THE AMERICAN UNIVERSITY OF BEIRUT

IN VIVO CHARACTERIZATION OF THE UNDERLYING IMMUNOLOGIC MECHANISMS OF DISEASE MODULATION IN THE CONTEXT OF *TOXOPLASMA GONDII* AND INFLUENZA A VIRUS CO-INFECTIONS

by
NAJAT KHODR BDEIR

A thesis

submitted in partial fulfillment of the requirements of the degree of Master of Science to the Department of Experimental Pathology, Immunology and Microbiology of the Faculty of Medicine at the American University of Beirut

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AMERICAN UNIVERSITY OF BEIRUT

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Title: *In vivo* characterization of the underlying immunologic mechanisms of disease modulation in the context of *Toxoplasma gondii* and influenza A virus co-infections

Toxoplasma gondii (T. gondii), an obligate eukaryotic intracellular protozoan parasite, is the causative agent of toxoplasmosis. In immunocompetent individuals, *T. gondii* infections are usually asymptomatic; however, some patients may present with flu-like symptoms and sometimes lymphadenophathy. Contrarily, in immunocompromised patients, the infection may be life threatening. Another spectrum of the disease is congenital toxoplasmosis, which may lead to abortion, or severe fetal outcomes including mental retardation, hydrocephaly, microcephaly, chorioretinitis and impaired vision in newborn infants.

Influenza A virus (IAV) is a major cause of acute respiratory tract infections in humans, which occasionally cause pandemics. Severe influenza infections are characterized by complications like pneumonia, encephalitis, and secondary bacterial pneumonia. Infection with *T. gondii* has been shown to modulate the outcomes of disease in the context of infection with other pathogens like *Helicobacter felis, Trichinalla spiralis, and Mycobacterium avium.* However, no studies investigated the disease outcome and underlying immunologic mechanisms of IAV infection in the context of acute toxoplasmosis. Therefore, the aim of this study is to characterize the effect of co-infections and the underlying immune response with these two pathogens in mice.

Six-to-eight weeks old female BALB/c mice were intraperitoneally infected with tachyzoites of a type II *T. gondii* strain followed by the intranasal inoculation with IAV or vice versa. Mouse survival and weight change were monitored over the course of three weeks of infection. Acute toxoplasmosis was verified by western blot. Real time PCR and plaque assay (for IAV) were used for quantification of parasitic and viral loads within the lungs and peritoneal lavage, respectively, during the acute toxoplasmosis stage. In addition, quantitative real time PCR was used to quantify expression of pro and anti-inflammatory cytokines in the in the spleen and lungs of singly infected and co-infected mice on days 2 and 5 after IAV infection. Last but not least, the impact of the co-infections on the progression to chronic toxoplasmosis was assessed by quantifying the bradyzoite marker (BAG-1) in the brains of mice singly infected with the parasite or in the context of co-infection. To the best of our knowledge, no study investigated the outcome and immune mechanism involved in the case of a pre-existing acute *T. gondii* infection followed by a subsequence infection with IAV. Our results indicate that an acute infection with *T. gondii* followed by IAV infection few days later results in an aggravated disease outcome. Co-infected mice also displayed reduction of spleen IFN-γ, TNF-α, and IL-6, which led to attenuated tachyzoite clearance and delayed progression into chronic toxoplasmosis.

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CHAPTER I

INTRODUCTION

A. Toxoplasma gondii

1. Origins and history

Toxoplasma gondii (T. gondii) is an obligate intracellular protozoan parasite discovered by Nicolle and Manceaux in North Africa (Nicolle and Manceaux 1908) and by Splendore in Brazil (Splendore 1908). The genus name is derived from the Greek word: *toxon,* meaning "arc" or "bow", referring to the crescent shape of the organism, and the latter designation: plasma, meaning "life".

In 1937, Albers Sabin and Peter Olitsky established the first *in vitro* culture system of *T. gondii* and hypothesized transmission through ingestion of contaminated meat by the parasite (Sabin and Olitsky 1937). In 1939, the parasite was recognized as a human pathogen and the cause of a congenital disease (Wolf, Cowen, and Paige 1939). Two years later, acquired toxoplasmosis was described in a six-year-old boy who was admitted to the hospital for a headache after being hit with a baseball bat. The boy developed an enlarged spleen, neurological abnormalities, lymphadenopathy, and eventually died after thirty days of illness (Sabin 1941). Numerous reports described the identification of parasitic tissue cysts in brain autopsy slides (Kean and Grocott 1947) leading to the development of the Dye- test by Sabin and Feldman for the diagnosis of *T. gondii* (Sabin and Feldman 1948).

2. Taxonomy

T. gondii belongs to the phylum Apicomplexa, which includes many pathogenic protozoa of human and animals including *Plasmodium* spp, *Cryptosporidium* spp, and *Eimeria* spp. *T. gondii* is considered the pathogen of choice for studying the phylum apicomplexa due to its easy propagation and culture, as well as the efficiency in its genetic manipulation (knock-out, conditional knock-out, *crispr cas9*….) (Kim and Weiss 2004; Shen et al. 2017).

T. gondii is the only species in the *Toxoplasma* genus and belongs to the family Sarcocystidae in the class coccidian (Tenter and Johnson 1997). All members of coccidia share an obligate intracellular nature and a conserved way of invasion actively involving the apical complex of these parasites (Katris et al. 2014). This class of intracellular pathogens also has the ability to infect a wide spectrum of hosts (Dubey 2009). *T. gondii* is prevalent in many species and has a wide range of intermediate hosts (Lambert 2009). Figure 1 depicts the classification of some important apicomplexan parasites.

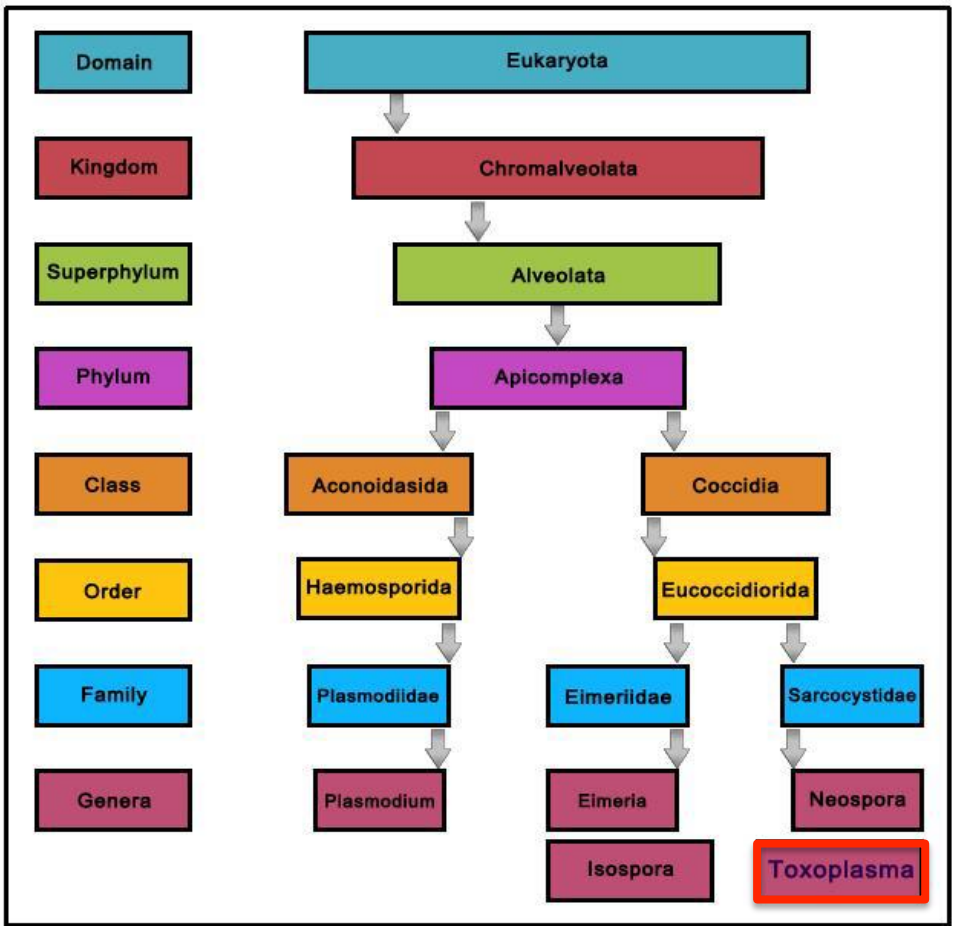

Figure 1. Classification of some of the most important apicomplexan parasites. Toxoplasma is highlighted in a red box. Adapted from http://www.ncbi.nlm.nih.gov/Taxonomy

3. Stages of T. gondii life cycle

The life cycle of *T. gondii* includes three infective stages: 1) the tachyzoite, which is the rapidly invasive and replicating stage within the intermediate host (Frenkel 1973); 2) the bradyzoite, which is the slowly dividing stage in the tissue cysts (Frenkel 1973); and 3) the sporozoite, which is the environmental stage found inside oocysts shed in felines' feces and responsible for the transmission of the infection from the definitive host to the broad range of intermediate hosts (Dubey et al. 1997; Speer and Dubey 1998). An overview on each stage will be summarized below.

a. The tachyzoite

The tachyzoite is the most extensively studied stage of *T. gondii*. This invasive stage is crescent shaped and is approximately 2 x 7 μm in size (Dubey, Lindsay, and Speer 1998). Structurally, tachyzoites consist of various organelles and inclusion bodies including polar rings, rhoptries, apical rings, micronemes, endoplasmic reticulum, mitochondria, ribosomes and others (Sheffield and Melton 1968; de Souza and Souto-Padrón 1978) (Figure 2a). Tachyzoites have the ability to penetrate any vertebrate nucleated cell and become incased within a parasitophorous membrane. Once within the vacuole, they divide by endodyogeny forming rosettes (Figure 2b) and continue to replicate until the host cell ruptures, thus releasing the parasites, which consequently infect neighboring cells (Goldman, Carver, and Sulzer 1958).

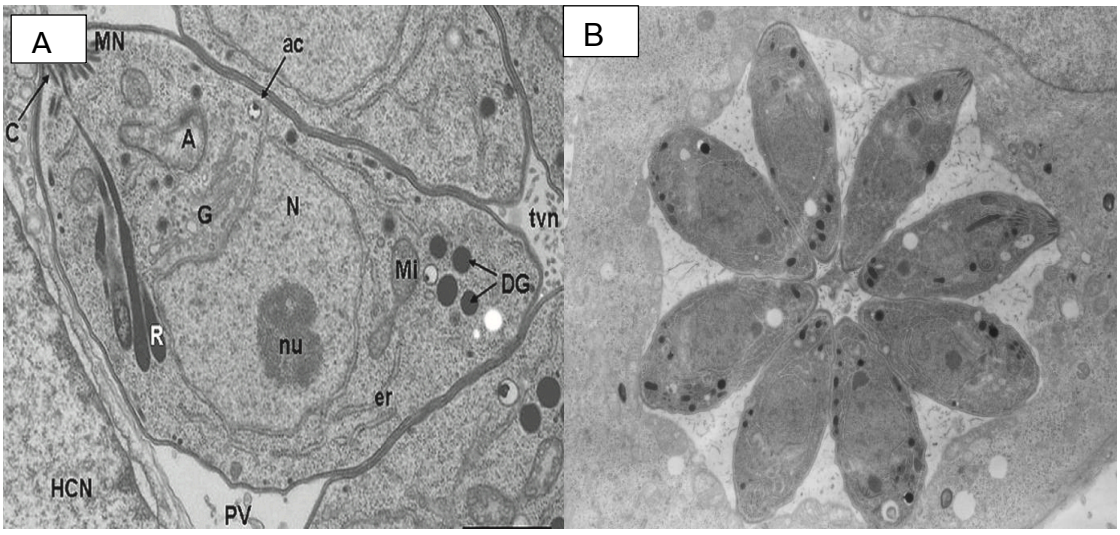

Figure 2: Structure and organization of *T. gondii* tachyzoites. (A) Electron micrograph of a *T. gondii* tachyzoite in the parasitophorous vacuole (pv) of a human foreskin fibroblast. Note A, apicoplast; C, conoid; DG, dense granule; ER, endoplasmic reticulum; G, Golgi body; HCN, host cell nucleus; MN, micronemes; Mi, mitochondria; N, nucleus; NU, nucleolus; R, rhoptry (Clicher Jean-Francois Dubremetz). (B)| Rosette of 8 tachyzoites inside the parasitophorous vacuole (Clicher Jean-Francois Dubremetz)

b. The bradyzoite

Under the tight control of the host immune system, the rapidly dividing tachyzoites transform into slowly replicating bradyzoites that mostly form inside cysts within the brain and skeletal muscles of the intermediate host (Figure 3a) (Dubey, Lindsay, and Speer 1998). The bradyzoite stage is the hallmark of the chronic phase of toxoplasmosis and lasts for the whole lifespan in immunocompetent hosts. Bradyzoite cysts range in size from 50 μm to 70 μm and may contain up to 2000 bradyzoites that are, as the tachyzoites, crescent shaped but more rounded and approximately 1.5 μm x 7 μm in size (Dubey, Lindsay, and Speer 1998; Fortier et al. 1996). Structurally, bradyzoites are more slender than tachyzoites, have a posteriorly located nucleus, and several amylopectin granules |(Figure 3b) (Fortier et al. 1996).

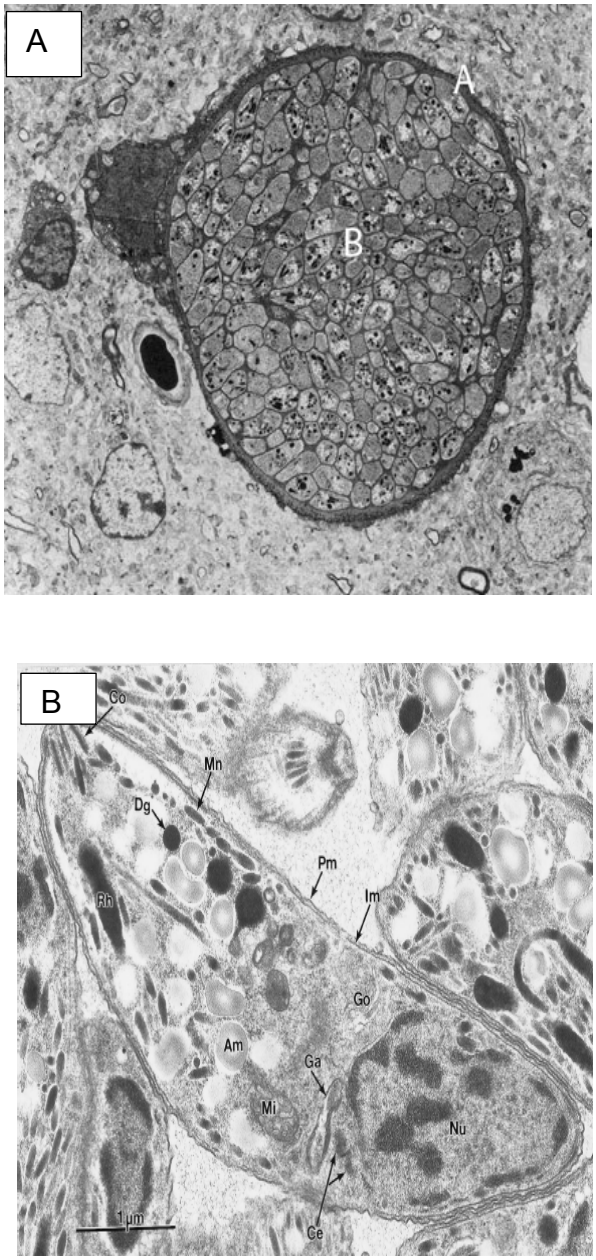

Figure 3*:* The structure and organization of *T. gondii* bradyzoites. (A) Transmission electron micrograph of a tissue cyst in the brain of a mouse with chronic toxoplasmosis. A, cyst wall; B, bradyzoites (Cenci-Goga et al. 2011) (B) Electron micrograph of a *T. gondii* bradyzoite Am, amylopectin granule; Ce, centrioles; Co, conoid; Dg, electron-dense granule; Ga, Golgi adjunct (apicoplast); Go, Golgi complex; Im, inner membrane complex; Mi, mitochondrion; Mn, microneme; Nu nucleus; Pm, plasmalemma; Rh, rhoptry.(Dubey, Lindsay, and Speer 1998)

c. The sporozoite

T. gondii sporozoites are the infective stages presenting inside sporulated

oocysts that are shed in cat feces. These forms are responsible for the transmission of the parasite to an intermediate host upon ingestion of contaminated food or water. Sporozoites are 2 by 6 to 8 μ m in size with a subterminal nucleus. Structurally, sporozoites are similar to tachyzoites, but are more abundant in micronemes, rhoptries, and amylopectin granules.

4. Transmission and life cycle

Intermediate hosts, including humans, become infected with *T. gondii* through 1) ingestion of unwashed vegetables (O. Liesenfeld 1999), or meat products contaminated with oocysts or bradyzoite cysts, respectively (Jacobs, Remington, and Melton 1960; Dubey, Lindsay, and Speer 1998, 199), 2) congenitally by vertical transmission (Wolf, Cowen, and Paige 1939), and 3) rarely through blood transfusion (Herwaldt 2001) and organ transplantation (Ryning et al. 1979). The complete life cycle of *T. gondii* involves a sexual cycle that is restricted to the feline gut epithelium and an asexual cycle that can occur in a broad spectrum of intermediate hosts (Jackson and Hutchison 1989)(Figure 4).

