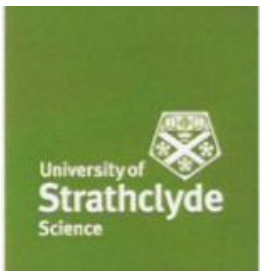

Sex-influenced histopathological changes and the immunity during *Toxoplasma gondii* infection

By

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Declaration

‘I declare that, except where specifically indicated, all the work presented in this report is my own and I am the sole author of all parts.’

Signed: _____

Date: _____

Abstract

Studies indicate female mice are more susceptible to *T. gondii* infection in comparison to male mice. The following studies were undertaken to enhance our understanding about the influence of sex in the immune response during *T. gondii* infection and the disease outcome in BALB/c mice. Early parasite replication and immunological events were studied in parallel through applying an in vivo imaging system (IVIS) with luciferase expressing *T. gondii* and a cytometric bead array to quantify key immunological mediators. The results confirmed female mice to be more susceptible to acute infection, as determined by higher mortality rates and weight loss compared with males. However, conflicting with expectations female mice had lower parasite burdens during the acute infection than male mice. Female mice also exhibited a significantly increased production of MCP-1, IFN-γ and TNF-α than male mice. These results suggest that a stronger immune response in females results in better parasite control, but has detrimental effects on health and mortality. Brain tissue of male and female mice chronically infected with *T. gondii* cysts were compared by using histopathological examination and RNAseq technologies to determine transcriptomic changes. The data indicate that female mice had increased pathology and developed more tissue cysts. The transcript analysis indicate that infected females had increased transcript levels of genes associated with PI3K/AKT/mTOR pathway, T cell exhaustion and apoptosis/pyroptosis. Consistent with these observations, female mice developed greater levels of DNA fragmentation as determined by TUNNEL and increased expression of NLRP3 in their brains. Overall, the results suggest that female mice mount a stronger immune response that controls parasite levels, but causes increased weight loss and mortality. In addition, the increased inflammation in the early stages of infection might account for the increased markers of T cell

exhaustion that correlates with increased pathology in brains of female mice during *T. gondii* chronic stage.

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Abbreviations

Name	Abbreviation
Adaptor molecule apoptosis-associated speck-like protein	ASC
Androgen receptor	AR
apoptotic protease-activating factor 1 (Apaf-1)	Apaf-1
B-cell lymphoma 2(Bcl-2)	Bcl-2
Bone marrow-derived	BMM
Chemokine (C-C motif) ligand 2	CCL2
C-terminal caspase recruitment domain	CARD
Damage-associated molecular patterns	DAMP
Differentially expressed DG	DG
endoplasmic reticulum stress	ERS
Estradiol 17beta	E2
Estrogen receptors	ERs
Haemotoxylin and Eosin	H&E
In vivo imaging system	IVIS
Indoleamine 2,3-dioxygenase	IDO
Influenza A virus	IAV
Interferon γ	IFN-γ
Interleukin 10	IL-10
Interleukin 12	IL-12
Interleukin 6	IL-6
Lipopolysaccharides	LPS
Monocyte chemoattractant protein 1	MCP-1
Netrin	Ntn1
Nicotinamide adenine dinucleotide	NAD+
orthogonal projections to latent structures-discriminate analysis	OPLS-DA
PI3 kinases	PI3K
Principal Component Analysis	PCA
Principal Component Analysis	PCA
Toxoplasmic encephalitis	TE

Chapter 1 .General introduction

1.1 Introduction

1.1.1 Overview of *Toxoplasma gondii*

Toxoplasma gondii (T. gondii) is perhaps the most successful intracellular parasitic organism and its distribution is worldwide. It is capable of infecting all warm-blooded animals including humans (Innes, 2010). *T. gondii* infects about one-third of the world's population in all continents. Based on clinical observations the vast majority of immune-competent individuals infected with *T. gondii* are asymptomatic or experience mild self-limiting illness (Halonen and Weiss, 2013). However, highly virulent strains of *T. gondii* can cause severe ocular disease even in immune-competent adults (Innes, 2010). While, immunocompromised individuals such as AIDS patients, may experience toxoplasmic encephalitis associated with infection, *T. gondii* is also medically important in other immunocompromised individuals such as patients undertaking cancer therapy or organ transplantation (Halonen and Weiss, 2013). If a women is infected during pregnancy *T. gondii* may cause congenital toxoplasmosis, abortion and neonatal mortality (Weiss and Dubey, 2009).

1.1.2 History

In 1908, while Nicolle and Manceaux were working on leishmaniasis research in the laboratory of Charles Nicolle at the Pasteur Institute in Tunis, they discovered *T. gondii* in tissues of a rodent, Ctenodactylus gundi. Initially they thought this organism to be *Leishmania*, but soon recognized that they had found a new protozoan parasite and called it *Toxoplasma gondii* (Nicolle, 1908). They named it *Toxoplasma* based on the morphology of the tachyzoite and bradyzoite phases of the parasite which are arc shaped (Nicolle, 1909): toxo is Greek for bow. In fact, the correct name for the parasite should have been *Toxoplasma gundi* as Nicolle and Manceaux had wrongly identified the host animal as Ctenodactylus gondi (Dubey, 2008). Fifteen years after this

discovery, the first toxoplasmosis case in humans was described by Janků *(1923)*, an ophthalmologist in Prague, who identified parasitic cysts in the retina of an 11 month-old infant with congenital hydrocephalus (Janku, 1923). Later, Levaditi.,(*1928)* suggested these parasitic cysts in the retina of the 11month-old infant were *T. gondii* cysts and also proposed a possible relationship between toxoplasmosis and congenital hydrocephalus (Levaditi, 1928). In fact, the definite clinical impact of *T. gondii* remained unidentified until 1939 when Wolf and Cowen in New York City described a fatal case in an infant with infantile granulomatous encephalitis, and believed the child to be congenitally infected with *T. gondii* (Wolf et al., 1939). The completed life cycle of *T. gondii* was determined in 1969-70. It was demonstrated as an obligate intracellular protozoan, and member of the phylum Apicomplexa, subclass Coccidian (Hutchison et al., 1969, Frenkel et al., 1969), capable of infecting all warm blooded animals including humans as one of its many intermediate hosts (Kim and Weiss, 2008).

1.1.3 Biology

1.1.3.1 *T. gondii* Life cycle

The full discovery of the *T. gondii* life cycle explains the prevalence of the parasite world wide since the primary hosts for it are domestic cats (Dubey, 2009). All non-feline warm-blooded animals including humans are the intermediate hosts of the parasite. It has three infectious forms in nature, namely, tachyzoites [asexual forms], tissue cysts surrounding bradyzoites [found mostly in the brain and muscle], and oocysts comprising sporozoites [the product of sexual recombination]. The invasive form in humans and other mammalian hosts is the tachyzoite which is also the form that is responsible for cellular and tissue damage. Tissue cysts holding bradyzoites serve as reservoirs of infection in intermediate hosts and thus play a key role in

parasite transmission and latent infection. Faecal oocysts containing sporozoites are only formed in the definitive hosts (Montoya and Liesenfeld, 2004) and they are extremely infectious and survive in the environment for months or even years (Black and Boothroyd, 2000).

Oocysts are transmitted into multiple kinds of intermediate hosts, almost all warm-blooded animals, from mammals to birds through contaminating their food or water (Robert-Gangneux and Darde, 2012). Any warm-blooded animal that ingests these infectious oocysts will become a host for the asexual cycle. However, if the ingesting animal is a cat, the bradyzoites continue through an invasive and division process into sexual stages. Bradyzoites divide profusely in the intestinal epithelial cells of cats producing schizonts that release merozoites that form male and female gametes (Black and Boothroyd, 2000). Male gametes are bi-flagellated and capable of swimming to locate and fertilize female gametes which then develop to form the oocyst which are discharged into the intestinal lumen (Hill et al., 2005). Successful infection of felines leads to the shedding of more than 100 million oocysts in the faeces after a period of 3 to18 days (O'Dell et al., 1996). Any warm-blooded animal that ingests these infectious oocysts will become a host for the asexual cycle. Therefore, the parasite will undergo its asexual multiplication within the intermediate hosts. After infection, oocysts containing sporozoites or tissue cysts containing bradyzoites differentiate into tachyzoites within the intestinal epithelium of the host (Robert-Gangneux and Darde, 2012). Tachyzoites rapidly replicate by endodyogeny and disseminate throughout the host's body (Halonen and Weiss, 2013). Tachyzoites differentiate into bradyzoites, which form tissue cysts about 7 to 10 days post-infection and are presumed to remain in the host throughout life, mostly in the brain and muscular tissues (Robert-Gangneux and Darde, 2012) **(Figure 1.1)**.

1.1.3.2 *T. gondii* transmission route

T. gondii is optionally heterogeneous in its infectivity and has obtained several possible routes of transmission within and between diverse host species. Three forms of *T. gondii*, tachyzoites, bradyzoites in tissue cysts and sporozoites oocysts are all able to infect definitive and intermediate hosts (Tenter, 2009). The main transmission sources of *T. gondii* in humans are undercooked or raw meat and unwashed vegetables contaminated with tissue cysts or oocysts. In addition, blood transfusion contaminated with tachyzoites within blood cells, or organ transplants from infected donors contaminated with tissue cysts can be rare sources of infection (Tenter et al., 2000). However, the variation in prevalence of *T. gondii* within a population appears to be associated with the hygiene and eating behaviours (Montoya and Liesenfeld, 2004).

Congenital transmission is an additional transmission route, which occurs when the foetus is infected with *T. gondii in utero*. It was firstly described in humans by Wolf in 1939 and later it was established that many animal species including rodents, sheep and goats can also transmit disease in this manner (Hill and Dubey, 2002). A study estimated that 30–60% of pregnant women in Europe are infected with *T. gondii* because of undercooked meat consumption (Kijlstra and Jongert, 2008). A possible scenario that accounts for congenital transmission is that the parasite reaches the placenta and then invades and proliferates within the placenta cells before sequentially entering the fetal circulation and therefore infecting the fetal organs (Remington et al., 2011) **(Figure 1.1).**

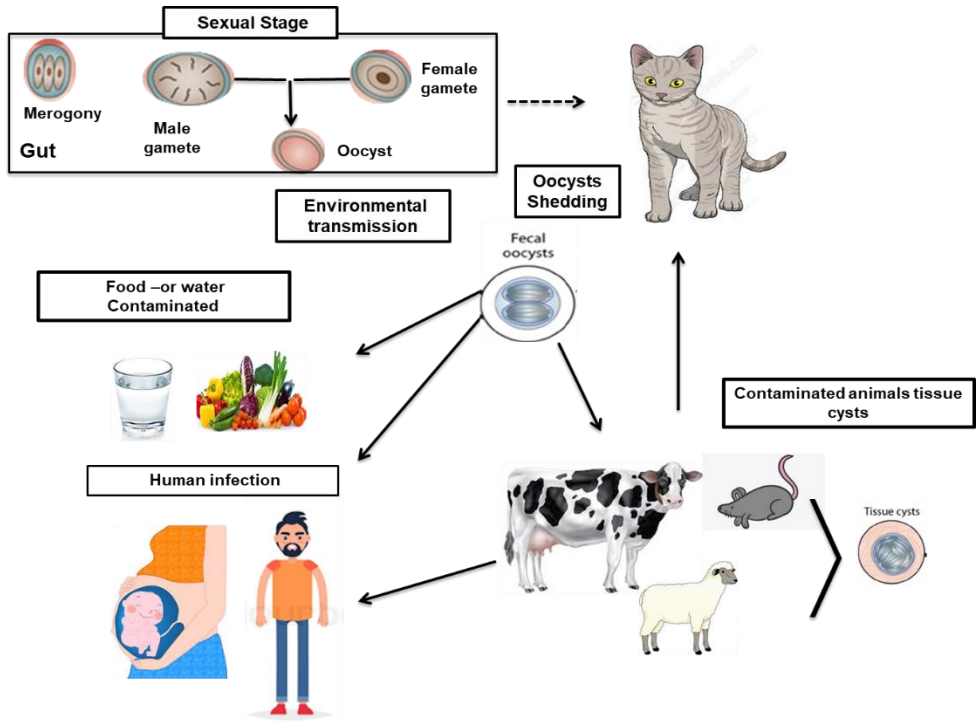


Figure 1.1 The life cycle of *T. gondii* and transmission routes

Sexual replication of *T. gondii* takes place in cats as the definitive host. After replication of merozoites within enterocytes of the cat, male and female *T. gondii* gametes are generated within the host cells. The fusion of gametes will form oocysts which are shed in cat faeces. Asexual replication happens within the intermediate host, ingestion of tissue cysts by feeding may cause transmission to either other intermediate hosts or re-initiate the sexual stage if it is a cat. Contamination of food and water with oocysts causes infection of intermediate hosts, and also congenital toxoplasmosis occurs if the infected host is a pregnant woman. The acute infection is recognized by tachyzoites that are distributed throughout the body. Tachyzoites transform to bradyzoites within tissue cysts that are characteristic of the chronic stage of infection.

1.1.3.3 *T. gondii* genotypes and geographical distribution

The genus *Toxoplasma* has only one species, *T. gondii*. Early studies that aimed to understand population genetics of *T. gondii* reported a remarkable clonality with the majority of strains falling into 3 clonal lineages (Howe and Sibley, 1995, Dardé, 2004). It is now recognised that this was an oversimplification as a result of poor sampling. Now, genotyping outcomes of the strains gathered from human and animals around the world demonstrate an abundant genetic range. These studies, indicate that the distribution of *T. gondii* genotypes differ significantly with geographical areas (Lehmann et al., 2006). As yet more than 200 genotypes have been identified in the *T. gondii* database (Cheng et al., 2017). The most common strain of *T. gondii* is isolated from North America and Europe, and it falls into one of the three originally defined clonal lineages, referred to as types I, II and III (Howe and Sibley, 1995). However, a slight level of genetic variance just 1 to 2% at the DNA sequence level accompanies the three main lineages (Robert-Gangneux and Darde, 2012). In addition, isolations from different continents such as South America, Africa and Asia revealed multi-chromosome genotypes (Lehmann et al., 2004). For example, in South America *T. gondii* strains are genetically different, included types Br I, Br II, Br III, and Br IV. Also a current study has indicated that Chinese 1 [ToxoDB#9] genotype is the common type in China (Shen and Wang, 2015).

