AMERICAN UNIVERSITY OF BEIRUT

A MURINE MODEL TO STUDY TOXOPLASMA GONDII AND A SECONDARY INFLUENZA A VIRUS INFECTION

by
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submitted in partial fulfillment of the requirements
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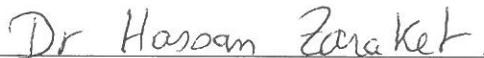

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AN ABSTRACT OF THE THESIS OF

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Title: A Murine Model to Study Infection with *Toxoplasma gondii* and a Secondary Influenza A Virus Infection.

Toxoplasma gondii is an obligate intracellular protozoan parasite that can infect a wide variety of animals, including one third of the human worldwide population. It causes a- to mild symptomatic disease in immunocompetent patients but may become fatal in immunocompromised patients and in fetus of primo-infected pregnant women.

Influenza A virus (H1N1) is a negative-sense single stranded RNA virus belonging the *Orthomyxoviridae* family. Influenza A viruses (IAV) is a significant cause of morbidity and mortality in human population worldwide. Influenza A/H1N1 virus emerging from pigs caused a pandemic in 2009 and continues to cause infections worldwide. Like other flu causing viruses, H1N1 causes the typical flu symptoms including: sore throat, cough, fever, chills and some body aches. AN infection with influenza virus may also lead to complications such as pneumonia and cardiac complications.

As both *T. gondii* and IAV infections are common, and since the risk of infection with either pathogen is extremely high, it is intriguing to investigate the outcome of secondary infection with IAV following a pre-existing acute *T. gondii* infection.

To the best of our knowledge, no study investigated the outcome of a pre-existing acute *T. gondii* infection followed by a secondary infection with IAV.

We used 6-8 week old female BALB/c mice which were intraperitoneally injected with type II *T. gondii* parasites, followed by IAV intranasal inoculation. Mice were sacrificed at days 3 and 5 post infection with IAV, and those either infected with *T. gondii* infected with both *T. gondii* and IAV were verified for acute toxoplasmosis by Western Blot. Parasitic and viral loads in mice organs were quantified by quantitative Real-Time PCR.

We have shown that 10% of mice infected with IAV alone and 10% of those infected with *T. gondii* alone die by day 22. Interestingly, an infection with *T. gondii* followed by IAV the next day has shown 60% mortality by day 22, while an infection with *T. gondii* followed by IAV 4 days later has shown 40% mortality. At the molecular level, the parasitic load in the peritoneum on days 3 and 5 post-infection with IAV was higher in co-infected mice than in those infected with the parasite alone. This may explain the higher lethality in mice subjected to secondary infection with IAV, when compared to an infection with either pathogen.

In conclusion, we have shown that an infection with *T. gondii* followed by IAV is more lethal than an infection with either pathogen in mice, which is very alarming to patients, especially since infection with both pathogens if very common in our country as in the world.

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CHAPTER I

INTRODUCTION

A. Toxoplasma gondii

1. Origins

The protozoan parasite *Toxoplasma gondii* was described for the first time in 1908 by Charles Nicolle and Louis Manceaux, while working in Tunisia. They isolated the parasite from the tissues of *Ctenodoactylusgundi*, an animal that was originally used for research on another flagellated parasite: *Leishmani*a (Nicolle and Manceux, 1908). In parallel, and in the same year, the parasite was also isolated from the tissues of a rabbit by Alfonso Splendore (Splendore, 1908). It was named based on its shape, where the first part of its name originates from the Greek word "toxo", which translates to bow, and refers to the crescent shape, while the second part, "plasma", means life.

Several cases of *Toxoplasma*-like infections were reported in animals and humans between 1908 and 1937. In 1914, Castellani was the first to report a *Toxoplasma*-like parasite in the blood smear of a 14-year old boy who had died after suffering from fever, anemia and splenomegaly (Castellani, 1914). Later on, congenital toxoplasmosis, which results from transplacental parasite transmission, was first described in a three-day old newborn girl suffering from encephalomyelitis, seizures and chorioretinitis. This was described after an autopsy that revealed the presence of *Toxoplasma gondii* in her brain (Wolf, Cowen, & Paige, 1939a; Wolf, Cowen, & Paige, 1939b).

Over the years, it was shown that the immune response to *T. gondii* involves T-cells acting intracellularly, as well as some antibodies acting extracellularly (Frenkel,

1988). The importance of T-cell mediated immunity was highlighted by Frenkel

(Frenkel, 1988), when he proved that mice lacking a thymus fail to mount the proper

immune response against the parasite. Later on, it was shown that the initial immune

response following parasite entry, takes place in the intestine, with the infected host

mounting a Th1 response, characterized by the production of IFN-γ (Chardès et al.,

1993; Chardès, Buzoni-Gatel, Lepage, Bernard, & Bout, 1994; Denkers & Gazzinelli,

1998; O. Liesenfeld, 1999).

2. Taxononomy

Toxoplasma gondii belongs to the phylum *Apicomplexa*, which includes

several other intracellular parasites such as the human pathogens *Plasmodium*, which is

the causative agent of malaria, and *Cryptosporidium*. *Apicomplexa*, along with

Dinoflagellates and Ciliates belong to the superphylum Alveolata, which includes a

number of clinically relevant human pathogens (Figure 1) (reviewed in Lentini et al.,

2015) .
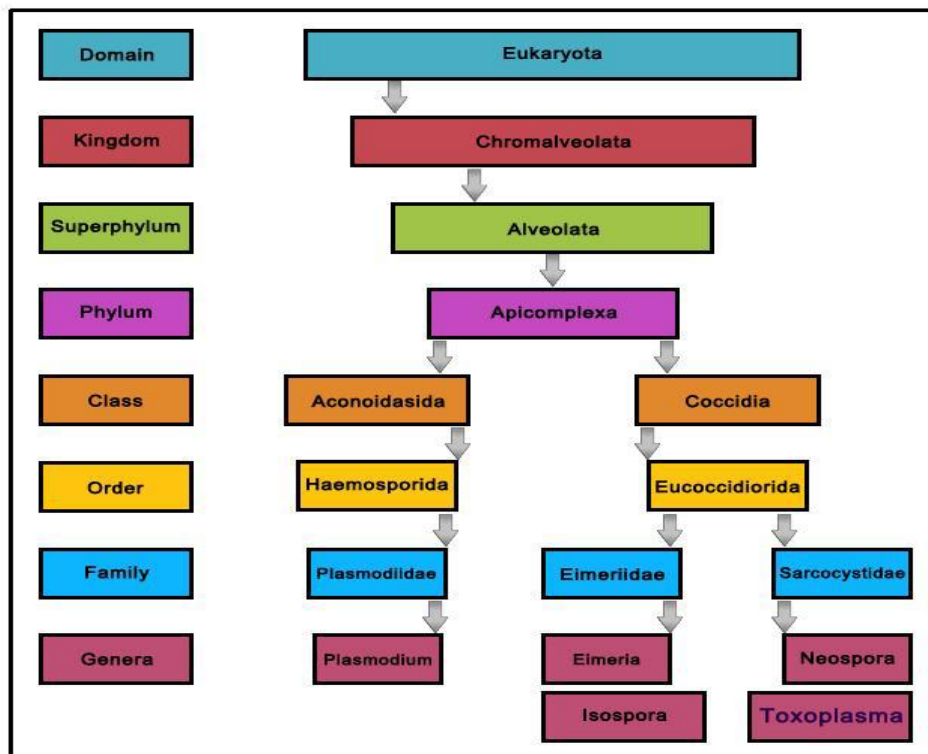
Figure 1: Taxonomy. Retrieved from http://www.ncbi.nlm.nih.gov/Taxonomy

Despite some differences between the members of the phylum *Apicomplexa*,
such as the target host cell type, the common feature among all these parasites is that
they possess a polarized cellular structure as well as a complex organization of their
cytoskeleton and organelles at their apical end. These organelles form the apical
complex and allow all parasites in this phylum to successfully invade their host
cells(Besteiro, Dubremetz, & Lebrun, 2011; Black & Boothroyd, 2000). Their mode of
invasion is conserved and involves a sequential discharge of the namely micronemes,
rhoptries and dense granules (Besteiro et al., 2011; Carruthers & Sibley, 1997).

3. Developmental stages of Toxoplasma with emphasis on tachyzoites

The life cycle of *T. gondii* comprises three morphologically distinct infective
stages: the sporozoites, the fast-replicating tachyzoites and slow-replicating bradyzoites.

These infective stages share a number of common features: they are all crescent-shaped, with a pointed apical end in which secretory organelles are concentrated, and a more round posterior end (Robert-Gangneux & Dardé, 2012) (Figure 2). Since we only worked on the tachyzoite stage in our study, this prompted us to only describe its characteristics.

Tachyzoites are also known as trophozoites or endozoites. These are the fast replicative stage of the parasite that are able to infect virtually any nucleated cell. They are responsible for the acute phase of the infection and replicate by endodyogeny (where budding is synchronous with nuclear division), to later exit the cell by lysis (Hunter & Sibley, 2012; Lentini et al., 2015; Robert-Gangneux & Dardé, 2012). Tachyzoites replicate inside a parasitophorous vacuole which does not fuse with the endolysosomal system, helping the parasite survive inside the cells and evade the host immune system (El Hajj et al., 2007; Hunter & Sibley, 2012; Robert-Gangneux & Dardé, 2012).
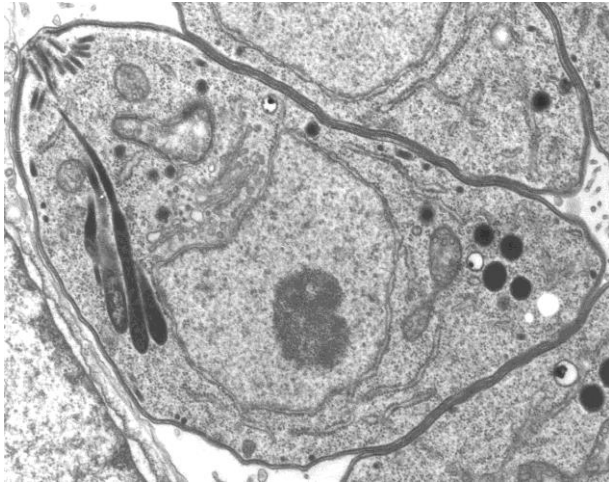
Tachyzoite stage of *T. gondii*
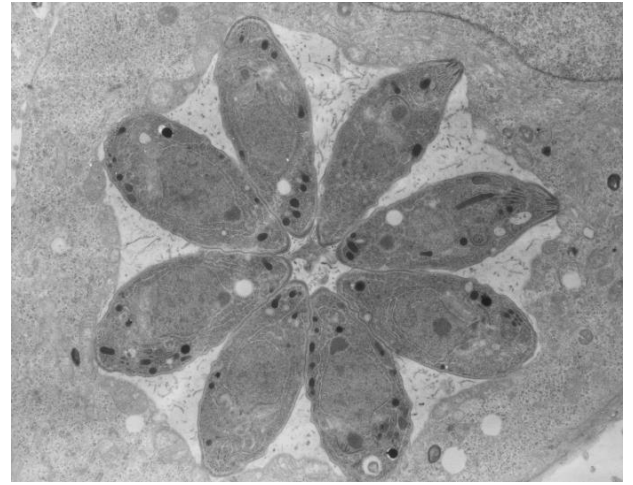
Rosette of 8 tachyzoites inside the parasitophorous vacuole

Figure 2: Electron microscopy of *Toxoplasma gondii* tachyzoites (Clicher Jean-Francois Dubremetz).

4. Life cycle of Toxoplasma gondii

Toxoplasma gondii is an obligate intracellular parasite and represents one of the most common zoonotic pathogens (Blanchard et al., 2015; Weiss & Dubey, 2009). *T. gondii* has a heteroxeneous life cycle, which remained unidentified until the 1960s (Hutchison, Dunachie, Siim, & Work, 1969; Sheffield & Melton, 1970).