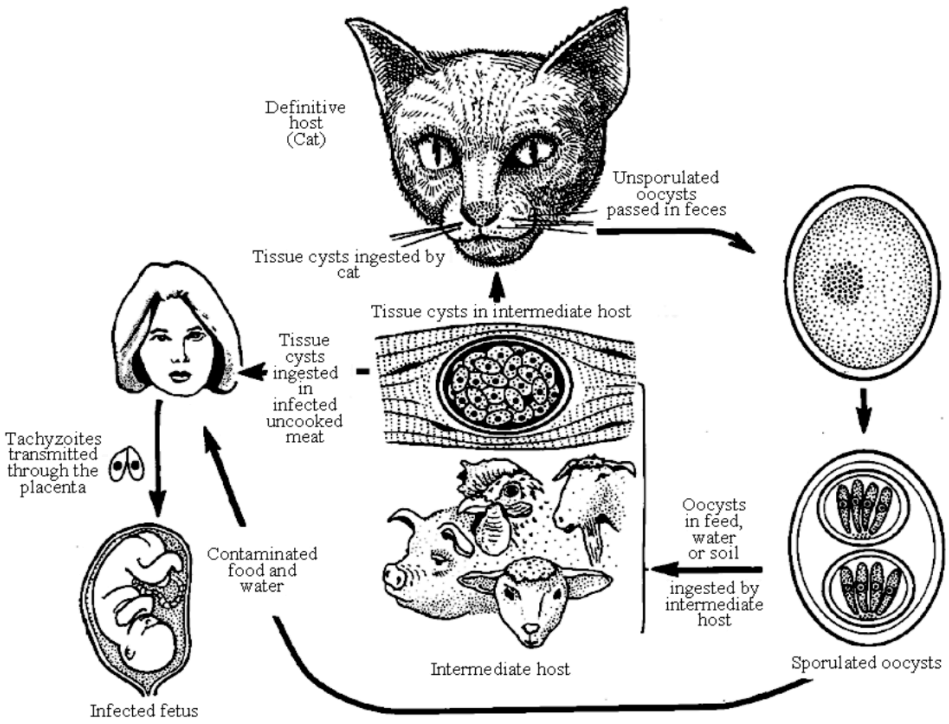

Figure 4: The life cycle of *T. gondii*. Sporozoites represent the infectious stages of *T. gondii.* They are contained in sporulated oocysts shed in cat feces. Sporulated oocysts are accidentally ingested mainly in contaminated food, by an intermediate host. Vertical transmission may also occur from infected mother to fetus (Dubey, Lindsay, and Speer 1998)

a. The sexual cycle

The sexual cycle of *T. gondii* begins when a domestic cat or any other member of the *Filedae* family ingests bradyzoite cysts from an infected prey, which has acquired *T. gondii* through accidental ingestion of oocysts from the environment. Upon ingestion, bradyzoites are released leading to the dissociation of the cyst wall by the action of gastric proteolytic enzymes. Within approximately two days, bradyzoites infect enterocytes within the ileum of the definitive host and undergo a number of asexual multiplications leading to the formation of merozoites and schizonts (Frenkel 1973; Dubey, Lindsay, and Speer 1998). Following this event, micro and macrogametes are formed and fuse by gametogony leading to the formation of an oocyst, which is released

from the intestinal epithelium of the definitive host by disruption of the enterocyte wall. The unsporulated oocyst excreted within cat feces undergoes maturation and sporulation under the appropriate temperature and humidity in the external environment. As a result, an infective sporulated oocyst containing two sporocysts thatinclude four sporozoites each, will be formed (Frenkel 1973; Dubey 1998).

b. The asexual cycle

The asexual or intermediate life cycle begins upon the ingestion of sporulated oocysts in contaminated food or water. Digestive enzymes within the gastrointestinal tract mediate the release of sporozoites, which then invade the intestinal epithelium and differentiate into the rapidly dividing tachyzoite stage (Dubey 1997). The latter can rapidly replicate by endodyogeny within any nucleated cell and disseminate throughout the intermediate host tissues (Sheffield and Melton 1968). Approximately 5 days post-ingestion of oocysts, tachyzoites begin to differentiate into bradyzoites under the tight control of the host immune system. This leads to the formation of cysts, predominantly within the central nervous system and skeletal muscles (Frenkel 1973). Bradyzoite cysts within the brain are known to appear as early as 2 weeks post *T. gondii* infection, and reach their maximum peak within 4 weeks subsequent to infection (Chew et al. 2012). These bradyzoites may then differentiate back to tachyzoites and initiate acute infection within the new intermediate host, thus completing the asexual cycle (Dubey 1997).

5. Genetics of T. gondii

Despite the opportunity for genetic recombination in the feline host, *T. gondii* strains differ by 1% or less at the DNA sequence level (Saeij, Boyle, and Boothroyd 2005). *T. gondii* presents eight strains according to their virulence. However, the most commonly used classification was proposed by Howe and Sibley who described three

clonal lineages known as types I, II, and III, based on restriction fragment length polymorphism (RFLP) analysis of the polymorphic surface antigen 2 (SAG2) (Howe et al. 1997; Sibley and Boothroyd 1992).

These strains display a different lethal dose LD_{50} (which corresponds to the dose of parasites needed to kill 50% of infected animals) in mouse models. Accordingly, these strains elicit different host immune responses (Saeij, Boyle, and Boothroyd 2005). Type I strains are the most virulent with an LD_{50} =1 (Sibley and Boothroyd 1992), where one parasite is sufficient to generate high parasitic loads and a strong Th1 response (Melo, Jensen, and Saeij 2011).

In contrast type II strains incur intermediate virulence with an LD_{50} ranging between 10^2-10^3) and have been reported as the most commonly isolated type from humans worldwide (Sibley and Boothroyd 1992) .

Type III *T. gondii* strains are associated with the lowest virulence with an LD50= 1>10^5 parasites (Saeij, Boyle, and Boothroyd 2005). These strains are largely found in animals and may occasionally be associated with human toxoplasmosis *(Howe et al. 1997).*

6. Clinical manifestations and complications

In immunocompetent individuals, *T. gondii* infections are usually asymptomatic although some patients might have mild headaches, myalgia, and lymphadenopathy (Dubey, Prakask, and Beattie. 1988). Contrarily, in immunocompromised patients, the infection may be life threatening, whereby symptoms include apathy, dementia, motor seizures, ataxia, hemiparesis, coma, and ultimately leading to death if not treated (Luft and Remington 1992). Another spectrum of the disease is congenital toxoplasmosis,

where the severity of clinical outcome is inversely related to gestational age in primo infected women. *T. gondii* infection during the first trimester results in spontaneous abortion, while infection during later trimesters may result in chorioretinitis, intracranial calcifications, and hydrocephalus (McAuley 2014).

7. T. gondii prevalence

Toxoplasmosis in one of the most common worldwide zoonosis. The parasite is estimated to affect a third of the world's population as indicated by the presence of specific IgG (Montoya 2002; Wam et al. 2016). It is worth noting that seroprevalence varies between countries and even within the same country depending on diet, social and cultural habits, geographic factors, routes of transmission, and climate (Studenicová, Bencaiová, and Holková 2006; Wam et al. 2016). The highest prevalence has been reported in warm and humid areas in tropical Africa and Latin America (Pappas, Roussos, and Falagas 2009). Higher seroprevalence rates in certain communities may be attributed to the consumption of raw meat products (Cook et al. 2000).

Furthermore, studies have shown seroprevalence to be more prominent in lower socioeconomic groups than in the middle and upper class groups. Higher socioeconomic communities have improved hygienic conditions, filtered water supplies, and clean feeding sources for cats. These factors contribute to a decrease in the *T. gondii* seroprevalence in such communities (Robert-Gangneux and Dardé 2012).

A retrospective study conducted in 2010 on sera from hospitals and laboratories within Beirut revealed that the seroprevalence of *T. gondii* IgG antibodies ranged between 55 and 67%, whereas IgM antibodies were reported in nearly 7% of the

screened samples (Bouhamdan et al. 2010). This indicates a significant spread of the parasite among the Lebanese population and a high incidence of acute infections.

8. The immune response to T. gondii

a. Innate immunity

Primarily, the host immune response to *T. gondii* compromises an interaction between the profilin-like protein (TgPRF) on the surface of the parasite with TLR-11 on dendritic cells, prompting the production of IL-12 by the activation of MyD88 pathway (Scanga et al. 2002). A deficiency in the MyD88 adapter protein results in complete loss of resistance to acute infection in mice systemically or orally infected with *T. gondii*, likely due to the dysfunctional production of IL-12 (Sukhumavasi et al. 2008; Scanga et al. 2002, 88). However, there are strain dependent differences in MyD88 dependence (Hunter and Sibley 2012), whereby both type I and III parasite strains induce STAT3/6 constitutive activation and MyD88-dependent IL-12 production (Melo, Jensen, and Saeij 2011). In contrast, type II parasite strains stimulate NF-kB translocation and MyD88-independent IL-12 production by the interaction of TNF receptor associated factor 6 (TRAF-6) with IL-1R associated kinase 1 (IRAK1) to mediate signaling downstream of MyD88 (Rosowski et al. 2011).

Monocytes or macrophages also induce the production of IL-12 as well as TNF-α, by the interaction of glycosylphosphatidylinositol (GPI) anchored proteins with TLR-2 and TLR-4 (Hunter and Sibley 2012) . The deletion of individual TLRs has modest effects on host susceptibility to infection, implying that multiple TLRs are involved in *T. gondii* immune recognition (Ricardo T. Gazzinelli and Denkers 2006).

It has been revealed that the central control mechanism for *T. gondii* growth requires IL-12 stimulation of adaptive $CD4^+$ and $CD8^+$ T lymphocytes, as well as innate type natural killer cells (NK) to release IFN-γ (R T Gazzinelli et al. 1993).

IFN-γ stimulates the production of reactive oxygen and nitrogen intermediates, which lead to damage and growth impediment of the parasite within macrophages (Adams et al. 1990). Studies have also demonstrated the presence of an IFN-γ dependent, inducible nitric oxide (iNOS) independent mechanism for resistance against toxoplasmosis. This pathway relies on the IFN-γ dependent activation of immune related GTPases (IRGs), which leads to clearance of *T. gondii* from multiple cell types (Scharton-Kersten et al. 1997).

b. Adaptive immunity

Processes leading to the stimulation of adaptive immunity against *T. gondii* are less understood than mechanisms activating the innate immunity. Within the parasitophorous vacuole, *T. gondii* is shielded from mechanisms of antigen processing and presentation (Blanchard and Shastri 2010). Moreover, cells harboring the parasite have been demonstrated to display reduced expression of major histocompatibility complex (MHC) molecules (C. G. Lüder et al. 1998; C. G. K. Lüder et al. 2003; Hunter and Sibley 2012). Despite these evasion mechanisms, infection with type II *T. gondii* induces the activation of dendritic cells and a strong $CD8^+$ T cell response, while type I parasites induce a weaker response (Tait et al. 2010). Prominent antigens presented on MHC class I molecules include dense granule GRA6, GRA4, GRA7, GRA3, GRA2 as well as rhoptry protein ROP7, ROP5, ROP16, and others (Hunter and Sibley 2012; Grover et al. 2014; Cao et al. 2015). These antigens are polymorphic, which might explain the strain-dependent effects on adaptive immunity. Antibodies mediate

resistance to secondary *T. gondii* infection (Sayles, Gibson, and Johnson 2000; Dupont, Christian, and Hunter 2012) and assist in clearance of parasites during acute infection. IgM initially acts on egressing tachyzoites by blocking cell invasion, mediating agglutination and enabling complement activation (Dupont, Christian, and Hunter 2012).

b. Immune response in *T. gondii* type II

It has been well demonstrated that different types of *T. gondii* induce diverse immunological responses (Melo, Jensen, and Saeij 2011). Infection with type II parasites activates an early immune response leading to the massive production of pro-inflammatory cytokines (e.g. IL-12, IFN-γ, …) early after infection. Cytokines produced by the early response prime the immune environment towards a Th1 type response and activate several T cell subtypes including NK, Th17, and Th1 cells (Figure 5) (Denkers and Gazzinelli 1998) .

Genetic mapping identified a dense granule protein known as GRA15, which activates the NF-kB pathway independently of MyD88, leading to its translocation into the nucleus, and the eventual massive production of IL-12 and pro-inflammatory cytokines such as IL-1 β, IL-18, and IL-17 in type II strains (Rosowski et al. 2011). IL-12 activates NK and T cells to produce IFN- γ, which is involved in the production of reactive oxygen and nitrogen intermediates, destruction of the PV, and autophagy (Melo, Jensen, and Saeij 2011).

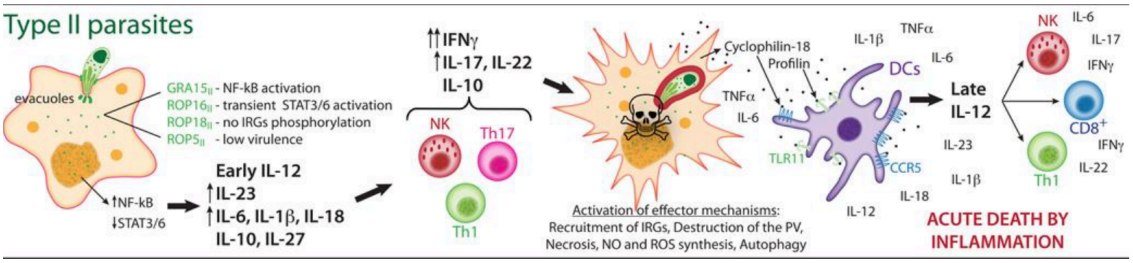

Figure 5: The immune response to *T. gondii* type II *(Melo, J*ensen, and Saeij 2011). Type II *T. gondii* secrete different proteins to modulate the host immune responses including GRA15, GRA 14, ROP18, ROP5, leading to early activation of NF-κB in the infected cells and massive production of pro-inflammatory cytokines early after infection.

B. The influenza virus

1. Classification and antigenic types

The influenza virus is a negative sense, single stranded, and segmented RNA virus belonging to the family of *Orthomyxoviridae.* Four influenza types (A-D) have been identified based on the nucleoprotein (NP) and matrix (M1) proteins (Webster et al. 1992; Bouvier and Palese 2008; Noda and Kawaoka 2010). These types have different degrees of pathogenicity, host specificity, and antigenic variation. Influenza A viruses (IAVs) have the broadest host range and infect a variety of animals including humans, pigs, and birds. Aquatic birds are considered the source for all IAVs in other species (Webster et al. 1992). Influenza B viruses infect humans and occasionally seals (Baigent and McCauley 2003; Noda and Kawaoka 2010), while influenza C viruses benignly infect humans, pigs and dogs (Yuanji (Kuo Yuanchi) et al. 1983; Noda and Kawaoka 2010). Influenza D has been recently identified to infect cattle and pigs (CITE).

IAV is further classified into subtypes based on the genetic and structural properties of its surface glycoproteins: the hemagglutinin (HA) and the neuraminidase

(NA). HA and NA exist in 16 and 9 genetically distinct subtypes, respectively. However, only three HA subtypes (H1, H2, and H3) and 2 NA (N1 and N2) have caused human epidemics (Noda and Kawaoka 2010; Bouvier and Palese 2008; Fouchier et al. 2005).

2. Virion structure and organization

Influenza viruses are pleomorphic and may either be spherical (100 nm in diameter) (Stanley 1944; Noda and Kawaoka 2010; Bouvier and Palese 2008) (Figure 6, or filamentous with a length often beyond 300 nm (Chu, Dawson, and Elford 1949). The lipid envelope of IAV is traversed by tetrameric matrix protein (M2) ion channels and studded with HA and NA glycoprotein spikes with a one to four ratio; this envelope overlays a matrix (M1) protein that encloses the virion core (Noda and Kawaoka 2010; Bouvier and Palese 2008) (Figure 6).

Internal to the M1 matrix, each of the 8 influenza RNA segments is associated to multiple copies of the arginine rich protein NP and the viral transcriptase consisting of three RNA polymerase components PB1, PB2, and PA to form the ribonucleocomplex (RNP) (Table 1). IAV genome encodes for at least 16 known proteins (Table 1) (Noda and Kawaoka 2010; Bouvier and Palese 2008; Cheung and Poon 2007).