1.1.4 Toxoplasmosis outcomes

Toxoplasmosis is the medical term for the diseases caused by *T. gondii* infection. The clinical outcomes of toxoplasmosis largely depend on the parasite virulence and host's genetic background, sex, and immunological status (Guy, 2014). Most of *T. gondii* infections in human are asymptomatic (Hill et al., 2005). Although, 80-90% of cases of toxoplasmosis in immunocompetent individuals are undetected,10-20% of

cases may develop acute infection with observable symptoms. These symptoms are generally self-limiting and normally resemble a mild to severe flu or glandular fever-like illness, although in rare occasions ocular toxoplasmosis with acute infection might occur. Ocular disease as a consequence of adult acquired disease is normally associated with south American lineages. However, immunocompromised individuals such as HIV patients may develop severe or life-threatening illnesses due to either acute infection or reactivation of a past latent infection (Guy, 2014). For example, encephalitis is the most common clinical outcome of toxoplasmosis in immunosuppressed patients (Luft and Remington, 1992).

Toxoplasmosis can be congenitally acquired when a woman contracts the infection during pregnancy. The severity of congenital infection varies depending on which semester when maternal-infection occurs. Infection during the first trimester tends to be more severe than the second or third trimester of pregnancy (McAuley, 2014). The congenital infection may compromise the embryonic developmental process causing severe complications, such as encephalitis, neurological disease, mental illnesses, visual inflammatory disorders, cardiovascular abnormalities or even abortion (Oz, 2014).

Infection with *T. gondii* results in a chronic infection due to the ability of the parasite to form tissue cysts, which are normally located in the CNS and musculature of infected mammals. While the cysts remain predominantly latent for the lifetime of the host, reactivation of the cysts can result in life-threatening toxoplasmic encephalitis in immunocompromised patients. It has also been reported that *T. gondii* is an important trigger for cryptogenic epilepsy, and potentially involved in the etiology of schizophrenia and behavioural alterations in the host (Suzuki et al., 2010).

1.1.5 Histopathology of toxoplasmosis

During the development of acute infections, *T. gondii* affects different tissues and organs such as the liver, lungs and spleen frequently causing severe lesions and noticeable histopathological alterations within the host (Hill et al., 2005). In addition, the parasite can be detected within the eyes, heart, and the central nervous system. (Atmaca et al., 2013).

1.1.5.1 Splenic histopathology

Toxoplasmosis within the splenic pulp results in inflammation and tissue necrosis in humans (Remington et al., 2011). A study by Hazíroğlu and others., (*2003*), in rabbits infected intraperitoneally with *T. gondii*, found that the spleen morphology was swollen, and pale yellow with necrotic foci around 1-2 mm, and the parasite invasion was distributed diffusely all over the spleen with impacts on its diameter and size. Macroscopically, after the 4th day of infection the spleen showed general lesions that were focally disseminated. During the acute infection, lesions normally increase gradually and differentiation of inflammatory cell types occurs, and also a few tachyzoites are normally present within severe focal necrotic regions (HaziroĞLu et al., 2003). When ICR (CD-1, Outbred) mice were infected intraperitoneally with 10 cysts per mouse, the histopathological observations found splenic lymphatic nodules damaged or missing, red pulp expanded and white pulp atrophied with hyperaemia (Wu et al., 2011).

1.1.5.2 Interstitial pneumonia

Pulmonary toxoplasmosis is the second or third most common systemic infection associated with immune-depressed individuals such as AIDS patients after brain and cardiac cases (Martinez-Giron et al., 2008, Cheepsattayakorn and Cheepsattayakorn,

2014). Moreover, pulmonary toxoplasmosis can be significantly critical during organ transplants and may lead to the development of reactivation of previously latent infection (Fricker-Hidalgo et al., 2005). Macroscopically, the appearance of the lungs during pulmonary toxoplasmosis is congested, with regions of consolidation as well as infiltration of inflammatory lymphocytes (Martinez-Giron et al., 2008). In pulmonary toxoplasmosis areas of necrosis can be observed with numerous extracellular as well as intracellular tachyzoites within leukocytes and macrophages (Vijayan, 2013).

1.1.5.3 Hepatitis

Studies in mice by using intravital microscopy imaging analysis have found that *T. gondii* invasion into the liver might occur progressively via the surface of sinusoidal epithelial cells to the Kupffer cells, finally invading and expanding into the cytoplasm of hepatocytes (Frevert et al., 2005). Histopathological analysis of liver in inbred mice infected orally with 10 tissue cysts revealed that replication of tachyzoites within the hepatocytes initiates necrosis, leading to necrotic foci complemented by infiltration of inflammatory mononuclear cells (Pinheiro et al., 2015). A study by Atmaca., et al., (*2013*) in normal Swiss albino mice infected intraperitoneally with *T. gondii* RH strain tachyzoites, found the parasite within hepatocytes 4 and 7 day post-infection resulting in activation of a number of hepatic stellate cells (Atmaca et al., 2013). Hepatic stellate cells play important roles during parasitic infection via developing and forming fibrosis and cirrhosis progression (Rastogi et al., 2012) and interface within the sinusoidal space with antigen presenting cells (APCs) such as dendritic cells and Kupffer cells, indicating their possible influence on APC functions during the infection (Ichikawa et al., 2011).

1.1.5.4 Toxoplasmosis Encephalitis (TE)

The brain is the last organ to be reached by tachyzoites owing to its immunological privileged position, and the blood–brain barrier (BBB) (Pinheiro et al., 2015). The use of diverse transgenic parasites combined with reporter mice and intravital imaging as well as in vitro models of the BBB suggest three mechanisms for parasite invasion through the BBB: (i) Paracellular entry whereby extracellular tachyzoites transmigrate through tight junctions between endothelial cells; (ii) Transmigration of infected leukocytes across the endothelial cell layer the "Trojan horse" mechanism; (iii) Transcellular entry whereby endothelial cells become infected, enabling release of tachyzoites in the neural parenchyma (Corcino et al., 2019, Mendez and Koshy, 2017, Lachenmaier et al., 2011). The Waree group., *(2007)* investigated the histopathological changes of chronic *T. gondii* infection in ICR mice, and their microscopic observations showed that the inflammatory response mainly involved mononuclear cells located within lesions and close to tissue cysts. Also the histopathological characteristics linked with chronic toxoplasmosis in the brain can be congestion of the meninges with mononuclear cell infiltration and some perivascular cuffing (Waree et al., 2007). A study using Swiss albino mice intraperitoneally infected with 20 *T. gondii* cysts showed that activation of astrocytes and microglia/macrophages is a major occurrence in toxoplasmic encephalitis (TE). Neuronal degeneration, necrosis and glial proliferation can be observed throughout the brain as result of toxoplasmosis (Dincel and Atmaca, 2016).

1.1.6 Immune response to *T. gondii*

The immune response to *T. gondii* infection is distinctive and heterogeneous due in large part to the high level of heterogeneity in genetic backgrounds of hosts. In addition, *T. gondii* can infect multiple tissue types such as the central nervous system

and the placenta and each have their specific immune responses. The extra levels of complexity of *T. gondii* immunity are further compounded by the diverse virulence of different strains and the frequency of infection. For about 60 years, *T. gondii* has been a critical model pathogen for understanding the murine immune system in resistance to intracellular pathogens (Tait and Hunter, 2009). However, the majority of laboratory studies have used type II strains with intermediate virulence to simplify the study of the immune response in mice throughout the acute and chronic stages of infection. These in vivo studies, particularly analysing infection in mice deficient in several immune effector genes have contributed significantly to understanding immunity to *T. gondii* (Hunter and Sibley, 2012) **(Figure 1.2)**.

1.1.6.1 Early immune response to *T. gondii*

Intermediate hosts normally become infected with *T. gondii* following ingestion of tissue cysts or oocysts. The parasites then multiply and spread within the intestinal cells causing host cell lysis releasing tachyzoites that distribute throughout the host's different organs and tissues (Tait and Hunter, 2009). When *T. gondii* manage to cross the epithelial barrier and reach the lamina propria, they interact with resident macrophages, dendritic cells (DCs) and intraepithelial lymphocytes (Buzoni-Gatel et al., 2006). During parasite invasion of host cells, three sequential waves of proteins are excreted into the host cell from 3 parasite organelles, identified as the micronemes, dense granules and rhoptries. These proteins can modify host cell activity and influence the development of an immune response directed against the parasite (Lim et al., 2012). In addition, *T. gondii* in the intracellular environment is able to modify the lipid composition of the parasitophorous vacuole (PV) creating a specialized intracellular organelle. The PVs enable the transport of essential nutrients

from the host cells to the parasite, as well as inhibiting lysosomal synthesis that would cause parasite lysis and death (Cesbron-Delauw et al., 2008).

T. gondii-induced IL-12 production in dendritic cells (DCs) was dramatically reduced in mice lacking MyD88, an adaptor molecule in TLR signalling pathways, or in mice lacking CCR5 (Scanga et al., 2002). Stimulation with cyclophilin-18 from *T. gondii* induced the expression of CCR5 in DCs (Aliberti et al., 2000). Profilin-like protein of *T. gondii*, is essential for host cell invasion and active egress from cells and was identified as a ligand of TLR11 (Plattner et al., 2008, Yarovinsky et al., 2005). Both cyclophilin-18 and profilin ligands stimulated IL-12 production in $CD8\alpha^+$ DCs and $CD8\alpha^-$ DCs. In addition to TLR11, TLR12 recognizes profilin-like protein and is essential for IL-12 production, particularly in plasmacytoid DCs (pDCs) (Koblansky et al., 2013).

Although humans do not express either TLR11 or TLR12, human monocytes produce pro-inflammatory cytokines in response to *T. gondii* infection, suggesting that other TLRs in humans recognize different components of *T. gondii* to produce IL-12 in antigen-presenting cells. Additional types of TLRs might be involved in the *T. gondii* immune response such as TLR7 and TLR9 that recognize RNA and DNA derived from *T. gondii*, respectively (Andrade et al., 2013). It is also possible that nucleic acid recognition by endosomal TLRs (including TLR8 in humans) act as danger signals of parasite invasion and induces IL-12 production in human cells. *T. gondii* also has HSP70 molecules and GPI-achored molecules that can act as ligands for TLR2 and 4 (Aosai et al., 2006, Debierre-Grockiego et al., 2007). Foreign nucleic acids, the essential signature molecules of invading pathogens that act as danger signals for host cells, are detected by endosomal nucleic acid-sensing toll-like receptors (Sasai et al., 2018) **(Figure 1.2)**.

1.1.6.1.1 Cytokines involvement response in *T. gondii*

Many in vivo studies in mice have shown that regulating of *T. gondii* needs early production of the pro-inflammatory cytokines like IL-12 that stimulates natural killer (NK) cells and CD4+ T cells and CD8+ T cells to produce IFN-γ (Hunter and Sibley, 2012). The production of IL-12 depends on APC activation shortly after *T. gondii* infection in mice (Mason et al., 2002) . Studies on Bone-marrow-derived macrophage (BMDM) have found IL-12 and IFN-γ are the key elements in an early defence mechanism against *T. gondii* by promoting parasite death, controlling tachyzoite population load and tachyzoite-bradyzoite developmental conversion. For example a study found that parasite growth was not controlled through infection in mice lacking IL-12 or IFN-γ resulting in the host's death (Braun et al., 2013). IFN-γ is a key mediator in resistance to *T. gondii* due to its role in the activation of a range of antimicrobial events within the host cell causing limitation of parasite replication. For example, an in vitro study in human fibroblasts found that IFN-γ modifies cell metabolism leading to tryptophan degradation in fibroblasts and iron starvation (Hunter and Sibley, 2012). Furthermore, IFN-γ activates macrophages to produce nitric oxide (NO) that regulates intracellular parasite proliferation. This also leads to inducing parasite transformation to the slow growing bradyzoite form which in turn will develop into tissue cysts in the central nervous system and in the muscles, and consequently the chronic phase of infection (Miller et al., 2009). Inducible nitric oxide synthase (iNOS) is expressed upon *T. gondii* infection because it is an IFN-γ inducible gene. Mice deficient for iNOS have been reported to have a normal response to the acute phase of infection, but show increased lethality during parasite expansion and severe pathology caused by the increased parasite burden in the brain (Khan et al., 1997). However, in another murine model, ablation of NO using aminoguinadine has been shown to result in increased mortality during the acute phase of infection

(Woods et al., 2013). NO produced via iNOS activation, prevents the fatal exacerbation of progressive toxoplasmic encephalitis (TE). IFN–γ also stimulates the robust expression of indoleamine 2,3-dioxygenase (IDO), which mediates tryptophan degradation to inhibit the growth of *T. gondii* in several human cell lines including fibroblasts, glioblastoma cells, retinal pigment epithelial cells and macrophages (Sasai et al., 2018).

1.1.6.1.2 Immune cell involvement during early response to *T. gondii*

Monocyte

Activated cells of monocyte lineage have long been understood to possess potent microbicidal activity against *T. gondii* and other intracellular pathogens. CCR2-dependent inflammatory monocytes activated by IFN-γ and expressing Ly6C have been identified as important effectors of resistance to *T. gondii*. After oral infection, these cells are recruited into the intestinal mucosa, where they appear to control infection through direct parasite killing, such as producing high amounts of TNF-α, inducible NO synthase (iNOS), and reactive oxygen intermediates (ROS), which directly contribute to the control of the parasite burden in the host (Dunay et al., 2008, Karlmark et al., 2012). Chemokine receptor CCR2 and MCP-1-deficient mice fail to recruit inflammatory monocytes, that results in a loss of parasite control and acute susceptibility to *T. gondii* infection (Dunay and Sibley, 2010). Monocytes, also in response to soluble *T. gondii* antigens produce IL-12 therefore, stimulating IFN-γ production by T cells in vitro (Dupont et al., 2012).