The complete life cycle of the parasite involves a definitive host belonging to the *Felidae* family, and multiple intermediate hosts (Figure 3), which are virtually all mammals including humans, as well as reptiles and birds (Chadwick et al., 2013; Robert-Gangneux & Dardé, 2012; Weiss & Dubey, 2009).
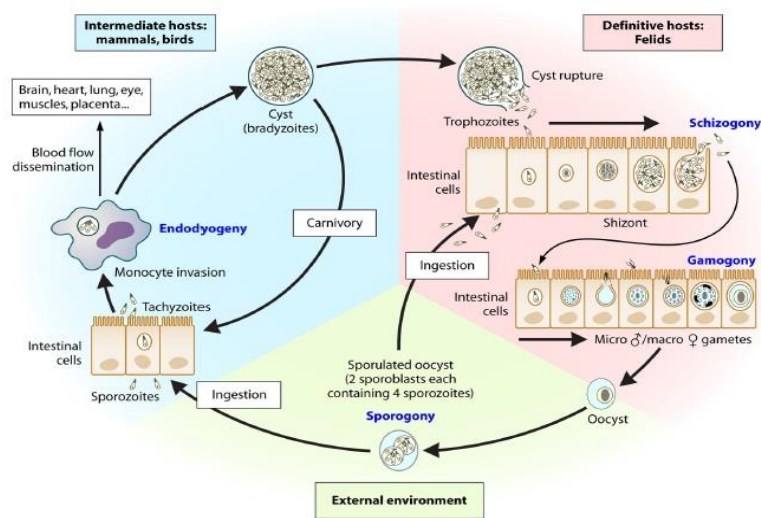
Figure 3: *Toxoplasma gondii* **life cycle (Robert-Gangneux & Dardé, 2012).**

  a. <u>Sexual life cycle</u>

The sexual stages take place only in the in the gut epithelium of cats or other

species of the *Felidae* family (Denkers & Gazzinelli, 1998). Definitive hosts acquire the

infection usually by feeding on an infected prey, through ingestion of tissue cysts. Up to

two days post-infection, bradyzoites settle within the enterocytes of the definitive host's

ileum and undergo a finite number of asexual cycles leading to the production of

merozoites contained in schizonts (Robert-Gangneux & Dardé, 2012). This step is

followed by gametogony, resulting in the production of macrogametes and

microgametes, which are the female and male gametes respectively. The products of

gamete fusion are liberated from the cat's enterocytes through membrane disruption,

and shed in cat feces as unsporulated oocysts (Robert-Gangneux & Dardé, 2012). Under

the adequate conditions, oocysts take one or two days to sporulate. Sporulatedoocysts

contain two sporocysts, each having four haploid sporozoites, which are the infective

entities (Figure 3). Predation and ingestion of tissue cysts by cats complete the parasite's life cycle (Hunter & Sibley, 2012).

b. Asexual life cycle

The asexual stages of *T.gondii* take place when an intermediate host ingests sporulatedoocysts from a contaminated surface. Enzymes present in the gastro-intestinal tract of the host lead to sporozoite liberation, followed by their transformation into tachyzoites, which multiply rapidly and are the responsible for the acute phase of infection. Tachyzoites are capable of infecting any nucleated cell and crossing the blood-placenta barrier, leading to congenital toxoplasmosis in the fetuses of primo-infected mothers. Following a proper immune response, tachyzoites are cleared from the tissues, and switch to bradyzoites that survive within cysts, protecting them from the immune response (Denkers & Gazzinelli, 1998). Bradyzoites are confined mostly in the central nervous system and the skeletal muscles, and upon loss of immunity, will reactivate into tachyzoites (Denkers & Gazzinelli, 1998) (Figure 3).

5. *Modes of transmission of Toxoplasma gondii*

Initial infection with *T. gondii* can be acquired through three main routes (Figure 4): either by ingestion of tissue cysts found in raw or undercooked meat, or by ingesting food or water contaminated with sporulated oocysts, shed in cat feces, or congenitally (O. Liesenfeld, 1999; Verma & Khanna, 2013). Ingestion of contaminated oocysts or tissue cysts is known as horizontal transmission, whereas transplacental transmission is known as vertical transmission.

Evidence suggests that infections acquired by the ingestion of oocysts are more

severe than those acquired by the ingestion of tissue cysts (Dubey, 2004). Moreover, sporulatedoocysts are more resistant to the external environment and harsh conditions, and can maintain infectivity for prolonged periods of time (Robert-Gangneux & Dardé, 2012).

Congenital toxoplasmosis, which takes place when tachyzoites cross the blood placental barrier, occurs in primo-infected mothers and can lead to complications, the severity of which is highly dependent on the trimester of pregnancy in which the infection occurs (Robert-Gangneux & Dardé, 2012).
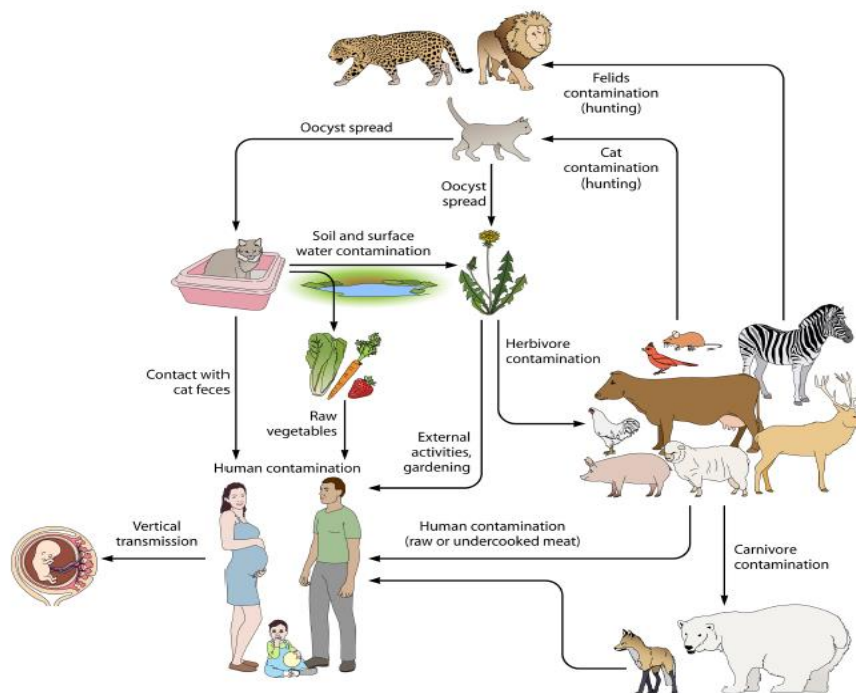
Figure 4: Modes of transmission of *Toxoplasma gondii* (Robert-Gangneux & Dardé, 2012).

6. Prevalence of Toxoplasma gondii

Until now, *T. gondii* infection has been described in more than 300 animal species (Chadwick et al., 2013; Robert-Gangneux & Dardé, 2012).*T. gondii* is an

abundant pathogen, estimated to infect about a third of the world's population (Weiss & Dubey, 2009). Its seroprevalence in humans, which can be measured by the specific levels of IgG against the parasite, ranges between 10 and 70 %, depending mainly on the geographical region (Blanchard et al., 2015; Furtado, Smith, Belfort, Gattey, & Winthrop, 2011). However, the rate of infection is dictated by several factors, such as the host's age, immune status, dietary habits and exposure to animals (Stoicov et al., 2004).

In Lebanon, a retrospective study on sera from hospitals and private laboratories was performed in 2010 and showed that the seroprevalence of IgG anti-*Toxoplasma* antibodies was 55 and 67%, respectively, while the seroprevalence of anti-*Toxoplasma* IgM antibodies was 6.7 and 6.8%, respectively (Bouhamdan, Bitar, Saghir, Bayan, & Araj, 2010). This study has also shown that exposure to the parasite increases with age (Bouhamdan et al., 2010). This study dates back to 2010 and no new studies assessing the seroprevalence have been performed since then.

7. Clinical manifestations of Toxoplasma gondii

The outcomes of an infection with *T. gondii* vary based on both parasite genotype and host immune status (Hunter & Sibley, 2012). In a normal host, an infection with *T. gondii* was originally thought be to asymptomatic (Weiss & Dubey, 2009). However, it has been proven that an infected individual shows some symptoms limited to fever, fatigue and lymphadenopathy (Denkers&Gazzinelli, 1998). Furthermore, 10-20% of normal individuals who are infected may develop chorioretinitis, myocarditis and polymyositis (Weiss & Dubey, 2009). However, the serious consequences are usually manifested in newborns which acquire the parasite

congenitally, as well as in immunosuppressed individuals.

a. Congenital toxoplasmosis

Clinical manifestations of congenital toxoplasmosis depend on the stage of
pregnancy during which the infection is acquired (Weiss & Dubey, 2009). If a mother is
infected for the first time with *T.gondii* during the first and second trimesters of
pregnancy, a spontaneous abortion or a stillbirth are the usual outcome of infection. If
the pregnancy does come to term, an infection during the first trimester leads to the
highest severity of disease, as the infants will experience a variety of neonatal diseases,
such as microcephaly, hydrocephaly, hearing loss, chorioretinitis and psychomotor and
mental retardation (Weiss & Dubey, 2009). If the mother is infected with the parasite
during the last trimester of pregnancy, the infection will cause subclinical diseases with
delayed manifestations in the infant, which may take months or years before they appear
(Weiss & Dubey, 2009). In short, the risk of vertical transmission is much higher as the
pregnancy advances, but the outcomes are more severe during the early developmental
stages of the fetus (Furtado et al., 2011).

b. Neurotoxoplasmosis

In immunosuppressed patients or those with a defective T-cell response, such
as people with AIDS, transplant patients or patients who are taking corticosteroids or
cytotoxic drugs, *T. gondii* can cause encephalitis, a disseminated infection and ocular
toxoplasmosis and may even lead to death (Blanchard et al., 2015; Furtado et al., 2011;
Weiss & Dubey, 2009). Treatment of toxoplasmosis is not capable of eliminating cysts,
which is why an immunocompromised patient has to remain on anti-toxoplasmosis

drugs for the entire duration of immunosuppression (Sturge & Yarovinsky, 2014).

Reactivation in AIDS patients can cause cerebral toxoplasmosis or toxoplasmic

encephalitis, as well as a variety of non-specific symptoms such as fever, dementia and

seizures (Blanchard et al., 2015; Furtado et al., 2011). It is worth noting that

toxoplasmosis in these individuals is most likely due to parasite reactivation upon

immunosuppression, and rarely due to a newly acquired infection. However, incidents

of newly acquired infections may occur if a transplant patient receives a contaminated

organ, and this has been reported in heart, kidney and liver transplant patients who were

seronegative and received organs from seropositive donors (Weiss & Dubey, 2009).

 c. Ocular toxoplasmosis and others

Ocular toxoplasmosis is another disease manifestation that may occur due to

contracting the parasite congenitally or postnatally. The typical disease outcome is the

formation of lesions in the posterior pole of the eye (Commodaro et al., 2009).

Several mental diseases have also been associated with *T. gondii* infection. A

study by Torrey et. al has found that the presence of anti-*Toxoplasma* antibodies is an

intermediate risk factor associated with the development of schizophrenia, where the

most common disease manifestation is hallucinations (Torrey, Bartko, & Yolken, 2012;

Yolken & Torrey, 2008). However, the mechanism by which the parasite causes these

neurological alterations is still unknown.

8. Diagnosis of Toxoplasma gondii

The diagnosis of *T. gondii* usually relies on serology, whereby changes in

antibody types and titers are used to determine the stage of infection. In 1968,

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Remington et. al were the first to suggest that congenital toxoplasmosis may be diagnosed by determining the presence of specific IgM antibodies against *T. gondii* in cord blood or in infant serum (Remington, Miller, & Brownlee, 1968). This logic was based on the fact that IgM antibodies can cross the blood-placental barrier, whereas IgG cannot. Later on, it was well documented that the first antibody to be detected following infection is IgM. IgM antibody titers start to rise 5 days post-infection and peak one to two months afterwards. On the other hand, IgG antibody titers rise during the first two weeks post-infection, peak three to six months later, and remain detectable for life (Hunter & Sibley, 2012; O. Liesenfeld et al., 1997).Currently, a simple and easy diagnostic tool for *T. gondii* is known as the agglutination test. It was developed by Fulton in 1965 and improved by Desmonts and Remington and further by Dubey and Desmonts, who named it "modified agglutination test (MAT)" (Weiss & Dubey, 2009)

However, PCR is the most used technique in the diagnosis of clinical toxoplasmosis, and has become widespread ever since Burg and his partners reported a successful amplification of the B1 gene from only one tachyzoite (Weiss & Dubey, 2009).

9. Genetics and virulence of Toxoplasma gondii

T. gondii can infect virtually all mammalian nucleated cells, which rendered it easy to propagate *in-vitro*(Black & Boothroyd, 2000). The full genetic characterization of the genome was finalized in 2005 (A. Khan et al., 2005). *T. gondii* genome is haploid, allowing for an easy genetic manipulation, especially knock-outs (Black & Boothroyd, 2000). It consists of $8*10^7$ base pairs on eleven chromosomes.

