells tend to grow in clusters and form clumps as they appear to grow on top of one another in the central region of the cell mass. These also have short, truncated processes and are bigger than the differentiated cells. Undifferentiated cells proliferate continuously, express immature neuronal markers and lack mature neuronal markers. These are considered to be reminiscent of immature catecholaminergic neurons (Kovalevich et al., 2013; Şahin et al., 2021).

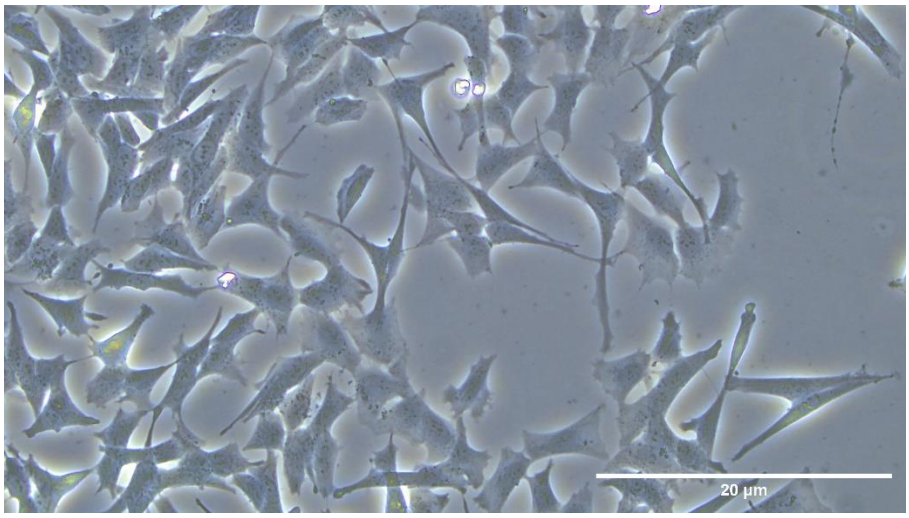

Figure 5: *IMAGE OF AN UNDIFFERENTIATED SH-SY5Y CELL CULTURE. Captured using Leica DMi1 inverted microscope (Leica microsystems) 20x objective and processed using the FIJI software. Scale represents 20µm.*

SH-SY5Y cells can become mature neuron-like cells by different treatments with differentiation-inducing agents such as retinoic acid (RA), phorbol esters or dibutyryl cyclic AMP (Kovalevich et al., 2013; Shipley et al., 2016). These differentiated cells become mature neuron-like cells that exhibit numerous but randomly distributed processes or become distinctly polarized, depending on the differentiation induction method. When differentiated, the cells express some changes like the change of the truncated processes to longer processes and polarized bodies (Kovalevich et al., 2013; Shipley et al., 2016). The differentiation process also induces a decrease in the proliferation rate, as cells are withdrawn from the cell cycle and an increase in the activity of neuron specific enolase, the dominant enolase enzyme present in neuronal and neuroendocrine tissues (Kovalevich et al., 2013; Shipley et al., 2016). The different methods of differentiation for the SH-SY5Y cells have different outcomes, depending on the method used, the cells would exhibit one phenotype or another. For example, the use of RA may result in a cholinergic or dopaminergic phenotype while other methods like the use of phorbol esters can result in adrenergic phenotype (Kovalevich et al., 2013).

The fact of whether the mature neuron-like cells created from SH-SY5Y cells have the same function as neurons or not is still unclear. But there is evidence that supports that differentiated cells are more excitable and their membrane potential increases relative to undifferentiated cells. Although the K^+ and Na^+ currents have also been observed in undifferentiated cells (Şahin et al., 2021).

3. Hypothesis and objectives

The brain is one of the most important organs for *Toxoplasma gondii* tissue cyst formation, however it is one of the less studied *in vitro* due to a lack of good models. The hypothesis for this project was that *T. gondii* invades and establishes an infection in SH-SY5Y cells and human foreskin fibroblasts (HFF) cells (the standard cells used frequently in *in vitro* cell culture assays for *T. gondii*) equally. Also, a secondary hypothesis was that during *T. gondii* infection SH-SY5Y cells change their expression of neuron-specific genes, and decrease the secretion of cytokines.

Based on the hypothesis, the objective of this work was to shed light into the infection for *T. gondii* in neuronal cells by comparing the infection in the neuronal SH-SY5Y cells to HFF. This aimed to set a starting point to, in the future, be able to determine if SH-SY5Y cells are a good *in vitro* model for study of *T. gondii* infection in the brain.

The secondary focus of the study was setting the base for a later study on the differences in neuronal behaviour with and without infection by comparing a group of neuron-specific genes and cytokines and determine if there were any changes in their expression.

4. Materials and methods

4.1. Cell cultures

4.1.1. SH-SY5Y cultures

The SH-SY5Y cells (gifted by Dr. Ben Pickard, University of Strathclyde) were stored at -80ºC in a cryogenic tube and were quickly defrosted and maintained in DMEM F12 media supplemented with 1% of L-glutamine, 1% PenStrep (Penicillin/Streptomyocin) and 10% FBS. Freshly thawed cells were initially cultured in a 25cm^2 culture flask and stored in an incubator at 37ºC, 5% CO_2 until confluent.

For cell maintenance and passaging, the cell media was removed and washed with PBS. Once this was done, TrypLE™ Express Enzyme (1X) (Gibco™, ThermoFisher Scientific) was added to the culture flask and it was left in the incubator for 3 mins. Once this time had elapsed, the cells were checked on the microscope to determine if they were properly detached. Supplemented DMEM media was added to inactivate the trypsin and split into fresh flasks.

For the creation of the cell stocks a new freezing media was created by supplementing DMEM F12 with 1% of L-glutamine, 1% of PenStrep and 10% FBS and 10% of DMSO. The cells were detached of the culture flask using the same method explained above, but when resuspending the cells after the centrifugation 1mL of freezing media was used for each culture flask. In total, 4 freezing tubes with 1mL of cell solution each were frozen.

4.1.2. Human foreskin fibroblasts (HFF) culture

The HFF cells (acquired from ATCC®) were cultured in a 75cm^2 culture flask containing DMEM media supplemented with 10% FBS and 1% L-glutamine and maintained in an incubator at 37ºC and 5% CO_2. These cells were kept in continuous culture and were sub-cultured once its confluence was around 90%. For subculture, the cells were treated as the SH-SY5Y cells explained before, with the difference that HFF cells were replated and maintained in 60mm TC grade petri dishes and the media used for these is the one specified for these cells.

4.1.3. *Toxoplasma gondii* RH-GFP cultures

The RH-GFP strain of *T. gondii* (gifted by Prof. Markus Meissner, LMU Munich), is a strain of T. gondii that is labelled with green fluorescence protein (GFP) that helps to simplify the

immunofluorescence staining process. The RH-GFP were cultured in 60mm TC grade petri dishes with a confluent monolayer of HFF cells (host cells) and supplemented DMEM media. The cultures were kept at 37ºC and 5% CO_2 and were replated once the host cells had been almost lysed.

4.2. Invasion/replication assays

For the invasion/replication (I/R) assay, circular glass coverslips were added to a 24 well plate. To help the SH-SY5Y cells attach to the coverslips, fibronectin diluted in PBS to 1ug/ml was added and incubated on the coverslips for 1 hour at RT. The SH-SY5Y and HFF host cells were added to the wells and incubated until there was a confluent monolayer.

The harvesting of the parasites was done manually by scraping the cells from the surface of the dish and syringing through a 26G needle 5 times and counting of the parasites using a haemocytometer and using the equation stated below.

$$Number\ of\ cells/_{mL} = \frac{number\ of\ cells\ counted}{number\ of\ squares\ counted} \times dilution factor \times 10{,}000$$

For the I/R assay, $1x10^5$ mechanically lysed RH-GFP were added to the host cells and left for incubation during 1h at 37ºC and 5% CO_2.