Neutrophils

Neutrophils rapidly home to sites of infection, phagocytose pathogens and release anti-microbial granules. However, neutrophils can also release immunoregulatory

cytokines and chemokines, suggesting that they may also participate in modelling immunity (Nathan, 2006). In vitro studies have observed that *T. gondii* triggers neutrophil synthesis of CC chemokine ligands CCL3, CCL4, CCL5, and CCL20, chemokines that are key factors in recruiting and activating DCs in response to *T. gondii* (Bennouna et al., 2003). Recently, these cells have been found to release chromatin and granule-associated neutrophil extracellular traps (NET) that ensnare and kill microbes. It has been established that NET release occurs in response to *T. gondii* in both human and mouse neutrophils during tachyzoite co-culture. NET entrapment of *T. gondii* could disable the parasite from infecting host cells and establishing infection (Abi Abdallah et al., 2012). Neutrophils are recruited during response to *T. gondii* infection and their ablation causes increased susceptibility to infection in vivo (Denkers et al., 2012). A study suggested that CXCR2 chemokine receptor is required for early neutrophil recruitment and it plays a key protective function in resistance to *T. gondii*. A study in CXCR2-/- mice showed reduction in TNF-α and IFN-γ levels. These mice had increased parasites in their peritoneal cavity during acute infection and increased numbers of brain cysts during chronic infection compared with wild type control mice (Del Rio et al., 2001). Although neutrophils are recruited in abundance in response to infection with *T. gondii* and their depletion is associated with increased susceptibility, in vivo neutrophil function remains controversial. Accumulation of these cells in the intestinal mucosa following infection could be a response to bacteria that translocate from the lumen to the sub-epithelium during *T. gondii* infection rather than a host response to the parasite itself (Heimesaat et al., 2006). It has been argued that neutrophils have no role in defence during *T. gondii* infection, at least in mouse models. The reason for the uncertainty is that in vivo antibody-mediated depletion protocols long used to remove neutrophils are now understood to also eliminate inflammatory monocytes because of common

expression of Gr-1 (Ly6C/G). Moreover, depletion studies using a neutrophil-specific anti-Ly6G antibody failed to find a protective effect for these cells on resistance to *T. gondii* infection (Dunay et al., 2010). However, the CXCR2 study was carried out with CXCR2 knockout mice on a BALB/c genetic background, unlike antibody depletion studies that were performed on a C57BL/6 background. It is possible that the role of neutrophils during *T. gondii* infection varies depending on mouse strain, host species, or parasite lineage (Denkers et al., 2012).

Macrophage

The macrophage is an essential cell in the initial immune response against *T. gondii* in murine infections (Andrade et al., 2013) and is a main producer of pro-inflammatory cytokines like IL-12, IL-1 and TNF-α that are important in response to *T .gondii* infection (Bogdan and Rollinghoff, 1999). Furthermore, an in vitro study in *T. gondii*-infected murine bone marrow-derived macrophages (BMDMs) indicated that the cell surface receptor CD40 is an important factor against parasitic infection due to its major role in macrophage activation. Therefore, initiation of IL-12 production and autophagy, are important mechanisms in controlling parasitic proliferation (Morgado et al., 2014). IL-12 is an important factor in the initiation of T cell-dependent and independent activation of macrophages, and plays a key role in Th1 differentiation (Ismail et al., 2017). Moreover, invasion of Lewis rat macrophages by *T. gondii* will activate the NLRP1 inflammasomes that are major components of the innate immune system resulting in rapidly induced programmed cell death (pyroptosis) as well as production of IL-1β and IL-18 and therefore inhibition of parasite replication (Wang et al., 2019) **(Figure 1.2)**.

Dendritic cells (DCs)

There is increasing in vivo evidence identifying key roles for dendritic cells (DCs) during *T. gondii* infection, most significantly it has been found that DCs are responsible for early production of IL-12 and presentation of microbial antigen to effector cells (Lambert et al., 2009). Maintaining and generating DCs largely depends on the ligand for the receptor tyrosine kinase fms-like tyrosine kinase 3 (flt3). Several studies have established that Flt3L−/− mice have increased susceptible to acute toxoplasmosis compared with wild type mice (Dupont et al., 2015). Moreover, DCs produce IL-12 via activation of the chemokine receptor CCR5 and this is MyD88 adaptor-mediated. It has been found that CCR5- deficient animals demonstrate high susceptibility to *T. gondii* infection. Also, it is well known that MyD88-deficient mice infected with *T. gondii* experience uncontrolled parasite growth and concomitant with significant reduction in IL-12 levels (Scanga et al., 2002). It has also been found that mice deficient in the transcription factor Batf3 (Basic Leucine Zipper ATF-Like Transcription Factor 3) that is involved in DCs development, die as result of *T. gondii* infection and experience extreme IL-12 deficiency, decreased CD8+ T cell responses, and high parasite proliferation (Mashayekhi et al., 2011).

In in vivo experiments DCs have lately been recognized as systemic transporters (Trojan horses) of *T. gondii* tachyzoites as result of their migratory behaviour and tolerance to parasitic infection. *T. gondii* tachyzoites exploit DC migration to escape clearance or to initiate infection (Lambert et al., 2009). Lambert et al. *(2006)* also demonstrated that *T. gondii* infection influences DC migration in vivo, by comparing the distribution of infected and uninfected (LPS stimulated) murine bone marrow-derived DC injected into mice. They found that *T. gondii*-infected DCs caused rapid increase in prevalence of parasites to distant organs and in aggravation of infection

in comparison with uninfected DCs. This study also showed that adoptive transfer of *T. gondii*-infected DC resulted in more rapid distribution of parasites to distant organs and in exacerbation of infection compared with infection with free parasites (Lambert et al., 2006). Moreover, during *T. gondii* infection, DCs transport microbial antigens from the site of infection to the spleen where the T cells gathered. Therefore, DCs trigger the polarisation of the T cells via production of IL-12 to initiate the activation of CD4+ and CD8+ T cells which are key factors in long-term immunity against *T. gondii* infection (Miller et al., 2009) **(Figure 1.2)**.

NK cells

Acute and chronic *T. gondii* infection induces NK cell activities in mice (Gigley, 2016). A study by the Goldszmid group using mice injected with anti- AsialoGM1 to diminish NK cells, prior to infection, found that after 72 hours the anti-AsialoGM1 treatment significantly reduced the frequency of DCs into infection sites. This might suggest the importance of NK cell in resident monocytes differentiation into DCs (Goldszmid et al., 2012). Also*, T. gondii*-stimulated NK cells initiate inflammatory DC differentiation in the early phase of infection causing increased T cells activation (Dupont et al., 2012). In addition, increased IL-12 production by DCs is mediated by NKG2D, a receptor on human and murine NK cells that plays an important function during *T. gondii* infection. C57BL/6 treated with Ab to NKG2D demonstrated higher parasite burden in the tissues, and higher mortality rates to infection (Guan et al., 2007). Moreover, NK cell-derived IFN-γ is key in both controlling inflammatory cell dynamics and driving the differentiation of resident monocytes during the immune response to the pathogen (Goldszmid et al., 2012) **(Figure 1.2)**.

1.1.6.2 Late Immune Responses to *T. gondii*

The early innate response stimulates a strong acquired type-1 response including CD4+ T cells and CD8+ T cells, that play key roles during toxoplasmosis (Nickdel et al., 2004). The importance of adaptive immune responses has been confirmed by the increased susceptibility of patients who are deficient in T and B cell function. Also, mice lacking in B cells, CD4+ T cells or CD8+ T cell demonstrate increased susceptibility to *T. gondii* during the chronic phase of infection (Dupont et al., 2012). Activation of the adaptive immune response produces a significant number of parasite-specific CD4+ and CD8+ T cells that generate IFN-γ within the parasite invasion sites (Halonen and Weiss, 2013).

1.1.6.3 T cells subsets

It was mentioned earlier that during *T. gondii* infection, T cells perform several important regulatory roles in mediating the resistance, since the severe toxoplasmosis cases are associated with decrease in T cell numbers (Dupont et al., 2012). In addition, mouse models deficient in CD4+ T cells showed a rise in susceptibility to infection during the chronic phase (Johnson, 2002). CD4+ T cells phenotypically can be differentiated into several subsets depending on required biological roles and their stimulator (O'Shea and Paul, 2010). CD4+ T precursor cells via IL-12 can be induced to differentiate and proliferate as Th1 cells that produce IL-2 and IFN-γ (Johnson, 2002). Moreover, Th2, Th17, and T regulatory cells (Treg) subsets are additional types of cell subsets found during parasitic infection (Huber and Pfaeffle, 1994).

Th1 and Th2 cells

The most important subtypes of CD4+ T cells that are involved in *T. gondii* infection are Th1 and Th2 cell, due to their key roles in controlling the balance between cell-

mediated and humoral immune responses. During *T. gondii* infection a T helper 1 (Th1) immune response is elicited. This provides a strong, protective immune response and is characterised by dendritic cells (DC) producing IL-12. The production of IL-12 leads to the differentiation of CD4+ T cells into Th1 cells expressing the transcription factor T-bet and the secretion of IFN-γ (Ahmed et al., 2017). Th1 cells produce IFN-γ as well as TNF-α cytokines stimulating the activation of macrophages which have been shown in vitro to mediate the killing of tachyzoites (Oksanen et al., 2014).

In vitro studies have shown that the Th2 cytokine IL-4 induces converting of B cells to plasma cells that are vital in modulating parasitic infections (Moens and Tangye, 2014). A study in IL-4 knockout mice infected with *T. gondii* found that the rate of mortality was significantly greater in knockout mice than in naive controls (Roberts et al., 1996). In contrast, another study in IL-4-/- mice also infected with *T. gondii* found that these mice were more resistant to infection than WT mice as measured by significantly reduced mortality and small intestine pathology. Significantly, plasma IL-12 and IFN-gamma levels were higher in IL-4-/- mice (Nickdel et al., 2004). However, the role of IL-4 during *T. gondii* infection is complex since the studies using mice deficient in IL-4 infected with *T. gondii* showing both disease protective and exacerbatory roles being identified (Nickdel et al., 2004). Mice lacking in IL-10 showed increased mortality as result of overproduction of pro-inflammatory cytokines during *T. gondii* infection (Suzuki et al., 2000) **(Figure 1.2)**.

Regulatory T cells (Treg)

Regulatory T cells (Treg) provide immune homeostasis and tolerance by actively preventing pathological and physiological impact of inflammatory immune responses (Olguin et al., 2015). Deletion of Treg cells in resistant BALB/c mice infected

intraperitoneally with *T. gondii* cysts caused an increased mortality associated with enhanced IFN-γ levels in mice sera (Tenorio et al., 2011). Treg cells typically express the interleukin-2 (IL-2) receptor alpha chain (IL2Rα), and treatment of *T. gondii*-infected susceptible C57BL/6J mice with anti-IL-2 complexes caused an increased Treg-cell function and survival, with simultaneous reduction in disease severity (Akbar et al., 2015, Olguin et al., 2015). These studies show that Treg cells are critical mediators of the immune response during *T. gondii* infection (Tenorio et al., 2011).

Th17 cells

Th17 cells are an additional subset of effector T cells producing the cytokines IL-17, IL-21, and IL-22 and they are involved in the inflammatory response in human toxoplasmosis. IL-23 secreted by innate immune cells is capable of stimulating the polarisation and expansion of IL-17-producing T cells (Silva et al., 2014). A study found that IL-17R signalling plays a protective role, since mice lacking IL-17R died more rapidly following to *T.* gondii infection (Kelly et al., 2005). An experiment by Guiton et al., (*2010*) used IL-17RA–deficient mice and IL-17A neutralizing antibody in C57BL/6 mice that were infected orally with 30 cysts of *T. gondii* 76K-strain. These mice were more resistant to *T. gondii* infection, presented fewer inflammatory alterations and less tissue injury in the ileum, liver, spleen, and brain, compared with wild type C57BL/6 mice, indicating that IL-17A is involved in tissue pathology during *T. gondii* (Guiton et al., 2010).

CD8+ T cells

The essential nature of CD8+ T cells during *T. gondii* infection was firstly indicated by Parker and colleagues study (1991). They found that adoptive transfer of T cells in BALB/c mice infected orally with *T. gondii* significantly increased their survival (Parker

et al., 1991). Once activated they undergo differentiation into short-lived effector and memory precursor effector cells. As effector cells, CD8+ T cells exert immune pressure on the parasite via production of inflammatory cytokines and through their cytolytic activity. When immune control has been established, the parasite develops into chronic infection regulated by the memory CD8+ T population (Gigley et al., 2011). Mice lacking CD8+ T cells exhibit failure to clear infection (Denkers et al., 1997), and depletion of CD8+ T had a greater effect on mortality of chronically infected animals than depletion of CD4+ T cells (Gazzinelli et al., 1992). Moreover, chronically infected susceptible mice strains show reduced numbers of intracerebral CD8+ T cells with toxoplasmic encephalitis (TE) development (Deckert-Schlüter et al., 1994). Combined, these studies underline the important role of CD8+ T in controlling *T. gondii* infection during the chronic stage of infection. CD8+ T cells during chronic infection are a major source of IFN-γ which is critical for mediating protective immunity to *T. gondii*, by controlling parasite reactivation (Suzuki, 2002). CD8-mediated cytotoxicity most likely plays an important role in controlling chronic toxoplasmosis (Bhadra et al., 2011). A study demonstrated that when CD8+ T were transferred into infected immunodeficient (SCID) animals that have already developed large numbers of cysts, CD8+ T cells are capable of eliminating cysts by a perforin-dependent mechanism (Suzuki et al., 2010). Based on these findings, IFN-γ and cytotoxicity appear to be the critical effector mechanisms for CD8-mediated control of this protozoan. CD8+ T cells are vital in maintaining chronic toxoplasma infection under control, therefore highlighting them as a dominant component of long-term immunity that is required to preserve the parasitic reactivation process in suppression status (Gigley et al., 2009). A previous study by Bhadra et al., (*2012)* using C57BL/6 mice which are susceptible to TE showed that CD8+T cells at the late stage of chronic toxoplasmosis display progressive exhaustion of functionality, enhanced apoptosis,

and weak recall response along with high expression of PD-1 an inhibitory receptor: a phenomenon referred to as CD8+ T cell exhaustion. CD8+ T cells exhaustion provides a new insight into the pathogenesis of chronic toxoplasmosis and is a potential pathway for developing immunotherapy against this parasite (Bhadra et al., 2012) **(Figure 1.2)**.