T. gondii isolates fall into three major multilocus genotypes I, II and III. These

genotypes were established using isolates from Europe and the United states (Robert-Gangneux & Dardé, 2012). Type I strain is the most virulent type, where the mouse lethal dose 50 (MLD_{50}) corresponding to the dose necessary to kill half the infected mice, is about 10 parasites. Types II and III are less virulent strains, and their $MLD_{50}s$ are 100 and 1000 parasites, respectively (Robert-Gangneux & Dardé, 2012).

10. Tachyzoite-specific surface antigen SAG-1: a marker of acute toxoplasmosis

Upon infection with *T. gondii*, tachyzoites stimulate the immune system to initiate an immune response against the parasite, including one of its surface antigens: SAG-1 (Velge-Roussel et al., 1994). Even though *T. gondii* has several surface antigens, SAG-1 is the most important, accounting for 5% of all *T. gondii* proteins.

SAG-1 gene was completely cloned by Brug and his colleagues in 1988. It has a length of 1.1 kb and is composed of 336 amino acids. The gene is translated into a 30 kDa protein, which is why it SAG-1 is also known as P30 (Burg, Perelman, Kasper, Ware, & Boothroyd, 1988). SAG-1 protein undergoes many post translational modifications, mainly glycosylation, resulting in a mature GPI-surface anchored protein (Burg et al., 1988; Velge-Roussel et al., 1994).

Various *T. gondii* antigens are known. Nonetheless, SAG-1 immunogenicity and reactivity with immune components makes it the best choice for diagnosis of an acute infection as well as vaccine research (Dupouy-Camet, Gavinet, Paugam, & Tourte Schaefer, 1993; Sun et al., 2013). The high IgG, IgM and IgA titers induced by SAG-1 give it a very important role in diagnostic procedures, especially that it is the marker of choice in the verification of acute infection using Western Blot in research

laboratories(Harning, Spenter, Metsis, Vuust, & Petersen, 1996; Siachoque, Guzman, Burgos, Patarroyo, & Gomez Marin, 2006).

11. Host immune response to Toxoplasma gondii

T. gondii is an obligate intracellular parasite, which allows it to evade the immune system (Blader & Saeij, 2009). The parasite internalizes inside macrophages and dendritic cells, hijacking them to spread to various sites, leading to the down regulation of pro-inflammatory cytokines such as IFN-γ and IL-12 (Figures 5 and 6), and the activation of anti-apoptotic mechanisms and secretion of the anti-inflammatory IL-10 cytokine, thus securing a safe spread to the tissues (Blader & Saeij, 2009; Melo, Jensen, & Saeij, 2011). *T. gondii* infection is distinguished by a vigorous immune response mediated by a Th-1 response and characterized by high levels of INF-γ, which are essential for the control of both the acute and chronic stages of infection in the murine model (Suzuki, Orellana, Schreiber, & Remington, 1988, Yap & Sher, 1999). To avoid a severe Th-1 response that may lead to severe immunopathology, a Th-2 response is needed. This response is characterized by the production of IL-10, to down-regulate the immune mediated effects of IFN- γ and IL-12 (Figures 5 and 6) (Gazzinelli et al., 1996; Suzuki et al., 2000).
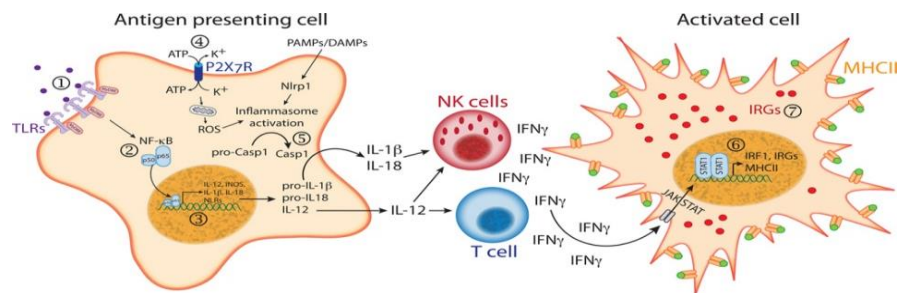
Figure 5: Host cell responses that can be modulated by *Toxoplasma gondii* (Melo et al., 2011).
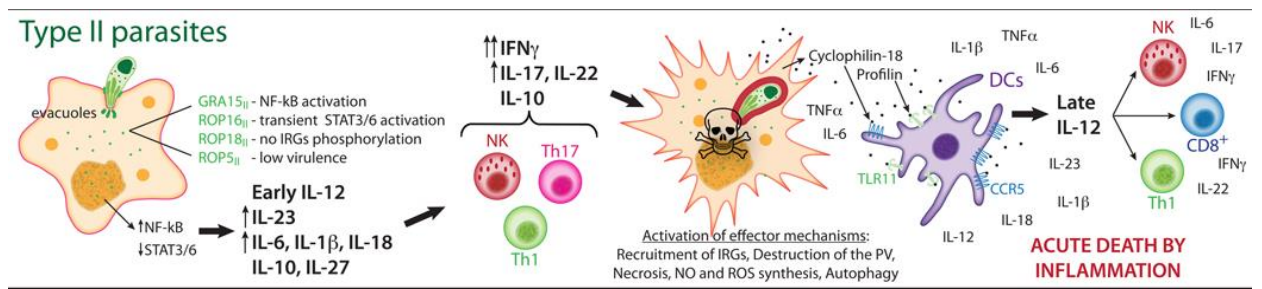
Figure 6: How *Toxoplasma* type II strain modulates host immune pathways (Melo et al., 2011).

B. Influenza A virus

1. Classification and antigenic types

Influenza is a contagious respiratory disease with a global burden due to seasonal outbreaks and occasional pandemics. It is also an economic burden because of the loss of productivity as well as the cost of medical care (Mak, Jayawardena, & Poon, 2012).

Influenza viruses belong to the *Orthomyxoviridae* family and are divided into four types: A, B C, and D. These types exhibit different degrees of antigenic variations, host specificity, and pathogenicity (Couch, 1996; Mak et al., 2012). Type A is known to undergo higher rate of evolution and exhibits more antigenic variations than the other influenza types, which is why it has been further categorized into subtypes based on the hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins (Bouvier & Palese, 2008; Mak et al., 2012). Type A is also known to infect a wide range of hosts, including humans, birds and swine, while types B and C exclusively infect humans, and type D has recently been identified in swine, cattle, sheep, and goats (Ferguson, 2009).

Influenza viruses exhibit two characteristic spike proteins: the hemagglutinin (HA) and the neuraminidase (NA), which are the basis for influenza A virus

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classification. There are 16 HA and 9 NA known subtypes that can be found in avian species. Novel HA and NA have been also discovered in bats (Bouvier & Palese, 2008; Mak et al., 2012). However, of all these diverse subtypes, only H1N1, H2N2 and H3N2 have been able to establish stable lineages in humans in the past century (Bouvier & Palese, 2008; Mak et al., 2012).

The standard nomenclature of influenza viruses includes the virus type, the species from which it was isolated (in case it was a non-human species), the location where it was isolated, the isolate number, the isolate year and the HA and NA subtypes in case of influenza A viruses (e.g. A/Puerto Rico/08/1934 H1N1 virus) (Bouvier & Palese, 2008).

2. Viral structure and genetic composition of influenza A viruses

The genome of the influenza virus is composed of eight negative sense single stranded RNA segments numbered in descending order of length (Figure 7) (Bouvier & Palese, 2008; Mak et al., 2012).
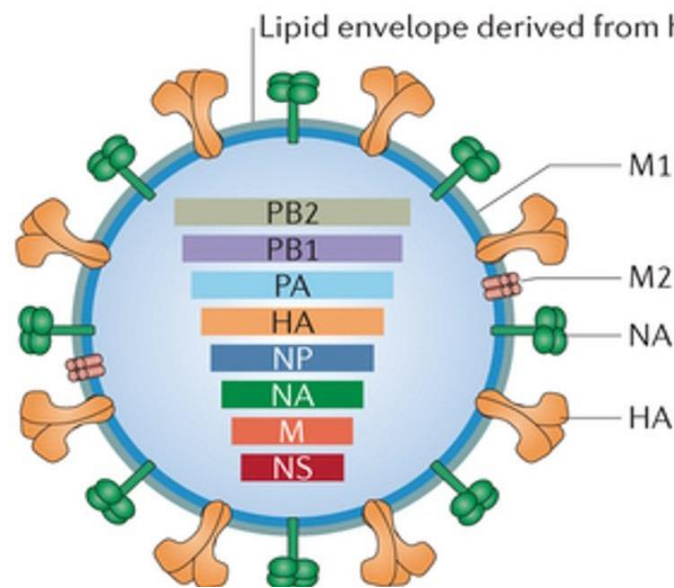
Figure 7: Structure and genetic makeup of the influenza virus(Shi, Wu, Zhang, Qi, & Gao, 2014).

The segmented genome of influenza virus facilitates genetic reassortment

events which drive the evolution of the virus(Marsh, Hatami, & Palese, 2007). This

genome encodes for at least eleven proteins, but the virus must rely on host machinery

for several aspects of its life cycle, which is discussed in the next section (König et al.,

2010).

Influenza viruses are spherical with a diameter of 80 to 120 nm, or filamentous

with a length often exceeding 300 nm (Bouvier & Palese, 2008; Couch, 1996). The

virion is studded with the spikes of the HA and NA glycoproteins that project from the

host-cell derived viral cell membrane, and matrix M2 ion channels transverse the lipid

envelope (Bouvier & Palese, 2008). The envelope and these integral membrane proteins

overlay the matrix M1 protein, which encloses the virion core (Bouvier & Palese,

2008). Internal to M1, the nuclear export protein (NEP) or nonstructural protein 2

(NS2), the ribonucleoprotein (RNP) complex, consisting of the heterotrimeric viral

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RNA-dependent RNA polymerase proteins and viral RNA (vRNA) coated with nucleoprotein (NP), can be found (Bouvier & Palese, 2008; König et al., 2010; Rossman & Lamb, 2011). The polymerase is composed of two polymerase basic and one polymerase acidic subunits (PB1, PB2 and PA respectively) (Figure 7) (Bouvier & Palese, 2008).

Table 1: The genomic segments of influenza A/Puerto Rico/8/1934 H1N1 virus and their respective encoded proteins (Bouvier & Palese, 2008).

Segment	Encoded protein(s)	Protein function
1	PB2	Polymerase subunit, mRNA cap recognition
2	PB1 PB1-F2	Polymerase subunit, RNA elongation, endonuclease activity Pro-apoptotic activity
3	PA	Polymerase subunit, protease activity
4	HA	Surface glycoprotein, major antigen, receptor binding and fusion activities
5	NP	RNA binding protein, nuclear import regulation
6	NA	Surface glycoprotein, sialidase activity, virus release
7	M1 M2	Matrix protein, vRNP interaction, RNA nuclear export regulation, viral budding Ion channel, virus assembly and uncoating
8	NS1 NEP/NS2	Interferon antagonist protein, regulation of host gene expression Nuclear export of RNA

3. Life cycle

The first step of virus infection is binding to the host cell membrane. This is achieved by the hemagglutinin (HA) spike proteins which bind to sialic acid residues on cell surface glycoproteins and glycolipids (Bouvier & Palese, 2008; Gambaryan et al.,

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2006; Gamblin et al., 2004). Virus attachment is followed by virus entry, whereby upon endosomal acidification, the M2 proton-selective ion channel mediates proton entry into the virion core, causing the ribonucleoprotein (RNP) core to dissociate from the M1 protein and allowing RNP release into the cytoplasm (Bouvier & Palese, 2008; König et al., 2010; Rossman & Lamb, 2011). Afterwards, viral RNA synthesis takes place. Following release into the cytoplasm, RNPs are transported to the nucleus, where synthesis of both the capped polyadenylated messenger RNA (mRNA) and the vRNA segments, which form the genome of the newly synthesized virions, takes place. The viral RNA-dependent RNA polymerase uses the negative sense vRNA to synthesize mRNA that is subsequently used for protein synthesis, and complementary RNA (cRNA) which is eventually transcribed into more vRNA (Bouvier & Palese, 2008). Nuclear export of capped and polyadenylated viral mRNA is achieved through the interaction of M1 with both vRNA and NP and with NEP, in order to mediate the export of M1-RNP into the cytoplasm via nucleoporins (Bouvier & Palese, 2008). The synthesis of viral proteins commences through the binding of viral mRNA on host cell ribosomes. Subsequently, the assembly of the viral structural proteins and assembly of the viral genomes take place. A fully infective viral particle is one that contains all eight RNA gene segments. Finally, budding must take place at the cell membrane. M1 interacts with both the plasma membrane and the nuclear protein, which is part of the RNP, providing structure and support for the viral membrane (Rossman & Lamb, 2011). NA, which has a sialidase activity, cleaves the bonds between HA and sialic acid residues at the cell surface and the viruses are released. NA also removes sialic acid residues from the virus envelope to prevent viral aggregation and enhance infectivity (Palese & Compans, 1976; Palese, Tobita, Ueda, & Compans, 1974).