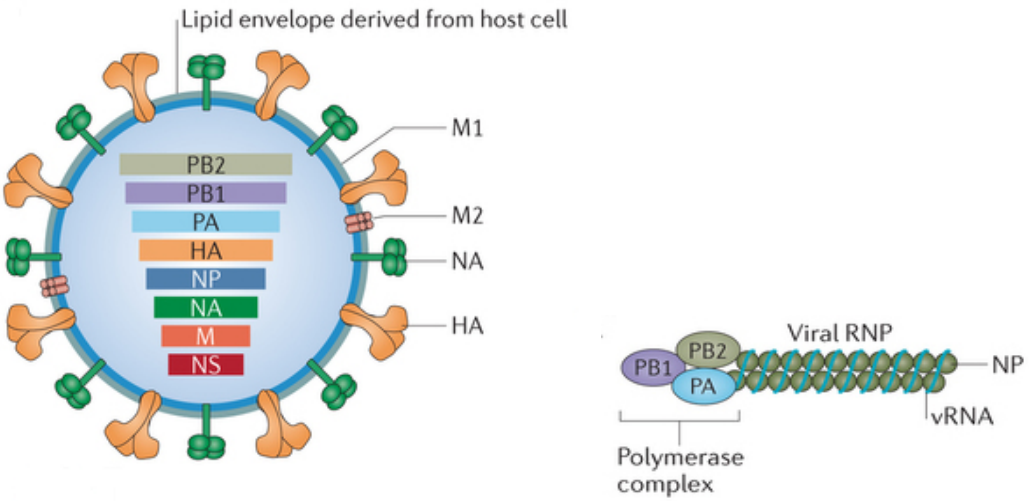

Figure 6: Structure and composition of the influenza A virus (Shi et al. 2014) IAVs are enveloped, single-stranded, RNA viruses that contain eight gene segments that encode 16 proteins. The non-structural segment encodes the nuclear export protein NS2 and the host antiviral response antagonist NS1; the matrix segment encodes the matrix protein M1, the ion channel protein M2; the hemagglutinin (HA) segment encodes the receptor-binding glycoprotein HA; and the neuraminidase (NA) segment encodes NA. In addition, nucleoprotein (NP) and the components of the RNA-dependent RNA polymerase complex (PB1, PB2 and PA) are expressed from their respective genome segments.

Table 1: Summary for the roles of the major viral proteins during the life cycle of the IAV with their respective coding genes (Adapted from: Insight into Influenza Viruses of Animals and Humans | Sanjay Kapoor | Springer 2017)

Genomic segment	**Protein**	**Role in the replication cycle**
4	HA	Surface glycoprotein, receptor mediated endocytosis
6	NA	Surface glycoprotein, viral release
5	NP	RNA binding protein, regulation of RNP import into the nucleus for replication and transcription
3	PA	RNA Polymerase subunit, endonuclease activity Regulation of RNP import into the nucleus for replication and transcription
2	PB1 PB1-F2	RNA polymerase subunit, endonuclease activity Regulation of RNP import into the nucleus for replication and transcription Involved in influenza-induced cell death
1	PB2	RNA polymerase subunit, mRNA cap recognition Regulation of RNP import into the nucleus for replication and transcription
7	M1 M2	Matrix protein, export of vRNP, assembly and budding Proton selective ion channel, endosomal fusion of viral envelope with endosomal envelope, assembly, and budding.
8	NS1 NS2/NEP	Interferon antagonist protein, interacts with various host factors Nuclear export of vRNPs, control for accumulation of vRNA and mRNA

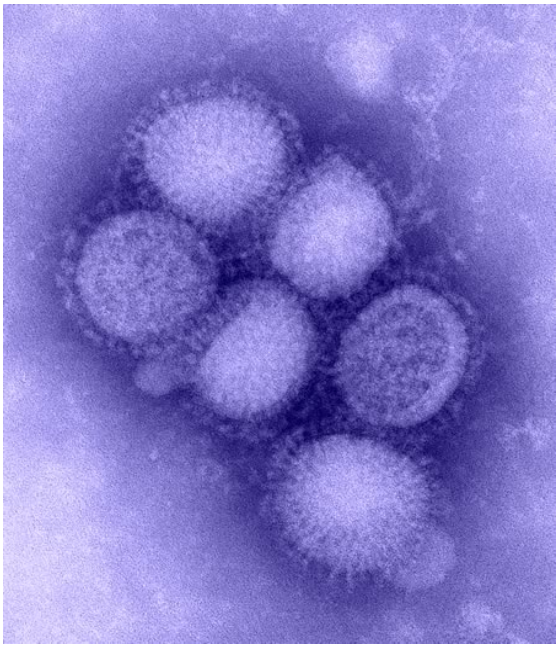

Figure 7: Electron micrograph of influenza A virus. A characteristic feature of the virus is its outermost layer of spike-like projections. These are the two viral surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), which are embedded in the lipid membrane of the viral envelope ("CDC H1N1 Flu | Images of the H1N1 Influenza Virus" 2017).

3. The replication cycle

Recognition of sialic acid residues on the host cell surface by viral HA glycoproteins is the first step for virus entry (Figure 8). Following attachment, virus particles are uptake into the endosomes. Low pH within the endosome triggers a conformational changes of the HA precursor polypeptide (HA0), exposing a fusion peptide that mediates merging of the endosomal membrane with the viral envelope. The drop in the endosomal pH also triggers the opening of the M2 ion-channel on the virus envelope. The influx of hydrogen ions through M2 further acidifies the viral core and disrupts interactions between NRPs and M1 matrix proteins (X. Sun and Whittaker 2013).

Subsequent to membrane fusion, a pore is formed, allowing the release of viral

RNPs into the host cell cytoplasm. Viral protein nuclear localization signals (NLSs) traffic the released RNPs into the host cell nucleus, where synthesis of viral mRNA for viral protein translation and vRNA genomes for viral progeny takes place (Wu, Sun, and Panté 2007). RNA-dependent RNA polymerase (RdRp) heterotimer, composed of PA, PB1, and PB2, mediates the synthesis of a complementary RNA strand (cRNA) from which it will subsequently transcribe more vRNA segments for progeny viruses and mRNA transcripts for viral protein synthesis. The mRNA transcripts are polyadenylated and capped then exported to the cytoplasm and translated using host ribosomal machinery. Packaging of viral components depends on sequence-specific packaging signals identified within the 5' and 3' noncoding and adjacent coding sequences on each of the eight viral segments (Hutchinson et al. 2010). Once components for viral assembly are in order, the virus will bud from lipid raft domains on the apical surface of infected cells.

Once budding is complete, the virus will remain attached to host cell sialic acid residues by HA glycoproteins. The latter association requires sialidase activity of the NA to release virions from the host cell (Iwatsuki-Horimoto et al. 2006; Bouvier and Palese 2008). NA is also thought to enhance viral infectivity by breaking down mucus in the respiratory tracts, thus allowing the virus to penetrate the respiratory epithelia (Matrosovich et al. 2004).

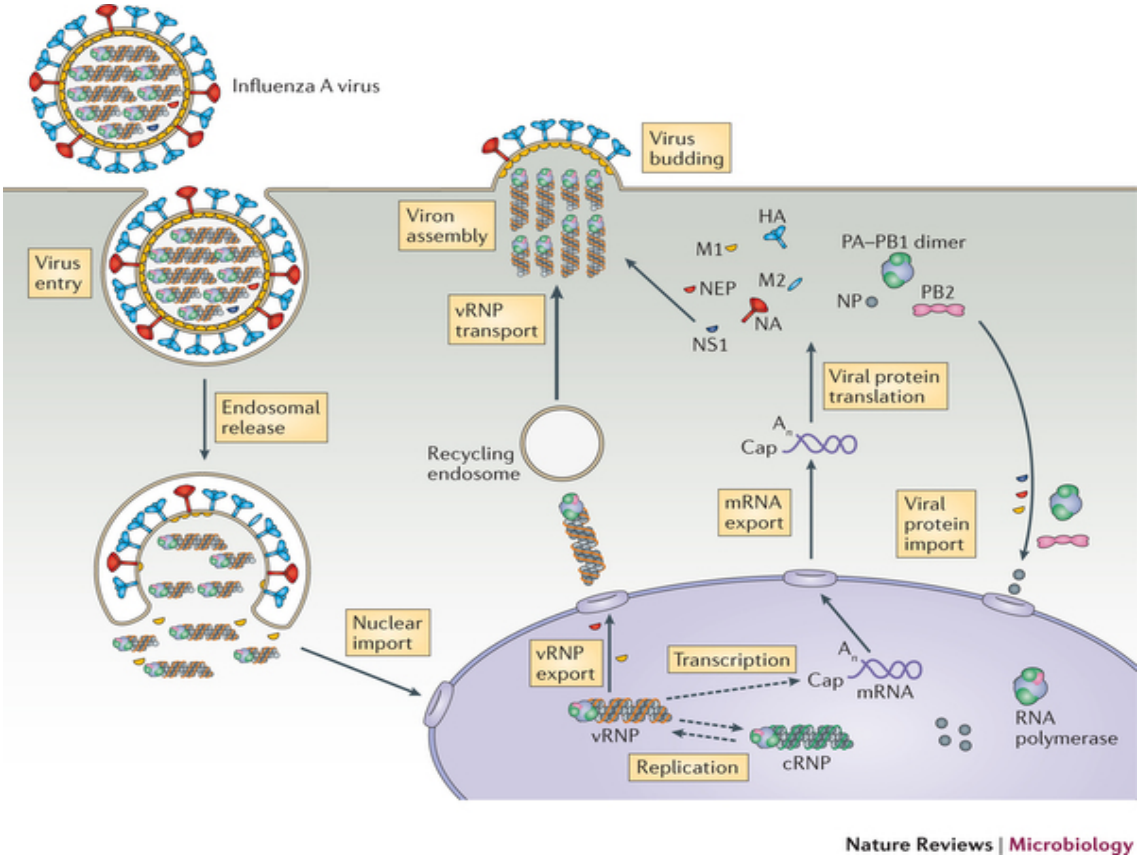

Figure 8: Influenza viral life cycle (Velthuis and Fodor 2016). Virus infection is initiated by binding viral hemagglutinin to sialic sialic acid residues on host cell receptors, followed by clathrin-mediated endocytosis. In the host cell, fusion of viral and endosomal membranes occurs at low pH, which enables the release of the segmented viral genome into the cytoplasm. The viral genome is subsequently translocated to the nucleus, where it is transcribed and replicated. Following synthesis in the cytoplasm, viral proteins are assembled into viral ribonucleoproteins (vRNPs) in the nucleus. Virus particles are assembled at the cell membrane, and the newly generated progeny virus buds into extracellular fluid.

4. Clinical symptoms and complications of influenza

Influenza is an acute self-limiting respiratory disease with prominent systemic symptoms (Cox and Subbarao 1999). The virus may be transmitted through aerosols, large droplets, or direct contact with secretions or fomites (Tellier 2006). The typical incubation time is 1-4 days with an average of 2 days (Cox and Subbarao 1999).

Uncomplicated influenza includes abrupt classical symptoms such as febrile

illness accompanied by malaise, myalgia, sore throat, non-productive cough and rhinitis (Nicholson 1992). Among children, additional clinical manifestations such as otitis media, nausea, and vomiting are common (Peltola, Ziegler, and Ruuskanen 2003; Ryan-Poirier 1995). Influenza illness is typically a self-limiting disease resolving within 3 to 7 days in the majority of people; however, cough and malaise may persist for over 2 weeks (Nicholson 1992).

The most frequent complication of influenza is pneumonia, which may either be primary viral or secondary bacterial pneumonia (Rothberg, Haessler, and Brown 2008). Primary viral pneumonia presents as an acute illness that does not resolve spontaneously. Cough may be productive and accompanied by persistent fever. Secondary bacterial pneumonia is most often caused by *Streptococcus pneumonia, Staphylococcus aureus, and Haemophilus influenzae* (Schwarzmann et al. 1971). In the case of a secondary bacterial infection subsequent recurrence of fever occurs 4-14 days after the resolution of initial influenza symptoms. This is accompanied by dyspnea, productive cough, and pulmonary consolidation (Rothberg, Haessler, and Brown 2008).

The risk for influenza complications and hospitalizations is especially high among people whom are over 65 years of age, young children, pregnant women, and people with underlying medical conditions (Rothberg, Haessler, and Brown 2008). Such conditions include obesity, heart disease, lung disease, diabetes, renal disease, etc. (Glezen, Decker, and Perrotta 1987).

5. Global influenza burden with a focus on the Middle East

Influenza is a contagious respiratory illness associated with a substantial global disease burden. The illness occurs in seasonal epidemics and may cause occasional

pandemics due to unpredictable antigenic shifts. Seasonal epidemics generally occur during the winter season for areas within temperate climate. In contrast, tropical regions exhibit an irregular pattern of outbreaks, which may occur during any time of the year. The World Health Organization (WHO) estimates an average of 3 to 5 million cases of severe illness from influenza resulting in 250 000 to 500 000 fatal illnesses worldwide per year and an estimated economic loss of 8 billion dollars in the US alone ("WHO | Influenza (Seasonal)" 2016) WHO reports have further indicated the central area of the Eastern Mediterranean Region (EMR) as an important area for influenza circulation. The EMR consists of 22 countries including Lebanon. The EMR countries are located under four of the eight global migratory flyways, and are therefore at risk of transmission of avian IAVs from migratory birds (natural reservoir) to local birds or animals that can potentially spread to humans (Kayali et al. 2013).

6. The immune response to influenza

a. Innate immunity

The first line of defense against influenza is mediated by innate immunity, which encompasses the combined effects of rapid innate cellular responses and the physical barriers such as mucus and collectins (van de Sandt, Kreijtz, and Rimmelzwaan 2012). Intracellular innate sensing of influenza is initiated due to an interaction of pattern-recognition receptors (PRRs) with viral RNA, the main pathogen associated molecular pattern (PAMP) of IAVs. This interaction yields the production of pro-inflammatory cytokines and type I interferons with important antiviral activity (Killip, Fodor, and Randall 2015) (Figure 10). Antiviral immunity conferred by type I interferons can be exerted due to the inhibition of viral protein synthesis within host cells, the induction of interferon stimulated genes (ISGs) that may hinder viral

replication (Matzinger et al. 2013), and the stimulation of dendritic cells (DCs) to enhance antigen presentation and thus contribute to initiating adaptive immune responses. Alveolar macrophages are also involved in antiviral innate immunity and harbor two distinct and competing functions. Once activated, alveolar macrophages phagocytose influenza-infected cells, thus limiting viral spread. This activation, however, prompts the production of nitric oxide synthase 2 (NOS2) and tumor necrosis factor alpha (TNF- α) which contributes to influenza-induced pathology (Oslund and Baumgarth 2011).

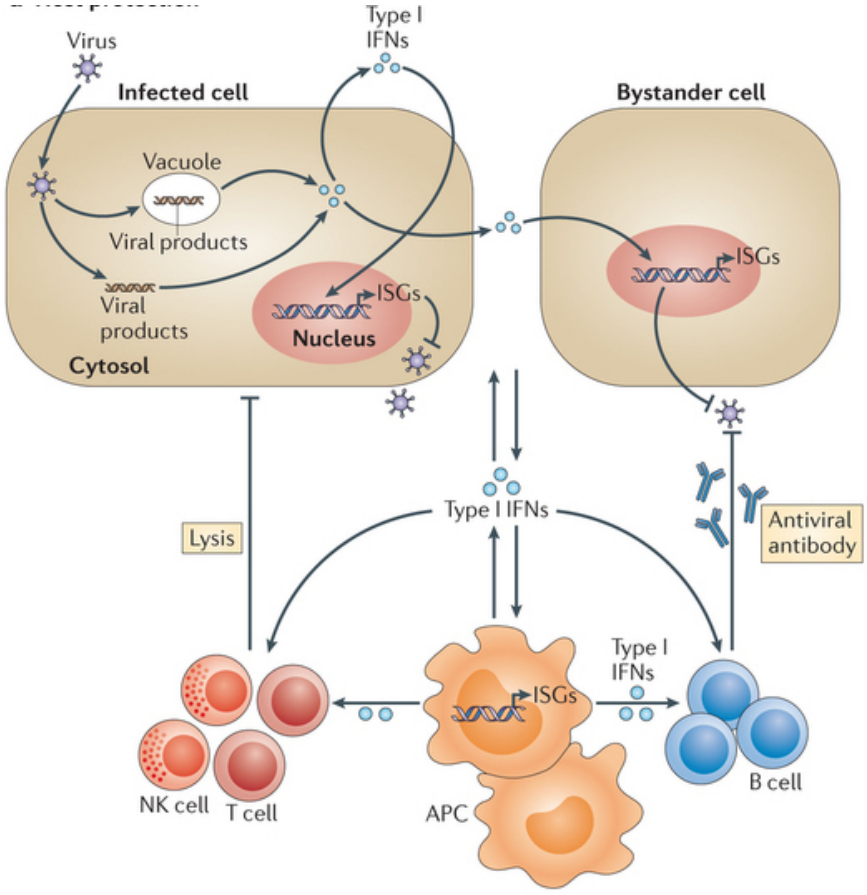

Figure 9: Interferon mediated immunity during IAV infections (McNab et al. 2015). Infected cells produce type I interferons (IFNs) in response to viral infection Feedback of type I IFNs onto infected and bystander cells leads to the induction of IFN-stimulated genes (ISGs), which function to block the viral replication cycle. Type I IFNs are also produced by, and act on, innate immune cells in response to viral infection and viral products. Type I IFNs acting on APCs can enhance the antigen-presenting function of these cells, and enhance antiviral function of adaptive immune cells.