1.1.6.3 Humoral immunity to toxoplasmosis

Host resistance to protozoan infections relies on both innate and acquired cell-mediated immune responses. Several in vivo reports have associated B cells and antibodies (Abs) in host survival and parasite clearance (Magez et al., 2008). Moreover, B cells can directly modulate T cell subsets and dendritic cells, and therefore influence adaptive immunity and the development of infection (Youinou, 2007). *T. gondii* infections produce not just immunoglobulin G (IgG) and IgM antibodies in serum of both humans and mice but also IgA antibodies (Sayles et al., 2000). The key role of antibody in immunity to *T. gondii* is confirmed by using B Cell-Deficient (μMT) mice. These mice promote apparently normal IFN-γ responses, but succumb within 3– 4 weeks post-infection of *T. gondii* associated with high parasite proliferation in the CNS (Kang et al., 2000). This growth in susceptibility is possibly due to absence of antibodies, as the passive transfer of antibodies conferred protection to B cell-deficient mice. Antibodies protective activity may be via a diversity of mechanisms. In vitro studies have discovered that antibodies opsonize parasites for phagocytosis, activate the classical complement pathway and also block invasion (Dupont et al., 2012).

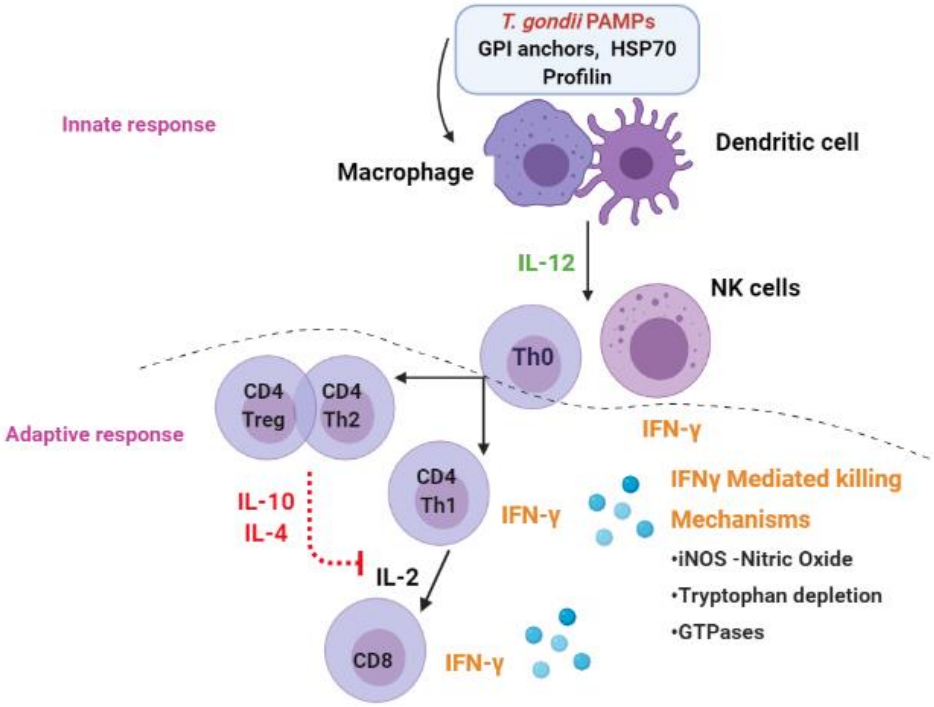


Figure 1.2 The immune response sequence following infection with *T. gondii*

Following infection of *T. gondii*, DCs and macrophages activated and produce IL-12. Therefore, IL-12 directs T cells polarisation towards a Th1 and Th2 adaptive response along with IFN-γ produced by natural killer (NK) cells. Activated CD4+ Th1 and CD8+ T cells produce and initiate IFN- γ mediated killing mechanisms. While, Th2 response controls Th1 response by producing IL-4, and IL-10.

1.1.7 Immunity and sex differences

Around 15 million of the 57 million deaths that happen annually worldwide are directly linked to infectious diseases. The World Health Organization (WHO) has focused on studying and controlling infectious diseases around the globe, with significant attention paid to the influence of sex and gender on chronic infectious diseases, such as HIV and tuberculosis (Klein et al., 2010). Researchers have noticed that the immune response in male and female acts differentially during infection (Reardon, 2016). Increased evidence suggests that clinical trials on men are often not applicable to women and vice versa. It is clear that there are significant biological and health differences between males and females. Although the life span of women is longer than men, the illness rate of women is higher. While women experience more nonfatal conditions and disability, men deal with more life-threatening chronic diseases and normally die at a younger age. It is well known that women are more susceptible than men to autoimmune and rheumatologic diseases, anemia, thyroid conditions, gall bladder disorders, migraines, arthritis, eczema, upper respiratory infections, gastroenteritis as well as short-term infectious diseases. Men, on the other hand, experience higher frequencies of coronary heart disease, cancer, cerebrovascular disease, emphysema, liver cirrhosis, kidney disease, and atherosclerosis (Pardue and Wizemann, 2001) **(Table 1.1)**.

Table 1.1 Sex differences in the intensity, prevalence and severity of disease following microbial infections in humans [a]

	Intensity	Prevalence	Severity
Viruses			
HIV			
Influenza Virus(avian H7N9)	N.D		
Influenza Virus (2009 H1N1)	N.D		
MERS-CoV	N.D		
Hepatitis B Virus			
Bacteria			
Mycobacterium tuberculosis	N.D		
Legionella pneumophila	N.D		
Campylobacter jejuni	N.D		
Leptospira spp.	N.D		
Parasites			
Plasmodium falciparum			
Toxoplasma gondii	N.D		
Schistosoma mansoni			
Entamoeba histolytica	N.D		
Fungi			
Paracoccidoides brasilliensis			
Aspergillosis fumigatus	N.D		
Crptococcus neoformans	N.D		

	= Male Bias
	= No Observed Bias
	= Female Bias
N.D	**= Not Determined**

[a] Updated from vom Steeg LG, Klein SL (2016) SeXX Matters in Infectious Disease Pathogenesis. PLoSPathog 12(2): e1005374. doi: 10.1371/journal.ppat.1005374

1.1.7.1 Sex differences in immune response

In vertebrates, immunological differences among the sexes are well known. In general males show lower immune responses than females. While, females have stronger innate and adaptive (humoral and cellular) immune responses in comparison to males. These immunological differences between the sexes might explain why males and females vary in their responses against parasitic infections (Klein, 2004). The factors responsible for these sex differences can be attributed to biological factors (i.e., sex differences, such as genetic and epigenetic factors or sex hormones) and to psychosocial factors (Ortona et al., 2019).

1.1.7.1.1 Sex differences in innate immunity

Males and females are different in their innate immune responses, and several studies in both humans and rodents show that inflammatory immune responses are largely higher in females compared with males. That might be the reason why women are more likely than men to develop inflammatory rheumatic diseases like systemic lupus erythematosus and rheumatoid arthritis (Da Silva, 1995). In addition, the phagocytic activity in neutrophils and macrophages was found to be greater in women than men (Spitzer, 1999). Nevertheless, many studies have shown that in males the plasma concentrations of some pro-inflammatory cytokines, such as IL-6 and TNF-α, are higher after infection (Diodato et al., 2001). Studies in mice also have illustrated that differences in APCs that form the bridge to adaptive immunity are affected by sex. Thus, APCs from females are more effective at presenting peptides than APCs from males. The expression of MHC class II on astrocytes, microglia and endothelial cells is increased in females compared with male mice as result of infection (Klein, 2004).

1.1.7.1.2 Sex differences in adaptive immunity

Generally, women exhibit larger antibody responses than men. Both basal levels of immunoglobulin, and also antibody responses to some types of vaccines, are regularly higher in females than males (Klein et al., 2015). Further studies in humans have illustrated that cytokine responses of CD4+ T cells is often different between women and men (Wikby et al., 2008). Clinical observations show that men have lower CD4+ T and CD3+ T counts as well as lower Th1 responses than women (vom Steeg and Klein, 2016). A study in women chronically infected with HIV-1 demonstrated that they have higher levels of CD8+ T cell activation genes (Meier et al., 2009). Women also display higher cytotoxic T cells activity than men with up-regulated expression of antiviral and pro-inflammatory genes (Hewagama et al., 2009). In mice, cell-mediated immunity is increased in females with higher lymphocyte proliferation, faster wound healing, and elevated immunological intolerance to pathogen products than male mice (Klein et al., 2010). Female mice showed higher production of Th2 cytokine responses such as IL-4, IL-5, IL-13, IL-9 and IgG1 following *Trichuris muris* infection than males (Hepworth et al., 2010). When mice were infected with the parasite *Giardia muris*, females exhibited lower infection outcome and higher antibody production than their companion males, which suggests a functional advantage for higher humoral immunity in females (Klein, 2004).

In several situations, females normally experience boosted antiviral, inflammatory, and cellular immune responses, that are necessary for pathogen clearance, however that may cause expanded development of disease symptoms and outcomes in females after infection. In contrast, for other infectious diseases, an inability to efficiently clear or control a pathogen may amplify the severity of disease in males in comparison with females (vom Steeg and Klein, 2016). Future studies are required

to understand the mechanisms that account for sex differences in the pathogenesis of infectious diseases and to identify both microbial and host variables that determine the extent of these sex differences (Hewagama et al., 2009).

1.1.7.3 Factors responsible for sex differences in the immune response

Sex hormones

Previous studies show the role of sex hormones in influencing the immune response (Bouman et al., 2005). The difference in the concentrations and the type of the sex hormones in male and females during the life time brings alterations in the outcome of immune response. Sex hormones involve androgens (mainly testosterone), estrogen (mainly 17 beta-estradiol in the ovarian cycle), and progesterone. In plasma of adult males, testosterone concentrations remain approximately stable. However, in females there is a variation in sex hormones estrogen and progesterone during menstrual cycle (Chiu et al., 1996).

Sex hormones are known to affect proliferation/apoptosis of cells. As sex hormones are lipophilic they diffuse through cell membranes to gain access to their intracellular cognate receptors. Several studies indicate the presence of intracellular estrogen receptors (ER-α, and ER-β) in T cells and B cells , dendritic cells , and humans monocytes (Greene et al., 1984, Banchereau et al., 2000, Mor et al., 2003). In addition to classical intracellular receptors, membrane receptors have been identified for estrogen known as G protein-coupled estrogen receptor 1 (GPER1) (Boonyaratanakornkit and Edwards, 2007).

The sex hormones binding to their respective steroid receptors directly stimulate signalling pathways that are linked with the production of cytokines and chemokines (Klein and Roberts, 2010). For instance, there is correlation between serum levels of

estrogen and progesterone and the number of human peripheral blood cells actively secreting interleukin-2 (IL-2), IL-4, IL-6, IL-10, TNF-γ and IFN-α as determined in vivo (Verthelyi and Klinman, 2000).

Sex chromosome-linked genes

Several sex differences are likely affected by the inherent imbalance of gene expression due to being encoded on the Y and X chromosomes of the host (Stamova et al., 2012). Many genes that are encoded on the X chromosome regulate immune responses and play a key role in altering sex differences in progressing immune-related diseases. For instance, the Toll-like receptor 7 (TLR7) located on the X chromosome, can recognizes viruses with RNA or DNA , and it has greater expression levels in cells from female than male mice (Pisitkun et al., 2006). It is found that DCs isolated from women produce twofold of IFN-α in response to TLR7 ligands HIV-1 than DCs from men (Meier et al., 2009). Moreover, there is an increased activity of X-linked genes in blood immune cells in women compared with men following ischemic stroke (Stamova et al., 2012). In addition, previous studies have illustrated that Y-chromosome-linked polymorphisms can influence susceptibility to autoimmune disease that is sex-dependent in SJL/J mice (Spach et al., 2009). Understanding sex differences in the expression of X-linked genes is challenging due to additional compounding effects of sex hormones contributing to the differential gene expression.

1.1.7.4 Sex differences influence the immunity to parasites

Sex differences in prevalence and pathogenesis of parasitice infections have caught parasitologist attention for a long time, actually almost since the systematic study of parasites started near the beginning of the twentieth century (Addis, 1946). Parasitologist investigating animals gathered in the field, found it natural to pay

attention to the differences in parasitic prevalence and pathogenesis between the host sexes, and their observations were later carried out in laboratory trials. The literature is filled with observations about the different prevalence and severity of natural parasitic infections among males and females of many species, as well as humans. These differences are certainly because of many factors, such as the different exposure of each sex to many parasite infective stages (Roberts et al., 2001). In both humans and other animals, the incidence and severity of parasitic infections in general is greater in males than females. Many studies have shown that between the sexes immunological differences appear which might explain the relative high level of parasitism occurrence in men. While females normally have greater immune responses than males, this create something of a double-edged sword action as while it might favour control against infectious diseases, it could be harmful in terms of promoting the development of autoimmune diseases (Pardue, 2001).