4. Animal reservoirs

A major problem in controlling influenza viruses is the large number of animal reservoirs (Webby & Webster, 2003).

Except for the novel bat influenza virus, all influenza A viruses are believed to have evolved in the avian reservoir, i.e. aquatic birds. Gene reassortment has played a major role in the evolution of influenza A viruses, due to genome segment exchange by viruses coinfecting the same host cell (Rezza, 2004). Pandemic viruses can emerge as an outcome of reassortment between avian viruses or avian and mammalian (e.g. human and/or swine) viruses (Mak et al., 2012). For example, the 1957 pandemic was caused by a previously circulating A(H1N1) strain which incorporated the avian influenza H2 subtype of HA, the N2 subtype of NA and polymerase 1 (PB1) genes (Mak et al., 2012). Furthermore, the 2009 pandemic influenza A(H1N1) virus (H1N1pdm09) was a product of multiple reassortments between avian, human and swine influenza viruses (Smith et al., 2009).

Avian influenza viruses are either poorly able or completely unable to infect human hosts, with few exceptions including the highly pathogenic H5N1 and H7N9 viruses, which are able to spread directly from birds to humans. Avian influenza viruses have a preferential binding to α-2,3-linked sialic acids (SA), while human influenza viruses have preferential binding to α-2,6-linked SA (Mak et al., 2012). Pigs are proposed to be the mixing vessels where genetic reassortment between human, avian and swine flu viruses can occur because they possess receptors for both human and avian influenza A viruses (Mak et al., 2012).

5. *Annual outbreaks and pandemics*

Influenza viruses are capable of causing annual outbreaks, as well as pandemics.

Annual outbreak are driven by antigenic drifts, where viruses accumulate frequent amino acid changes at the antigenic sites of HA and can no longer be fully neutralized by the host's antibodies (Bouvier & Palese, 2008).

Pandemics are generally caused by influenza A viruses possessing an antigenically novel HA (and sometimes NA) spike protein (Bouvier & Palese, 2008; Mak et al., 2012). This phenomenon is termed as antigenic shift, and it may generate completely novel viruses to which humans are not immune. Several pandemics have been documented, such as those of 1918 (Spanish flu), 1957(Asian flu), 1968 (Hong Kong flu) and most recently the 2009 H1N1 (swine flu) pandemic (König et al., 2010; Mak et al., 2012). Pandemics occur at irregular and unpredictable intervals and are associated with high infection rates and varying severity. All three influenza viruses that caused the three previous pandemics were reassortant viruses (Neumann, Green, & Macken, 2010). Moreover, the most fatal pandemic of 1918, which was due to the acquisition of antigenically novel surface glycoproteins to which most of the world's population was immunologically naïve (Bouvier & Palese, 2008).

6. *Detection and diagnosis of influenza virus infections*

Since influenza virus has been known to cause outbreaks, surveillance is an absolute necessity. Furthermore, quick detection of the infection in humans allows infection control and the immediate initiation of the proper treatment (Poon et al.,

2005). The preliminary diagnosis of influenza usually relies on clinical symptoms but must be confirmed using other specific tests (Mak et al., 2012).

Diagnostic methods include serology, virus isolation and culture, rapid antigen tests as well as molecular techniques. The problem with serology is that it relies on antibody formation, but sometimes antibodies are not formed due to the extremely rapid disease progression. The virus can be cultured, and is known to grow on Madin-Darby canine cells (MDCKs) and embryonated chicken eggs. Nonetheless, culture is not a practical diagnostic method, as it does not yield results quickly enough. ,although it may be used for virus isolation and propagation to allow for investigations such as sequencing (Mak et al., 2012).

Molecular tests remain the most sensitive and widely used method in diagnosing influenza infections since they yield accurate results fairly quickly and at a moderate cost. These tests include reverse transcription polymerase chain reaction (RT-PCR), real time RT-PCR, loop-mediated isothermal amplification (LAMP), pyrosequencing and microarray.

RT-PCR is broadly used because the necessary primer sequences needed to amplify influenza RNA are readily available, and can be used as both a qualitative and quantitative method to assess the presence and absence of the virus, and the viral load respectively. Quantitative real time RT-PCR is more sensitive and can yield quantitative results in real time. But, this technique requires expensive reagents such as fluorescent dyes or probes (SYBR green and Taqman). In the case of diagnosis of influenza infection, both RT-PCR and real time RT-PCR mainly detect the M gene, which is a highly conserved gene (Mak et al., 2012).

7. *Symptoms and complications of influenza virus infections*

Influenza viruses are transmitted via aerosolized droplets and contact and are able to infect the ciliated epithelium of the airways (Hers, 1966). Influenza infection can cause disease ranging from mild symptomatic to severe illness associated with complications. Influenza virus is one of the main causes of morbidity and mortality in the world. It is estimated that seasonal infections with influenza virus can affect 5-20% of the population causing 3 – 5 million severe illnesses and 250,000 to 500,000 deaths each year (Layne, Monto, & Taubenberger, 2009; Thompson et al., 2003). Furthermore, the pandemics have claimed the lives of millions of individuals. For example, the 1918 Spanish flu has resulted in more than 50 million deaths over the course of two years (Rossman & Lamb, 2011).

Uncomplicated influenza infections are characterized by a sudden onset of symptoms 24 to 48 hours post-infection with the virus (Bouvier & Lowen, 2010). Symptoms of influenza infection include high fever ranging from 38 to 41 ℃, dry cough, sore throat, nasal congestion accompanied by discharge, malaise, lethargy and inflammation of both upper and lower respiratory tract (Bouvier & Lowen, 2010; Mak et al., 2012).

Complications of influenza virus infections are generally uncommon in healthy individuals, but may be exhibited in susceptible groups such as very young children, the elderly and the immunocompromised. Respiratory complications include pneumonia which may be primary viral or secondary bacterial (Bouvier & Lowen, 2010; Mak et al., 2012). Other complications include hemorrhagic bronchitis, and cardiac complications, which may be severe enough to cause death (Mak et al., 2012). Gastroenteric symptoms might also occur due to influenza but are not very common (Jain & Goldman, 2009).

Generally, patients with underlying conditions such as cardiovascular problems or asthma are more prone to serve complications and hospitalizations due to influenza. Additionally, pregnant women are at high risk of complications due to influenza infection (reviewed in Girard, Tam, Assossou, & Kieny, 2010). Furthermore, coinfection with the H1N1pdm09 virus and Dengue virus in Africa was found to worsen disease outcomes (Cohen et al., 2015). Coinfection with influenza A virus (IAV) and measles appeared to cause severe complications such as pneumonia and enteritis in infected individuals (Cohen et al., 2015).

8. Vaccines and antivirals

Vaccines are the first line of defense against influenza viruses (Mostafa, Kanrai, Ziebuhr, & Pleschka, 2016). Seasonal influenza vaccines are updated frequently to match the strains that are predicted to prevail in an upcoming influenza season. However, this approach is not very practical and carries the risk of miss-predicting the strain to be included in the vaccine due to antigenic variations in influenza strains each year (Altenburg, Rimmelzwaan, & de Vries, 2015). Moreover, vaccines are not always available, especially in third world countries, and vaccination guidelines vary by country, according to target populations and available resources.

Two classes of antivirals are available against influenza infections: neuraminidase inhibitors (NAIs) and adamantane ion-channel blockers. However, the high rate of resistance to the latter class deemed them unusable in a clinical setting(Hurt, Ho, & Barr, 2006). Resistance to NAIs has also been reported worldwide (Samson, Pizzorno, Abed, & Boivin, 2013). Importantly, influenza antivirals are most efficient when administered within 48 hours of symptoms onset. Antivirals treatment is

highly recommended for people with underlying conditions who are at risk of developing secondary complications, and people who are under two and above 65 years of age (Jain & Goldman, 2009).

9. Immunity to influenza A virus

The first line of defense that virus particles must overcome in the airways are mucous and antiviral peptides, which may inhibit viral attachment and entry into the cells (Chiu & Openshaw, 2015). In case the virus bypasses these defenses, epithelial cells can recognize the virus via TLRs (mainly TLR3), retinoic acid inducible gene-I (RIG-I) receptors and nucleotide oligomerization Domain (NOD)-like receptor family pyrin domain containing 3 (NLRP3), and initiate an immune response (Chiu & Openshaw, 2015; van de Sandt, Kreijtz, & Rimmelzwaan, 2012). These lead to the production of interferons α and γ, which trigger the adaptive immune response, as well a general antiviral state (Figure 8)(Chiu & Openshaw, 2015; Lambrecht & Hammad, 2012). Furthermore, primed T-cells migrate through lymph nodes and into the lungs, where clearance of virus-infected epithelial cells will take place (Altenburg et al., 2015). Moreover, these inflammatory mediators will lead to the recruitment of macrophages as well as NK cells. Macrophages are signaled by alveolar epithelial cells to the site of damage, and they secrete TNF-α and IL-6 (Figure 8) (Van de Sandt et al., 2012).

The accumulation of pro-inflammatory cytokines following an infection with influenza virus plays an important role in influenza-associated morbidity and mortality, as well as the pathological effects that are seen in the infected host (Cheung et al., 2002; de Jong et al., 2006; Kash et al., 2006).

In addition, a humoral response against the virus is also generated (Altenburg et al., 2015; Chiu & Openshaw, 2015; van de Sandt et al., 2012). The main antibody classes involved in immunity to influenza viruses are secretory IgA in the upper respiratory tract and IgG in the lower, and sometimes IgM (Chiu & Openshaw, 2015; van de Sandt et al., 2012). The HA protein is the primary target of the adaptive immune response against influenza virus infections (Neumann et al., 2010).
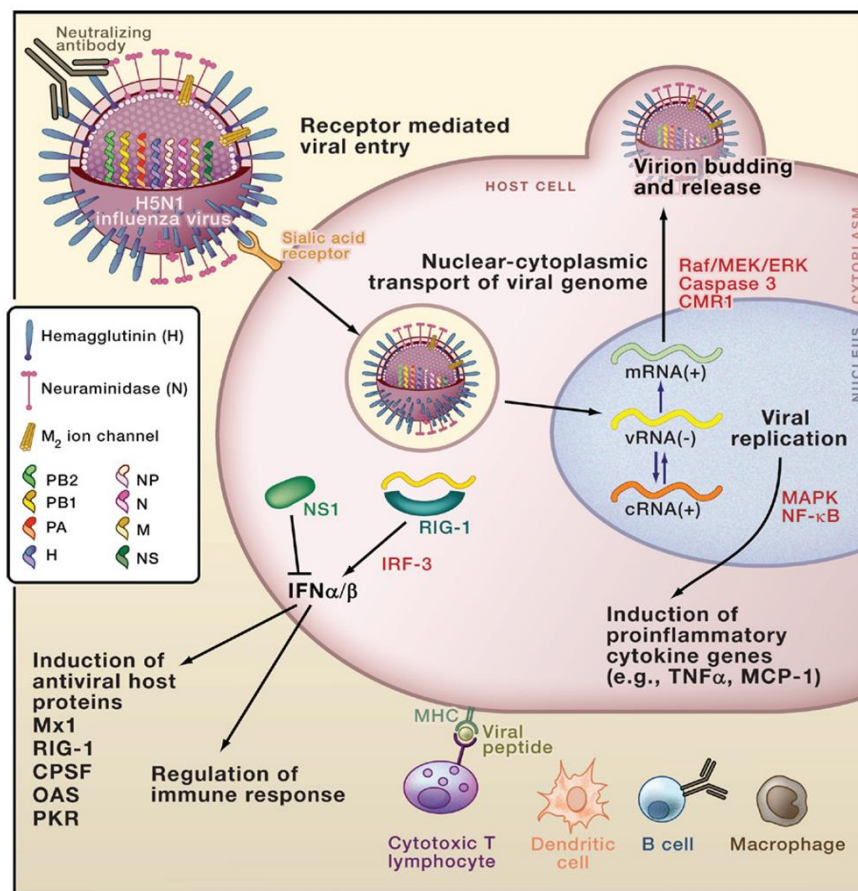
Figure 8 : Molecular basis of influenza virus pathogenesis (Salomon & Webster, 2009).

Since influenza infection, like a *T. gondii* infection, can initiate a Th-1 response in an infected host, it would be of interest to study the effect of an infection with these two pathogens on the immune system.