b. Cellular immunity

Infection with viruses leads to the induction of $CD4^+$ T helper (Th) cells that are distinguished based on their cytokine expression profiles. Th-2 cells produce the cytokines IL-13 and IL-4 thus promoting B-cell responses, while Th-1 cells produce IL-2 and IFN-γ, which prime the immune response towards cell mediated immunity (Kreijtz, Fouchier, and Rimmelzwaan 2011). Additional vital cytokines involved in cell mediated immunity against IAV include IL-6, an anti-inflammatory cytokine involved in neutrophil accumulation and neutrophil mediated viral clearance (Dienz et al. 2012)and IL-10, an anti-inflammatory cytokine with a potent role in the reduction of lung inflammation (J. Sun et al. 2009). Naïve $CD8^+$ cytotoxic T lymphocytes (CTLs) recognize and bind to viral epitopes associated with MHC class I molecules in the lymphoid tissues thus prompting their activation (van de Sandt, Kreijtz, and Rimmelzwaan 2012). Once CTLs reach the sites of infection, they recognize and eliminate virus-infected cells by cell lysis. Lytic activity is accomplished by the release of perforins that permeabilize the host infected cells, followed by the release of granzymes (GrA and GrB), which enter the cells and induce apoptosis (Metkar et al. 2008). GrA also displays non-cytotoxic effects, which include the cleavage of viral and host cell proteins thus preventing the efficient replication of influenza viruses (van Domselaar and Bovenschen 2011). CTLs also have the ability to induce apoptosis of virus-infected cells by Fas/FasL activation. In addition, CTLs produce cytokines, which enhance MHC antigen presentation by stimulating MHC expression thus enhancing antiviral activity. Post-infection memory CTLs found in lymphoid tissue become active upon subsequent influenza infections. Considering that CTLs during influenza virus

infections may be directed to either NP, M1, and PA proteins all of which are highly conserved, CTLs thus display a high degree of cross reactivity between different subtypes of the IAVs (Kreijtz, Fouchier, and Rimmelzwaan 2011).

c. Humoral immunity

Humoral immunity encompasses the production of antibodies directed against different viral antigens, primarily the HA protein (Figure 11). Antibodies targeted against the HA protein play a dominant role in virus neutralization by interrupting the binding of the HA glycoprotein spike to host sialic acid residue receptors and preventing viral attachment. HA antibodies may also contribute to phagocytosis of the viral particles by binding to Fc receptor- expressing cells (de Jong et al. 2000). Antibodies directed at NA also limit viral spread by inhibiting enzymatic activity and preventing the release of viral progeny (Rajendran et al. 2017). In addition, NP specific antibodies are able to activate complement mediated cells lysis of host-infected cells, through a mechanism yet to be clarified (Carragher et al. 2008). The occurrence of antigenic drifts and shifts, however, limits the effectivity of antibodies, leading to renewed host susceptibility (Laursen and Wilson 2013). Considering the mucosal tissues are the main entrance sites of influenza viruses, IgA and IgM are the main neutralizing antibodies to prevent pathogen entry and interrupt the viral life cycle. IgA antibodies are mainly directed against HA and NA viral glycoproteins. The isotype IgM is the hallmark antibody for primary immune response and initiates complement mediated neutralization of influenza viruses. However, in the event of a secondary infection, IgG is the predominant antibody involved in virus neutralization (Kreijtz, Fouchier, and Rimmelzwaan 2011).

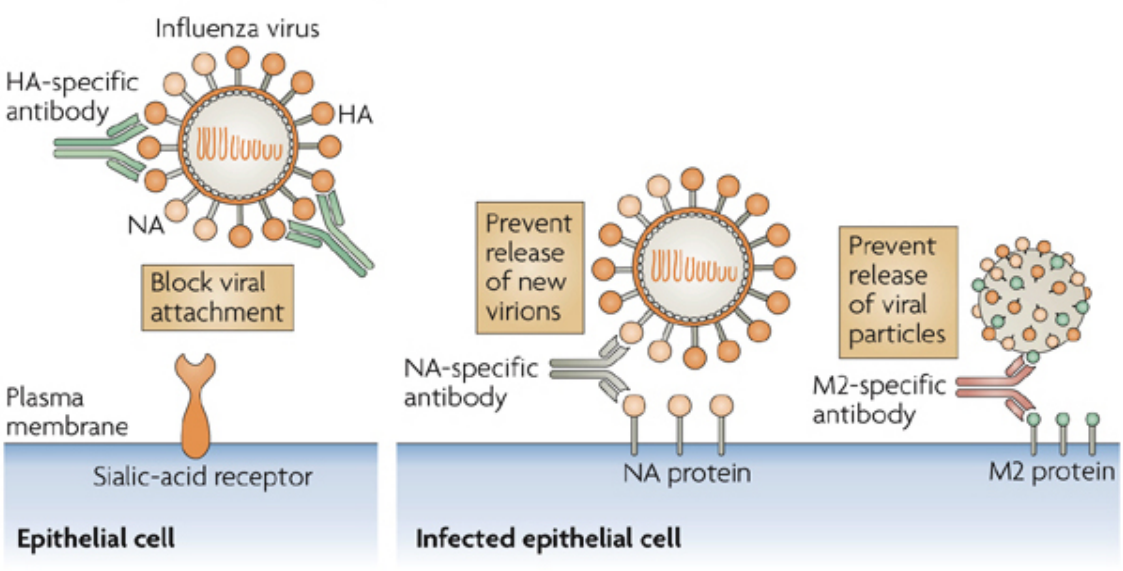

Figure 10: Antibody mediated immunity to influenza virus (Subbarao and Joseph 2007). Antibodies specific for HA block virus attachment, thereby preventing infection of cells, or they can prevent fusion. Antibodies specific for NA bind virus to the cell, thereby preventing the release of virions. Antibodies specific for M2 bind virus to the cell and prevent the release of viral particles into the extracellular fluid.

C. Coinfections

1. Definition

Co-infection is defined as the concurrent or sequential infection with two or more pathogens; which encompasses both acute and chronic stages (Bakaletz 2004; Pasman 2012). Over 800 million people are estimated to suffer from helminth co-infections (Hotez et al. 2007) and approximately one sixth of the global population is estimated to suffer from co-infections with globally important pathogens (Griffiths et al. 2011) such as HIV (Lawn 2004), malaria (Muturi et al. 2006), hepatitis virus (Sagnelli et al. 2004), influenza virus (Stefanska et al. 2013), *Leishmania* (Alvar et al. 2008) , and dengue virus (Pancharoen and Thisyakorn 1998). Unlike infections with a single pathogen, co-infections display a duel facet. On one side of the spectrum, the co-

existence of multiple pathogens may aggravate disease state and progression. On the other hand, coexistence of multiple pathogens may alleviate disease severity and progression (Ezenwa and Jolles 2011). Below, we summarize the results of studies which investigated co-infections involving *T. gondii* or IAV (Table 2).

Table 2: Summary for the disease outcome of some co-infection models.

Infection model	**Outcome**	**Reference**
T. gondii and Helicobacter felis	Aggravated disease outcome	(Stoicov et al. 2004)
T. gondii and Nippostrongylus brasiliensis	Unchanged disease outcome	(Oliver Liesenfeld, Dunay, and Erb 2004)
T. gondii and Plasmodium berghei	Attenuated disease outcome	(Settles et al. 2014)
Influenza A virus and *Trichinella Spiralis*	Attenuated disease outcome	(Furze, Hussell, and Selkirk 2006)
Influenza A virus and *Nematospiroides dubius*	Attenuated disease outcome	(Chowaniec, Wescott, and Congdon 1972)
Influenza A virus and *Pneumocystis murina*	Attenuated disease outcome	(Wiley and Harmsen 2008)
Influenza A virus and chronic *T. gondii*	Attenuated disease outcome	(O'Brien, Schultz-Cherry, and Knoll 2011)

2 Modulation of the immune response in mixed infections

a. *T. gondii* and *Helicobacter felis*

A study conducted by Stoicov et al. aimed to elucidate the underlying immune mechanisms that may occur due to an interaction between the two unrelated organisms: *Helicobacter felis* and *T. gondii*, which are two ubiquitous pathogens that can cause a spectrum of clinical diseases (Stoicov et al. 2004). Mice infected with *H. felis* showed higher mortality when challenged with *T. gondii,* as compared to control mice with *H. felis* infection alone. Further investigation demonstrated a blunted IFN-γ response to *T.*

gondii and higher parasite loads in co-infected mice compared with *T. gondii* single infection, co-infected mice also displayed areas of infarction and necrosis in the small bowel.

Markedly, *T. gondii* infection induced *H. felis* – specific IgG2a/IgG1 isotype switch and aggravated *H. felis* gastritis and mucosal damage. Dual infection also led to the alteration of *H. felis* colonization, whereby mice infected with *H. felis* alone displayed higher bacterial load than dually infected mice. Furthermore, investigation of cytokines at the level of gastric mucosa revealed a shift from *H. felis* Th-2 response to a Th-1 profile in co-infected animals. This study demonstrates how dual infection may alter the immune response to co-existing pathogens and lead to altered disease outcome and progression to not only one, but both pathogens (Stoicov et al. 2004).

b. *T. gondii* and *Nippostrongylus brasiliensis*

A study by Liensenfeld et al. in 2004 attempted to investigate whether infection with the helminth *N. brasiliensis* may affect *T. gondii* induced Th-1 immune response and immunopathology (Oliver Liesenfeld, Dunay, and Erb 2004).

Mice co-infected with *N. brasiliensis* and *T. gondii* did not have an altered course of infection when compared with control mice harboring *T. gondii* alone. Both co-infected and *T. gondii* singly infected animals produced high levels of IL-12 and IFN-γ, developed similar intestinal immunopathology, and died at the same time as mice singly infected with *T. gondii*. Markedly, the immune response to *N. brasiliensis,* was down regulated in co-infected mice, as demonstrated by reduced blood eosinophil levels and reduced IL-4 levels in the spleen and mesenteric lymph nodes compared to control mice with *N. brasiliensis* alone. In contrast, oral administration of *T. gondii*

lysate prior to *N. brasiliensis* infection had only a minor and short-lived effect on *N. brasiliensis* Th-2 responses (Oliver Liesenfeld, Dunay, and Erb 2004).

c. *T. gondii* and *Plasmodium berghei*

Considering that previous studies have demonstrated that animals with chronic *T. gondii* infection can survive subsequent lethal doses of viruses, bacteria or protozoa (O'Brien, Schultz-Cherry, and Knoll 2011; Ruskin and Remington 1968; Mahmoud, Warren, and Strickland 1976; Charest et al. 2000), a previous study by Settles et al. aimed to explore a potential protective effect that *T. gondii* may induce in an experimental cerebral malaria (ECM) murine model (Settles et al. 2014).

Chronic *T. gondii* infection followed by subsequent infection with *P. berghei* ANKA, a strain commonly used for the study of *P. berghei* induced ECM, significantly decreased mortality by 90% relative to mice singly infected with *P. berghei (Settles et al. 2014)*. Furthermore, *T. gondii* co-infection reduced *P. berghei* ANKA parasitemia and prevented the onset of ECM, whereby 95% of co-infected mice did not display ECM symptoms. To further elucidate the immune mechanism involved, mice were intravenously injected with soluble *T. gondii* antigens (STAg) at various times post *P. berghei* ANKA infection. Subsequently, vascular leakage and parasite sequestration were reduced in the brain of co-infected infected mice. Brain T- cell and IFN-γ levels were also reduced and a strong Th-1 response characterized by increased levels of serum IFN-γ, IL12, IL-10, MCP1, and IL-6 levels were observed in co-infected mice.

d. IAV and *Trichinella spiralis*

T. spiralis is a nematode parasite responsible for trichinosis with an enteric stage known to induce a Th-2 immune response (Grencis, Hültner, and Else 1991; Kelly et al. 1991). In contrast, infection with IAV is initially controlled by innate immune

mechanisms prior to activation of a strong Th-1 immune response.

A study conducted by furze et al, aimed to investigate the effects of the helminth *T. spirlais* on IAV infection (Furze, Hussell, and Selkirk 2006). For this purpose male NIH mice were infected with *T. spirlais* larvae then subsequently infected with IAV. Co-infection with the enteric stage *T. spiralis* enhanced recovery from IAV infection but had no effect on virus titer. Furthermore, co-infected mice displayed a marked reduction in early neutrophil influx into the broncheoaveolar lavage (BAL) accompanied by reduced lymphocyte infiltration, and reduced cytokine TNF-α and IL-10 levels. Histological examination further confirmed the amelioration of pulmonary inflammation by co-infection with *T. spiralis* (Furze, Hussell, and Selkirk 2006). The study demonstrates how dual infection may alter immune responses and lead to the amelioration of disease pathology.

e. IAV and *Nematospiroides dubius*

Nematospiroides dubius currently known as *Heligmosomoides polygyrus* is a naturally occurring intestinal nematode in rodents (Pritchard et al. 1984) . Mice infected with IAV, 7 and 21 days after *N. dubius* infection displayed decreased virus titers and lung consolidation compared to control mice with IAV infection alone (Chowaniec, Wescott, and Congdon 1972). In addition, co-infected mice displayed significantly lower viral antibody titers when higher numbers of *N. dubius* parasites were introduced. The decrease in viral antibody titers was hypothesized to be the result of reduced synthesis of antibodies in the parasitized host due to decreased antigenic stimulation from diminished viral replication, immunosuppressant effects of the parasite, and the competition between viral and parasitic antigens (Chowaniec, Wescott, and Congdon 1972).

f. IAV and *Pneumocystis murina*

Mice infected with *P*neumocystis *murina* followed by IAV infection 2 weeks later demonstrated an accelerated rate of viral clearance and an accelerated appearance of influenza specific neutralizing antibodies in serum and BAL (Wiley and Harmsen 2008). Furthermore, co-infected animals displayed reduced mortality and inflammatory cytokine levels in the BAL relative to control mice with IAV infection alone. There was no significant increase in $CD8^+$ and NK cells recovery from BAL of co-infected mice at the time where accelerated viral clearance was observed. In absence of an antibody response, virus clearance in co-infected animals showed no significant alteration relative to controls. These results indicate the role of influenza virus specific antibodies in viral clearance, which is dependent on the temporal association with the resolution of ongoing pneumocystis infection (Wiley and Harmsen 2008).

g. IAV and *T. gondii*

The increased rate of drug resistance raises an urgent need for the development of new therapies for the treatment of influenza infection (Hayden 2009; Ilyinskii, Thoidis, and Shneider 2008). One such therapeutic mechanism involves boosting the Th1 antiviral response by the use of non-harmful microbes (Pulendran 2004). A study conducted by O'Brien et al. attempted to identify a possible approach to enhance the antiviral Th1 type response during infection with a highly pathogenic avian influenza (HPAI) A/H5N1 through utilizing a chronic *T. gondii* infection (O'Brien, Schultz-Cherry, and Knoll 2011).

Mice chronically infected with *T. gondii* and challenged with a lethal dose of the HPAI H5N1 displayed enhanced survival and reduced lung virus titers when

compared with mice singly infected with H5N1. Furthermore, mice administered with non-infectious extracts of *T. gondii* (STAgs) prior to IAV infection demonstrated decreased viral titers and inflammation in the bronchium and alveolus compared to IAV control mice. In order to better understand the role of IFN-γ in STAg mediated protection, IFN-γ receptor knockout mice were infected with IAV followed by STAg injection. IFN-γ knockout mice did not survive IAV infection; hence suggesting an important role for IFN-γ in STAg mediated survival. Experiments also indicated NK cells as source for IFN-γ, which is both necessary and sufficient for survival.

The study of immune interactions between multiple pathogens in the framework of a complex host is of great interest given the high prevalence of co-infections on a global scale. The study by O'brien et al. examined the immune modulation that occurs from an infection with H5N1 IAV in concurrence with chronic *T. gondii* and demonstrated the role of STAg in ameliorating influenza induced pathology and illness. This study however, neither attempted to explore the effect of a concurrent infection between IAV and the acute stage of *T. gondii*, nor the effect of the co-infection on *T. gondii* induced immune response and pathology.

Considering that a chronic *T. gondii* infection was able to induce a protective effect on IAV infected mice, we speculated that the active immune response during acute toxoplasmosis might also have beneficial effects on the murine host.

A murine model for the co-infection of influenza virus A/Puerto Rico/8/34 strain with type II *T. gondii* tachyzoites was previously established at our lab (German, 2016). Surprisingly, the infection with 100 tachyzoites of *T. gondii* followed by IAV on the next day led to 60% mortality, while an infection with *T. gondii* then IAV four days

later led to 40% mortality. The parasitic load in the peritoneum of co-infected mice on days 3 and 5 was higher than that observed in *T. gondii* control mice. In contrast, viral load in co-infected mice was significantly lower than in IAV control mice (German, 2016). These data suggested that mice could be dying due to a worsening parasitic infection induced by the virus despite that attenuation of the viral infection by the parasite. The current study further examines the underlying immune response in co-infected mice and paves the way for further studies regarding the immune interactions that occur.

CHAPTER II

MATERIALS AND METHODS

A. Culture methods and titration

1. Parasite culture

T. gondii Type II strain (Pru *Δku80*) (kindly provided by Drs Maryse Lebrun and Jean Francois Dubremetz from France) was maintained by serial passages in Human Foreskin Fibroblasts (HFFs) grown in modified Eagle medium (DMEM) (Lonza) supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine 1% penicillin –streptomycin and kanamycin (Lonza). Media containing freshly egressed tachyzoites was collected and separated from host cell debris by centrifugation at 1200 rpm for 5 minutes. Parasites were counted on a haemocytometer under a light microscope and diluted to the desired count for subsequent mice infections.