1.1.7.5 Sex differences influence the immunity to *T. gondii*

In human, studies that linked sex differences and *T. gondii* infection are rare due to the fact that most 'healthy' adults are asymptomatic. However, among immunocompromised patients, *T. gondii*-induced encephalitis is more frequent among women than men (Klein, 2004). Studying a wide variety of inbred mice of different genetic backgrounds and major histocompatibility complex haplotypes revealed a significant difference in the susceptibilities of males and females to *T. gondii* infection (Roberts et al., 1995). This study found that female mice were more susceptible to acute infection, as defined by higher mortality rates, than male mice, however those female mice surviving to the chronic phase of infections harboured more cysts in their brains compare with surviving males. Male mice produce higher levels of TNF-α, IL-12, and IFN-γ than females during early infection (Roberts et al.,

1995). Roberts and his colleagues therefore investigated this observation in greater depth immunologically in the BALB/K mouse, a strain exhibiting moderate susceptibility to infection with *T. gondii*. Plasma TNF-α concentrations were increased in both male and female BALB/K mice on days 8 and 10 post-infection with males producing significantly greater concentrations than females. However, the first deaths happened at day 12 post-infection and these were among female mice. Further in vitro analysis of *T. gondii*-specific T-cell proliferative responses from day 15 post-infection and onwards showed significantly higher inflammatory responses in male mice compare with female counterparts. This difference was most obvious in splenocyte cultures initiated at day 15 post-infection, where full suppression of proliferation was observed in the splenocytes from female mice but not from male mice. Tissue culture supernatants analysis from these cultures exhibited differences in the quantities of IFN-γ and IL-10; male spleen cells produced greater concentration of IFN-γ during the early stages of infection than those from female mice. IL-10 production, however, peaked at highest concentrations in the cell cultures from both male and female initiated at day 22 post-infection. These results suggest that, in male mice, a prompt response to infection with high levels of IFN-γ and TNF-α. Also, IL-10 production might be essential in down regulating these potentially damaging inflammatory mediators. The poor survival rates and higher cyst burdens of female mice might be as a result of late response in terms of T-cell proliferation and IFN-γ production compared with their male counterparts (Roberts et al., 1995). Subsequent studies also found female severe combined immunodeficient (SCID) mice to have increased pathology in their brains and quantitatively different immune responses compared with male mice (Walker et al., 1997). This indicates that early innate immune events are different between the sexes and are likely determinants of disease

outcome. Importantly, these early studies did not measure parasite multiplication during the acute stages of infection.

1.1.8 Identification of key genes and pathways associated with sex differences during *T. gondii* infection

Studies in cancer occurrence found that males and females differed in the activation of several signaling pathways such as PI3K signaling and apoptosis (Natri et al., 2019). However, the influence of sex in these pathways during *T. gondii* has not been investigated, therefore, the following section briefly discuss these pathways.

1.1.8.1 Phosphoinositide-3 kinase PI3K signalling during *T. gondii* infection

Members of the phosphoinositide-3 kinase (PI3K) family control several cellular responses including cell growth, survival, cytoskeletal remodelling and the trafficking of intracellular organelles in many cells types and a variety of important immune functions (Koyasu, 2003). PI3Ks are enzymes which catalyse the phosphorylation of one or more inositol phospholipids in the 3-position of the inositol ring. Both the substrates and products of these reactions are membrane-captive phospholipids and the action of PI3Ks is to generate specific molecular messengers in the membranes upon which they act (Okkenhaug, 2013). There are four Class I PI3K isoforms (PI3K α, β, γ, δ), three Class II PI3K isoforms (PI3KC2α, C2β, C2γ) and a single Class III PI3K (PIK3C3). The four Class I isoforms synthesise the phospholipid 'PIP3'. PIP3 is a 'second messenger' used by many different cell surface receptors (Hawkins and Stephens, 2015) **(Table 1.1)**. This pathway is activated by a combination of ligands such as lipopolysaccharide (LPS), and various cell surface receptors such as TLRs, insulin receptor, estrogen receptor, and many cytokine receptors (Manukyan et al., 2010).

This pathway is activated during *T. gondii* infection and is important for the invasion and proliferation of the parasite in the host cell. The phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway play important roles in cell survival and apoptosis inhibition. *T. gondii* initiates these pathways in a dose-dependent manner via toll-like receptors TLR2 and TLR4 (Mammari et al., 2019). A previous study demonstrates that *T. gondii* activities host Gi-protein-dependent PI3K signalling to inhibit induction of apoptosis in infected macrophages (Kim and Denkers, 2006). Furthermore, *T. gondii* excreted-secreted antigens (ESA) have been found to inhibit Forkhead box P3 (Foxp3) a critical transcription factor, modulates regulatory T cells via the upregulation of PI3K signalling pathway in vitro (Chen et al., 2019). Studying the production of IL-23 and IL-12 in human monocytes during *T. gondii* demonstrated that, IL-23 production is positively regulated by PI3K, while IL-12 production is negatively regulated by PI3K (Quan et al., 2015). In addition, a similar study showed that *T. gondii* induced IL-12 and IL-23 production in Jurkat T cells was positively regulated by PI3K (Ismail et al., 2017).

Table 2.2 Catalytic and regulatory subunit of PI3Ks [a]

Class	Catalytic subunits	Regulatory subunits	Lipid products	Function
Class IA				
PI3Kα PI3Kβ PI3Kδ	p110α p110β p110δ	p85α/p55α/p50α P55γ p85β	PtIns (3,4,5) P_3	Cell signalling Growth and survival Glucose metabolism Immune response
Class IB				
PI3Kγ	p110γ	p101 , p84/87		
Class II				Membrane trafficking
	PIK3-C2a PIK3-C2b PIK3-C2g	-	PtIns (3)P	Cell signalling ,growth migration, and survival
Class III				Membrane trafficking Phagocytosis, cytokinesis
	Vps34-like	P150		

[a] Updated from (Ghigo et al., 2010)

1.1.8.2 Programmed cell death

Apoptosis, necrosis, and pyroptosis create the principal mechanisms by which programmed host cell death occurs. The term ‘‘programmed cell death’’ refers to genetically controlled or regulated forms of death, where the cell plays an active role in its own death (Lamkanfi and Dixit, 2010).

The immune system responds to infection by applying a diversity of means, from activation of pathways that promote host survival to stimulating programmed cell death. The cell death signalling pathways are activated within the infected cell to remove the intracellular niche of microbial replication and expose intracellular pathogens to extracellular immune surveillance. In addition, engulfment of dying cells

by macrophages and dendritic cells may stimulate activation of the adaptive immune system by presenting viral and microbial antigens to T cells (Albert, 2004). However, several pathogens use many strategies to manipulate host cell death and survival pathways to enhance their replication and survival. (Ashida et al., 2011). Pathogens modulate cell death activation by a variety of mechanisms, including (1) pore-forming toxins clearing the extracellular leakage of cellular components, (2) enzymes and effector proteins distributed into the host cytosol, and (3) antigens that target immune cells (Lamkanfi and Dixit, 2010).

Programmed cell death pathways are an active part of the host defence and control of against *T. gondii*. However, the parasite has developed multiple mechanisms to counter killing by the host cell death pathways in order to establish successful infection and therefore maintenance of chronic infection in the host (Halonen, 2015). Below, the molecular components and signalling pathways of apoptosis, and pyroptosis pathways will be described with their roles in the pathogenesis of *T. gondii*.

Apoptosis

Historically, apoptosis is known as an immunologically silent process due to the combined actions of caspases (Rongvaux et al., 2014). Apoptotic cell morphology is recognized by cytoplasmic shrinking, cell rounding, chromatin condensation, DNA fragmentation and membrane blebbing **(Figure 1.5)** (Fuchs and Steller, 2011, Taylor et al., 2008). Apoptosis is normally initiated by cell extrinsic pathways which are mediated by death receptors such as the Fas ligand or specific “death receptors” of the TNF receptor superfamily, or alternatively by cell intrinsic (mitochondrial) pathways that are activated by a variety of internal stimuli, such as growth factor deficiency, DNA damage, oxidative stress, as well as invasion by pathogens. Both of these pathways conclude in the activation of the effector caspases 3, 6 and 7. The

sequence of events that explain the extrinsic pathway of apoptosis are best described with the FasL/FasR and TNF-α/TNFR1 models. The binding of Fas ligand to Fas receptor results in the binding of the adapter protein FADD and the binding of TNF ligand to TNF receptor results in the binding of the adapter protein TRADD with recruitment of FADD. FADD then associates with procaspase-8 forming a death-inducing signalling complex (DISC), resulting in the auto-catalytic activation of caspase 8. Following, caspase 8 activation , the completing action of apoptosis is initiated (Elmore, 2007). In the case of the intrinsic pathway, the mitochondria are the core. When B-cell lymphoma 2(Bcl-2) family members, such as Bax and Bak, initiate a mitochondrial outer membrane permeabilisation (MOMP) that will cause the release of mitochondrial intermembrane space proteins, including cytochrome c. Once in the cytosol, cytochrome c binds an adaptor molecule called apoptotic protease-activating factor 1 (Apaf-1), directing its oligomerization to form a heptameric structure called the apoptosome (Yuan and Akey, 2013). The apoptosome then enrols and activates pro-caspase-9 that, in turn, cleaves and activates caspases-3 and 7 in mammals (Besteiro, 2015). As a result of apoptosis, cellular contents are compressed within membrane tied apoptotic bodies. If these bodies are not cleared, they will be subject to secondary necrosis (rupture), producing cytosolic damage-associated molecular patterns (DAMPs) to the extracellular environment that can induce robust type I interferon-mediated proinflammatory response (Gupta et al., 2009, Poon et al., 2014) **(Figure 1.3)**.

Apoptosis during T. gondii

T. gondii can interfere with host cell apoptotic machinery and stimulate either an anti- or pro-apoptotic response depending on the parasite virulence , load, and the host cell type (Contreras-Ochoa et al., 2013). Increased apoptosis during *T. gondii*

infection has been detected in mouse spleen cells (Gavrilescu and Denkers, 2001) and neuronal cells of BALB/c mice (el-Sagaff et al., 2005). Parasite organelles such as rhoptry proteins (ROPs) can initiate endoplasmic reticulum stress ERS-mediated apoptosis. For example, ROP18 has been found to contribute to mouse neural cell apoptosis through the ER stress-mediated apoptosis pathway (Wan et al., 2015). A number of studies propose that the parasite is controlling the apoptosis machinery at multiple levels: preventing the release of cytochrome c from mitochondria to the host cell cytosol (Keller et al., 2006), influencing the balance of pro- and anti-apoptotic BCL-2 family members (Carmen et al., 2006) and directly interfering with caspase processing and function (Vutova et al., 2007). A recent study by Graumann et al., (*2015*) using Jurkat T cells provides new understanding into the mechanisms of apoptosis inhibition by a type II *T. gondii* strain. Using a mitochondria-free in vitro reconstitution system, the authors show that there is a direct anti-apoptotic activity mediated by parasitic factors. More specifically, *T. gondii* protein lysates prevent the binding of pro caspase 9 to Apaf-1, in this manner suppressing caspase 9 activity and consequently caspase 3/7 activation. Therefore, in addition to being capable of inhibiting cytochrome c release from host mitochondria, the parasite can interfere with host apoptosis by directly preventing the recruitment of caspase 9 to the apoptosome **(Figure 1.3)** (Graumann et al., 2015). However, the conditions under which this occurs are rather complex and the mode of action is also probably dependent on the parasite strains and host cell types that are being considered (Besteiro, 2015).

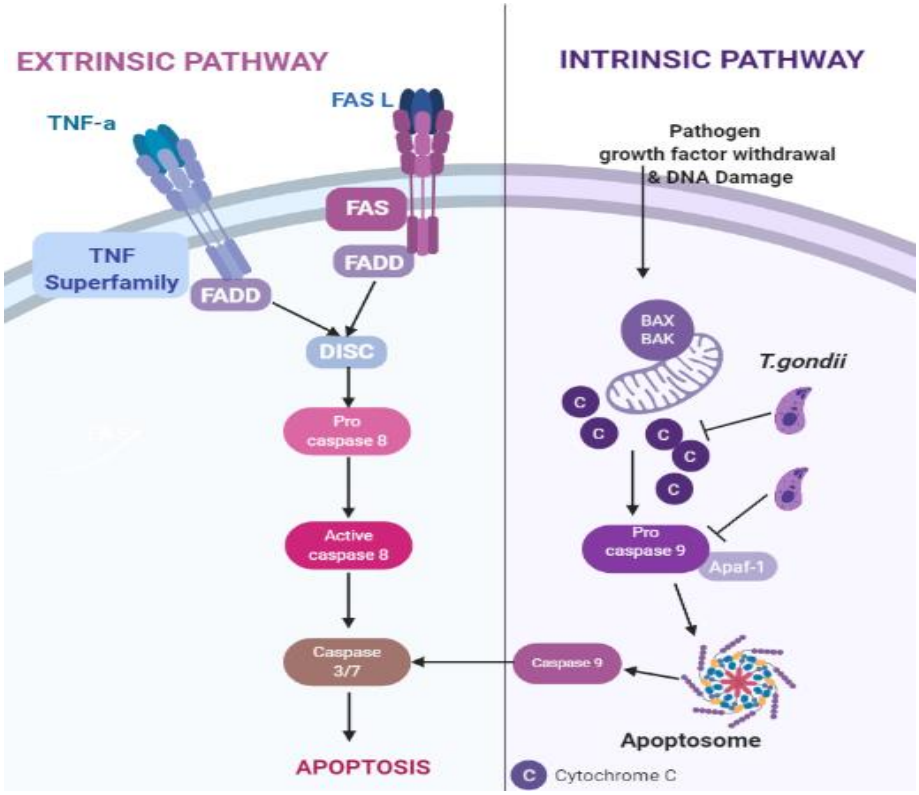


Figure 1.3 The apoptosis pathways, and potential interference of *T. gondii* with apoptosis

The extrinsic pathway is initiated by the binding of Fas ligand to Fas receptor as well as the binding of TNF ligand to TNF receptor that will recruit the adapter protein FADD then a death-inducing signalling complex (DISC) is formed resulting in activation of procaspase 8, followed by with 3/7 caspase activation, to initiate apoptosis. Intrinsic pathway activated by a variety stimuli cause Bax and Bak to activate mitochondrial outer membrane permeabilization (MOMP) leading to the release of cytochrome c that will bind to apoptotic protease-activating factor 1 (Apaf-1), to form the apoptosome. The apoptosome then activates pro-caspase-9 that, in turn, activates caspases 3/7. *T. gondii* control the apoptosis machinery by preventing the release of cytochrome c from mitochondria, or *T. gondii* proteins prevent the binding of procaspase 9 to Apaf-1, suppressing caspase 9 activity and the recruitment of the apoptosome.