C. Secondary infection

1. Definition

Human pathogens are common in the environment, and at any given time, a human being can be infected with one or more microbes. An infection with an organism following a previous infection with another is termed as secondary infection. The striking feature of secondary infections is that they may have unexpected consequences on an infected individual that completely differ from the outcomes of a single infection (Furze, Hussell, & Selkirk, 2006; Veldhoen & Heeney, 2014).

2. Modulation of the immune response in secondary infections

The immune response in case of a secondary infection can have a dual facet. One pathogen may lead to the exacerbation of the pathogenicity of the other, or it may stimulate the host immune response to fight against the other pathogen in a protective manner (O'Brien, Schultz-Cherry, & Knoll, 2011; Veldhoen & Heeney, 2014).

The Th-1 subset of T-helper cells is usually involved in cell-mediated immunity. Th1 cells drive the production of a subtype of macrophages known as M1, which causes a pro-inflammatory response. It is characterized by the production of pro-inflammatory cytokines such as IL-12. Th-2 cells drive the production of M2 macrophages, generating an immunomodulatory response (Martinez & Gordon, 2014; Reese et al., 2014),and the Th-2 response leads to the production of cytokines such as

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IL-4, IL-10 and IL-13, as well as serum immunoglobulin E (IgE) (Martinez & Gordon, 2014)). For that reason, types 1 and 2 responses are generally termed to be antagonistic.

A helminth infection is usually characterized by a strong Th-2 response, while bacterial, viral and intracellular parasites generally induce a Th-1 response (Oliver Liesenfeld, Dunay, & Erb, 2004; Reese et al., 2014; Stoicov et al., 2004).

Since secondary infections are fairly common, especially in the developing world, and since the majority of people who suffer from chronic infections usually harbor more than one infection, several studies have tried to elucidate the effect of a subsequent infection on a primary infection (Oliver Liesenfeld et al., 2004; Stoicov et al., 2004).

a. *Toxoplasma gondii* and *Listeria monocytogenes*

This study performed in the early 1960 by Ruskin and Remington was one of the first studies to demonstrate that immunity against a bacterial pathogen can be conferred by a protozoan. Swiss-Webster mice were inoculated intraperitoneally with different strains of the intracellular parasite *T. gondii* then inoculated intravenously with the bacteria *Listeria monocytogenes*. Mice singly infected with high doses of *L. monocytogenes* died from infection. In contrast, mice that were infected with *T. gondii* days or months before being challenged with the bacteria showed resistance to the bacterial infection, where they either survived the infection or exhibited prolonged survival (Ruskin & Remington, 1968).

The striking observation in this study is the fact that the highest immunity was conferred when the period between the parasitic challenge and secondary bacterial challenge was the shortest (Ruskin & Remington, 1968). When mice were

first challenged with an immunizing dose of *L. monocytogenes*, no protection against a subsequent *T. gondii* infection was observed (Ruskin & Remington, 1968). This study not only highlights that a parasite can have a protective effect on a subsequent bacterial infection, but also emphasizes the importance of the chronological order of infection on the disease outcome.

b. *Nippostrongylus brasiliensis* and influenza A virus

In the 1960s, several papers were published on the effect of a parasitic infection followed by exposure to IAV. *N. brasiliensis* is a parasite that infects the human host through the skin, migrates into the lungs and must be coughed up and swallowed to mature into adult larvae that can produce eggs shed in feces (Oliver Liesenfeld et al., 2004). It was observed that mice coinfected with *N. brasiliensis* and IAV on the same day showed an increase in viral titers and lung consolidation.

The study further showed that mice infected with *N. brasiliensis* followed by IAV twelve days later showed neither an increase in viral titer nor in lung consolidation. It was presumed that the presence of *N. brasiliensis* larvae in the lungs of infected mice at the same time as viral replication was taking place is the reason behind the observed results (Wescott & Todd, 1966).

c. *Toxoplasma gondii* and *Nippostrongylus brasiliensis*

Mice infected with *N. brasiliensis* followed by *T. gondii* did not show a different difference in mortality rate compared with mice infected with only *T. gondii*, nor did they show an increased number of parasitophorous vacuoles or inflammatory infiltrates in the ileum. Monoinfection with *N. brasiliensis* has been

shown to elicit a Th-2 response in infected individuals (Oliver Liesenfeld et al., 2004). While *T. gondii* has been shown to cause a Th-1 response in the infected host (Oliver Liesenfeld et al., 2004) that needs to be modulated by a Th-2 response to avoid immune induced damage, C57BL/6 mice dually infected with *N. brasiliens* and *T. gondii* exhibited a reduction in Th-2 markers compared to *N. brasiliensis* singly infected mice, and an increase in Th-1 markers compared to *T. gondii* singly infected mice. These results show that an infection with *N. brasiliensis* does not alter the protective immune response to *T. gondii*, and that *T. gondii* causes the down regulation of the immune response to *N. brasiliensis* (Oliver Liesenfeld et al., 2004)

When *T. gondii* infection was established in C57BL/6 prior to infection with *N. brasiliensis* two weeks later, total serum IgE levels, IL-4 and IL-5 levels were higher in *N. brasiliensis* infected mice compared to coinfected mice. When coinfection took place four weeks apart, a decrease in Th-2 markers was also observed in coinfected mice.

These sets of experiments show that *T. gondii* is capable of modulating a Th-2 response against a previously established and a developing *N. brasiliensis* infection, , and that host genetics cause different outcomes of infections (Oliver Liesenfeld et al., 2004).

d. <u>*Nematospiroides dubius* and influenza A virus</u>

Nematospiroides dubius is a parasitic nematode that can be found in the duodenum and small intestine of some rodents. The parasite has now been renamed to *Heligmosomoidespolygyrus.* Mice infected with *N. dubius* 14 days prior to their infection with IAV were shown to have decreased mortality, viral antibody titers

and decreased lung consolidation compared to non-parasitized controls.

Furthermore, the viral antibody titer in the lungs of coinfected mice significantly

decreased when a higher number *N. dubius* adult worms were injected. For that

reason, it was shown that a moderate *N. dubius* infection is required to produce a

significant decrease in viral antibody titers (Chowaniec, Wescott, & Congdon,

1972).

Competitions between viral and parasitic antigens or an

immunosuppressant activity of the parasite were suggested as potential underlying

mechanisms for the decrease viral antibody titers.

e. <u>*Toxoplasma gondii* and *Plasmodium berghei*</u>

Plasmodium berghei is a parasite that can cause malaria in mice, similar to

that cause in humans by *Plasmodium falciparum*. The virulent ANKA strain of *P.*

berghei causes experimental cerebral malaria (ECM), a fatal neurological condition

similar to cerebral malaria caused in humans by *P. falciparum*.

Mice chronically infected with *T. gondii* then infected with the virulent

ANKA strain of *P. berghei* were shown to have a prolonged survival compared to

mice infected only with *P. berghei* (Mengs & Pelster, 1982).

Similarly, 90% of chronically infected mice survived a subsequent

infection with a lethal dose of the ANKA *P. berghei* strain, and showed significantly

decreased parasitemia and signs of ECM (Settles, Moser, Harris, & Knoll, 2014).

Furthermore, treatment with *T. gondii* soluble antigen 2 (STAg), known to cause a

Th1 response, was shown to cause a rapid increase in IL-12 and a subsequent

increase in IFN-γ and macrophage chemoattractant protein 1 (MCP-1) in coinfected

mice, thus leading to the reduction of parasitemia and ECM (Settles et al., 2014).

f. <u>*Schistosoma mansoni* and murine -gammaherpesvirus-68</u>

Two common infections among humans are those with an intestinal helminth

and a herpesvirus. Intestinal helminths usually cause a Th-2 response, characterized by

the production of macrophages with the M-2 phenotype, and the production on IL-4 and

IL-13 (Reese et al., 2014). Parasitic infections usually modulate infection with other

pathogens, which is why Reese et al. suspected that a helminth infection might lead to

the reactivation of a latent gammaherpesvirus infection in mice. Indeed, mice latently

infected with the murine gammaherpesvirus-68 (MHV68) showed viral reactivation

upon infection with *Schistosomamansoni* eggs.

These results suggest that the Th-2 response induced by the parasite, namely

through the production of IL-4, is needed for viral reactivation. Moreover, it was

observed that IL-4 antagonizes the suppression of viral reactivation and replication by

IFN-γ (Reese et al., 2014).

g. <u>*Helicobacter felis* and *Toxoplasma gondii*</u>

Helicobacter pylori is a Gram-negative bacterium estimated to infect over half

of the world's population, and it is the leading cause of adenocarcinoma (Stoicov et al.,

2004). The bacterium cannot be eliminated by the immune system, and it establishes a

chronic infection of the gastric mucosa. The response to this organism may be a Th1 or

Th2 response, depending on several factors such as the host's genetics and coinfection

with other organisms which may shift the immune response on a way or another

(Stoicov et al., 2004)). BALB/c mice infected with *H. felis*, a bacterium causing symptoms in mice similar to those cause by *H. pylori* in humans, normally exhibit a Th2 response, thus showing elevated levels of IL-4 and IL-10 (Reese et al., 2014; Stoicov et al., 2004). However, when infected with *T. gondii*, whether prior to or following an established *H. felis* infection, coinfected mice exhibited elevated levels IFN-γ, IL-12 and IL-1β, and decreased levels of IL-4 and IL-10 as compared to mice infected with either pathogen. Furthermore, coinfected mice exhibited higher levels of gastric inflammation, and a significant increase in IgG2 *H. felis*-specific antibodies, and a simultaneous decrease in IgG1 antibodies, indicating a *Toxoplasma* induced isotype switching.

This study highlights the fact that the interplay between pathogens does not alter the clinical outcome of one disease, but rather of both (Stoicov et al., 2004).

h. Influenza A virus and *Trichinella spiralis*

Trichinella spiralis is a parasite that initially invades intestinal epithelial cells, and then disseminates to skeletal muscles. The enteric phase of *T. spiralis* is generally characterized by a Th-2 response, and the immune response becomes mixed in the systemic phase (Furze et al., 2006). Mice were first infected with *T. spiralis*, then with IAV at different time points. Mice infected with IAV during the enteric phase of disease (day 7 post *T. spiralis* infection) showed a quicker weight gain and were significantly heavier than controls. However, the number of viral particles in the lungs and the rate of viral clearance were not different from the IAV control group (Furze et al., 2006).

Additionally, in mice infected with IAV during the systemic phase of *T. spiralis* infection (60 days post infection), no differences were observed between mono- and coinfected mice (Furze et al., 2006).

This study showed a reduction in the inflammatory infiltrates in mice infected with IAV during the early stage of *T. spiralis* infection (day 7), quicker recovery and weight gain and a reduction of cytokines such as IL-10 and Tumor Necrosis Factor alpha (TNF-α) in the bronchoalveolar lavage (BAL), but no effect on the viral clearance. It also showed a decrease in $CD4^+$, $CD8^+$ and NK cells in the lungs of coinfected mice. It was also shown that the timing of the coinfection is critical, since no changes where observed after *T. spiralis* had encysted in muscle cells (day 60) (Furze et al., 2006).

i. <u>*Toxoplasma gondii* and influenza A (H5N1) virus</u>

Due to increased resistance to antivirals, and the viral evolution preventing therapeutic advances in anti-influenza vaccination, microbes that boost the Th1 antiviral response may be employed. As previously mentioned, *T.gondii* induces a strong Th1 response characterized by increased production of IFN-γ (Oliver Liesenfeld et al., 2004). The aim of the study conducted by O'Brien et al. in 2011 was to determine whether a chronic infection with *T. gondii* could protect mice against a normally lethal H5N1 infection, and if the administration of STAg following H5N1 infection could also have a protective effect.

C57BL/6J mice were given a single dose of 50 to 5000 wild type (WT), 73F9 or N28E2 parasite strains. After a month, brains were collected and homogenized to make sure the parasite had established chronicity through the formation of brain cysts.

The chronically infected mice were then challenged with a one mouse lethal dose 50 (MLD$_{50}$) of the lethal A/Hong Kong/483/1997 (HK/483) avian H5N1 influenza A virus (O'Brien et al., 2011).

80% of the mice infected with both the WT parasite and IAV survived, while all the mice infected with only IAV died. It was also shown that protection by the WT parasite against the virus is independent of the parasite inoculum dose. Mice infected with 73F9 parasites, which causes the formation of less cysts than the WT, required a higher inoculum dose (5000 parasites) to achieve protection. Mice infected with the N28E2 strain, which causes smaller brain cysts and fails to establish a proper chronic infection, were all protected by a low inoculum dose (500 parasites). However, when IAV was administered 4 months after *T. gondii* infection, only the mice infected with WT and the 73F9 strain were protected from the viral infection.