2. IAV propagation

The influenza A/Puerto Rico/8/34 virus strain (obtained from Dr. Richard Webby at St. Jude's children's hospital) was propagated in Madin Darbey canine kidney (MDCK) cells. One day prior to infection, cells were seeded at a density of $3*10^6$ cells per 175 cm^2 flask. On the next day, cells were inoculated with 0.01 MOI (multiplicity of infection) of the virus, then incubated for one hour with gentle rotation every 15 minutes. After incubation was complete, the inoculum was replaced with 25 ml of viral infection media (VIM) containing 0.2 g/ml TPCK-trypsin. Once extensive cytopathic effect occurred, the culture supernatant was collected and centrifuged at 10,000 rpm to clear cell debris. Media was then harvested, aliquoted, and stored at -80 °C for later use.

Viral titration was performed using plaque assay to determine viral titer.

3. Titration

MDCK cells were seeded at $8*10^5$ cells per well in a 6-well plate one day prior to the assay. Upon reaching confluency, cells were washed two times with PBS++ (phosphate buffered saline with calcium and magnesium). Wash media was then aspirated from the wells then 10-fold dilutions of viral stock were added to each well starting at 10^3 and reaching 10^8 fold dilutions. Plates were then incubated at 37 °C for 1 hour with gentle rotation every 15 minutes. Once incubation was complete, the inoculum was removed and cells were covered with 2 ml of 1% agarose nutritive overlay and dishes were left to incubate at 37 °C. Three days later, the agarose overlay was removed and cells were stained with crystal violet solution.

The viral stock was calculated as follows:

[Virus stock] (pfu/ml) = (observed plaque number x dilution power)/ volume of viral dilution used (ml)

B. In vivo experiments

1. Ethical statement

All murine protocols were approved by the Institutional Animal Care and Use Committee (IACUC number: #1604374) of the American University of Beirut. All animals were housed in specific facility with a 12h ON/OFF light cycle. Animals were sacrificed by cervical dislocation after deep anesthesia with isoflurane.

2. Survival evaluation:

For survival evaluation**,** Six to eight week-old female BALB/c mice were used. The following groups of mice were presented: 7 mice were infected intraperitoneally

with 250 tachyzoites of *T. gondii,*14 mice were intranasally inoculated with $2.4*10^5$ pfu of IAV, and 23 mice per group of co-infection were infected with both pathogens. Coinfection with the two pathogens was done either 1 or 4 days apart Experimental design for each group is presented (Figure 11). Mice were monitored for survival and weight loss over the course of 21 days and mice losing 30% of their body weight were euthanized by cervical dislocation for humane reasons. Survival experiments were repeated three times.

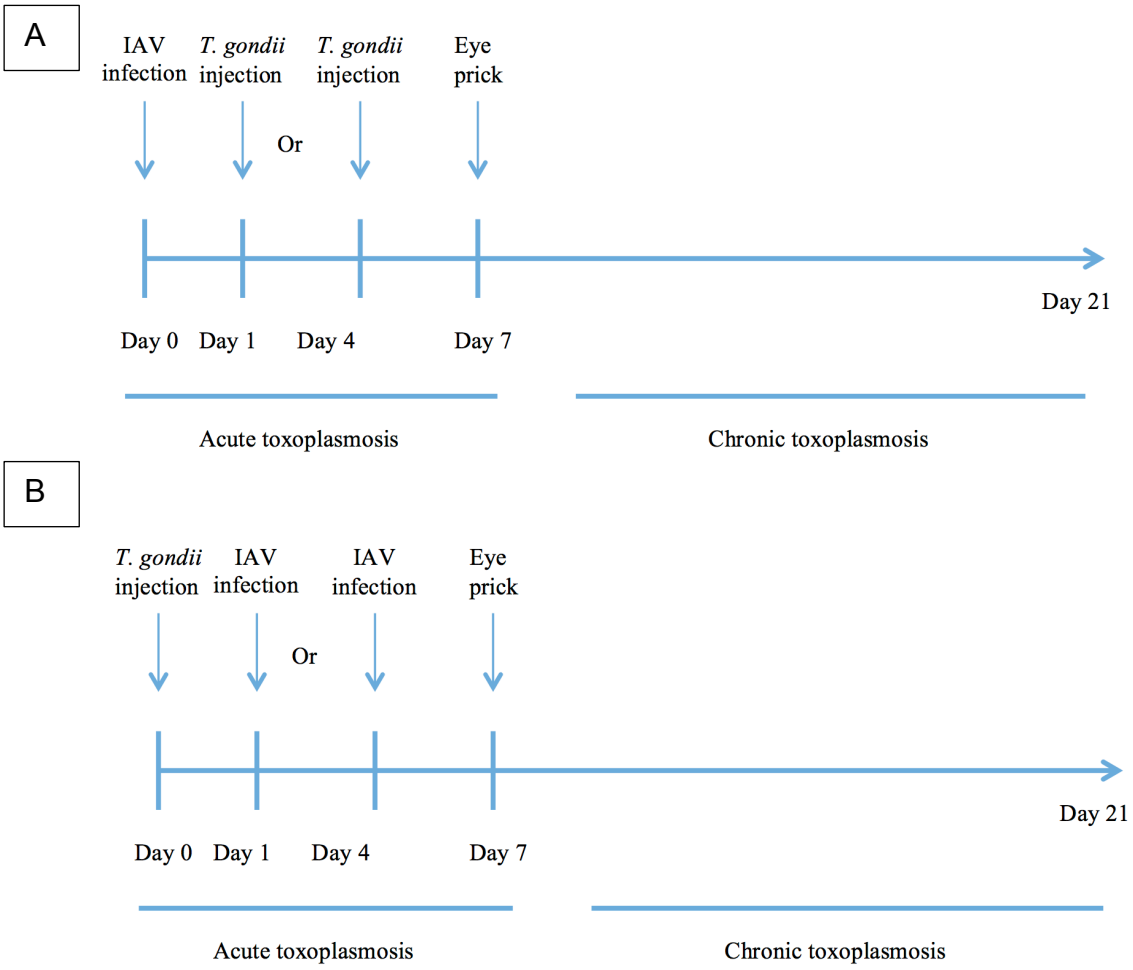

Figure 11: Timelines of infections for survival experiments. (A) Co-infection with *T. gondii* prior to IAV infection; (B) Co-infection with IAV prior to *T. gondii*

On day 7 post-infection, blood was collected by eye prick from *T. gondii* infected mice then western blot was performed using the sera of mice on tachyzoites extracts to check for the potential presence of Ig and thus for the verification of acute toxoplasmosis.

For determining the progression into chronic phase, surviving mice until week 4 were sacrificed and their brains were harvested. The bradyzoite marker (BAG-1) was used to quantify bradyzoites by real-time PCR as described later.

C. Verification of acute *T. gondii* infection by western blot

On day 7 post *T. gondii* infection, approximately 0.05 to 0.1 ml of blood was withdrawn from the retro-orbital sinus of each mouse infected with *T. gondii* alone or co-infected with IAV. Blood collection was done by the insertion of a hematocrit capillary tube through the conjunctiva membrane of the medial canthus of the right eye. Blood was centrifuged at 13,000 rpm for 15 min and sera were then collected.

Freshly egressed *T. gondii* tachyzoites were obtained from a T25 culture flask then boiled in Laemmli SDS-PAGE sample buffer and allowed to run on a previously prepared 12% polyacrylamide gel. Separated bands were then transferred onto a nitrocellulose membrane overnight at 30V. The following day, transfer strips were blocked and incubated with 10 µl of the previously isolated mouse sera over night at 4°C. Strips were then washed and incubated with anti-mouse HRP (Horseradish peroxidase)-conjugated secondary antibodies (1:5000) and a luminal chemiluminescent. Seropositivity was confirmed by the observation of a characteristic complex banding pattern produced by autoradiography.

D. Titer determination and cytokine study

Groups of 6-8 week old female BALB/c mice were infected according to Figure 12. Three to five mice from each group were then sacrificed on days 2 or 5 post IAV infection. Lungs and spleen were harvested and peritoneal lavage was collected for RNA extraction and cytokine profile. RNA extraction was performed using the Trizol method. Parasitic and viral titer were determined by quantification of the tachyzoite specific marker SAG-1 and the IAV M gene in the mice peritoneal lavage and lungs, respectively. IAV titer in the lungs was also determined by plaque assay as previously described.

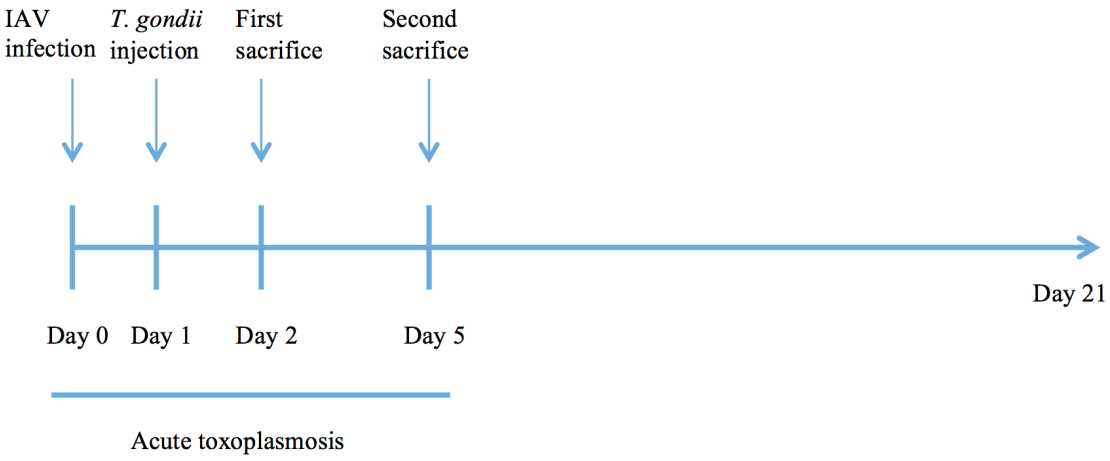

Figure 12: Experimental timeline for determining virus and parasitic loads and the underlying inflammatory responses

E. RNA extraction

Total RNA was extracted using the Trizol extraction method. Briefly, 1ml of Trizol were added to the peritoneal lavage pellets or harvested organ homogenates followed by a brief vortex, mixture was left to stand for 5 minutes. 200 μ L of

chloroform were added and the mixture was vortexed for 1 minute, followed by centrifugation 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 500 μ L of isopropanol. After centrifugation at 15000g for 10 minutes, the obtained pellet was washed twice with 70% ethanol and the RNA pellet was allowed to air-dry for few minutes to remove excess ethanol. The obtained precipitated RNA was resuspended in 40 μ L of nucleases-free water. RNA concentration was then quantified using the Nanodrop (ND-1000) spectrophotometer.

F. cDNA synthesis

For cDNA synthesis, the reaction mixture was prepared as presented in Table 3. The mixture was then incubated for 5 min at 25°C, 60 min at 42°C, then for 5 min at 70°C.

Table 3: Components and volumes of the cDNA synthesis reaction

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

G. Quantitative real time PCR (qRT PCR)

Syber green-based qRT PCR was performed using the BIORAD machine (CFX384). The mix contained 2 µL of template cDNA (100 µg/ µL), 0.5 µL (0.1µM) of each primer mix (forward and reverse), 5 µL buffer containing 20 mM Tris-HCl , 100

mM KCl, 7 mM MgCl2, 0.4 mM each dNTP (dATP, dCTP, dGTP, TTP), stabilizers, 0.05 unit/ml Taq DNA Polymerase, JumpStart Taq antibody, SYBR Green, and 2.5µL of RNase free water. The mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene to which all the expression results of the target genes were normalized. Primer sequences utilized and their corresponding annealing temperature are given in Table 4.

The PCR reaction consisted of a DNA denaturation step at 95°C for 3 minutes, followed by 40 cycles (denaturation at 95°C for 15 seconds, annealing at each primer's specific 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 normalized to GAPDH values. The threshold cycle (Cq) corresponds to the cycle at which there is a significant detectable increase in fluorescence.

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

Δ Cq = Cq (target gene) – Cq (GAPDH reference gene)

$\Delta\Delta$Cq = ΔCq (test group) – ΔCq (control group)

Table 4: Sequences and annealing temperatures of utilized primers in qt RT PCR.

Primer		**Sequences (5'-3')**	**Annealing temperature (^{o}C)**
GAPDH	F	5'-CATGGCCTTCCGTGTTCCTA-3'	56.2
	R	5'-CCTGCTTCACCACCTTCTTGAT-3'	
BAG-1	F	5'-GTCGGGCTTGTAATTACTCGGG-3'	60.5
	R	5'-GCGGAGAAAGTGGACGATGG-3'	
SAG-1	F	5'-ACTCACCCAACAGGCAAATC-3'	56.6
	R	5'-GAGACTAGCAGAATCCCCCG-3'	
IFN-γ	F	5'-TGGCTCTGCAGGATTTTCATG-3'	58.6
	R	5'-TCAAGTGGCATAGATGTGGAAGAA-3'	
TNF-α	F	5'-CCACGTCGTAGCAAACCACC-3'	59.9
	R	5'-GGTGAGGAGCACGTAGTCGG-3'	
IL-6	F	5'-CCATCCAGTTGCCTTCTTGGG-3'	58.1
	R	5'-GGTCTGTTGGGAGTGGTATCCT-3'	
IL-10	F	5'-ATAACTGCACCCACTTCCCA-3'	59.2
	R	5'-TGGACCATCTTCACTACGGG-3'	

H. Statistics

All data from *in vivo* experiments were analyzed in Microsoft Excel, as averages with standard deviations. Statistical significance was also analyzed using Microsoft Excel's two-tailed Student's t-tests and 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. IAV infection subsequent to acute toxoplasmosis increases host mortality compared to singly infected mice

1. T. gondii followed by IAV on day 1

We first wanted to address the effect of preexisting *T. gondii* infection on IAV infection outcome. Mice were infected by *T. gondii* on day 0, then subsequently by IAV on day 1 (Figure 11). In order to confirm that an active infection with *T. gondii* took place, serum IgG antibodies against the parasite can be detected one week post-infection by western blot using *T. gondii* antigens. A complex banding pattern indicates an infection with *T. gondii* (Erlich et al. 1983). In our infection model, 95-98% of the sera from *T. gondii* infected groups yielded a complex banding pattern with a major band at 30 kDa corresponding to the SAG-1 antigen indicating successful infection (Figure 13).

None of the mice infected with the parasite alone lost weight (Figure 14 A) or succumbed to infection (Figure 14 B). In sharp contrast, mice singly infected with IAV or with *T. gondii* followed by IAV alone displayed severe weight loss reaching its trough on day 7 in case of singly infected mice. Whereas surviving mice co-infected with the parasite and IAV one day apart displayed delayed recovery. (Figure 14 A). Mice singly infected with IAV alone displayed 50% mortality, whereby 7/14 mice died on days 6 and 7 with a median survival of 14 days (Figure 14 B). Similar mortality rate was observed in the context of co-infection of *T. gondii* then IAV on next day, with a median survival time of 18 days recorded whereby mortality occurred 6 to 15 days post infection (Figure 14 B). Hence, no significant change in survival was recorded in co-

infected mice relative to our IAV control.

2. T. gondii followed by IAV on day 4

We then wanted to assess whether a delay of 4 days between *T. gondii* and the subsequent IAV infection would affect mice survival. In this context, mice displayed a rapid reduction in body weight soon after IAV was introduced and resulted in pronounced mortality, whereby survival was a mere 26%. This implicates a more aggravated disease outcome when time between infections is increased (Figure 14 B). In addition, the median survival time for co-infected mice was 11 days whereby mortality occurred between days 6 and 15. Therefore, when IAV infection occurs 4 days apart from *T. gondii* infection mortality is increased and the median survival time is reduced by 3 days compared IAV single infection.

B. Infection with *T. gondii* subsequent to IAV infection does not alter disease outcome in mice

1. IAV followed by T. gondii on day 1

Since our results showed no impact of *T. gondii* on a subsequent IAV infection the next day, we wondered whether IAV infection taking place one day previous to *T. gondii* infection would impact mice survival. Mice singly infected with IAV displayed rapid weight loss (Figure 15 A) and 50% mortality, whereby 7/14 mice died on days 6 and 7 (Figure 15 B). Upon infection with IAV one day prior to *T. gondii* infection, mice displayed rapid weight reduction until day 5 (Figure 15 A) and 44% mortality was recorded with deaths occurring between days 5 and 11 (Figure 15 B). Surviving mice co-infected with the parasite and IAV one day apart displayed delayed recovery (Figure 15 A). Hence, the survival of mice was not altered due to co-infection compared to the IAV single infection control.

2. IAV followed by T. gondii on day 4

We then wanted to examine whether a longer delay between IAV and a subsequent *T. gondii* infection would impact mice survival. Mice infected with *T. gondii* 4 days subsequent to IAV infection displayed substantial weight reduction (Figure 15 A) and 34% mortality, with deaths occurring between days 5 and 10 (Figure 15 B). While mice survival seemed to be barely altered in the co-infected group, mice failed to recover as quickly as IAV singly infected mice as can be deduced from the weight loss profile (Figure 15 B).