After this incubation, the coverslips were extracted from the wells and plunged into a falcon tube containing sterile PBS to remove all non-invaded parasites from the surface of the host cells, and replaced into new wells with new media and left for 24h at 37ºC and 5% CO_2. After which, the cells were washed with 1mL pf PBS, fixed with 250µL of 4% PFA for 15 minutes and washed again with PBS.

4.2.1. Immunofluorescence

For the visualization of the parasites in the cultures, a immunofluorescence stain was performed. After fixation, the cells were first blocked and permeabilized with a solution of PBS-Triton-X100 (0.02%) and BSA (2%) in deionized water. AlexaFluor$_{647}$-Phalloidin in a 1:400 dilution and primary antibody SAG1 mouse in a 1:200 dilution, both in blocking buffer were added, incubated for an hour in a wet chamber and washed with PBS. After the SAG1 primary antibody a secondary antibody AlexaFluor568 goat-anti-mouse (1:200) was also added and incubated and washed as the primary antibody, in order to be able to identify the outer membrane of the parasites. The coverslips were then mounted on a microscope slide using DAPI Fluoromount-G® (SouthernBiotech).

4.2.2. Imaging

Fluorescent images of the parasite vacuoles were captured with a Nikon Eclipse 80i epifluorescence wide-field microscope using a 100x PlanApochromat oil immersion objective. Images were acquired using the MicroManager software and processed using FIJI software.

For the invasion assay, 15 random fields of view were taken from 3 coverslips, and the number of vacuoles was counted. This was assessed for two biological replicates.

For the replication assay, images of 100 vacuoles were taken for each of the 3 coverslip and the number of parasites in each of them was counted for 3 coverslips of two biological replicates.

4.3. Plaque assay

For the plaque assay, HFF and SH-SY5Y cells were each seeded in a well of a 6 well plate and cultured until confluent at 37ºC and 5% CO_2. After this time, 1000 freshly lysed RH-GFP parasites

were then inoculated into each well. These cultures were left to incubate unperturbed at 37ºC and 5% CO_2 for 5 days. After this incubation period, the cells were fixed with ice-cold methanol for 10min and stained with 1:2 dilution of trypan blue in deionized water.

When *T. gondii* replicates and egresses from the host cell, the host cell dies. As the cell cultures were left unperturbed, the egressed parasites would infect the nearest cells, creating voids in the monolayer of host cells that are called plaques. 15 of these plaques formed by repeated cycles of the asexual lifecycle of the *T. gondii* were imaged with the Leica DMi1 inverted brightfield microscope (Leica microsystems) using the 5x objective. The images were analysed using FIJI software where a line was drawn using the freehand tool around the edge of the plaque. The pixel to micron ratio was calculated and added to the image properties prior to quantification. Then the area in μm^2 was measured using the Analyse Particle function by the software.

4.4. RNA Extraction and cDNA synthesis

For the RNA extraction of a confluent monolayer of SH-SY5Y cells the Qiashredder (Qiagen, Hilden, Germany) was used to isolate the RNA and the outcome was purified using the RNeasy® Mini Kit (Qiagen, Hilden, Germany). The procedures were performed using the manufacturer's instructions for each kit. And concentration was calculated using the Qubit™ 4 fluorometer (Invitrogen, ThermoFisher Scientific) with the Qubit™ RNA assay kit (Invitrogen, ThermoFisher Scientific) a machine that calculates the concentration of RNA in the sample using a colorimetric reaction and a standard curve. This procedure was carried out using the manual provided by the manufacturer. The concentration was between 800- 900ng/mL in all cases.

The cDNA synthesis was done using the High-capacity cDNA Reverse transcription kit with the addition of RNAse inhibitor (Applied Biosystems, ThermoFisher Scientific) using the instructions provided by the manufacturer and the Biometra Tone thermal cycler (Analytikjena). The cDNA concentration was then calculated using the Qubit™ 4 fluorometer (Invitrogen, ThermoFisher Scientific) with the Qubit™ DNA assay kit (Invitrogen, ThermoFisher Scientific).

4.5. Gradient PCR analysis

In the PCRs the genes; Indoleamine 2,3-Dioxygenase 1 (IDO), Tryptophan 2,3-Dioxygenase (TDO), Macrophage Migration Inhibitory Factor (MIF), IL-1β, nitric oxide synthase 2 (iNOS 2) and Toll Like Receptor 4 (TLR-4) for SH-SY5Y cells were amplified. For Toxoplasma specific genes, the genes SAG1 and RH MIF were amplified, as well as GAPDH as housekeeping gene for SH-SY5Y cells. All primers are described in table 1 and were purchased from Invitrogen, ThermoFisher Scientific.

Table 1: *Genes and primers used for the PCRs.*

Gene	Direction	Sequence (5'-3')	Tm (ºC)	Size (bp)
GAPDH (housekeeping gene)	Forward	TCGACAGTCAGCCGCATCTTCTTT	64.7	104-344
	Reverse	GCCCAATACGACCAAATCCGTTGA	63.7	
IDO	Forward	GCCTGATCTCATAGAGTCTGGC	60.0	119
	Reverse	TGCATCCCAGAACTAGACGTGC	62.6	
TDO	Forward	CAGGTGCCTTTTCAGTTGCTGAC	62.6	138
	Reverse	GTAGTGATAGCCTGAGGAACCAC	60.2	
MIF	Forward	AGAACCGCTCCTACAGCAAGCT	64.0	122

	Reverse	GGAGTTGTTCCAGCCCACATTG	62.0	
IL-1b	Forward	ATGATGGCTTATTACAGTGGCAA	58.5	132
	Reverse	GTCGGAGATTCGTAGCTGGA	59.0	
iNOS 2	Forward	TTCAGTATCACAACCTCAGCAAG	58.7	207
	Reverse	TGGACCTGCAAGTTAAAATCCC	58.8	
TLR-4	Forward	CCCTGAGGCATTTAGGCAGCTA	62.1	126
	Reverse	AGGTAGAGAGGTGGCTTAGGCT	62.0	
SAG1 (*Toxoplasma* specific)	Forward	GCTGTAACATTGAGCTCCTTGATTCCTG	64.2	356
	Reverse	CCGGAACAGTACTGATTGTTGTCTTGAG	63.8	
RH MIF (*Toxoplasma* specific)	Forward	TCTTGAAGGACGCCGAAAAAG	59.1	156
	Reverse	AACTGGTGATGCCTCCAATGG	60.6	

The primers were first tested doing gradient PCRs to check that the cells are able to express these genes and to check the optimal annealing temperature for each primer. This was done using the DreamTaq Green PCR Master Mix (2X) kit (ThermoFisher Scientific) and following the protocol provided by the manufacturer with 0.5µL of cDNA (around 10ng of cDNA) and 100mM of each primer. The PCR products were run on by electrophoresis in a 1% agarose gel with a 0.006% of ethidium bromide for 40min at 120V. Then, the gels were visualised using the G:BOX gel reader (SYNGENE).

4.6. Enzyme-linked immunosorbent assay (ELISA) IL-6 and IL-1β quantification

Two ELISAs, one for IL-1β and one for IL-6 were made with three cultures of SH-SY5Y cells: one unperturbed, one stimulated with lipopolysaccharide (LPS) and one infected with *T. gondii*. For this, the cells were seeded at a density of $4x10^4$cells/well in a 96 well plate. At t=0h 100µL of media was extracted from the LPS stimulation and *T. gondii* infection conditions and 100µL of treatment media was added into these wells. For the LPS stimulation a stock solution of 2g/mL of LPS was made in DMEM media and used for the stimulation of the wells. For the *T. gondii* infection, 20,000cells/well were added into each well with 100µL of DMEM media. The 200µL media of the cells was collected at various times (t=0h, 6h and 24h) to be analysed by ELISA. The ELISAs assays were made using the Human IL-1β Uncoated ELISA kit (Invitrogen, ThermoFisher Scientific) and Human IL-6 Uncoated ELISA kit (Invitrogen, ThermoFisher Scientific), respectively, and following the protocol provided by the manufacturer.