Pyroptosis

Although, apoptosis was the first well-recognised programme of eukaryotic cell death, (Fink and Cookson, 2005), other cell death programmes include a caspase 1-dependent programmed cell death (also known as pyroptosis) have been described. Pyroptosis is triggered by a variety of microbial infections (Bergsbaken et al., 2009). Pyroptosis leads to the release of inflammatory cytokines hallmarks associated with inflammasome activation (Man et al., 2017). Caspase 1 is a protease that processes the inactive precursors of interleukin 1β (IL-1β) and IL-18 into mature inflammatory cytokines (Fantuzzi and Dinarello, 1999). Moreover, caspase 1 activation can result not only in the production of activated inflammatory cytokines, but also rapid cell death characterized by plasma-membrane rupture and release of proinflammatory intracellular contents (Brennan and Cookson, 2000, Fink and Cookson, 2006). It is well recognised that pyroptosis plays a significant role in resisting microbial establishment as well as contributing to the pathogenesis of autoinflammatory and autoimmune diseases, such as neurodegenerative diseases (Yuan et al., 2018).

Pyroptosis is morphologically and mechanistically different from other forms of cell death as Caspase 1 dependence is a specific feature and mediating factor of pyroptosis (Bergsbaken et al., 2009). Although, pyroptosis is a programmed process of cellular self-destruction mediated by caspases, which is similar to apoptosis, the mechanism, characteristics and outcome of pyroptosis are different from apoptosis (Bergsbaken et al., 2009). Caspase 1 that is involved in pyroptosis activation is not involved in apoptosis, since caspase 1-deficient mice have no defects in apoptosis and develop normally (Li et al., 1995). Moreover, damage of mitochondrial integrity and release of cytochrome c does not occur during pyroptosis (Jesenberger et al., 2000). Caspase 1-mediates the processes by which plasma-membrane pores form

to disperse cellular ionic gradients, water influx, cell swelling and, ultimately, osmotic lysis and release of inflammatory intracellular contents (Fink and Cookson, 2006) . Although, cleavage of chromosomal DNA is a lethal incident that is often assumed to identify apoptotic cell death (Fink and Cookson, 2005), during pyroptosis, cells undertake DNA damage and become positive in the Terminal deoxynucleotidyl transferase dUTP nick end labeling assay (TUNEL) (Brennan and Cookson, 2000, Watson et al., 2000). However, the nuclear morphology of pyroptotic cells is distinct from apoptotic cells (Watson et al., 2000). Moreover, the nucleus in pyroptotic cells reveals chromatin condensation (Miao et al., 2011) **(Figure 1.4) (Figure 1.5)**.

Mechanism of pyroptosis

Pyroptosis is driven by the activation of the inflammasome, a cytosolic multiprotein complex responsible for the release of IL-1β and IL-18 (Tang et al., 2019). Activation of the inflammasome occurs, after pattern recognition "sensor" proteins such as Toll-like receptors (TLRs) or nucleotide-binding (NOD)-like receptors (NLRs), detect the presence of pathogens, their products, or danger signals (Lamkanfi and Dixit, 2012). Once a ligand binds to cytosolic receptors NLRs, Apoptosis-associated speck-like protein (ACS) containing a C-terminal caspase recruitment domain (CARD) that acts to bind NLRP proteins, such as NLRP3 with procaspase-1 to form a multiprotein complex termed an inflammasome. Activation of inflammasomes triggers self-cleavage and activation of procaspase-1 to an active protease, which then cleaves cytosolic pro-IL-1β and pro-IL-18 into their active forms (Quan et al., 2018, Compan et al., 2015). Activation of the inflammasome also drives cleavage of the pro-pyroptotic factor gasdermin D to form pores on the host cell membrane and cause the lytic death of the cell (Man et al., 2017) **(Figure 1.4)**.

Activation of inflammasomes during T. gondii

T. gondii infection promotes a defensive inflammasome-mediated response that limits parasite load and proliferation (Zamboni and Lima-Junior, 2015). A study found that the NLRP1 inflammasome was activated by *T. gondii* infection in rat macrophages from different inbred strains causing rapid cell death, inhibition of parasite proliferation and IL-1β/IL-18 production (Cirelli et al., 2014). A study by Gorfu et al., (*2014)* in NLRP1-deficient mice found that increased parasite loads and acute mortality during *T. gondii* infection. The same study found that mice lacking in NLRP3 infected with T*. gondii* had declined in levels of IL-18 production, and increased parasite replication, and death (Gorfu et al., 2014).

Purinergic P2X7 receptor in innate and adaptive immune responses. It is widely expressed by different immune cells. During innate immune response, damage-associated molecular patterns (DAMPs) or pathogen-associated molecular pattern (PAMPs) stimulate Toll-like receptors (TLRs) activation resulting in adenosine triphosphate (ATP) release which can activate P2X7 receptor (Savio et al., 2018). Activation of P2X7R promotes inflammasome formation and IL-1β release (Miller et al., 2011, Wiley et al., 2011). A study by Quan and colleagues., (*2018)* in a non-transformed human fetal small intestinal epithelial cell line infected with *T. gondii* found that P2X7R mediates activation of the NLRP3 inflammasome by forming a complex with the adaptor protein ASC and pro-Casp-1 inducing IL-1β maturation and production as well as inhibiting *T. gondii* proliferation (Quan et al., 2018). Collectively, these observations indicate the important role for NLRP1 and NLRP3 in controlling host resistance to *T. gondii* infection **(Figure 1.4)**.

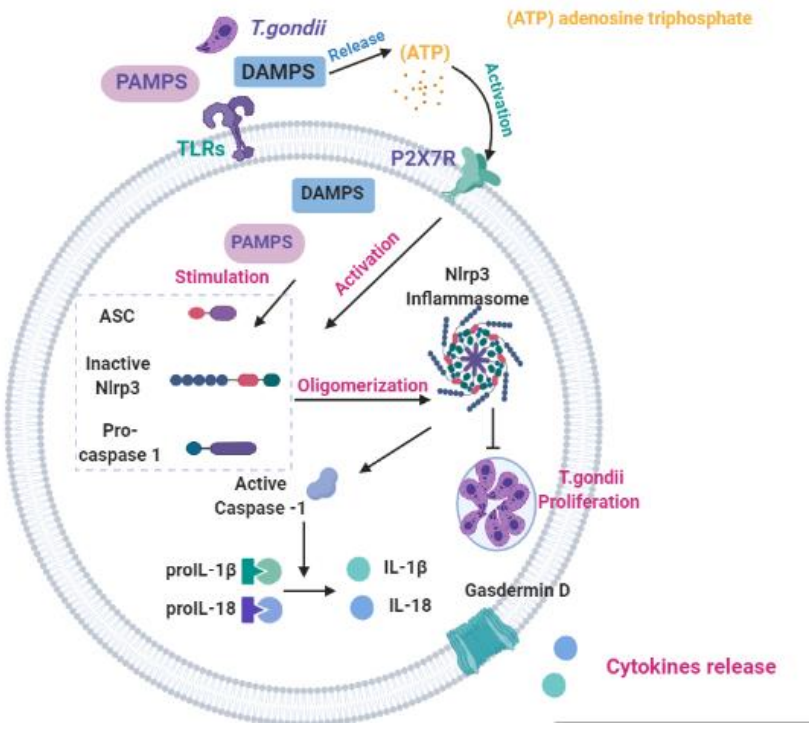


Figure 1.4 The Pyroptosis pathway, and the activation of inflammasomes by *T. gondii*

Activation of Toll-like receptors (TLRs) by PAMPS & DAMPS leads to trigger Apoptosis-associated speck-like protein (ACS) and binding NLRP proteins, such as NLRP3 with procaspase-1 to form a multiprotein complexes inflammasome. Activation of inflammasomes triggers self-cleavage and activation of procaspase-1 to an active protease, which then cleaves cytosolic pro-IL-1β and pro-IL-18 into their active forms. Activation of the inflammasome also initiates cleavage of the pro-pyroptotic factor gasdermin D to form pores on the host cell membrane and cause the lytic death of the cell and cytokine release. P2X7R activated by ATP mediates of the NLRP3 inflammasome inducing IL-1β and IL-18 maturation and production, therefore inhibiting *T. gondii* proliferation.

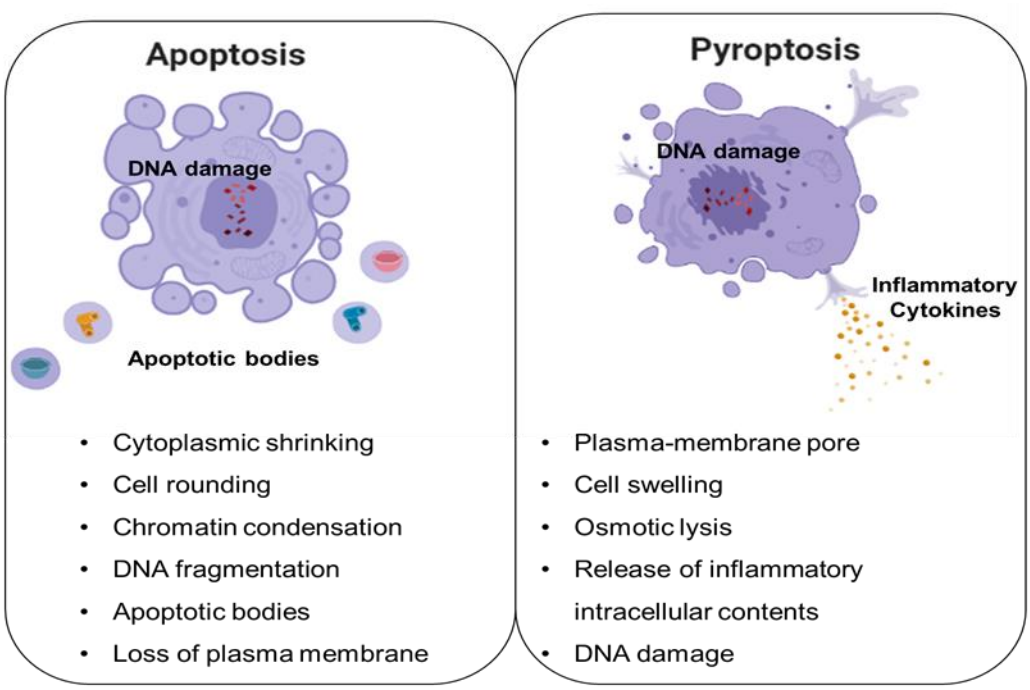


Figure 1.5 The morphological differences between apoptosis & pyroptosis

1.2 Research aim

The aim of this research is to better understand how sex contributes to the immune response and outcome of *T. gondii* infection in mice. The early events in immune competent mice have been poorly characterised and the relative contribution of parasite replication and inflammatory/immunological events remain to be fully elucidated. Recent advances in technology should now allow a reappraisal of sex differences in the pathogenesis of murine *T. gondii* infection. Thus the studies described herein aim to further our knowledge of sex differences in the response to *T. gondii* infection through use of an in vivo imaging system (IVIS) and luciferase expressing *T. gondii* to (i) quantify early *T. gondii* multiplication, (ii) quantify immunological mediators during infection using a bead array. In addition, by using modern 'omics' technologies studies will (iii) determine transcriptomic changes, in male and female mice chronically infected with *T. gondii* and (iv) correlate these with histopathological changes and validate key findings using specific histological staining techniques.

1.3 Research hypothesis

These studies will explore the hypothesis that female mice mount a stronger inflammatory response than male mice and this accounts for their relatively greater mortality.

Chapter 2 . Materials and Methods

2.1 Ethical approval

All animal procedures followed the guidelines from The Home Office of the UK Government. All the work was done under the Home Office licenses: PPL60/4568, “mechanism of control of parasite infection” authorization by the University of Strathclyde Animal Welfare Ethical Review Board. Experimental groups contained 3-5 mice per group. Statistical analysis was performed by Prims 8 statistical analysis software (Graphpad Prism, USA).

2.2 Experimental design

BALB/c mice were bred in house at the Strathclyde Institute of Pharmacy and Biomedical Sciences, Glasgow, UK under the establishment license issued by The Home Office of the UK Government under the Animals [Scientific Procedures] Act 1986 with approval by the University of Strathclyde ethical review board. Mice were housed under specific pathogen free conditions in polypropylene cages (13cm×35cm), containing Ecopure flakes and sizzle nest bedding (SDS Services) with access to water and CRM mouse chow (SDS Services) *ad libatim*. Mice were maintained under a 12-hour light-12 hours’ dark period.

For experimental groups, male and female mice were used between 8-11 weeks old. During the experimental course the animals were weighed daily and scored for signs of illness. Animals exceeding humane thresholds were euthanized.