Since survival of an influenza infection is mediated either by reducing pathogen burden or limiting the pathogen-induced immune damage, the levels of IAV in the lungs were measured (O'Brien et al., 2011). Three days following infection with IAV, viral titers were significantly decreased by 3 log in mice inoculated with the WT and the higher dose of the 73F9 strain, while the lower dose of this mutant decreased viral titers by 2 log. The higher inoculum dose of N28E2 decreased viral titers at 3 and 7 days p.i., while the lower inoculum dose failed to decrease titers at either time point.

The second part of the study aimed to test whether STAg administration post-influenza infection could have a protective effect. The reasoning behind it was that STAg causes an innate immune response similar to that caused by viable parasites (O'Brien et al., 2011). Mice were first inoculated with one MLD$_{50}$ of HK/483 H5N1 and STAg at 2 and 4 days p.i. 70% of the mice receiving STAg survived the influenza

infection. Additionally, 5 days p.i, lung viral titers were significantly decreased and histopathology revealed less bronchial and alveolar inflammation, similar to that of non-infected animals. Furthermore, when cytokine profiling was performed, only IFN-γ levels were increased in chronically infected mice as well as mice receiving STAg, which can be attributed to infection with *T. gondii* or STAg administration (O'Brien et al., 2011).

Since secondary infections are common occurrences, especially viral and parasitic ones, it is of particular interest to study how the immune response is modulates and how the survival is affected in instances of secondary infections. The results published by O'brien et al. are particularly interesting, since they shed light on changes that occur during concurrent infection with two very common pathogens. They also highlight the importance of STAg administration following IAV infection, which could be helpful in fighting IAV infections, especially with the emergence of strains that are resistant to antiviral treatments.

Based on that study, and on the fact that both *T. gondii* and IAV infections are characterized by a Th-1 response, we aimed to establish and characterize a murine model of infection between these two pathogens. This model aims to help us study the effect of pre-existing acute *T. gondii* and a subsequent infection with H1N1 IAV on host morbidity and mortality. We chose to study the acute phase of toxoplasmosis since it involves an active immune response to the parasite, which we speculated to be beneficial for viral clearance. This study allowed us to study how the parasitic infection with *T. gondii* would modulate the subsequent viral infection. To the best of our knowledge, this is the first study to assess these parameters, and may lead the way to

further studies assessing different parameters as well as different chronological orders of infection.

CHAPPTER II

MATRIALS AND METHODS

A. Experimental design

1. *Parasite culture*

Toxoplasma gondii tachyzoites from type II strains (kindly provided by Drs Maryse Lebrun and Jean Francois Dubremetz from France) were maintained by serial passages in Human Foreskin Fibroblasts (HFFs) grown in Dulbecco's modified Eagle medium (DMEM) (Lonza) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine 1% penicillin–streptomycin and kanamycin (Lonza).Parasites were counted on a haemocytometer under a light microscope and diluted properly for subsequent mice infections.

2. *Virus propagation and titration*

The influenza a virus A/Puerto Rico/8/34 strain was obtained from Dr. Richard Webby from St. Jude's children's hospital.

a. Virus propagation

For virus propagation, growth influenza virus stock was grown on Madin Darby canine kidney (MDCK) cells. Cells were seeded at a density of 3×10^6 cells per 175 cm^2 flask, one day prior to infection. Infection was performed in minimal volume of serum free medium to allow for more appropriate viral adsorption. Cells were incubated for 1 hour as such with gentle rotation every 15-20 minutes before the addition of 25ml of fresh virus infection media containing 0.2μg/ml TPCK-trypsin

(VIM). Cells were harvested 48-72 hours post infection, when cytopathic effect (cell lysis) usually occurs. The medium was then harvested and centrifuged at 10,000 rpm to clear from the cell lysates and stored at -80ºC until further use. Viral titration was performed using plaque assay in 6-well plates.

b. Titration

MDCK cells were seeded at the density of $5x10^5$ cells per well in 6-well plates, one day prior to the assay, to allow the formation of a homogeneously confluent monolayer on the day of the assay. For the assay, cells were washed 2-3 times with PBS++ (phosphate buffered saline with Calcium and Magnesium). Ten-fold serial dilutions of the virus stock were made in serum free medium. The wash medium was then removed from the cells and 0.2 ml of each virus dilution was added onto the wells starting with a 10^3 fold dilution and reaching a 10^8 fold dilution. Each dilution was done in duplicates. Plates were incubated at 37ºC with gentle rotation every 15-20 minutes, for an hour. The cells were then covered with 3ml of freshly prepared 1% nutritive agarose overlay. The dishes were left on the bench for 10 minutes for solidification and incubated at 37ºC. 72 hours later, the agarose overlay was removed and cells were fixed/stained with crystal violet staining solution (0.5% crystal violet, 10% ethanol, 20% formaldehyde). The virus stock titer was then calculated as follows:

[Virus stock] (pfu/ml) = number of plaques produced x dilution power
Volume of virus dilution used (ml)

3. *In-vivo experiments*

6 to 8 week old female BALB/c mice were used, following approval from the Institutional Animal Care and Use Committee at the American University of Beirut. The

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mice were subjected to a 12-hour light and dark cycles. Mice were anesthetized using

isoflurane and sacrificed by cervical dislocation.

a. <u>Survival evaluation</u>

Following the timeline (Figures 9 and 10), groups of ten mice each were

infected according to the below table:

Table 2 : Doses and time of injections

Group	Injection	Dose	Day
1	PBS	30µL	1
2	*Toxoplasma gondii*	100 parasites	0
3	H1N1 (PR8)	30µL(2.4*10^5 pfu)	1
4	*Toxoplasma gondii*	100 parasites	0
	H1N1 (PR8)	30µL(2.4*10^5 pfu)	1
5	*Toxoplasma gondii*	100 parasites	0
	H1N1 (PR8)	30µL(2.4*10^5 pfu)	4

Injection (IP)
T. gondii
(type II)
100 parasites

Intranasal
infection
H1N1 (PR8)
$2.4*10^5$ pfu

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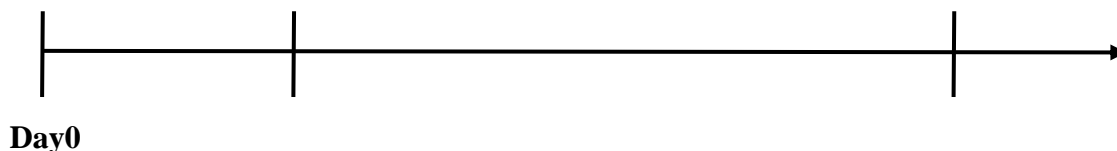
Day0

Figure 9: Timeline of infection of T. gondii on day 0 and H1N1 (PR8) on day 1 post
***Toxoplasma* infection**

Injection (IP)
T. gondii
(type II)
100 parasites

Intranasal
infection
H1N1 (PR8)
$2.4*10^5$ pfu

Termination:
21 days post
influenza
infection

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├──────────────┼─────────────────────────────────┼──────────────────►
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Day0   **Day 4**   **Day 26**
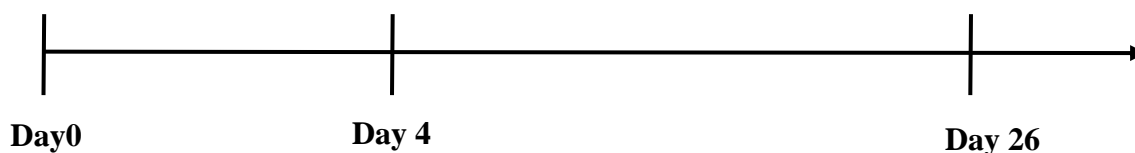
Figure 10: Timeline of infection of *T. gondii* on day 0 and H1N1 (PR8) on day 4
post Toxoplasma infection.

Mice were monitored for survival and weight loss. Mice losing 30% or more of

their initial body weight, animals were sacrificed for humane reasons. Survival curves

were plotted using GraphPad Prism.

To measure the virus titers and parasite load in infected mice, mice from each

group were sacrificed on days 3 and 5 post IAV infection, lungs were harvested and

peritoneal lavage was performed. Lungs were used to measure virus titers whereas the peritoneal lavage was used to quantify parasite load.

b. <u>Verification of acute *Toxoplasma gondii* infection by Western Blot</u>

Prior to mice sacrifice, approximately 0.1 ml of blood from the retro-orbital sinus of each mouse infected with *T.gondii* alone or coinfected with H1N1 (PR8) virus, was collected by inserting a hematocrit tube through the conjunctiva membrane of the medial canthus of the right eye. The blood samples obtained were centrifuged for sera extraction.

Type II *T. gondii* tachyzoites were obtained from a culture T25 flask for boiling in Laemmli SDS-PAGE sample buffer and running on previously prepared 12% polyachrylamide gels covered with 4% stacking gel in order to separate the protein bands, which were then transferred onto a nitrocellulose membrane overnight using a BioRad transfer unit at 30V. 5% non-fat dry milk in Wash Buffer composed of 15 mM Tris HCL (pH 8), 150 mM NaCl and 0.05% Tween 20 was utilized for blocking for 1 hour. Blood samples obtained from mice were centrifuged at 13,000 rpm for 15 minutes and 10µl of obtained sera were used extracted from the mice in 1 ml blood samples in 1 ml of 5% non-fat milk dissolved in wash buffer were used to incubate the strips and to reveal seropositivity. Incubation was carried out overnight at 4^0C. The strips were then washed and incubated with anti-mouse HRP (Horseradish peroxidase)-conjugated secondary antibodies (1:5000 dilution, Santa cruz, sc-2031, lot: B2212). Secondary anti-mouse antibodies 1:5000 dilution and a luminal chemiluminescent (Santa cruz, sc-048) was used to confirm seropositivity. This chemiluminescent substrate binds to the

secondary antibodies producing autoradiography detectable by luminescent light. Any seronegative mouse was discarded from our study.

c. Total RNA extraction

Total RNA was extracted using the TRIzol extraction method. Briefly, 1mL of Trizol was added to the peritoneal lavage pellets or lung homogenates. The mixture was incubated for 5 minutes at room temperature followed by the addition of 200μL of chloroform. The mixture was vortexed for 1 minute, followed by incubation for 1 minute at room temperature and centrifuged at 15000g for 10 minutes. After obtaining a clear aqueous-organic interphase, the upper phase containing the total RNA was gently taken, transferred into a new Eppendorf tube and precipitated by adding 0.7V isopropanol. After centrifugation at 15000g for 10 minutes, the pellet was washed twice with excess pure ethanol and centrifuged for 10 minutes at 15000g. RNA pellet was then spun down to remove excess ethanol and allowed to air-dry for few minutes to remove any excess of ethanol. The obtained precipitated RNA was resuspended in 30μL of RNAse/DNAse free water. RNA concentration was then quantified using the nanodrop (ND-1000) spectrophotometer at an absorbance of 260 nm.

d. cDNA synthesis

3μg of total RNA were used to prepare cDNA in a total volume of 20μL. Components of the cDNA synthesis kit provided by Thermo (#K1622) were added in the same order indicated in the table below (table 3) as per the manufacturer's recommendations. The mixture was gently mixed and incubated for 5 minutes at 25ºC

followed by 60 minutes at 42ºC, then for 5 minutes at 70ºC and the reaction was stopped at 4ºC.

Table 3 : Components of cDNA preparation process and volumes involved.

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

e. <u>Quantitative Real Time PCR (q RT PCR)</u>

Syber green based qRT PCR was performed using the BIORAD machine (CFX96 Optics Module, Serial No. 785BR04788). SAG-1 and M primers (table 4) were chosen to detect and quantify the tachyzoite specific marker SAG-1 and the influenza M gene in the mice peritoneal lavage and lungs, respectively.

The mouse glyceraldehyde-3-phosphate dehydrogenase(GAPDH) was used as a reference gene for quantification. GAPDH specific primers (table 4) were used to detect and quantify GAPDH transcripts, to whichall the expression results of other candidate genes were normalized as described by Walkeret al (Walker et al., 2013).

In qRT-PCR, individual reactions were prepared with 0.25μM of each of the reverse and the forward primers, 150ng of cDNA and SYBR Green PCR Master Mix to afinal volume of 11μl. PCR reaction consisted of a DNA denaturation step at 95°C for3minutes, followed by 35 cycles and 45 cycles for SAG-1 and M respectively (denaturation at 95°C for 15 seconds, annealing at each primer'sspecific temperature for 60 seconds, extension at 72°C for 30 seconds). For each experiment,reactions were performed in duplicates and expression of individual genes was normalizedto GAPDH values. The Threshold cycle (Ct) corresponds to the cycle at which there is asignificant detectable increase in fluorescence.