4.7. Statistical analysis

The data of the replication assay was plotted using Microsoft Excel 2021 onto a bar graph. For the analysis of the results of the plaque assay and invasion assay, superplots were made to be able to analyse the different data points for each replicate and the mean values in the same graph and identify the difference in the results. For this the data was submitted to the Hyugene data plotting website (huygens.science.uva.nl/SuperPlotsOfData/) and the graph was automatically provided with the standard deviation of the values. The statistical analysis (means, t tests and standard deviations) for all the data was performed using Excel. P values are represented in the graphs as * (P<0.05), ** (P<0.01), *** (P<0.001) and **** (P<0.0001).

5. Results

As *T. gondii* has the ability of invade and infect all nucleated cells been more prone to form cysts in the brain, this work was aimed to test if SH-SY5Y cells were able to be infected and if so, if the

infection cycle was similar to that in HFF. So, it was set up to do two sets of assays, first a set of *in vitro* assays were done and later, a set of gene expression and ELISA assays were also carried out to check if *T. gondii* infection caused any changes in the expression of SH-SY5Y cells.

5.1. Invasion/replication assays

5.1.1. Invasion assay

The first of the *in vitro* assays made was the invasion assay. The purpose of this assay was the determination of whether or not SH-SY5Y cells could be infected and if both cultures have the same invasion ratio in order to determine if the posterior assays are comparable.

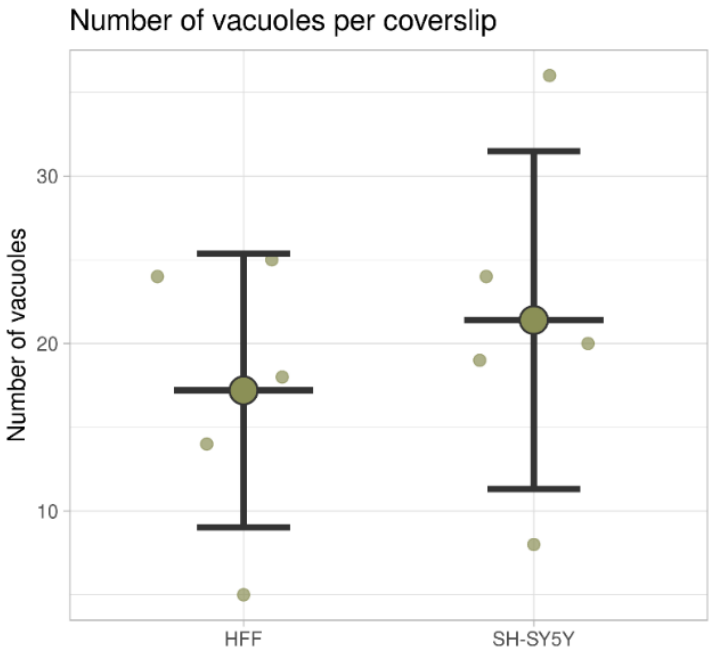

Figure 6: *INVASION OF TOXOPLASMA GONDII IN HFF AND SHSY5Y CELLS SH-SY5Y. The vacuoles of 15 random fields of view were counted per coverslip. Data presented as mean±SD (n=2, 2 or more technical replicates). Image made using huygens.science.uva.nl/SuperPlotsOfData/.*

The results of the invasion assay seen in figure 6 showed that there was no statistical difference between the SH-SY5Y cell cultures and the HFF cultures for the number of parasites that invade the cells. However, there is a tendency towards a higher invasion rate in SH-SY5Y cells.

5.1.2. Replication assay

Once the possibility of infection of SH-SY5Y cells was clear, the next step was to analyse the replication of the parasite within these cells. This assay evaluates the rate of replication in SH-SY5Y and determine if it is faster in SH-SY5Y cells than in HFF cells, by counting the number of parasites per parasitophorus vacuole, as seen in figure 7. This can help understand if *T. gondii* replicates better in SH-SY5Y cells.

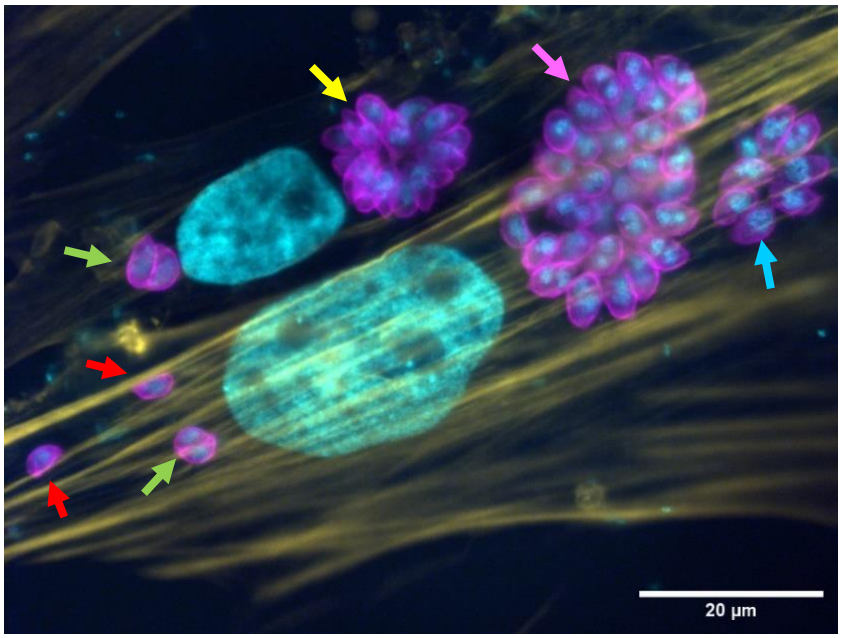

Figure 7: *EXAMPLE IMAGE OF PARASITOPORUS VACUOLES WITH DIFERENT NUMBERS OF PARASITES IN HFF CELLS. In cyan: DAPI, in magenta: SAG1 stained with AlexaFluor568 and in yellow: actin stained with Phalloidin647. Red arrow shows single parasite vacuole, green arrow shows two parasites vacuole, blue arrow shows 8 parasites vacuole, yellow arrow shows 16 parasites vacuole and pink arrow shows 32+ parasites vacuole. This image was taken using an Olympus IX71 Inverted Fluorescence Microscope (Olympus) and a 100x objective. Scalebar represent 20µm.*

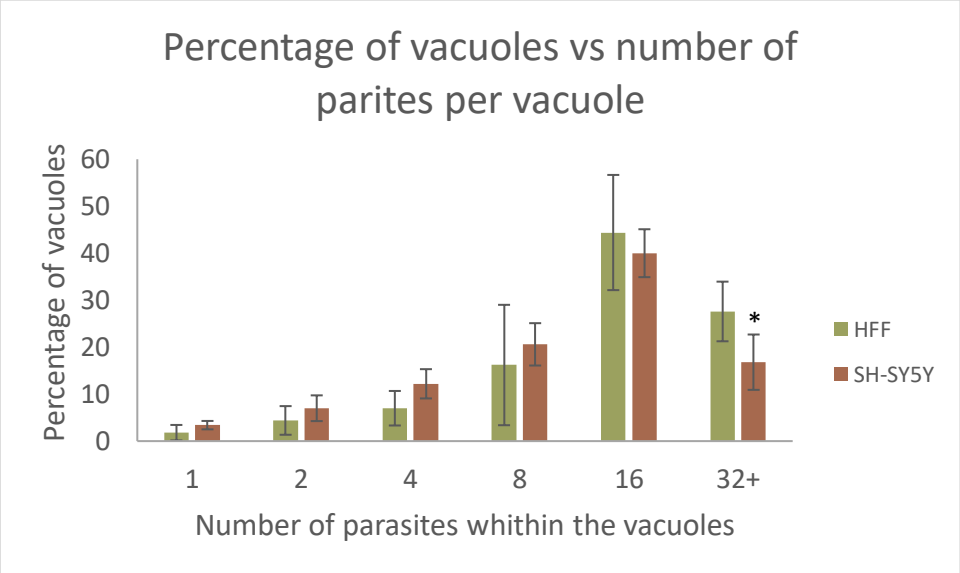

Figure 8*: PERCENTAGE OF VACUOLES WITH DIFFERENT STAGES OF REPLICATION IN SH-SY5Y AND HFF CELLS. The cells were immunostained and 100 vacuoles were imaged per technical replicate. Data shown as mean±SD (n=2, 2 or more technical replicates). *: p<0.05 (T test), percentage of vacuoles in each stage in SH-SY5Y cell culture significantly different from the percentage of vacuoles in HFF cell cultures in the same stage.*

The number of parasitophorous vacuoles with small number of parasites in them (1-8 parasites) is higher in SH-SY5Y cells than in HFF cells, as seen in figure 7. Higher number of parasites in a parasitophorous vacuole are less common in SH-SY5Y cells, and the difference becomes significant for the 32+ parasites vacuoles.