C. IAV but not *T. gondii* load is increased in the context of co-infection

Since the infection model in the order IAV subsequent to *T. gondii* resulted in prominent disease aggravation, we decided to focus on elucidating the underlying immune mechanisms modulating survival within this infection model. For this purpose, we speculated that the aggravated disease outcome might be attributed to enhanced viral and parasitic replication. Considering that lungs are the target site for viral replication, viral titer was determined by plaque assay of lung supernatants. Because of the commonly used intraperitoneal mode of injection of *T. gondii* tachyzoites in mice, the peritoneum is the privileged site for macrophage recruitment and tachyzoite replication. Hence peritoneal lavage was also collected and qt RT PCR was performed to determine the parasitic load.

Mice co-infected with *T. gondii* and IAV one day apart displayed significantly higher viral titer relative to IAV singly infected mice on day 2 after IAV infection (Figure 16). In contrast, mice infected with *T. gondii* followed by a subsequent IAV infection on day 4 did not display a significant difference in lung virus titers compared to IAV single infection (Figure 16).

On day 5 post IAV infection, a subset of mice infected with *T. gondii* followed by IAV on day 1 displayed viral clearance, while the rest displayed viral titer similar to IAV control (Figure 16). Similarly, no significant difference was recorded between mice infected with *T. gondii* followed by IAV on day 4 and IAV alone control mice (Figure 16). Our results implicate an increased viral replication in the context of mixed IAV and *T. gondii* infection occurring one day apart.

As previously demonstrated, mice co-infected one day apart did not display a change in survival rate relative to IAV control, however, these mice showed elevated viral replication. Furthermore, mice co-infected 4 days apart displayed a more aggravated disease outcome, yet did not demonstrate a significant change in viral titer. Hence, we conclude that disease outcome in the context of co-infection cannot be solely attributed to virus replication and the host immune response could be responsible for this outcome.

But we first wanted to rule out whether disease modulation is due to alteration of parasitic load in co-infected mice. Therefore, peritoneal lavage was collected from mice singly infected with *T. gondii* or co-infected with *T. gondii* and IAV. Parasitic load was evaluated by quantifying the tachyzoite marker SAG-1 by qRT PCR. At day 2 post IAV infection, no significant change in the parasite load was recorded in the co-infected mice relative to mice singly infected with *T. gondii* regardless of the duration between infections (Figure 17). In addition, no significant change in parasite load was observed on day 5 between *T. gondii* control and co-infected mice, suggesting that the parasite replication is not altered as a result of co-infection (Figure 17).

Considering these results, we speculated that the immune response initiated in the advent of co-infection is responsible for the rise of viral titer and not the direct effect

of the co-existing pathogens themselves. Hence an *in vitro* model of co-infection was designed whereby IAV was titrated in the presence of different concentrations of *T. gondii* tachyziotes on MDCK cells. Cells singly infected with *T. gondii* did not show any cytopathic effect and hence no disturbance of the monolayer was noted. In the context of coinfection, plaques indicated no significant change in virus titer relative to a single infection with IAV (Figure 18). We hence confirmed that the elevation of viral replication in mice co-infected one day apart is not the result of the co-existing pathogens on one another but rather might be a result of the immune environment induced.

In order to further understand the underlying mechanisms governing host survival and viral replication, we decided to focus our study on the assessment of the host immune response in the context of IAV and *T. gondii* co-infection. On a scientific level, the study of co-infection will increase our understanding of pathogen-pathogen interactions within the complex setting of a host. Many studies have identified *T. gondii* as a protective pathogen during co-infection (Settles et al. 2014; Santiago et al. 1999; Mahmoud, Warren, and Strickland 1976), however this role is highly dependent on the induced immune environment, whereby studies have indicated aggravated outcomes resulting from a hyperactive immune response (Stoicov et al. 2004). In the context of *T. gondii* and IAV, studies have indicated a protective immune response against IAV resulting from the expression of IFN-γ and its modulatory role on other cytokines (O'Brien, Schultz-Cherry, and Knoll 2011), we hence focused our study on the assessment of some vital pro- and anti-inflammatory cytokines which may have significant role in modulating disease outcome.

D. Co-infection induces a decrease in pro-inflammatory IFN-γ levels in the spleen of mice

In the context of acute *T. gondii* infection, IFN-γ activates effector mechanisms including the induction of reactive oxygen species, tryptophan degradation and autophagy (Melo, Jensen, and Saeij 2011). IFN- γ also plays a role in viral clearance by stimulation of antigen presentation and upregulation of major histocompatibility proteins MHC-I and MHC-II (Ramos and Fernandez-Sesma 2015).

IFN- γ was not detectable within the lungs of singly infected and co-infected mice on days 2 and 5. Hence, IFN- γ does not contribute to host survival within mice singly infected with IAV. However, in the context of the spleen, IAV singly infected and co-infected mice displayed decreased levels of IFN- γ expression relative to *T. gondii* control mice (Figure 19), implicating virus-induced reduction of spleen IFN-γ expression in co-infected animals. However, on day 5 post IAV infection, no IFN- γ expression was detectable in the spleens of the control and co-infected groups (Figure 19). Reduced IFN- γ levels may have a role in reduced control of parasitic replication, however, parasite titers were not significantly altered between co-infected mice relative to *T. gondii* control, implying a possible compensation provided by other cytokines. We hence decided to look at TNF- α, a vital cytokine for the control of both IAV and *T. gondii* infections.

E. Co-infection induces an increase in the pro-inflammatory TNF-α levels within the lungs but not the spleen

During toxoplasmosis, TNF-α acts as a vital inflammatory cytokine that is produced mainly by macrophages following IFN-γ induction (Dupont, Christian, and Hunter 2012). Moreover, TNF-α synergizes with IFN-γ to initiate the production of

reactive nitrogen species to impede viral growth (Chang, Grau, and Pechère 1990). In the context of IAV infection, TNF-α acts as a mediator of lung inflammation and immunopathology, whilst having important anti-influenza activity that is important for viral clearance and disease resolution (Suzuki et al. 2000).

A dramatic elevation in TNF- α expression was observed in the lungs of mice infected with *T. gondii* followed by IAV one day apart compared to IAV singly infected mice (Figure 20). However, mice infected with IAV 4 days after *T. gondii* infection showed no significant change in TNF- α expression relative to IAV control mice.

In sharp contrast, *T. gondii* control mice showed no detectable expression of TNF- α in the lungs on day 2 (Figure 20). On day 5, TNF- α levels decreased in all groups relative to day 2, although co-infected mice maintained higher transcript levels than singly infected mice (Figure 20). On day 2 after IAV infection, mice singly infected with IAV or co-infected with *T. gondii* and IAV one day apart displayed significant decrease in TNF-α expression in the spleen compared to the *T. gondii* control group. However, TNF- α transcript levels at day 5 are significantly higher in co-infected mice relative to our *T. gondii* alone control (Figure 21).

The observed elevation of TNF- α within the lungs of mice co-infected one day apart indicates the hyperactivity of this antiviral cytokine in the immune response against IAV, which may enhance viral clearance, and hence contribute to host survival despite the elevated virus titer.

F. Co-infection induces a decrease in IL-6 transcript levels within the lungs and spleen

IL-6 is a co-stimulatory cytokine molecule which plays an important role in controlling parasite growth (Jebbari et al. 1998). In case of IAV infection, IL-6 promotes neutrophil accumulation and neutrophil-mediated virus clearance (Dienz et al.

2012).

On day 2 after IAV infection, our results indicate reduced expression of IL-6 in the lungs of mice singly infected with *T. gondii* relative to IAV control mice (Figure 22). In contrast, co-infected mice do not display significant alteration of IL-6 transcript levels in the lungs, implicating that the presence of *T. gondii* in co-infected mice does not affect IAV-induced IL-6 expression (Figure 22). On day 5 after IAV infection, IL-6 expression levels decrease in co-infected mice, eventually reaching levels comparable to *T. gondii* control (Figure 22). On day 2 after IAV infection, co-infected mice displayed reduced IL-6 levels within the spleen relative to *T. gondii* control. This decrease, however, is recovered on day 5, whereby all mouse groups displayed similar IL-6 transcript levels (Figure 23). Considering that levels of IL-6 expression within the lungs and spleen were not significantly altered, we decided to investigate IL-10 as another anti-inflammatory cytokine, which might better explain the immune response induced and the corresponding outcome on host survival.

G. Co-infection induces an increase in IL-10 transcript levels within the lungs and spleen

IL-10 is a potent anti-inflammatory cytokine that acts by controlling IL-12 induced Th-1 type immune responses during *T. gondii* infections (Lu, Huang, and Kasper 2003). In addition, studies have demonstrated the role of IL-10 as an important anti-inflammatory response for the reduction of virus-induced lung pathology (Cunningham 2009).

On day 2 after IAV infection, our results indicate elevated levels of IL-10 expression in lungs of *T. gondii* control mice compared to mice infected with IAV alone

(Figure 24). Similarly, co-infected mice displayed higher IL-10 expression, implicating *T. gondii* induced IL-10 expression in co-infected animals. However, on day 5 after IAV infection, both co-infected and singly infected mice were recorded to have decreased IL-10 expression levels (Figure 24).

On day 2 post IAV infection, IAV singly infected mice and co-infected groups did not display a change in IL-10 transcript levels within the spleen relative to mice with *T. gondii* alone. In sharp contrast, IL-10 levels dramatically increased for all groups on day 5 post IAV infection. This increase however, is more pronounced in co-infected animals (Figure 25). Hence, the elevation of IL-10 levels within the lungs and spleen of co-infected animals might act as a buffer to balance the effects of inflammatory cytokines and contribute to host survival in co-infected animals.

H. Co-infection induces a decrease in BAG-1 expression levels

Since in a previous study by Gigley et al. it was shown that a co-infection between an attenuated *T. gondii* strain (cps-1-1) and the type II ME49 strain of *T. gondii* was able to provide protection against chronic toxoplasmosis (Gigley, Fox, and Bzik 2009), we then wanted to monitor the progression to chronic toxoplasmosis in the context of co-infection by IAV and *T. gondii*. The bradyzoite specific marker (BAG-1) was quantified using qRT PRC in the brain lysates of mice sacrificed 4 weeks after *T. gondii* infection. A significant decrease in BAG-1 expression was noted in all co-infected mice groups relative to mice infected with *T. gondii* alone (Figure 26). We hence conclude that the immune response induced in the context of IAV and *T. gondii* co-infection, is protective against chronic toxoplasmosis by partially restricting the progression into the chronic phase through the inhibition of bradyzoite encystment.

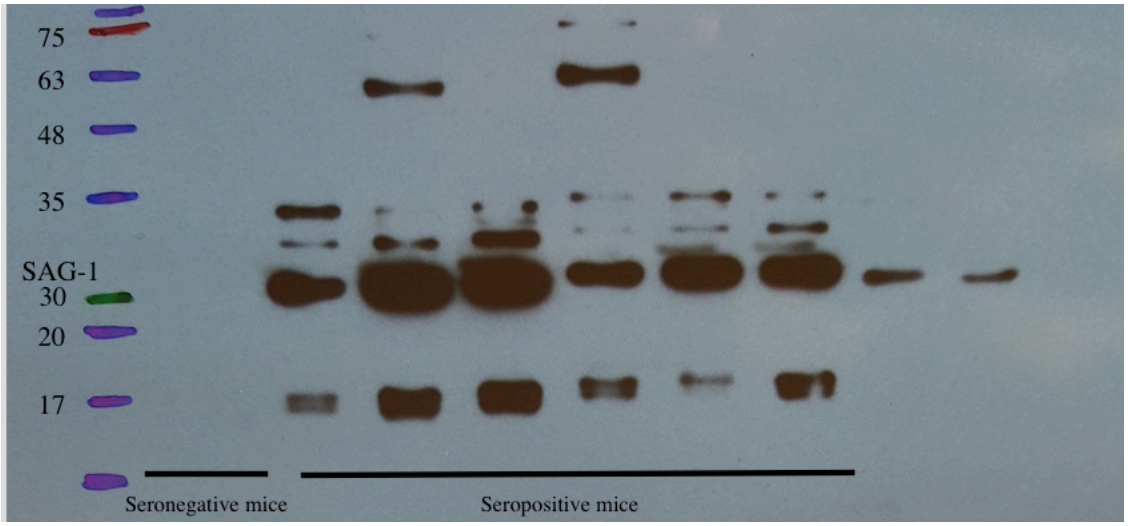

Figure 13: Verification of acute *T. gondii* infection. BALB/c mice were infected, intraperitoneally with *T. gondii* alone, infected with IAV followed by *T. gondii* on days 1 or 4, or with *T. gondii* followed by IAV on days 1 and 4. Sera were collected from *T. gondii* mono-infected and co-infected mice. Western blot was performed to verify acute infection. A complex banding pattern indicates seropositivity for acute *T. gondii* infection.

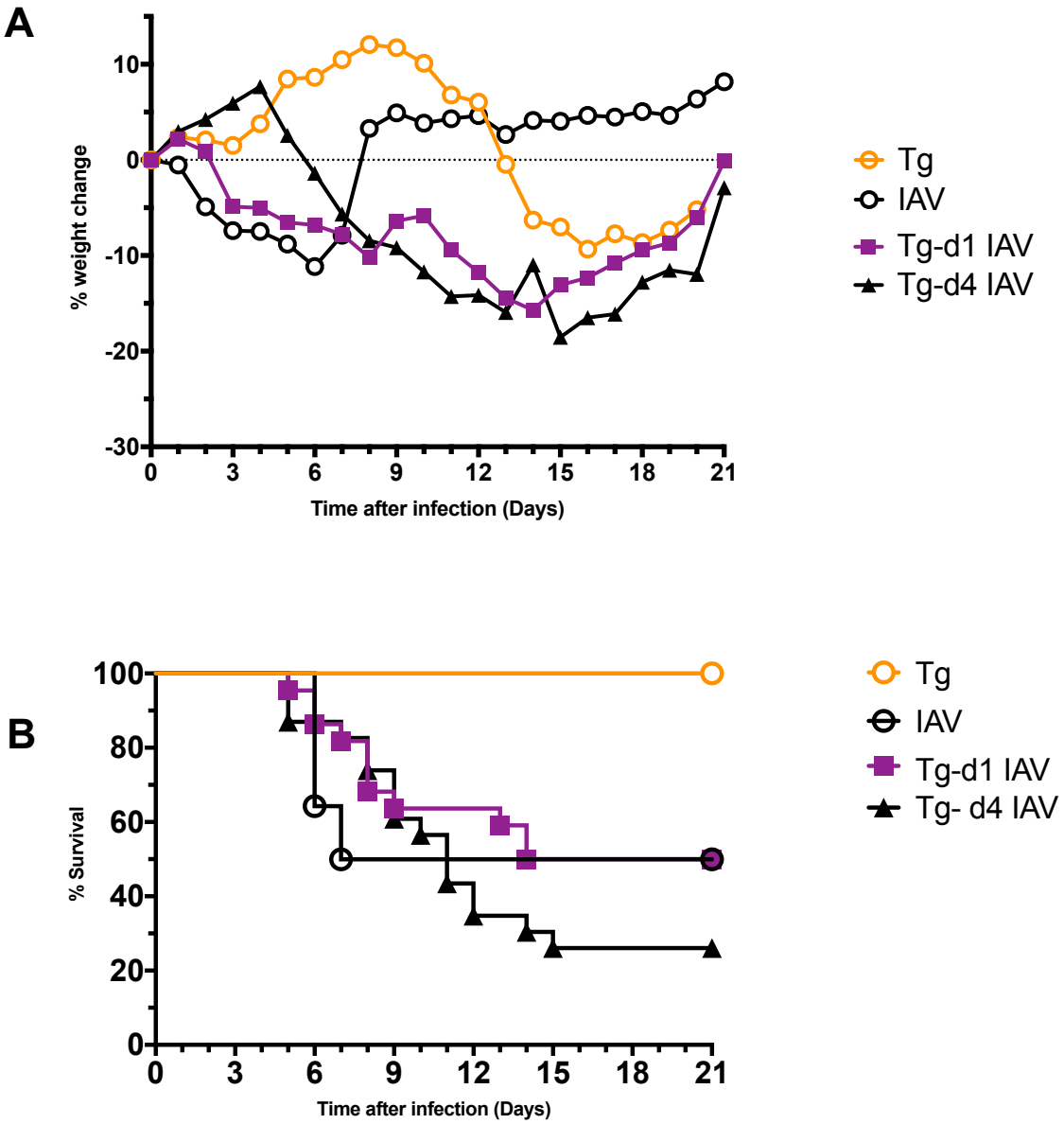

Figure 14: Weight change (A) and survival rate (B) for mice infected with *IAV* subsequent to *T. gondii.* BALB/c mice were infected intraperitoneally with *T. gondii* alone, intranasally with IAV alone, or infected with *T. gondii* followed by IAV either on days 1 or 4. Mice were monitored for 21 days post *T. gondii* infection. (co-infected mice have total n=23 per group, IAV control micehave n=14, and *T. gondii* control mice have n=7).