The expression of each target gene was then calculated using the Livak method (Schmittgen & Livak, 2008) (expression ratio=$2^{-\Delta\Delta Ct}$), where:

$\Delta Ct = Ct_{\text{(target gene)}} - Ct_{\text{(GAPDH reference gene)}}$

$\Delta\Delta Ct = \Delta Ct_{\text{(test group)}} - \Delta Ct_{\text{(control group)}}$

This method is widely used for relative gene expressionanalysis. In the $2^{-\Delta\Delta Ct}$ method, it is assumed that both the target gene and the reference gene are amplified with an efficiency nearing 100% and within 5% of each other.

Table 4: Forward and reverse primers used in this study.

Primer	5' ➜ 3'sequence
Mouse GAPDH Forward Primer	5'-CATggCCTTCCgTgTTCCTA-3'
Mouse GAPDH Reverse Primer	5'-CCTgCTTCACCACCTTCTTgAT-3'
Mouse SAG-1 Forward Primer	5'-ACT CAC CCA ACA ggC AAA TC 3'
Mouse SAG-1 Reverse Primer	5'-gAg ACT AgCAgA ATC CCC Cg-3'
Influenza A M gene Forward Primer	5'-GAC CRA TCC TGT CAC CTC TGA C-3'
Influenza A M gene Reverse Primer	5'-AGG GCA TTY TGG ACA AAK CGT CTA-3'

f. <u>Statistics</u>

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

CHAPTER III

RESULTS

A. Infection with *Toxoplasma gondii* followed by a secondary influenza A virus increases host weight loss and mortality as compared to single infection with either pathogen

During the acute phase of infection with *T. gondii*, the immune system inhibits tachyzoite replication and induces the parasite switch into bradyzoites, which are responsible for the chronic phase infection. That is why mice can normally survive *T. gondii* acute infection.

Infection with influenza A virus in mice is characterized by weight loss or slower weight gain than non-infected mice, and may sometimes even lead to death (Trammell and Toth, 2011).

Since a chronic infection with *T. gondii* was shown to confer a protective effect against a lethal H5N1 infection(O'Brien et al., 2011), we wanted to check the effect of an acute *T. gondii* infection followed by an H1N1 infection on host morbidity and mortality.

Mice infected with either pathogen or both pathogens, or mock infected with PBS displayed different rates of weight loss and mortality .All mice infected with PBS survived (Figure 11) and gained about 40% of their initial body weight (Figure 12) by the end of the survival evaluation period.

Single infection with *T. gondii* showed that all mice survived until day 18, when one death was recorded (Figure 11). Although weight loss in not a marker of

Toxoplasma infection, it was recorded. Mice gained weight, however the rate was slower than the mock infected group (Figure 12).

Single infection with H1N1 (PR8) showed that all mice survived, with the exception of one mouse dying at day 8 (Figure 11). Mice gained about 18% of their initial body weight, on average, by the end of the study, which is less than half the observed weight gain in the mock infected group (Figure 12). This may be attributed to the increased severity of infection.

Strikingly, infection of mice with *T. gondii* then H1N1 (PR8) the next day led to 60% death (Figure 11), where three mice died at day 6 post-infection with IAV, one at day 7 p.i with IAV and two at 8 days p.i with IAV. These coinfected mice showed a significant drop in average weight during the first days. However, the weight gain observed after day 8 is attributed to the surviving mice (Figure 12).

Infection of mice with *T. gondii* then H1N1 (PR8) on day led to a 40% death rate by the end of the survival experiment (Figure 11), where two mice died at day 11 p.i with IAV, one at day 14 p.i with IAV and one at 15 days p.i with IAV. The mice infected with both pathogens showed a drop in weight between days 7 and 15 days, however the weight gain observed after day 15 is attributed to the surviving mice (Figure 12).

B. Infection with *T. gondii* and influenza A virus the next day significantly increases the parasitic load in the peritoneum and significantly decreases the viral load in the lungs compared to single infection

The pattern observed in the survival experiment led us to further investigate the molecular basis underlying the increased mortality following coinfection with *T. gondii* and H1N1 the next day.

The mice infected with *T. gondii* alone or infected with both *T. gondii* and IAV were tested for seropositivity. Briefly, on days 3 and 5 p.i. with IAV, blood sample collection using retro-orbital puncture from the Medial Cathenus of each infected mouse was performed, and served for verifying the successful acute infection by western blotting (Figure 13). In case of seropositivity against *T. gondii* infections, it was documented (Erlich, Rodgers, Vaillancourt, Araujo, & Remington, 1983) that a complex pattern of immunoreactivity in which most of the antigens of tachyzoites (with molecular weights of 75, 70, 65, 54, 38, 31, 29, 28, 26, 24 and 6 kDa) react with serum IgG from acutely infected mammals, in our case mice. The majority of sera from all tested mice showed a complex profile of bands with a major band showing at 30 kDa and corresponding to the SAG-1 (P30) antigen; our negative control serum was taken from a non-infected mouse and didn't show any reactivity with tachyzoites. We obtained seronegative mice; these were discarded from the study.

Following the verification of the acute infection, peritoneal lavage was performed on mice infected with *T. gondii* alone or coinfected with H1N1. We also harvested lung samples from mice infected with H1N1 alone or from mice coinfected with both *T. gondii* and the virus. Parasite burden was evaluated by quantifying the tachyzoite marker SAG-1 whereas the viral burden was assessed through the quantification of the M gene.

At day 3 p.i. with IAV, the levels of SAG-1 were sevenfold higher in the peritoneum of mice infected with both pathogens with respect to mono-infected mice (p-value = 0.0033) (Figure 14 a), while the levels of M gene in the lungs of mice infected with both *T. gondii* and IAV were significantly reduced (p-value = 0.002) (Figure 14 b).

At day 5 p.i. with IAV, the levels of SAG-1 were more than twofold higher in the peritoneum of mice infected with both pathogens (*p*-value 0.087812) with respect to mono-infected mice (Figure 15 a), while the levels of M gene in the lungs of mice infected with both *T. gondii* and IAV were significantly reduced (p-value = 0.00968) (Figure 15 b).

These results could suggest that the mice are dying due to the increased parasitic load in the peritoneum, while the viral replication is suppressed in the lungs of mice infected with both pathogens.
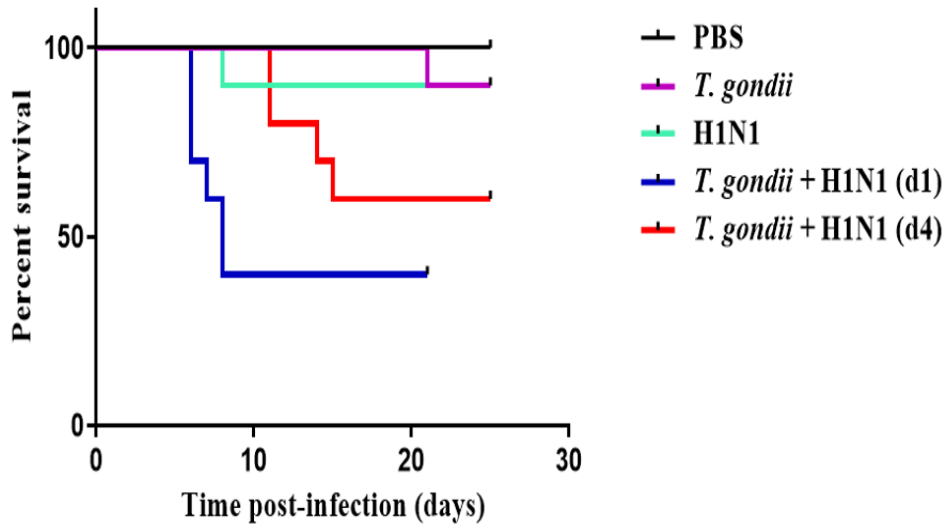
Figure 11: Survival of mice infected with PBS, T. *gondii*, H1N1 (PR8) or H1N1 (PR8) one day, or four days post T. *gondii* infection. Groups of ten mice each were infected with either *T. gondii* or H1N1 or with H1N1 one day or four days post *T. gondii* infection. Survival was monitored and recorded for 21 days post H1N1 infection. A group of ten mice was mock-infected with PBS. The results are expressed as percent survival of the initial starting number.
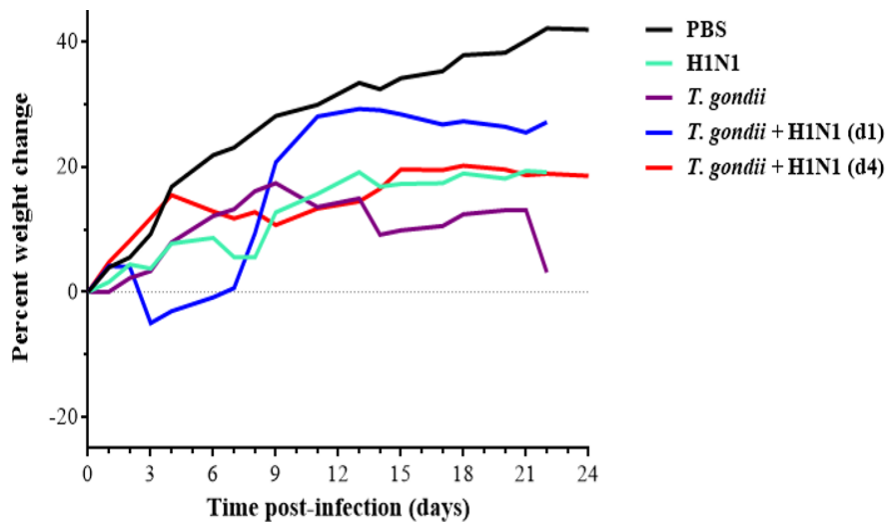
Figure 12: Weight change of differently infected mice groups. Groups of ten mice each were infected with either *T. gondii*, H1N1, H1N1 one day post *T. gondii* infection or H1N1 four days post *T. gondii* infection. A group was mock infected with PBS. Average weight change was calculated and plotted. The results are expressed as average percent difference in weight with respect to the initial starting weight of each group.