5.2. Plaque assay

Once it was clear that the SH-SY5Y cells were able to be infected by *T. gondii* and the replication of the parasite was evaluated, the question of the expansion of the infection was arisen. For

this, the plaque assay was done. In it, the cells were left unperturbed and the death of the host cells formed the holes in the cell culture monolayer (plaques), the area of which was calculated using Fiji and manually outlining of each plaque as seen in figure 9.

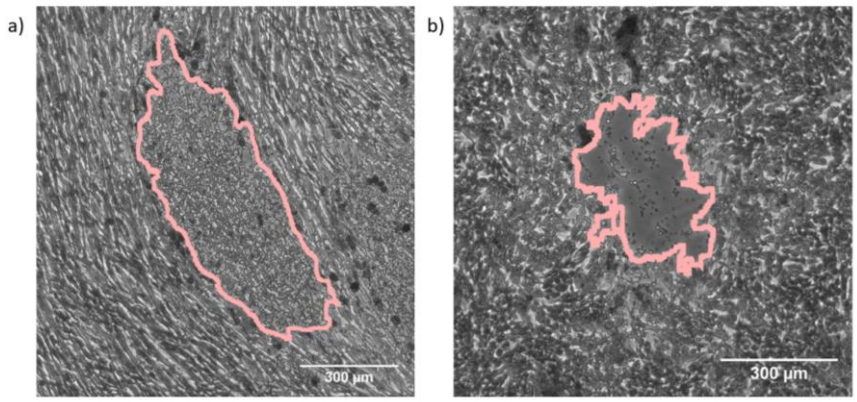

***Figure 9**: EXAMPLES OF THE PLAQUES AND THE MANUALLY OUTLINED AREA CALCULATED. a) Image of a plaque formed by the replication of T. gondii in a confluent monolayer of HFF cells b) Image of a plaque formed by the replication of T. gondii in a confluent monolayer of SH-SY5Y cells. In pink, the drawing of the outside of the plaque that was used to measure the area of the plaques made with the software FIJI (scalebar represents 300µm). Images made using Leica DMi1 inverted microscope (Leica microsystems) 20x objective and processed using the FIJI software (1pixel=0.621µm).*

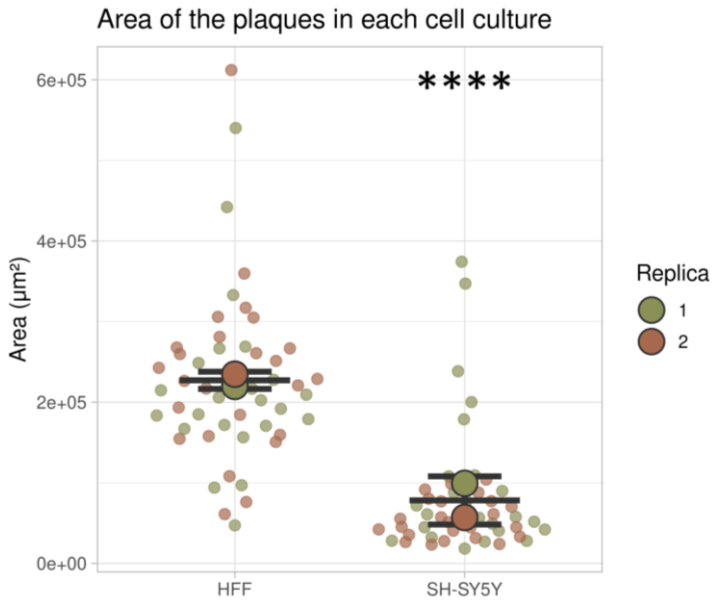

***Figure 10:** AREA OF THE PLAQUES IN SH-SY5Y CELLS AND IN HFF CELLS SORTED BY BIOLOGICAL REPLICATES. The area of the plaques in SH-SY5Y cells is significantly smaller than the area in HFF cells. In green the areas of the plaques for replicate 1 and the mean value, In brown the area of the plaques for replicate 2 and the mean value. Data shown are mean±SD (n=2). Image made using huygens.science.uva.nl/SuperPlotsOfData/. ****: p<0.00001 (T test), significantly different from HFF.*

For the area of the plaques, it can be seen in figure 9 that there was a significant difference between the area of the plaques in the HFF culture and the SH-SY5Y culture, as the plaques of the HFF culture doubled the size of the ones in the SH-SY5Y culture. Also, it can be seen that he replicates did not follow any specific pattern and the results for both replicates are mixed together. There were bigger plaques on both cultures that double the size of the mean. These were some plaques that seemed two different plaques that were joined on development, but if the difference between the two plaques could not be determined it was counted as one.

5.3. Gene expression

5.3.1. Gradient PCR

After knowing that the infection of the SH-SY5Y cells was possible, some qPCR analysis were proposed in order to be able to determine if the gene expression neuron-specific genes in SH-SY5Y cells was modified by *T. gondii*. The primers were first tested to ensure the cells expressed the genes and, for this, gradient PCR were made to stablish the optimal annealing temperatures for the primers to have successful qPCR in the future.

Table 2: *Results of the genes tested and the optimal annealing temperatures for the primers.*

GENES	ANNEALING TEMPERATURES
GAPDH	60ºC
IDO	59ºC
TDO	55ºC
MIF	57ºC
IL-1β	53ºC
iNOS2	51ºC
TLR-4	59ºC
SAG1	58ºC
RH MIF	57ºC

For the gradient PCR results shown in table 2 and annex 1, it can be seen that all the genes tested worked for the SH-SY5Y cell cultures. In this case, TDO, IL-1β and iNOS2 were tested with LPS stimulated SH-SY5Y cells, as these cells do not express these genes if not induced by an external factor, as previous work with SH-SY5Y cells showed (L. Yang et al., 2020) and as seen in figures 14, 17 and 19 in annex 1, used as controls.

5.4. ELISA

Given that the SH-SY5Y cells produce IL-1β when infected with *T. gondii*, as checked in the gradient PCR analysis, the question of the modulation of the production of cytokines by *T. gondii* was raised, due to the fact that it was also stated by previous studies (Sedighi et al., 2021). The production of cytokines from the SH-SY5Y cells was analysed in order to try to understand how can *T. gondii* evade the immune response inside the organism and if the modulation of the production of cytokines plays a role in it. For this, the cells were cultured and given different treatments (no treatment, LPS and T. gondii) to assess the IL-6 and IL-1β production.