2.3 Maintenance Human Foreskin Fibroblasts (HFFs) culture

Human Foreskin Fibroblast (HFF) cells were grown in a 75cm2 tissue culture flask (TPP, Switzerland). Confluent flasks of HFF cells were split by removing media and washed with 5 ml of Phosphate-buffered saline (PBS). Three milliliters of Triple E (Invitrogen, Inchinnan, UK) was added and incubated for approximately 10 minutes and maintained at 37°C in humidified conditions and in the presence of 5% (v/v) CO_2. Following incubation period, the cells were checked by microscopy and remaining attached cells were dislodged by gentle shaking. The cell suspension was transferred to a tube containing 25 ml growth media (10% Fetal Calf Serum (FCS) (Gibco, Thermo Fisher Scientific, UK), 1% Penicillin/ streptomycin solution (Sigma-Aldrich, UK), 1% L-Glutamine (Gibco, Thermo Fisher Scientific, UK), 1% Amphotericin B (Sigma, Irvine, UK) and Dulbeccos Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific, UK)) and centrifuged for 5 minutes at 1400 rpm. The supernatant was discarded and the pellet of cells was re-suspended in 50ml. Cells were seeded into now flasks in 10ml aliquots and incubated at 37°C in humidified conditions with 5% CO_2.

2.4 *T. gondii* Maintenance

2.4.1 II *Prugniaud T. gondii* transfected with the firefly luciferase gene

The Prugniaud *T. gondii* type II strain genetically modified to express the Firefly Luciferase (FLUC) gene was utilized and maintained by frequently passing into HFFs primary cells line (as mentioned above). Once HFFs monolayer were 80% confluent the flask, 500 µl of *T. gondii* (containing approximately 93 x 10^5/ml tachyzoites) were added along with freshly prepared media and then maintained at 37°C in a humidified condition and in 5% CO_2. *T. gondii* was harvested when a large number of intracellular parasites could be observed within the HFFs monolayer. Cells were first washed with

PBS and then the flask was scraped using a 30cm cell scraper (TPP, Trasadingen, Switzerland) and then washed with 10 ml sterile (PBS) (Lonza, UK), the suspension passed 10 times through a 21-gauge blunt needle (BD, Drogheda, Ireland) in order to release intracellular parasites from the host cells. The cell suspension was centrifuged at 1400 rpm for 5 minutes and the pellet was resuspended in 1ml of PBS (Lonza, UK/). Finally, the number of parasites was counted by haemocytometer and cell concentration adjusted to the required dose for infections.

2.4.2 Harvesting of *T. gondii Beverley* (RRA) strain

Beverley cysts were maintained *in vivo* at the Strathclyde Institute of Pharmacy and Biomedical Sciences, by continual passage of infective brain tissue homogenates through outbred CD1 albino mice. In Brief, after sacrificing the infected mouse by CO_2 inhalation, the brain was removed and homogenised by passing repeatedly through a 21 blunt needle, containing 2ml of sterile PBS (Lonza, UK), to gain an even suspension. 15µl of brain suspension was put on a microscope slide and the cysts counted by light microscopy. The suspension was diluted in PBS to adjust the concentration of cysts needed for experimental studies. New stock CD1 mice were infected with 10 tissue cysts by oral gavage.

2.5 Acute infection model

2.5.1 In vivo imaging and set up IVIS Spectrum 200 Series

2.5.1.1 Preparing Luciferin

The D-luciferin potassium salt (Caliper Lifesciences) solutions were prepared fresh prior to imaging experiments. 15mg/ml solution were prepared in filtered PBS.

2.5.1.2 In vivo Imaging procedure

Starting on the day fourth post infection *in vivo* imaging was carried out using the IVIS Spectrum. The group being imaged was injected intraperitoneally with 200µl of Luciferin as prepared previously. Mice were anaesthetised with a 2.5%-3.5% isoflorane/oxygen mix. Once the animals were anaesthetised, they were relocated to the imaging chambers ventrally to the nose cones that attached to the manifold inside the imaging chamber so that anesthesia could be maintained. At 20 minutes post-injection with D-Luciferin imaging was carried out with a 1-minute exposure, medium binning on an open filter. This was shown to establish the peak of the bioluminescent signal following conducting of D-luciferin solution (Woods, 2012)

2.1.4 Cytokines measurement in plasma collected during acute infection

To measure the cytokines Interferon gamma (IFN-γ), Monocyte chemoattractant protein 1 (MCP1) Interleukin 12 (IL-12p70), Interleukin 10 (IL-10), Interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF-α) by using the BD Cytometric Bead (Biosciences.BD, UK). The blood samples were collected from infected male and female mice, in addition to uninfected male and female mice at day 6 post-infection. Following the manufacturer's recommended protocol, the standard concentration solutions with a ten-point curve, were prepared by serial dilutions. Then 50µl of samples, standards capture beads suspension was added to wells of 96 filtered plate followed by incubation period of a 2 hours. Subsequently, 10µl of detection reagent solution was added to each well for a further hour. The supernatant was removed by applying the plate to a vacuum manifold. The beads were re-suspended in 200µl wash buffer and transferred into 5ml tubes to be read on the BD FACS Canto 2. Acquisition was undertaken using a BD FACSDiva™ software. (BD Biosciences, Oxford, UK), and results determined using Kaluza 1.3 Analysis Software to flow data analysis

(Beckman Coulter, UK). The reading in flow cytometry was done by my colleague Dr. Stuart Woods.

2.5.3 Weight percentage and survival rate for the acute model

During the infection course each mouse was weighed daily, and the percentage change from the initial start weight was calculated. The overall survival rate for each group was also calculated as a percentage.

2.6 Sex hormones mediate MCP-1/CCL2 production by BMMs in a dose-dependent manner

2.6.1 Producing mouse bone marrow-derived macrophages (BMMs)

Bone marrow cells were cultured from the femur and tibia bones of 6-8-week old BALB/c male mouse, and was sacrificed by cervical dislocation. The femur and tibia removed and cleaned from adherent tissues and washed with 70 % ethanol. The bones were cut at both ends and the bone marrow obtained inserting a 15 guage needle into the cavity and flushing each bone with complete Dulbeccos Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific, UK) containing of 20% Fetal Calf Serum (FCS) (Gibco, Thermo Fisher Scientific, UK), 2% Penicillin/ streptomycin solution (Sigma-Aldrich, UK), 2% L-Glutamine (Gibco, Thermo Fisher Scientific, UK). The eluted cells then collected, filtered using a 40µm cell strainer (Falcon, Fisher Scientific, UK) and centrifuged at 1400 rpm for 5 minutes. After that the supernatant was discarded the pellet resuspended in 10ml per bone of complete DMEM medium with addition of 30% of L cell conditioned media (containing Macrophage-colony stimulating factor (M-CSF) was added to the cell pellet. The cells were cultured in 10cm2 Petri dishes, 10ml of cells per plate. The dishes were maintained at 37°C in humidified conditions and 5% (v/v) CO_2. On day three 10 ml of fresh complete DMEM

was added to feed the macrophages. On day seven all of the media was replaced with 20ml fresh complete DMEM containing 30% L cell conditioned media. On day ten the cells were harvested by adding 5ml ice cold medium (RPMI-1640, Lonza, USA) and scraping them to allow adherent cells to detached. The cell suspension was centrifuged at 1400 rpm for 5 minutes. The pellet was resuspended in complete RPMI and cell count and was determined using trypan blue (Sigma, Dorset UK). The cell concentration was adjusted to 2 x 10^6 cells/ml. The phenotype of the cells was characterised using flow cytometry. Cells were plated in 96 well plates (TPP, Switzerland) at 2 x 10^5 cells/well.

2.6.2 Flow Cytometry

0.5x10^6 cells were resuspended in 100µl FACS Buffer (2 % Bovine Serum Albumin (Sigma, Dorset UK) in PBS (Gibco, Thermo Fisher Scientific, UK) containing specific anti-CD11b (FITC) (BD Pharmingen) and anti F4/80 (PE) (eBioscience) antibodies at 1/100 dilution and incubated for 30 minutes on ice, in the dark. Single stain and unstained controls were set up in parallel. Following incubation samples were washed with 5 ml FACS Buffer and centrifuged at 300g for 6 minutes. This was repeated a further 2 times. After the final wash the cells were resuspended in 300µl FACS Buffer for analysis. Analysis was carried out using BD FACS Canto II running FACSDiva immunocytometry system (BD Pharmingen). Voltages and system compensation were set up using unstained and single stain controls prior to samples being ran. Samples were run at a medium flow rate and 20,000 events collected per sample. Analysis was carried out using FlowJo software to calculate cell populations positive for CD11b and F4/80. This work was carried out by my colleague Dr. Kerrie Hargrave.

2.6.3 Sex hormones preparation of for BMMs treatment

β-Estradiol

50mg of β-Estradiol (Sigma-Aldrich, UK) was dissolved in 3 ml 100% Ethanol (Sigma-Aldrich, UK) with 61189µM concentration. To make 250µM as stock concentration 20.42µl of hormones solution was diluted in about 5000µl of complete medium. From the stock concentrations, the serial dilution was made as the following (62.5, 3.3, 15,6, 7.8, 3.8).

Testosterone

50mg of Testosterone (Sigma-Aldrich, UK) was dissolved in 1 ml 100% Ethanol (Sigma-Aldrich, UK) to make 17335µM stock solution. To make 250µM as stock concentration 20.42µl of hormones solution was diluted in about 5000µl of complete medium. From the stock concentrations, the serial dilution was made as the following (62.5, 3.3, 15,6, 7.8, 3.8).

2.6.4 Treatment of BMM cells with sex hormones and stimulation with *T. gondii* and LPS

Bone marrow derived macrophages were plated out with $4x10^5$ cells/well in a 96 well plate and allowed to adhere overnight. Cells were infected with FLUC *T. gondii* with$16x10^5$ cells/well. β-Estradiol or Testosterone were added at final concentrations as mentioned above. Cells were stimulated with LPS at a final concentration of 2µg/ml, the final volume in all wells was 200µl. All samples were plated out in triplicate with appropriate untreated, uninfected and unstimulated controls. Plates were incubated for 24 hours. at 37°C in a humidified condition of 5% (v/v) CO_2.

2.6.5 Measure MCP-1/CCL2 concentration from supernatant of BMMs treated with sex hormones and stimulated with *T. gondii* and LPS

After 24 hours of incubation period, MCP-1/CCL2 concentrations in the supernatant were measured by ELISA Sandwich (CCL2/JE/MCP-1 DuoSet ELISA, R&D Systems, UK). Following the manufacturer's recommended protocol, the capture antibody was diluted to the working concentration in PBS and used to coat 96-well microplate with 100µl per well. The plate then was sealed and incubated overnight at room temperature. Next day, each well aspirated and washed with wash buffer (0.05% Tween® 20 (Sigma-Aldrich, UK) in PBS, pH 7.2-7.4), this was carried out a total of three times. Each plate was blocked by adding 300µl blocked reagent diluent (1% BSA (PAA Cell Culture Company, Cambridge) in PBS) to each well and then incubated at room temperature for 1 hour. Plates were washed three times as before. Standards and samples were diluted in reagent diluent and 100µl of each was added to the appropriate wells. Plates were sealed with an adhesive strip and incubated for 2 hours at room temperature. Plates were washed and 100µl of detection antibody was diluted in reagent diluent at working concentration and added to each well. The plates were covered with adhesive strip and incubated for 2 hours at room temperature. Following washing, 100µl of Streptavidin-HRP was added to each well and then the plates were incubated in dark for 20 minutes at room temperature. After washing, 100µl of substrate solution consist of 10ml of sodium acetate (Sigma-Aldrich) pH 5.5, 100µl of TMB (Thermo Scientific), and 5µl of H_2O_2 (Sigma-Aldrich) was added into each well, and then incubated for 20 minutes in the dark. To stop the colorimetric reaction 50µl of stop solution which is 10% (v/v) H_2SO_4 (Sigma-Aldrich) was added to each well. The absorbance was immediately read, using a microplate reader set at 450 nm (Spectramax 190, Molecular Devices, USA).

2.7 Chronic infection model

2.7.1 Weight percentage and survival rate during chronic infection

During the infection course each mouse was weighed daily. and the percentage change from the initial start weight was calculated. The overall survival rate for each group was also calculated in percentage.

2.1.2 Histopathology analysis

Tissue sections were prepared for histopathological analysis and stained with Haematoxylin and Eosin using standard procedures at Queen Elizabeth University Hospital, Glasgow. Tissue analysis was overseen and verified by Dr. Fiona Roberts who is a Consultant Pathologist at the Queen Elizabeth University Hospital, Glasgow.

2.1.3 Brain sampling for RNA transcriptomic experiments

After sacrificing the animals, the brains immediately collected and divided sagittal into their 2 hemispheres. Tissues were completely submerged in a collection vessel containing RNAlater (Qiagen), approximately 10µl reagent per 1mg of tissue, and stored in -20°C until RNA extraction.

2.1.4 Isolation and extraction of whole RNA from mouse brain

To extract whole RNA from brain samples, the RNeasy purification kit (Qiagen) was used as specified in the manufacturer's instructions. RNA integrity was assessed using a Bio-analyser kit (Agilent RNA 6000 Nano Bio-analyser assay). The samples were stored at -80^{o}C until sent to GTCA Biotech (Germany) on dry ice for RNA sequencing data and transcriptomic analysis.