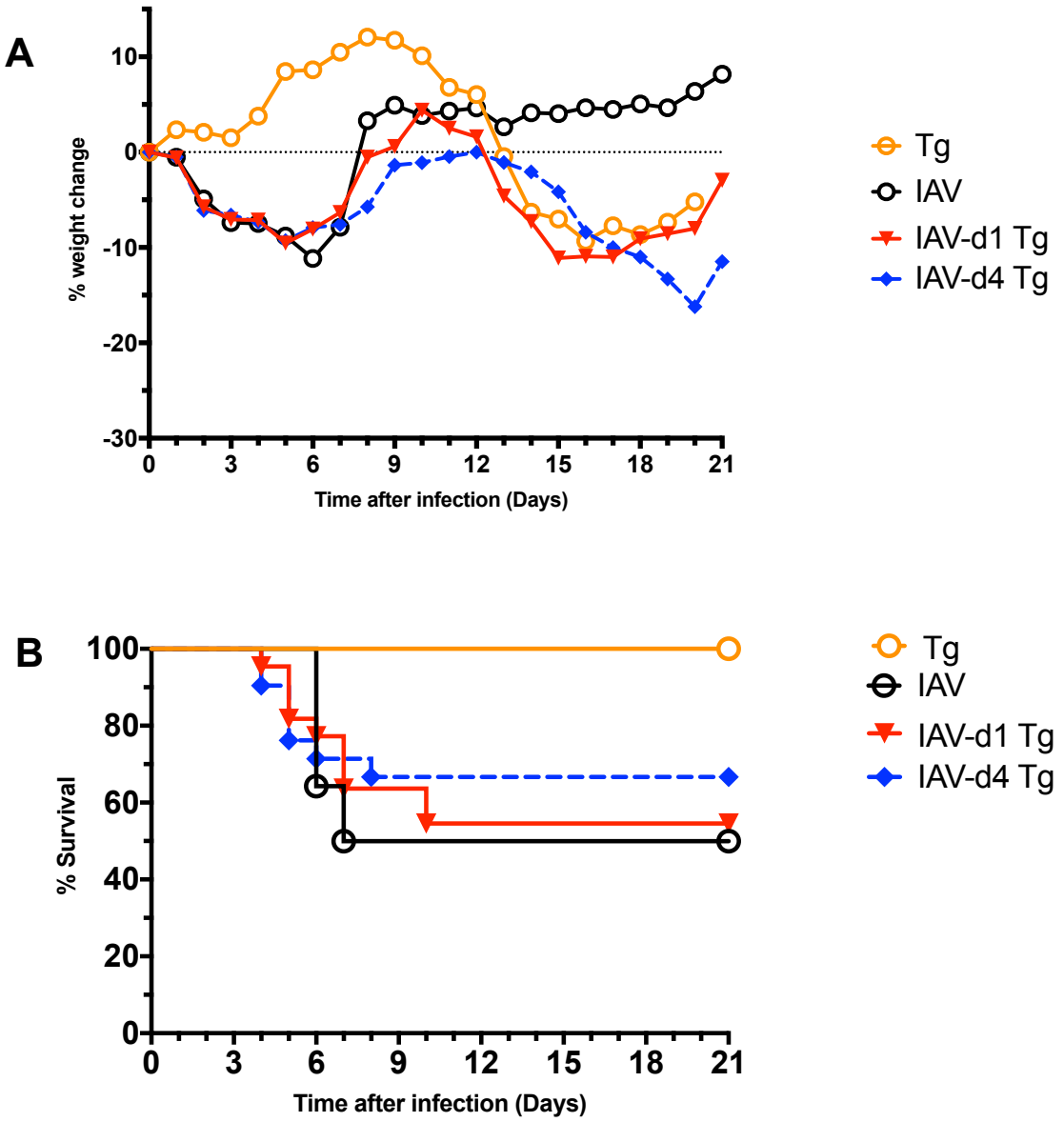

Figure 15: Weight change (A) and survival rates (B) for mice infected with IAV prior to *T. gondii.* BALB/c mice were infected intranasally with IAV alone, intraperitoneally with *T. gondii* alone, or infected with IAV followed by *T. gondii* on days 1 or 4. Mice were monitored for 21 days post *T. gondii* infection. Results are pooled from 3 experiments (co-infected mice have total n=23 per group, IAV control mice have n=14, *T. gondii* control mice have n=7).

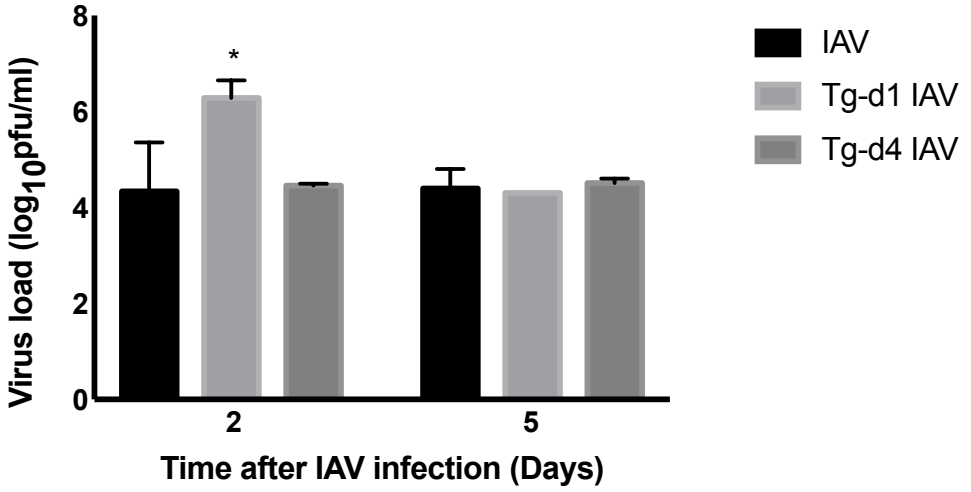

Figure 16: IAV replication is enhanced in the context of mixed *T gondii and IAV* infection administered 1 day apart. Mice were infected intranasally with IAV alone, intraperitoneally with *T. gondii* alone, or co-infected with *T. gondii* followed by IAV on days 1 or 4, (n=3-5 for each group). Virus titer was determined by plaque assay of BALB/c lung supernatant. Results are expressed as mean + SD, * 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.

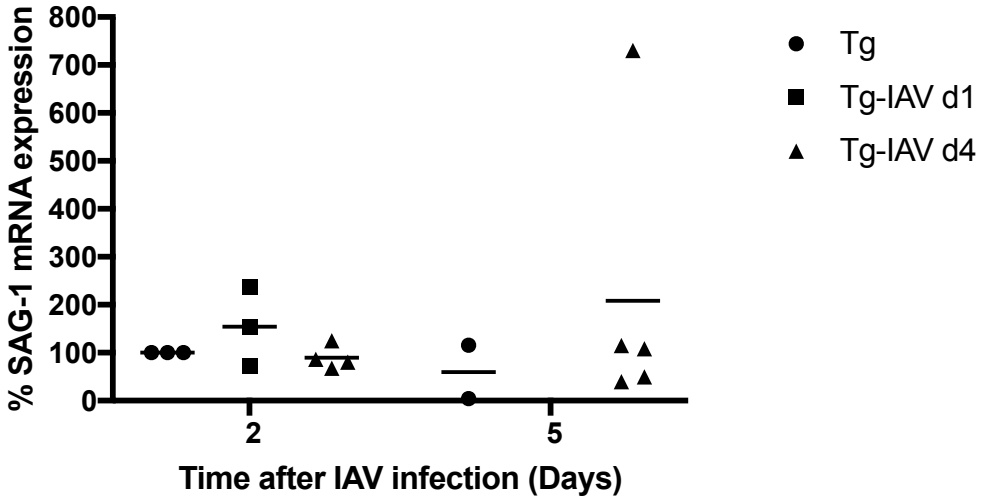

Figure 17: Mixed IAV and *T. gondii* infection does not alter parasite replication. BALB-c mice were infected intranasally with IAV alone, intraperitoneally with *T. gondii* alone, and co-infected with *T. gondii* followed by IAV on day 1 or 4, (n=3-5 for each group). Parasite titer in the peritoneal lavage was determined by qtRT PCR pellets. Results are expressed as mean + SD, * 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.

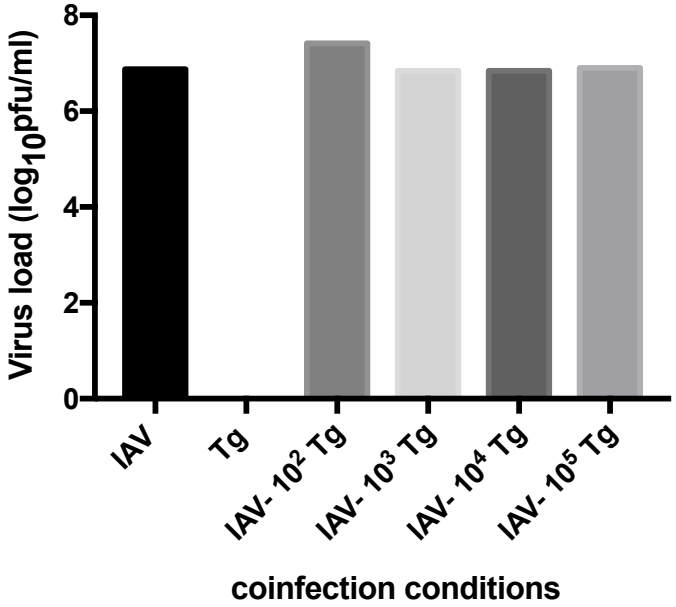

Figure 18: *T. gondii* does not alter IAV replication *in vitro*. MDCK cells were seeded in 6-well plates one day prior to infection. Upon reaching confluency, 100 µL containing increasing concentrations of *T. gondii* tachyzoites and 100 µL of serially diluted virus was added to each well. Plates were incubated for 1 hour followed by the addition of an agarose overlay. Plates were incubated for 3 days, and then stained to count plaques and determine the titer.

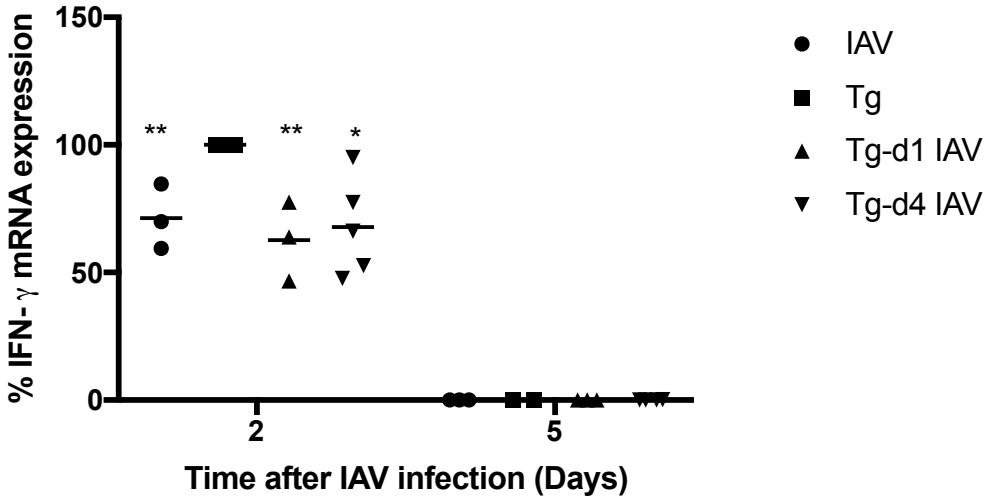

Figure 19: Mice infected with *T. gondii* followed by IAV the next day display decreased IFN- γ expression in the spleen. BALB/c mice were infected intranasally with IAV alone, intraperitoneally with *T. gondii* alone, and co-infected with *T. gondii* followed by IAV on day 1 or 4, (n=3-5 for each group). Parasite titer in the peritoneal lavage was determined by qtRT PCR pellets. Results are expressed as mean + SD, * 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.

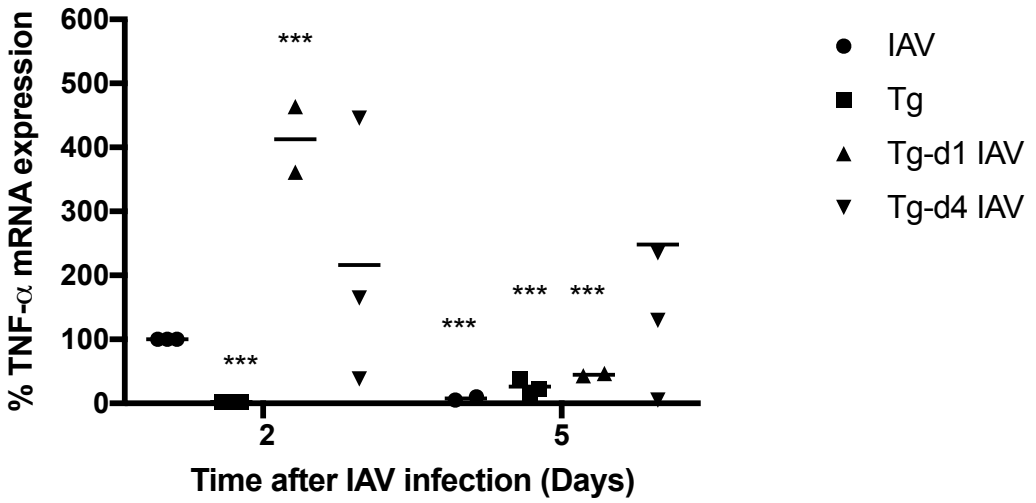

Figure 20: Mice infected with *T. gondii* followed by IAV the next day display an increased TNF-α cytokine expression in lungs. BALB/c mice were infected intranasally with IAV alone, intraperitoneally with *T. gondii* alone, or co-infected with *T. gondii* followed by IAV on days 1 or 4, (n=3-5 for each group). % expression was determined by qRT PCR and groups were compared to IAV control on day 2. Results are expressed as mean + SD, * 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.

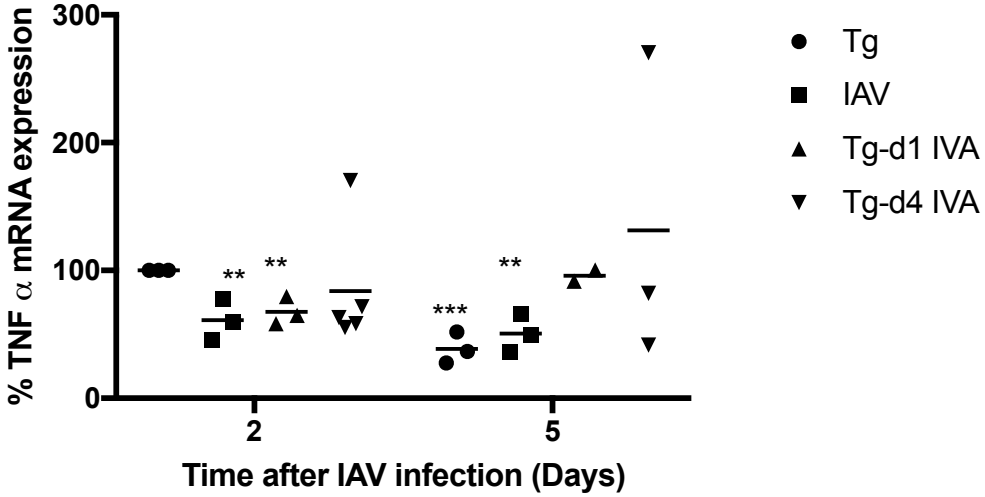

Figure 21: Mice infected with *T. gondii* followed by IAV the next day display decreased TNF-α cytokine expression in spleen. BALB/c mice were infected intranasally with IAV alone, intraperitoneally with *T. gondii* alone, or co-infected with *T. gondii* followed by IAV on days 1 or 4, (n=3-5 for each group). % expression was determined by qRT PCR and groups were compared to IAV control on day 2. Results are expressed as mean + SD, * 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.

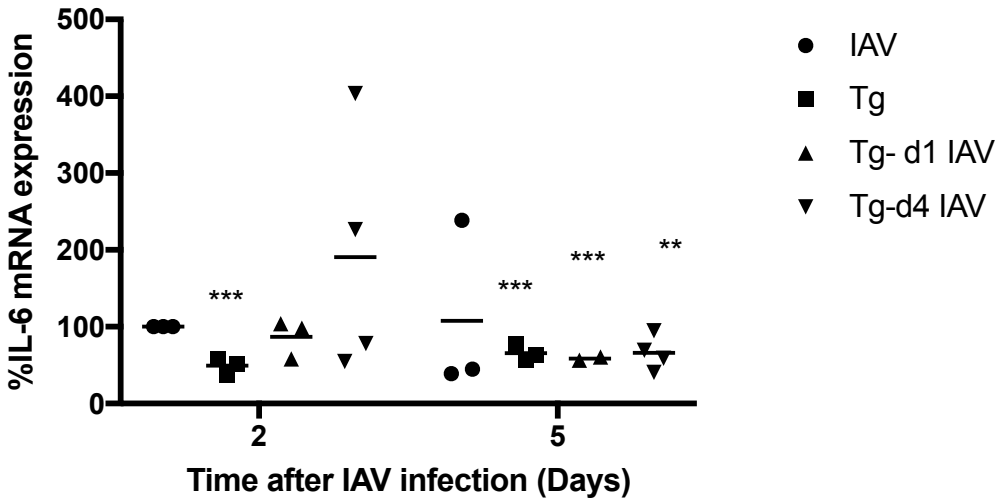

Figure 22: Mice infected with *T. gondii* followed by IAV the next day display unaltered IL-6 expression in the lungs. BALB/c mice were infected intranasally with IAV alone, intraperitoneally with *T. gondii* alone, or co-infected with *T. gondii* followed by IAV on days 1 or 4, (n=3-5 for each group). % expression was determined by qRT PCR and groups were compared to IAV control on day 2. Results are expressed as mean + SD, * 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.