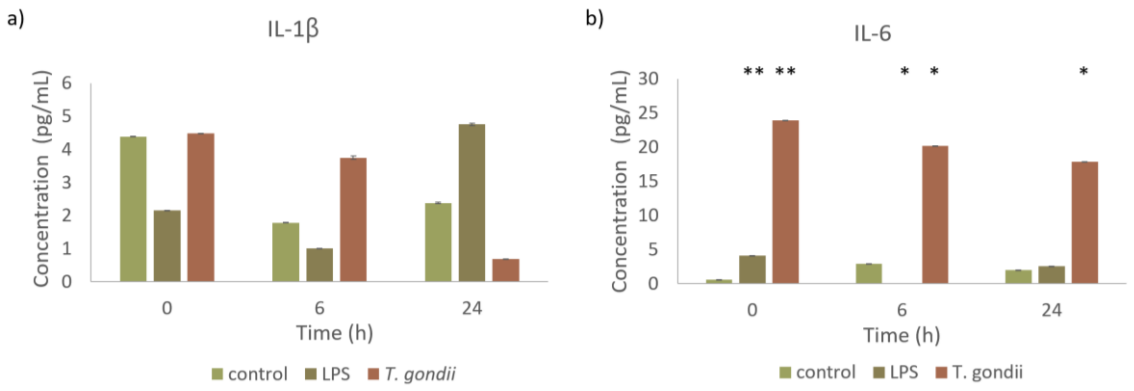

Figure 11: *SECRETION OF CYTOKINES IN SH-SY5Y CELLS WITH DIFFERENT TREATMENTS ANALYSED WITH ELISA. The stimulation of the cells was made by the addition of 100µL of 2g/mL of LPS for the LPS condition and the addition of 20.000parasites/well for the T. gondii condition. Data shown are mean±SD (n=1). a) Concentration of IL-1β (pg/mL) at each time (0h, 6h and 24h) for each condition. b) Concentration of IL-6 (pg/mL) at each time (0h, 6h and 24h) for*

*each condition. From the T test statistical results: *: p<0.05, values significantly different from control; **: p<0.01, T. gondii stimulation values significantly different from control.*

The levels of cytokines for both, IL-6 and IL-1β had a tendency to decrease with time in the *T. gondii* infected group and in the control group for IL-1β, as it can be seen in figure 10. Although the difference in cytokine production between the control group and different groups was not statistically different for IL-1β production. At t=0h the cytokine production was similar for *T. gondii* infection and for the control and it was decreased in LPS stimulation. At t=6h, the cytokine secretion by the LPS stimulation group continued to be decreased compared to the control, but the *T. gondii* infected cells' cytokine secretion was higher than the control, although not significant. Lastly, at t=24h, the LPS stimulated cells produced more IL-1β than the control group and the *T. gondii* infected cells produced less cytokines than the control, both without significance.

For IL-6 production, a statistical significance could be seen between control and LSP stimulation and *T. gondii* infection. The *T. gondii* infection group had a statistical increase compared with the control in all the times, but it decreased with time. In comparison, for the LPS stimulated group, this increase could just be seen at t=0h, then at t=6 there was a decrease and finally at t=24h there was another increase but without statistical significance.

It is likely that some contamination may have occurred, as the concentration of IL-1β is high for all the conditions at t=0h. The sample collection for this time was just after the addition of the stimulation, so the concentration of cytokines should be low, as these cells do not produce IL-1β if not in a dangerous situation.

6. Discussion

This work was aimed to show if SH-SY5Y cells could be a good *in vitro* model for *T. gondii* infection studies in neurons. For this, *in vitro* assays to determine the infection ratios, the replication rate and the infection expansion were made, and cytokine secretion and gene expression was looked at in order to assess the effects of the infection of the cells. The results showed that there were some differences in the *T. gondii* infection of SH-SY5Y compared to HFF cells. These differences could be caused by the structure of the cells as HFF morphology is different to the one of SH-SY5Y cells.

First, the fact that the infection seemed to be similar in both cultures showed that the parasites did not have a preference or higher affinity for infection of one type of cells. As it was expressed by previous studies in the matter (Schlüter & Barragan, 2019). This also gave a common start point for the interpretation of the results as it showed that both cell cultures had the same probabilities of infection and that the differences between them were not caused by a difference on the infection ratios of the cells.

The replication assay showed that the SH-SY5Y cells had more parasitophorus vacuoles with smaller number of parasites. This could mean either two things, one could be that the replication in SH-SY5Y cells was slower than in HFF cells, but the fact that there were some 32+ vacuoles present in the samples was a sign that that was not the case. Another thing that could be happening is that the parasitophorus vacuoles egressed faster, and there was a higher number of new infections. The last case is the one that seems more probable, as the number of vacuoles with 16 parasites was similar and there were some 32+ vacuoles in the samples. It was expected that the majority of the vacuoles were around the 16 parasites stage, as the parasites replicate within a cell in vitro in 6 to 8 h (Black & Boothroyd, 2000), and both of the cell cultures presented

the majority of the vacuoles in this stage. The fast egression of the parasitophorous vacuoles could be explained by the difference in size between SH-SY5Y cells and HFF. SH-SY5Y cells are smaller and this could have impacted the number of parasites that could healthily reside within these cells before the need for egression.

For the formation of plaques, it could be seen that the HFF cell cultures infected with *T. gondii* had bigger sized plaques than those seen in the SH-SY5Y cell cultures. As said before, the infection of both cell cultures seemed to be equivalent, what means that the difference in the plaque size was not a cause of a slower or lesser infection of SH-SY5Y cells compared to the HFF cells. The difference on the plaque size could be related to the size of the cells as HFF cells tend to have a bigger area than SH-SY5Y cells. Also, the egression rate of the parasites could also be affecting the size of the plaques. The egression made the cells burst and the plaques to form, but in the case of SH-SY5Y cells, the egression of the parasites liberated less parasites than the egression in HFF cells, because as discussed before, the egression happened earlier in the endodyogenic cycle. This means that the number of parasite that were liberated and free to invade new cells was smaller and thus, the number of cells infected from each egression was smaller. Which in turn means that even though the egression occurred faster, the number of infected cells after it was smaller. The differences between the SH-SY5Y cultures and the HFF cultures in terms of the spreading of the infection were not that big as HFF have a bigger number of cells infected but it took more time for the parasites to egress and SH-SY5Y cells had less infections per egression but the egressions were faster. This leads to the theory that the differences between the plaque sizes resided in the size of the cells. SH-SY5Y cells are smaller than HFF cells and in the same area there can fit more SH-SY5Y cells than HFF cells. This would mean that the spreading of the infection was the same, but the effects of this spreading were less visible in the SH-SY5Y cultures because of the number of cells that were in them. Additionally, the fact that the data extracted for both replicates was mixed together and did not show any pattern ensures the reproducibility of the assay, given that both replicates showed similar results.

All of these three assays reassure that there was an infection in SH-SY5Y cells and that SH-SY5Y cells follow the infection cycle until the egression stage more or less at the same rate as the HFF cells.

For the gene expression assays it has been proved the functionality of these primer pairs and the expression of these genes in the SH-SY5Y cells. Although it is yet to be studied the comparisons between the quantitative expression on uninfected SH-SY5Y cells and infected SH-SY5Y cell cultures in future projects. The cytokine secretion on the other hand had an interesting pattern as the production of IL-1b and IL-6 was clear for infected SH-SY5Y cells, as some studies had previously shown (Sedighi et al., 2021), but their secretion seemed to decrease as time went on. This could mean that the cells could produce cytokines when exposed to a danger, but *T. gondii* had some mechanism to decrease their production in neuronal cells, and thus, to evade the immune response on the brain, as some early studies also showed (Blanchard et al., 2019). This was quite interesting as it is known that *T. gondii* resides in the brain of animals and can form cysts in this organ. The ability of *T. gondii* to be able to evade the secretion of these cytokines helps with the evasion of the immune response in the brain and would explain the ability of these parasites to persist in the brain.

It has to be taken into account that these results and theories are based on an n=2 for the invasion/replication and plaque assays and that a third replicate would be necessary in order to ensure a correct statistical representation of the data. For the ELISA, only one was performed

and the data that this provided must be completed with at least another replicate for it, as it does not have a statistical significance. Also, comparisons of the cell areas could be beneficial to be able to efficiently compare both cell types and reassure the theories.

7. Conclusion

From this study it can be concluded that the *T. gondii* infection of SH-SY5Y cells is similar to the infection in HFF cells. In this work it is proposed that the difference in both cell culture infections was driven by the cell size, as SH-SY5Y cells are smaller and thus were not able to hold as many parasites inside the cell. This resonates with the fact that the *T. gondii* asexual active cycle circled around faster and with the less noticeable impact of the cell death at a large scale.

The modification of the gene expression in the SH-SY5Y cells is yet to be determined, but in this work is reflected the expression of certain genes in this cell type. The cytokine modulation in this cell type with the infection of *T. gondii* is something that needs further study but would be an interesting approach for future projects. As in this work it has been brought into light that there can be a modulation of the secretion of IL-1β and IL-6 driven by the invasion of *T. gondii* in SH-SY5Y cells.