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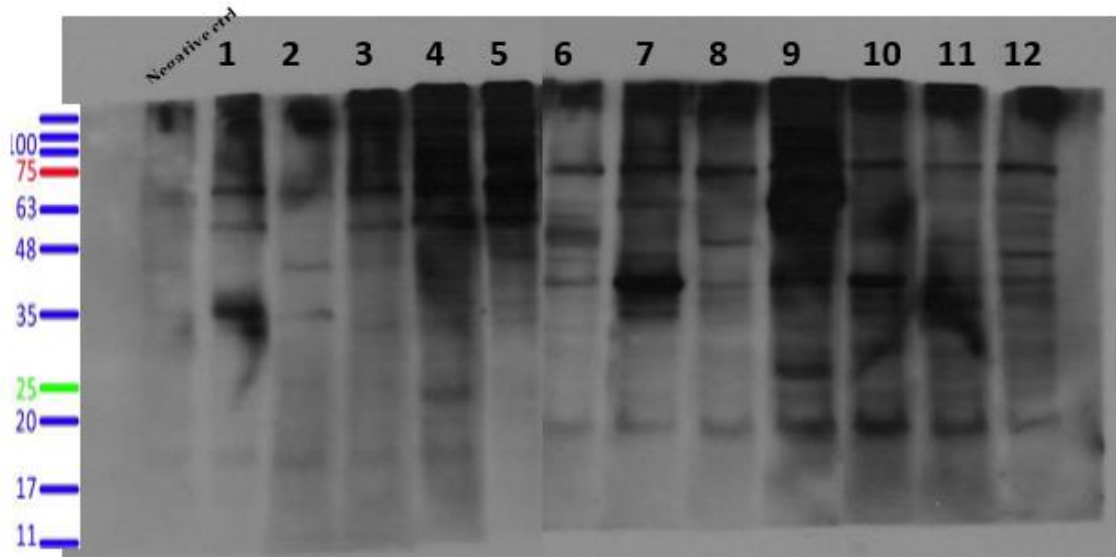
Figure 13: Verification of acute infection at day 3 p.i. with IAV by western blot.
Similar results were obtained for day 5 p.i. with IAV.
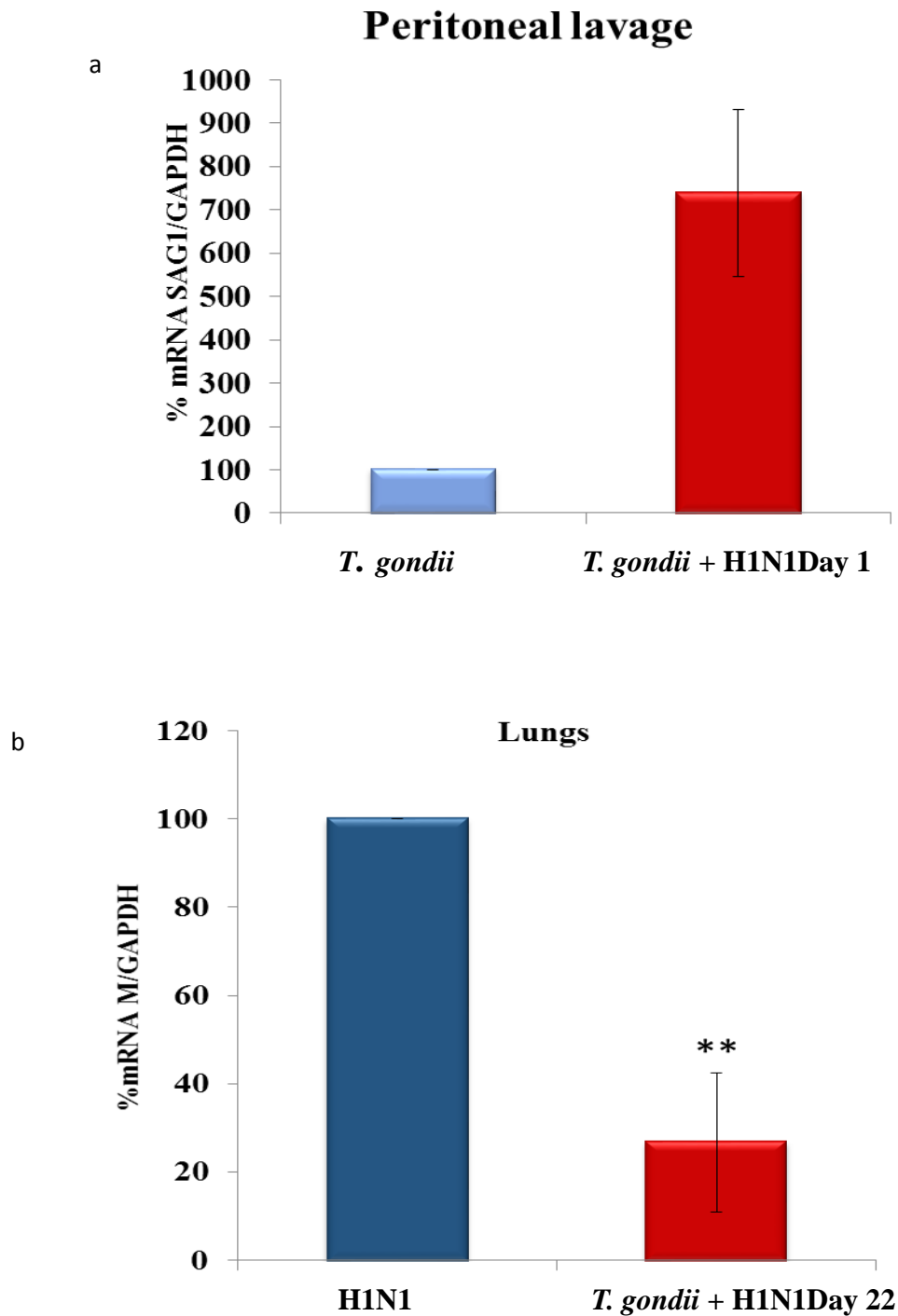
Figure 14: Parasitic and viral loads on day 3 post-infection with IAV. a. Relative expression levels of SAG1 in the peritoenum of BALB/c mice on day 3 post IAVinfection. b. Relative expression levels of M gene in the lungs of mice on day 3 post IAV infection. Mice were infected with either *T. gondii*followed by H1N1 on the following day. Control groups were either infected with *T. gondii* or H1N1. *p*-value: between 0.01-0.05 corresponds to *, between 0.01-0.001 corresponds to ** and less than 0.001 corresponds to *** using student's t-test.
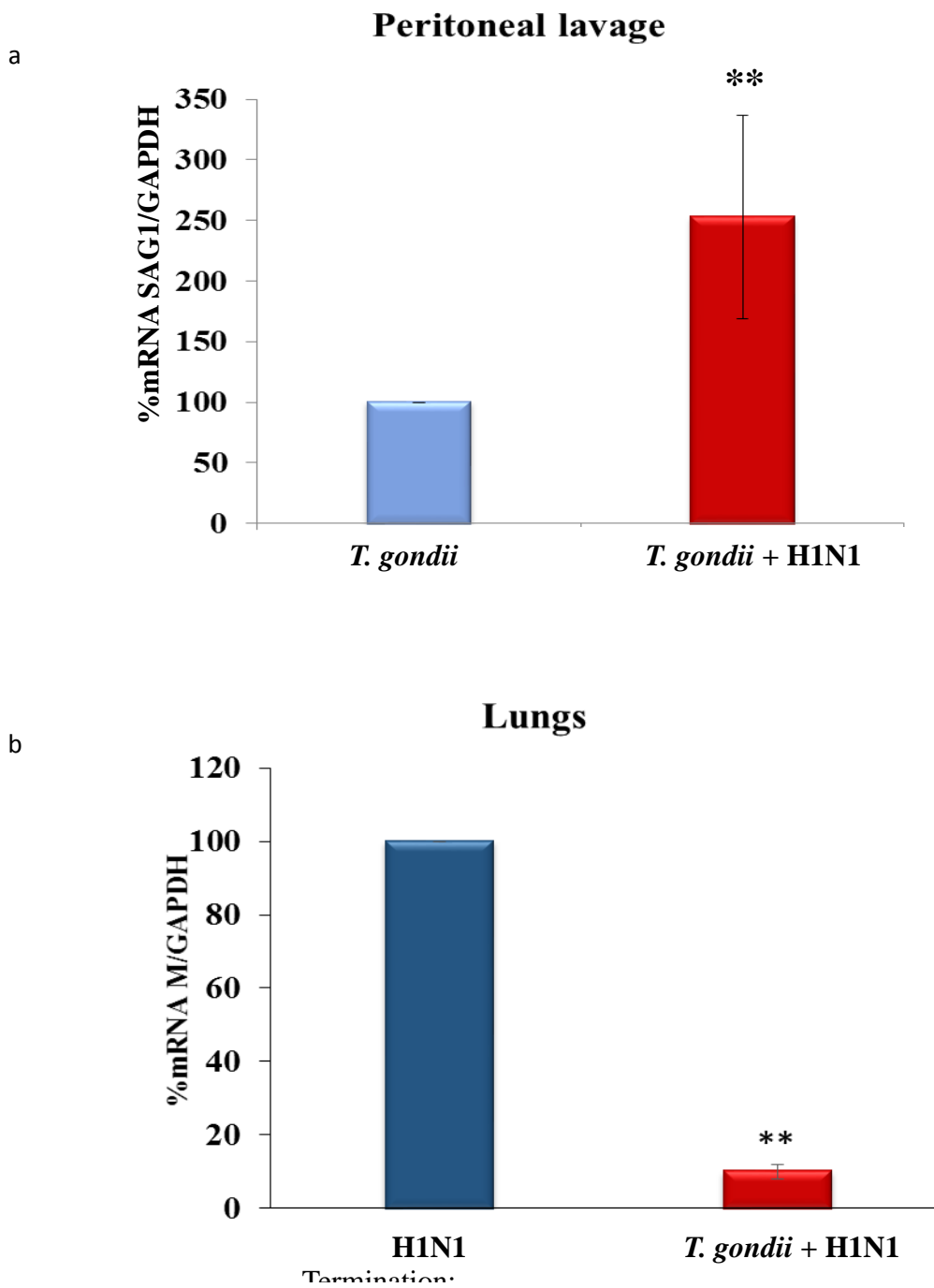
Figure 15: Parasitic and viral loads on day 5 post-infection with IAV. Relative expression levels of SAG1 in the peritoenum of BALB/c mice on day 5 post IAV infection. b. Relative expression levels of M gene in the lungs of mice on day 5 post IAV infection. Mice were infected with either *T. gondii* followed by H1N1 on the following day. Control groups were either infected with *T. gondii* or H1N1. *p*-value: between 0.01-0.05 corresponds to *, between 0.01-0.001 corresponds to ** and less than 0.001 corresponds to *** using student's t-test.

CHAPTER IV

DISCUSSION

The intracellular parasite *T. gondii* is one of the most common and successful zoonotic pathogens, mainly due to its ability to infect a wide variety of hosts, including humans (Blanchard et al., 2015). Approximately on third of the human population is estimated to have been exposed to it (Verma & Khanna, 2013)*.* The disease may have several clinical presentations, and this is mainly dictated by the host immune response(Blanchard et al., 2015; O. Liesenfeld, 1999; Verma & Khanna, 2013).Since type II strains are the most isolated clinically from patients (Ajzenberg et al., 2002), we focused on these strains to pursue our study. Furthermore, *Toxoplasma* is the model apicomplexan for experimental studies regarding host/parasite interactions.

Influenza A virus, the second pathogen involved in our study, is a very common virus that causes yearly outbreaks, as well as pandemics, and is a global health and economic burden (König et al., 2010; Mak et al., 2012). Influenza virus is prone to antigenic shifts and drifts, which may sometimes render the host's immunity and vaccines ineffective. Influenza virus is characterized by uncomplicated infections, but as with *T. gondii,* may cause severe complications in certain populations including the immunocompromised (Bouvier & Lowen, 2010; Mak et al., 2012).

Secondary infections are common phenomena, and the effect of infection with one pathogen on infection with another has been reported and studied at the phenotypical and the molecular level for several years (Chowaniec et al., 1972; Oliver Liesenfeld et al., 2004; O'Brien et al., 2011; Ruskin & Remington, 1968; Wescott & Todd, 1966). Particularly, O'brien et al. demonstrated that a chronic infection with *T.*

gondii could confer protection against a subsequent H5N1 influenza infection(O'Brien et al., 2011). To the best of our knowledge, there is not a single study elucidating the impact of an acute infection with *T.gondii* on a subsequent H1N1 influenza virus infection.

Our experiments revealed that infection with *T. gondii* followed by H1N1 infection led to a dramatic weight loss and higher death rate (Figures 11 and 12) when compared to single infection with either pathogen. This shows that an acute infection with *T. gondii* followed by IAV infections results in increased host mortality instead of conferring a protective effect and increasing survival as originally presumed.

To better understand the mechanism underlying increased mortality rate upon infection with both pathogens, we further investigated the parasitic and viral loads in infected hosts infected with both *T. gondii* and IAV with respect to mono-infected hosts. We showed that the parasitic load increases in the peritoneum, while the viral load decreases in the lungs of hosts infected with both pathogens, on days 3 and 5 p.i. with IAV (Figures 13 and 14).

The decrease in viral titers in mice infected with both *T. gondii* and IAV with respect to mono-infected mice is consistent with previous findings which have shown that a pre-existing infection with another pathogen may lessen the burden of a subsequent IAV infection (Chowaniec et al., 1972; Furze et al., 2006; O'Brien et al., 2011). However, none of the previous studies had assessed the load of the pathogen with which the host is first infected. For that reason, we speculate that *T. gondii* is indeed conferring a certain protection as indicated by lower viral load. Suppression of IAV replication maybe mediated by heightened IFN-γ and IL-12 due to active *T. gondii* infection which suppresses IAV.

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The increased mortality rate in mice infected with both pathogens may be attributed to the significant increase in the parasitic load in these mice. IAV has been shown to hamper secretion of IL-10 which is needed for clearance of *T. gondii* during acute infection (I. A. Khan, Matsuura, & Kasper, 1995; Schliehe et al., 2015). This might explain the uncontrollable increase in *T. gondii* parasitic load observed in the peritoneum in mice infected with *T. gondii* and IAV. Further studies will focus on elucidating the immunologic mechanisms underlying modulation of disease outcome in mice infected with *T. gondii* followed by a secondary IAV infection by performing a cytokine profiling of these mice and comparing it to that of mono-infected ones.

In conclusion, studying the effect of an acute *T. gondii* infection of a subsequent influenza A virus infection is of clear benefit, as both pathogens are extremely common and widespread, and may both cause severe complications in immunocompromised hosts, especially that those hosts are at risk of reactivation of *T. gondii.* Also studies are needed to determine the real prevalence and burden of *T. gondii* and IAV in Lebanon and worldwide.

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