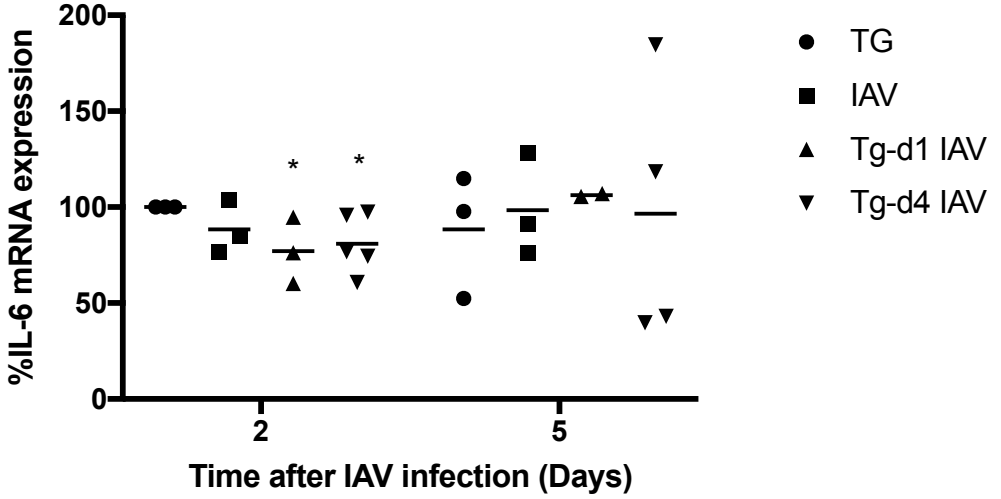

Figure 23: Mice infected with *T. gondii* followed by IAV display decreased IL-6 expression in the spleen. BALB/c mice were infected intranasally with IAV alone, intraperitoneally with *T. gondii* alone, or co-infected with *T. gondii* followed by IAV on days 1 or 4, (n=3-5 for each group). % expression was determined by qRT PCR and groups were compared to IAV control on day 2. Results are expressed as mean + SD, * 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

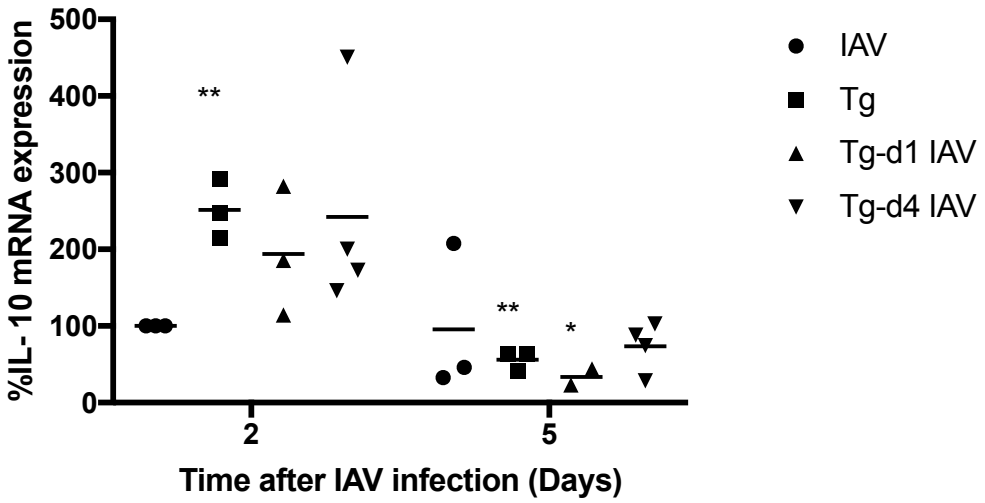

Figure 24:Mice infected with *T. gondii* followed by IAV the next day display increased IL-10 expression in the lungs. BALB/c mice were infected intranasally with IAV alone, intraperitoneally with *T. gondii* alone, or co-infected with *T. gondii* followed by IAV on days 1 or 4, (n=3-5 for each group). % expression was determined by qRT PCR and groups were compared to IAV control on day 2. Results are expressed as mean + SD, * 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

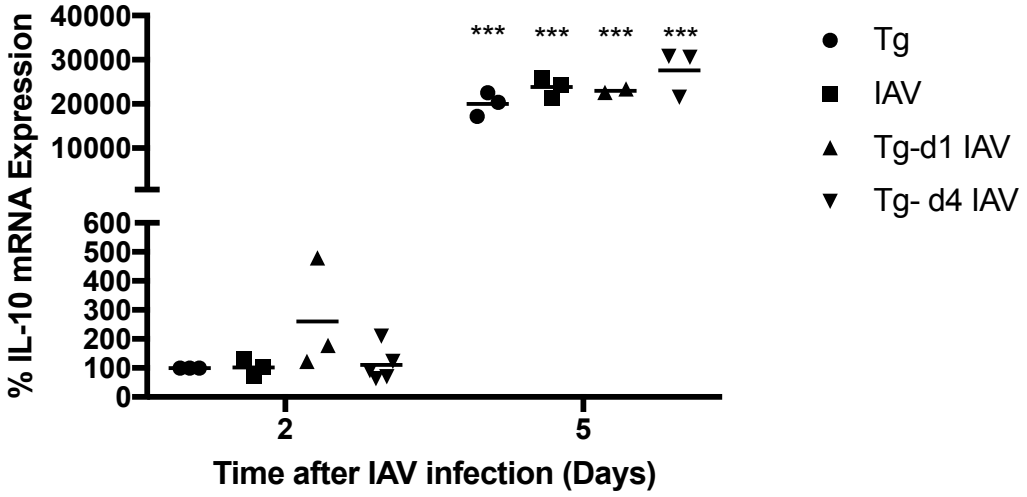

Figure 25: Mice infected with *T. gondii* followed by IAV display increased IL-10 expression in the spleen. BALB/c mice were infected intranasally with IAV alone, intraperitoneally with *T. gondii* alone, or co-infected with *T. gondii* followed by IAV on days 1 or 4, (n=3-5 for each group). % expression was determined by qRT PCR and groups were compared to IAV control on day 2. Results are expressed as mean + SD, * 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

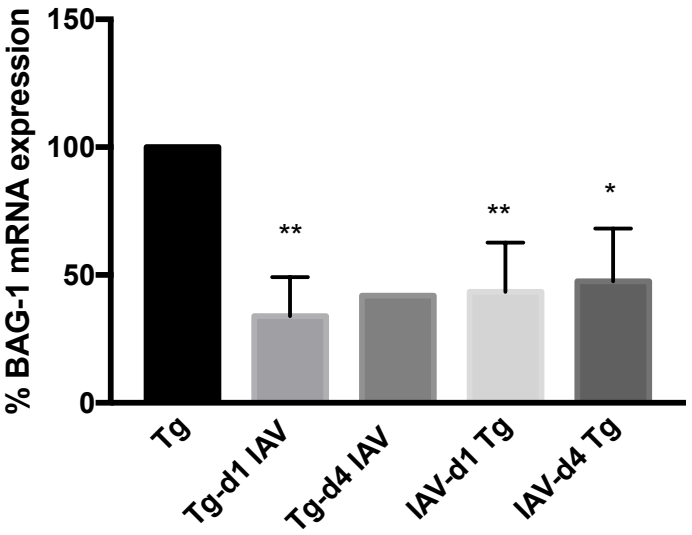

Figure 26: Infection with *T. gondii* followed by IAV or vice versa decreases BAG-1 expression in the peritoneal lavage. BALB/c mice were infected intranasally with IAV alone, intraperitoneally with *T. gondii* alone, co-infected with *T. gondii* followed by IAV on days 1 or 4, or co-infected with IAV followed by *T. gondii* on days 1 or 4. % expression was determined by qRT PCR and groups were compared to IAV control on day 2. Results are expressed as mean + SD, * 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 IV

DISCUSSION

One sixth of the global population is estimated to suffer from co-infections with globally important pathogens (Griffiths et al. 2011). Such pathogens include influenza, a major respiratory virus with serious disease burden (Rothberg, Haessler, and Brown 2008) and *T. gondii*, an obligate intracellular parasite with a wide range of intermediate hosts (Lambert 2009). O'Brien et al. previously demonstrated that a chronic infection with *T. gondii* can confer a protective effect against HPAI A/H5N1 infection (O'Brien, Schultz-Cherry, and Knoll 2011). In contrast, our results show that in the context of acute toxoplasmosis, a low pathogenicity IAV infection occurring within few days after *T. gondii* infection, results in an aggravated disease outcome. This outcome seemed to depend on the timing and order of infection since infected mice with IAV first then *T. gondii* did not display a similar disease burden when compared to IAV single infection. Moreover, the enhanced disease severity was not associated with prominent changes in virus or parasitic loads neither in the lungs nor in the peritoneal lavage, respectively, suggesting that the observed disease modulation is mediated by the host immune response.

Indeed, a similar enhancement of disease severity was previously demonstrated in the context of mixed infections between *T. gondii* and *H. felis* (Stoicov et al. 2004). This was attributed to a blunted IFN-γ response resulting in delayed parasitic clearance (Stoicov et al. 2004). Worth noting, IFN-γ is the major mediator of resistance to *T. gondii* infection (Melo, Jensen, and Saeij 2011). This cytokine acts by the activation of

effector mechanisms including tryptophan degradation (Fujigaki et al. 2002; Engin et al. 2012), autophagy (Krishnamurthy et al. 2017), and reactive oxygen and nitrogen species induction (Adams et al. 1990; Engin et al. 2012). IFN-γ also plays a vital role in IAV infections by inhibiting viral replication (Yuk et al. 2016) and up-regulating the expression of MHC I and MHC II (Killip, Fodor, and Randall 2015). In this study, mice infected with *T. gondii* then IAV 4 days apart had higher, albeit not statistically significant, parasitic loads on day 5 post-infection compared to mice singly infected with *T. gondii*. These mice also displayed significantly lower IFN-γ expression levels in their spleens on day 2 post-infection compared to *T. gondii* alone infected mice. Whereas mice infected with IAV alone had significant reduction in IFN-γ levels similar to co-infected mice. These findings suggest that IAV infection results in an attenuated IFN-γ production accompanied by a delayed clearance of the parasite in the context of co-infection with the virus and the parasite 4 days apart. Interestingly, when co-infections were only 1 day apart, mice were able to clear the parasite similar to those infected with *T. gondii* alone. This could be due to the smaller window of time between infections, which allowed mice to control *T. gondii* replication before IFN-γ was dampened due to the subsequent virus replication. In line with these findings suggesting delayed tachyzoite clearance, co-infected mice displayed significantly decreased abundance of the bradyzoite marker (BAG-1) suggesting a delayed or more controlled progression into the chronic stage of toxoplasmosis in mice with mixed infection relative to those infected with the parasite alone.

Surprisingly, we were not able to detect IFN-γ expression in the lungs on day 2 or 5 post IAV infection, indicating a possible IAV strain specific IFN-γ induction. Hence, IFN-γ does not induce an antiviral response in the strain of IAV used in our

infection model.

Several studies of IAV co-infections with other pathogens including fungi and neamtodes have described attenuated disease outcomes (Furze, Hussell, and Selkirk 2006; Wiley and Harmsen 2008; Chowaniec, Wescott, and Congdon 1972; O'Brien, Schultz-Cherry, and Knoll 2011). In contrast, our model of IAV and *T. gondii* co-infection revealed enhanced disease severity and increased viral replication. Hence, we assessed TNF-α, a major cytokine often examined in IAV co-infections with critical roles in promoting inflammation, increasing MHC class I protein expression on target cells, and boosting the effect of cytotoxic T-cells. Elevated expression of TNF-α has been demonstrated to induce severe lung immunopathology and ultimately death (Hussell, Pennycook, and Openshaw 2001). Furze et al. previously demonstrated amelioration of influenza-induced pathology in mice co-infected with *T. Spiralis* that was partially attributed to reduced inflammation because of a reduction of lung TNF-α in co-infected mice. In contradiction, recent emerging evidence has demonstrated an immune regulatory role for TNF-α (Damjanovic et al. 2011; Singh et al. 2007), whereby a study by Damjanovic et al. revealed that TNF- α plays a role in the control of lung inflammation by regulation of MCP-1. Our results indicated elevated expression of lung TNF-α and enhanced viral replication in mice co-infected one day but not four days apart. However, mice co-infected one day apart had higher survival relative to mice co-infected 4 days apart, and similar survival compared to mice with IAV alone. This suggests that the increase in TNF-α expression in lungs of co-infected mice might have an immune regulatory role that reduces inflammation and ultimately lead to the observed enhanced survival in mice co-infected one day apart.. Moreover, our results indicated a reduction of spleen TNF-α in synchrony with the reduction of IFN-γ in co-

infected mice. This dampened expression of IFN-γ and TNF-α in the spleen could lead to the delayed tachyzoite clearance as well as the delayed or more controlled progression into the chronic stage of toxoplasmosis. Indeed, TNF-α is an important inflammatory cytokine in the context of *T. gondii*, whereby it synergizes with IFN-γ for macrophage activation and the inhibition of parasite replication (Filisetti and Candolfi 2004).

The dramatic reduction in spleen IFN-γ and TNF-α on day 5 after IAV infection coincided with a dramatic increase in IL-10 cytokine levels in co-infected and singly infected mice. This is consistent with previous studies indicating that a heightened IL-10 response dampens IL-12 expression, and in turn leads to the reduction of IFN-γ and TNF-α expression (Couper, Blount, and Riley 2008). IL-10 counters the harmful effects of an exaggerated Th-1 inflammatory response which produces TNF-α, IFN-γ and NO during *T. gondii* infection (Filisetti and Candolfi 2004). Our results indicate that mice co-infected one or four days apart retain the anti-inflammatory effect of IL-10 on *T. gondii* induced pathology. IL-10 is also vital for the control of lung inflammation, whereby a previous study by Sun et al. demonstrated an aggravated pulmonary inflammation and increased mortality in IL-10 deficient mice (J. Sun et al. 2009). IL-10, however, does not have an effect on viral replication (J. Sun et al. 2009), thus limiting its role to the alleviation of virus-induced immunopathological inflammation. In this study, we noted an increase in IL-10 expression in co-infected mice and mice with *T. gondii* alone, relative to IAV singly infected mice. This indicates that the presence of *T. gondii* in co-infected mice enhances IL-10 expression. This elevation, however, was not strong enough to induce a reduction in lung TNF-α of co-infected mice.

Furthermore, we investigated the role of IL-6 as another anti-inflammatory

cytokine involved in both IAV and *T. gondii* infections. In the context of IAV, IL-6 plays an important role in neutrophil accumulation and neutrophil mediated clearance of viral infection (Dienz et al. 2012). Additionally, IL-6 is a costimulatory molecule with important implications during early *T. gondii* infection, leading to a reduction in parasitic growth (Jebbari et al. 1998). Our results showed a reduction of IL-6 levels within the spleen on day 2 post IAV infection; however, this decrease was transient, and IL-6 levels were recovered on day 5. These observation suggest that early IL-6 reduction coupled with reduced TNF-α and IFN- γ may have contributed to the delayed tachyzoite clearance in co-infected mice. Additionally, our results indicate that lung IL-6 levels in mice infected with *T. gondii* alone are significantly lower than those observed in IAV control mice. In contrast, co-infected mice do not display significant alteration of IL-6 mRNA expression, thus the presence of *T. gondii* maintains lung IL-6 expression in co-infected mice, counteracting IL-6 suppression due to IAV.

Our study provides important data that could be used to further elucidate the complex interaction between two clinically important pathogens, IAV and *T. gondii*. According to our findings, an acute infection with *T. gondii* followed by IAV a few days later, results in an aggravated disease outcome. This may be attributed to a reduction of spleen IFN-γ, TNF-α, and IL-6 levels in co-infected mice which led to delayed tachyzoite clearance and delayed progression into chronic toxoplasmosis. In addition, it is proposed that a synergy between peak cytokine production is required to maintain host survival, whereby co-infection when there is a shorter time between infections results in a less aggravated disease outcome.

Although transcription is the primary level at which gene expression is controlled, it does not always mirror the protein levels due to the action of repressors,

which may inhibit translation (Cooper 2000). Therefore,, follow-up studies are required to assess cytokine protein expression. Additionally a more holistic profile of cytokines using more times points is essential. Such studies should be ideally coupled with histopathological examinations of the lungs and spleen of co-infected mice to better understand the underlying mechanisms of disease modulation. Influenza infections can sometimes lead to unexplained complications in otherwise healthy individuals. The role of un-noticed co-infections with other pathogens like *T. gondii* as a cause of such complications should be further assessed clinically.

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