To be able to determine if the SH-SY5Y cells are a good model for the infection of *T. gondii* in the brain more studies are needed. In this work it has been demonstrated that undifferentiated SH-SY5Y cells have a similar infection pattern to HFF, but it has yet to be studied the differences with differentiated SH-SY5Y cells and the modulations in the gene expression for both neuronal cell cultures.

8. Acknowledgments

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10. Annex

10.1. Annex 1: gel images

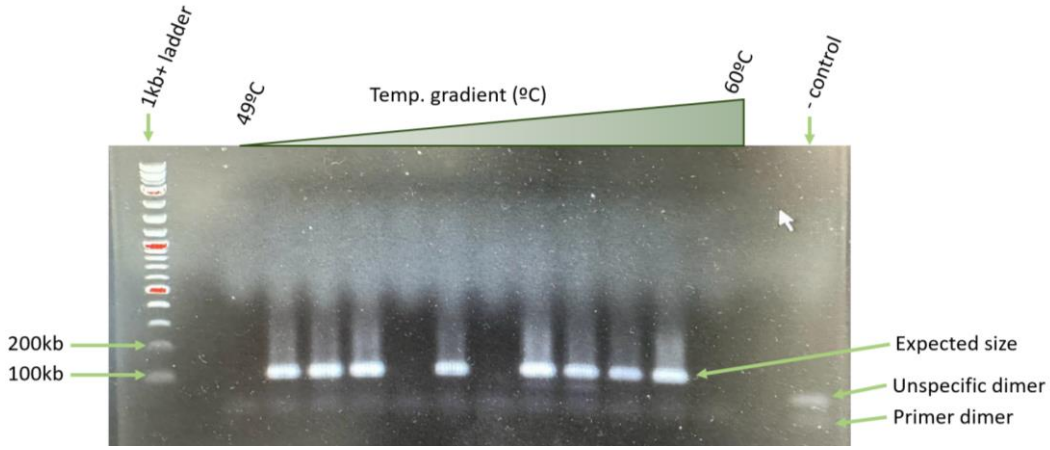

Figure 12: *GAPDH electrophoresis results for gradient PCR with SH-SY5Y cell cDNA. Range of temperatures from 49-60ºC, augmenting 1ºC each well from left to right. Last well is blank control.*

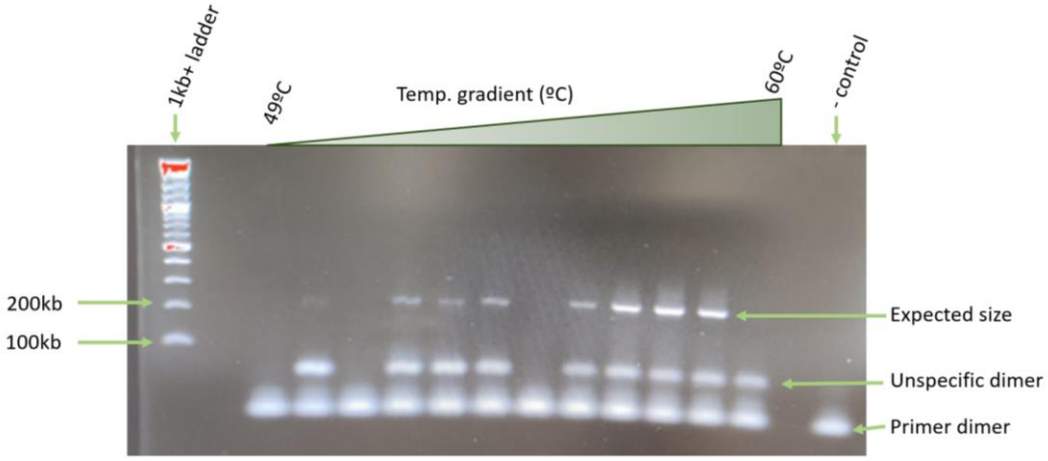

Figure 13: *IDO electrophoresis results for gradient PCR with SH-SY5Y cell cDNA. Range of temperatures from 49-60ºC, augmenting 1ºC each well from left to right. Last well is blank control.*

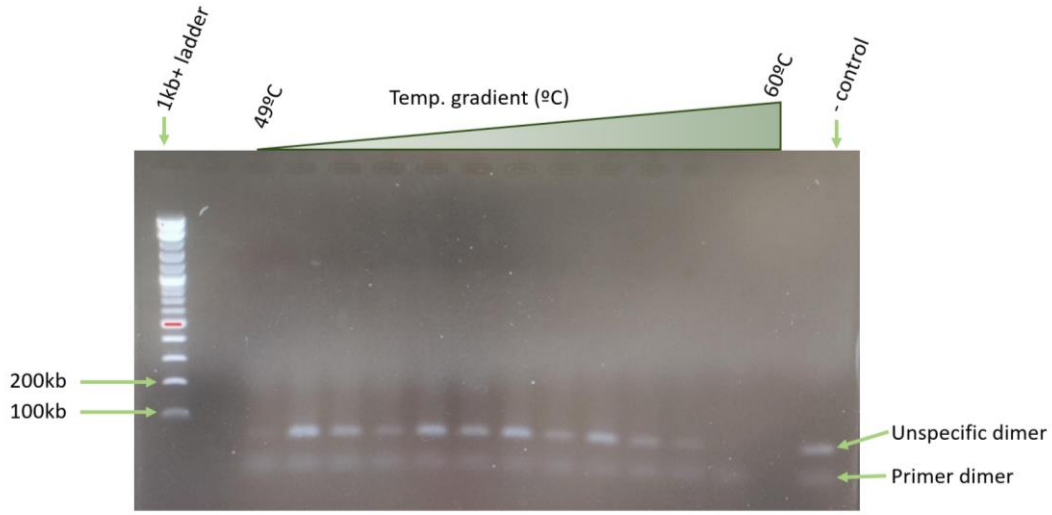

Figure 14: *TDO electrophoresis results for gradient PCR with SH-SY5Y cell cDNA. Range of temperatures from 49-60ºC, augmenting 1ºC each well from left to right. Last well is blank control.*

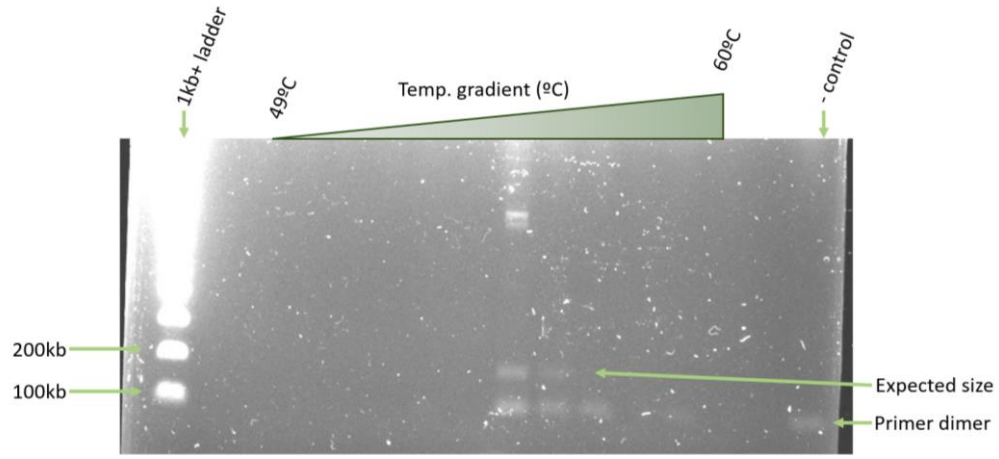

Figure 15: *TDO electrophoresis results for gradient PCR with LPS stimulated SH-SY5Y cell cDNA. Range of temperatures from 49-60ºC, augmenting 1ºC each well from left to right. Last well is blank control.*

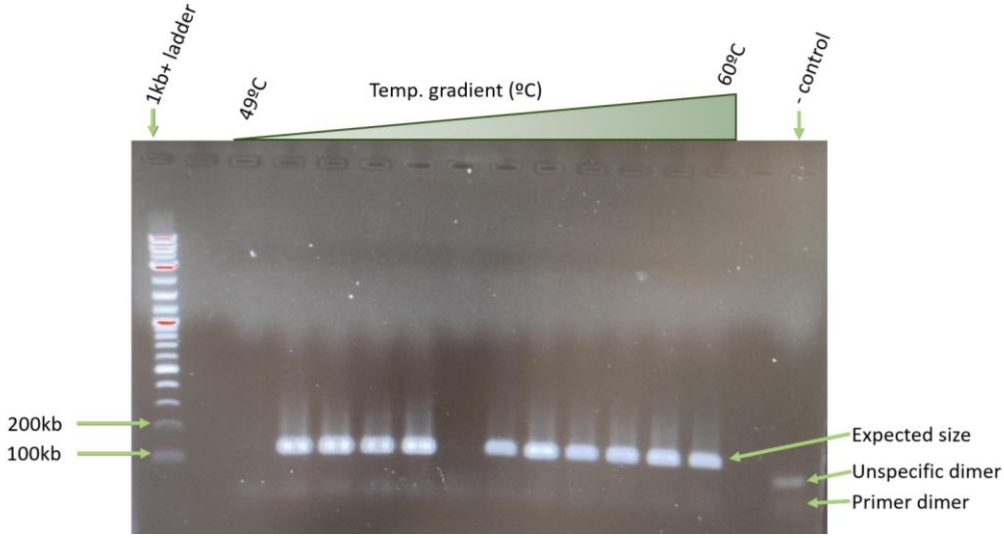

Figure 16: *human MIF electrophoresis results for gradient PCR with SH-SY5Y cell cDNA. Range of temperatures from 49-60ºC, augmenting 1ºC each well from left to right. Last well is blank control.*

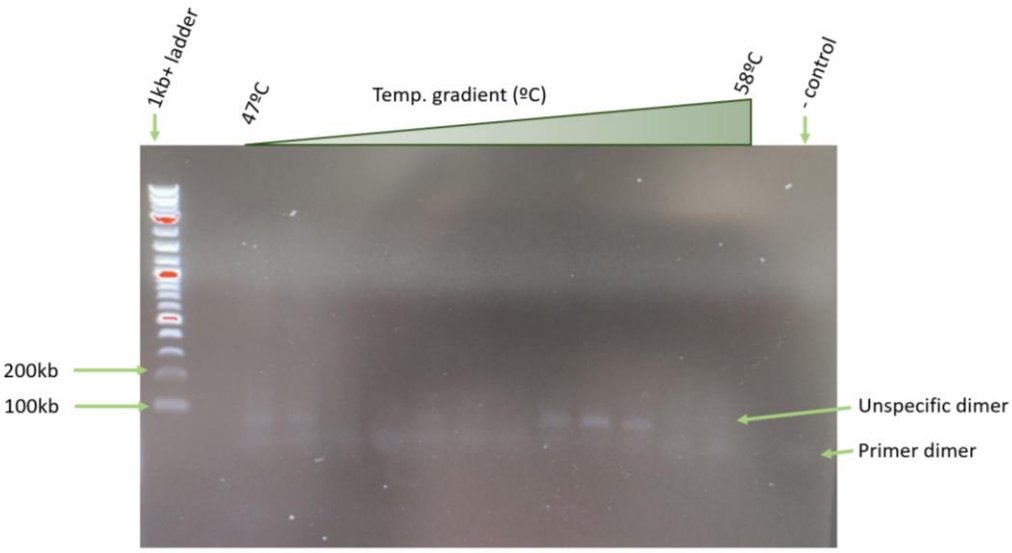

Figure 17: *IL-1β electrophoresis results for gradient PCR with SH-SY5Y cell cDNA. Range of temperatures from 47-58ºC, augmenting 1ºC each well from left to right. Last well is blank control.*

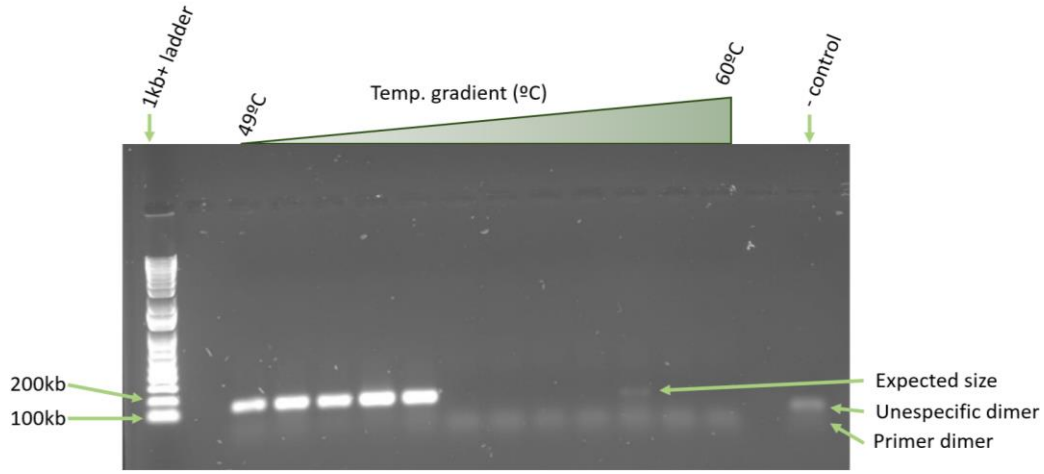

Figure 18: *IL-1β electrophoresis results for gradient PCR with LPS stimulated SH-SY5Y cell cDNA. Range of temperatures from 49-60ºC, augmenting 1ºC each well from left to right. Last well is blank control.*

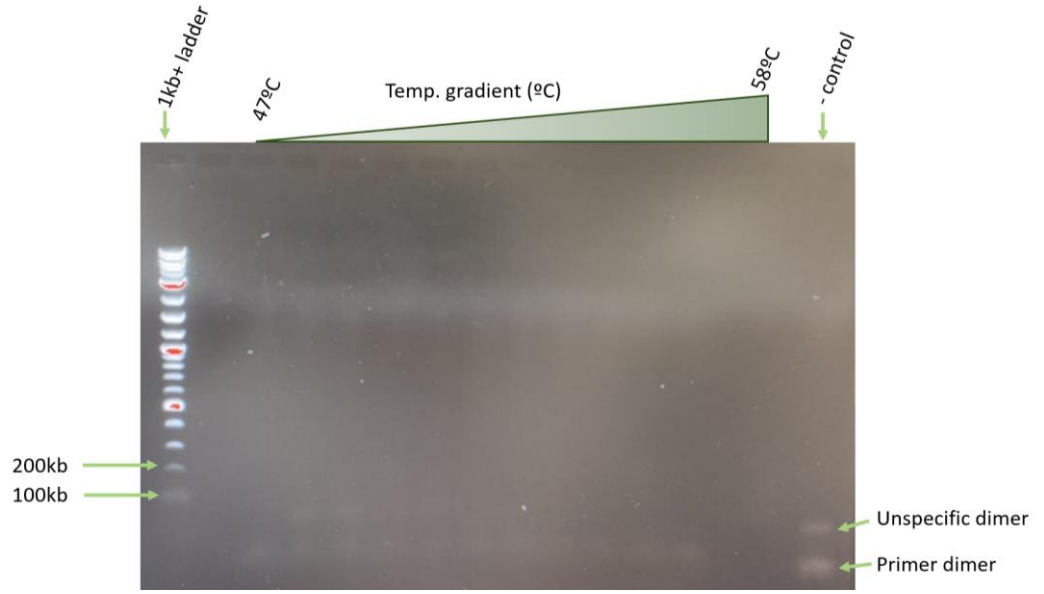

Figure 19: *iNOS2 electrophoresis results for gradient PCR with SH-SY5Y cell cDNA. Range of temperatures from 47-58ºC, augmenting 1ºC each well from left to right. Last well is blank control.*

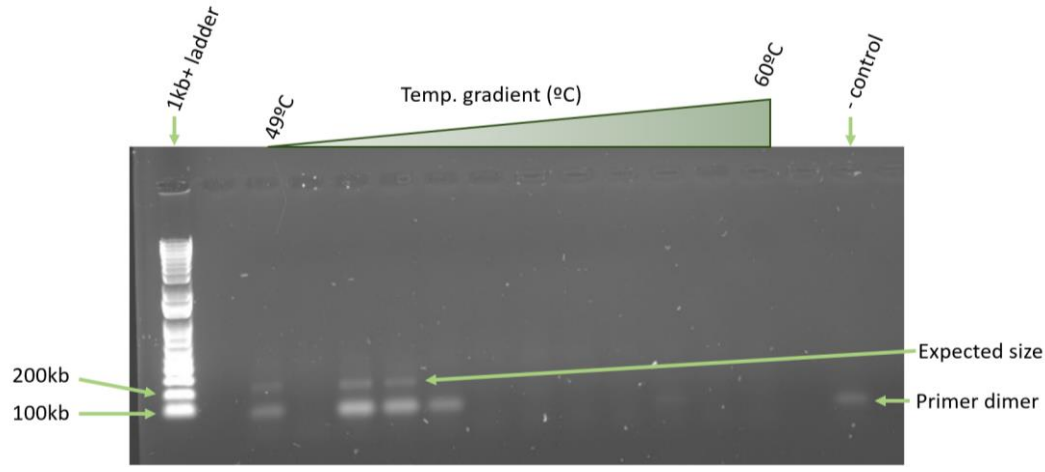

Figure 20: *iNOS2 electrophoresis results for gradient PCR with LPS stimulated SH-SY5Y cell cDNA. Range of temperatures from 49-60ºC, augmenting 1ºC each well from left to right. Last well is blank control.*

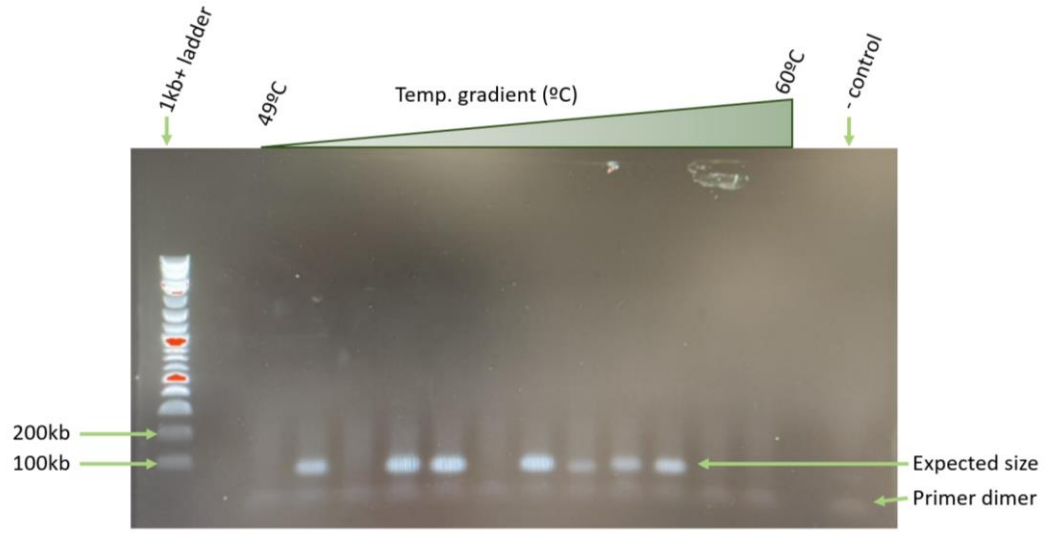

Figure 21: *TLR-4 electrophoresis results for gradient PCR with SH-SY5Y cell cDNA. Range of temperatures from 49-60ºC, augmenting 1ºC each well from left to right. Last well is blank control.*

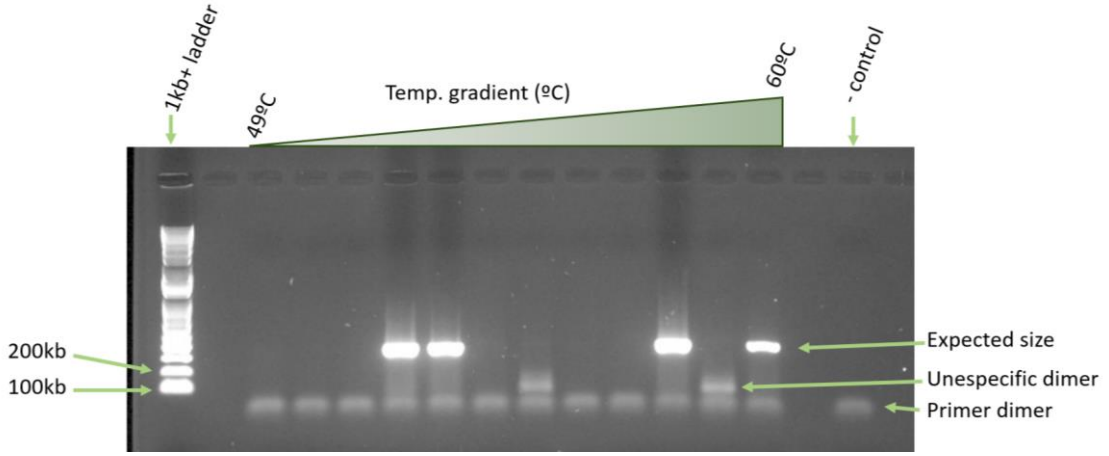

Figure 22: *SAG1 electrophoresis results for gradient PCR with T. gondii infected SH-SY5Y cell cDNA. Range of temperatures from 49-60ºC, augmenting 1ºC each well from left to right. Last well is blank control.*

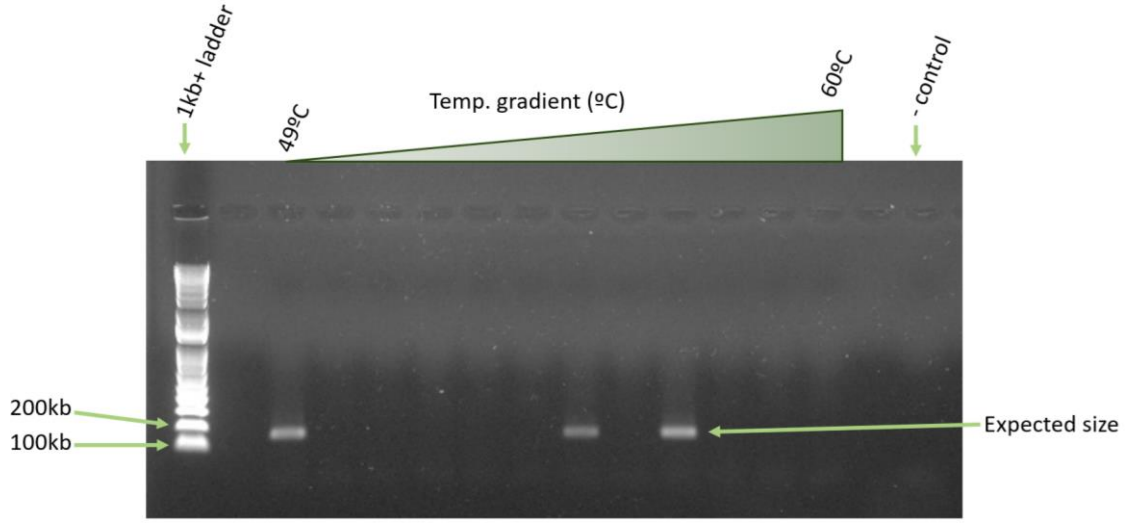

Figure 23: *RH-MIF electrophoresis results for gradient PCR with T. gondii infected SH-SY5Y cell cDNA. Range of temperatures from 49-60ºC, augmenting 1ºC each well from left to right. Last well is blank control..*