2.1.5 GATC expression analysis

Reference genome

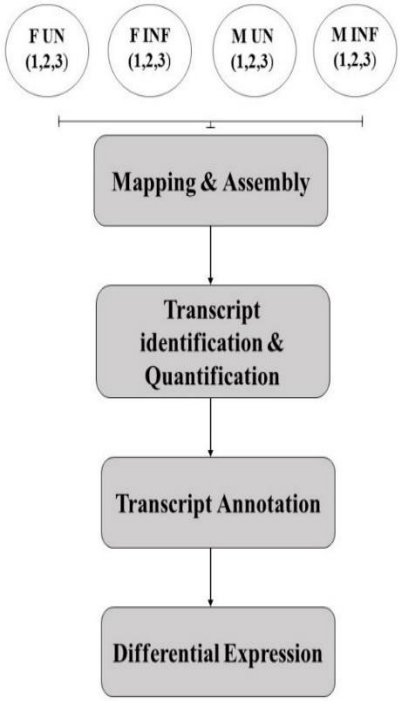
Organism: Mouse

Genome: mm10 / GRCm38, Ensembl

Annotations: v85 Ensembl

Workflow

Schematic chart of the data analysis



2.1.6 Expression Analysis

Expression analysis was performed at GATC. Briefly, the RNA-Seq reads were aligned to the reference transcriptome using Bowtie generating genome / transcriptome alignments. TopHat was used to recognize the possible exon-exon splice junctions of the primary alignment. Then Cufflinks was used to recognize and calculate the transcripts from the preprocessed RNA-Seq alignment-assembly. Following that, Cuffmerge was used to combine the recognized transcript pieces to full length transcripts and annotate the transcripts based on certain annotations. As a final point, combined transcripts from the groups of mice were compared using Cuffdiff to define the differential expression levels at transcript and gene level containing a measure of significance between samples / conditions.

2.1.7 Frozen brain tissues during the chronic infection

Brain tissues were collected, at day 30 post-infection for immunohistochemistry analysis, the half of the brain for each sample was embedded in Optimal cutting temperature compound (OCT compound) (Cellpath, Thermo Scientific, UK) prior to frozen sectioning on a microtome-cryostat (Shandon Thermo Fisher Scientific, UK). The frozen brain samples were sectioned at a 7 µm thickness then were replaced into adhesion slides (Thermo Scientific™ SuperFrost Plus™). The sides were store -20°C until used.

2.1.7.1 Detection of apoptotic cells in brains tissue

The TUNEL Assay Kit - HRP-DAB (abcam 206386) (previously called In situ Apoptosis Detection Kit (DAB) was used following the manufacturer's recommended protocol to detect DNA fragmentation in tissue sections:

Tissue fixation and hydration

For fixation step the slide was immersed in ice cold mixtures of 75% Acetone and 25% Ethanol for 15 minutes at room temperature and gently drained off excess liquid and carefully dry the glass. To help hold reaction volumes around the tissue, the tissue encircled using a waxed pen (ImmEdge Hydrophobic Barrier PAP Pen, Vector) and the wax allowed to dry for about 30 minutes. For hydration the slide immersed in Tris-buffered saline (1X TBS, 20mM Tris pH 7.6, 140mM NaCl) (Sigma, UK) for 15 minutes at room temperature and carefully dried the glass slides around the tissue.

Performing the TUNEL Assay

Proteinase K was diluted 1:100 in dH_2O, the entire tissue section covered with 100µl of Proteinase K and incubated for 10 minutes at room temperature. Slides were rinsed with 1X TBS for 5 minutes and excess liquid gently removed. The tissue section was covered with 100µl of 3% H_2O_2 diluted in methanol and incubated for 5 minutes at room temperature. The slide was rinsed with 1X TBS as before then the section covered in 100µl of TdT Equilibration buffer and incubated for 30 minutes at room temperature. The TdT Equilibration Buffer was carefully blotted.

The working TdT Labeling Reaction Mixture prepared as follows: for each tissue, a 1 in 40 dilution of TdT Enzyme was prepared in TdT Labeling Reaction Mix in a clean tube 40µl of TdT Labeling Reaction Mix was immediately applied onto each tissue section and covered the with a coverslip to assure even distribution. The slide was placed in a humidified chamber and incubate at for 1.5 hour at 37ºC. The coverslip was then removed and the slide washed in TBS solution for 5 minutes in a Coplin jar. The tissue section was then covered with 100µl of Stop Buffer and incubated at room temperature for 5 minutes. Rinsing step was repeated and the tissue blocked by 100µl

of Blocking Buffer for 10 minutes. Then 100µl of conjugate diluted in Blocking Buffer was applied into the tissue and incubated in a humidified chamber for 30 minutes at room temperature. Rinsing step was repeated. DAB solution prepared by adding 4µl DAB solution 1 to 116µl DAB solution 2, the tissue covered with 100µl of DAB solution and incubated for 15 minutes at room temperature. The slide was rinse gently with dH_2O and the entire tissue then covered with 100µl of Methyl Green Counterstain solution and incubated for 3 minutes. After draining off the counterstain the slide was placed in a Coplin jar and dipped 4 times into 100% ethanol. Followed by dipping the slides 4 times into 100% xylene (Shandon Thermo Fisher Scientific, UK). Finally, wiping excess xylene from back of slide and around tissue and a glass coverslip mounted using mounting media (Vector Laboratories, UK) over the tissue. The slide was examined by light microscope (Nikon Eclipse 50 I). A digital camera (Nikon Digital Sight DS-U3) linked with a computer has NIS Element F 3.2 imaging software was used for capturing the photos of examined sections. For microscopic examination blind processing was applied to prevent potential bias.

2.1.7.2 Detection for NLRP3 expression in tissue brain

Frozen sections of brain tissue prepared as shown above was used for immunohistochemistry staining in in order to detect NLRP3 expression. After tissue fixation by an ice-cold mixture of 75% acetone and 25% ethanol for 10 minutes, the section was outlined by wax and allowed to dry for 45 minutes. For rehydration, the slide was immersed in 1xPBS for 15 minutes. To block endogenous peroxidase activity, the section was immersed in 0.3% H_2O_2 diluted in methanol and incubated for 15 minutes. The slide was washed in x 5 minutes in 1xPBS. To prevent non-specific binding a blocking step was applied in tissue by mixture of 5% BSA and 10% normal donkey serum (abcam, UK) with 0.05% Tween20 diluted in 1xPBS for 15

minutes. The tissue was incubated with 1:200 of the Goat polyclonal to NLRP3 (abcam, UK) diluted in 1% BSA for 4 hours at 37ºC. The washing step was repeated and the secondary antibody of Donkey Anti-Goat IgG H&L (HRP) (abcam, ab205723) was diluted 1:2000 in 1% BSA of blocking buffer and incubated with tissue for 1 hour, followed by washing step as previously. For visualization, ImmPACT® AMEC Red Peroxidase Substrate kit was used (Vector, UK) and prepared as following: 5ml of ImmPACT AMEC Red Diluent mixed with 90µl of Reagent 1 and 80µl of Reagent 2 then incubated with the tissue for approximately 20 minutes. To stop the colorimetric reaction, the slide was immersed in 1xPBS for 1 minute. Finally, the slide was placed in a Coplin jar and counterstained in haematoxylin for 1 minute, which was followed by washing under running tap water for about 5 minutes. Excess water wiped from the back of slide and around tissue and a glass coverslip mounted using mounting media over the tissue. The slide was examined by light microscopy (Nikon Eclipse 50 I). A digital camera (Nikon Digital Sight DS-U3) linked with a computer has NIS Element F 3.2 imaging software was used for capturing the photos of examined sections. For microscopic examination blind processing was applied to prevent potential bias.

2.1.7.3 Detection for NLRP3 expression in Bone Marrow Macrophages (BMM)

After producing BMMs as described above, the cells were plated 1 x 10^6 cells/ml on 4 well chamber slides (Thermo Scientific Nunc) and incubated overnight at 37°C with media or media containing 2µg/ml of LPS in a humidified condition of 5% (v/v) CO_2. Cell culture medium was removed and the slides washed in PBS. Cell were fixed with ice cold mixtures of 75% acetone and 25% ethanol for 10 minutes. Slides were washed with PBS three times and incubated with cold methanol for 10 minutes. The washing step was repeated. The cells were incubated with mixture PBS and

0.05%Triton x-100 for 5 minutes. The cells were blocked with PBS, 1% BSA and 5% FCS for 1 hour. The cells were incubated with 1:200 of the Goat polyclonal to NLRP3 (abcam, UK) diluted in 1% BSA of blocking buffer for 4 hours at 37ºC. The washing step was applied as shown above. The secondary antibody of Donkey Anti-Goat IgG H&L (HRP) (abcam, ab205723) was diluted 1:2000 in 1% BSA of blocking buffer and incubated with cells for 1 hour. Then washing step was applied. For visualization ImmPACT® AMEC Red Peroxidase Substrate kit was used (Vector, UK) and prepared as following: 5ml of ImmPACT AMEC Red Diluent mixed with 90µl of Reagent 1 and 80µl of Reagent 2 then incubated with the tissue for approximately 20 minutes. To stop the colorimetric reaction, the slide was immersed in 1xPBS for 1 minute. A glass coverslip mounted using mounting media over the slides. The slide was examined by light microscopy (Nikon Eclipse 50 I). A digital camera (Nikon Digital Sight DS-U3) linked with a computer has NIS Element F 3.2 imaging software was used for capturing the photos of examined sections. For microscopic examination blind processing was applied to prevent potential bias.

2.8 Data Analysis

All the statistical analysis was done by Prism Software 8 (Graph Pad) and Mann-Whitney U test applied to identify P value ≥ 0.05. for means of 5 samples of each group.

2.9 Software

A commercially available software package, SIMCA version 14.0 (MKS UmetricsAB, Sweden) was used to perform Multivariate data analysis (MVDA).

Chapter 3 . Acute infection model

3.1 Introduction

Sex differences have been demonstrated to significantly influence the response to *T. gondii* infection in at least some mouse strains. Immune competent female BALB/K mice have been demonstrated to have increased weight loss and mortality compared with male mice when infected with *T. gondii* (Roberts et al., 1995). Furthermore, female severe combined immunodeficient (SCID) mice have been shown to have increased pathology in their brains and quantitative differences in their immune responses compared with male mice (Walker et al., 1997). Together these observations demonstrate that early events associated with innate immune response likely play a role in sex-dependent susceptibility and immunity to *T. gondii* infection. However, these early events in immune competent mice have been poorly characterized, in particular the potential influence of sex differences in early parasite replication, and inflammatory responses, have not been well studied.

However, since the original studies described above, advances in technology should now allow a reappraisal of this situation.

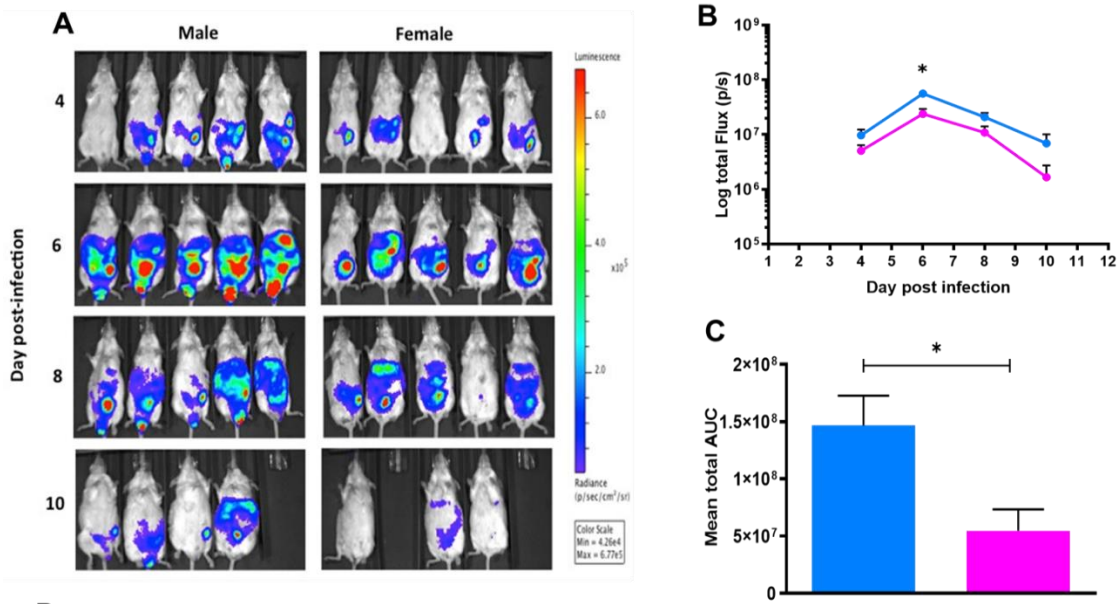
The studies described herein make use of more recently available technology to further our knowledge of sex differences in the response to *T. gondii* infection. Specifically, (i) through use of an in vivo imaging system (IVIS) and luciferase expressing *T. gondii*, parasite multiplication and location can be quantified at multiple timepoints in the same mice, (ii) multiple diverse immunological mediators can be quantified in small blood samples using a cytometric bead array, in male and female BALB/c mice infected with *T. gondii.*

3.2 Results

A total of 4 separate experiments were performed to determine the effects of sex on *T. gondii* infection. The data shown is from a single experiment, but the data has also been analysed collectively using a ratio paired t test.

3.2.1 Male mice have increased *T. gondii* multiplication compared to female mice

Five male and five female mice were infected intraperitoneally with $2x10^4$ type II Prugniaud tachyzoites, expressing firefly luciferase and imaged at 4, 6, 8 and 10 post-infections. A bioluminescent signal was visible by day 4 post-infection, originated exclusively from the abdomen of both sexes. Male mice also had a bioluminescent signal in their testicles. Bioluminescence increased in male and female mice over the course of the study, peaking at day 6. By day 10 the signal in mice was decreased and in some cases no longer detectable **(Figure 3.1 A)**. Quantitative analysis of parasite burdens was performed by calculating the log total light flux using LivingImage4.0 software and expressed as photons per second. The region of interest (ROI) in the data shown include the testicles, but similar data was obtained when the ROI was set to remove the signal from the testicles. In the single experiment shown, parasite burden was significantly higher among males in comparison to females in particularly at 6-day post infection when parasite numbers peaked **(Figure 3.1 B)**. In this experiment, the mean of total Area Under Curve (AUC) of the parasite burden during infection course between male and female groups was significantly higher in male mice than female mice **(Figure 3.1 C).** The data from the 4 individual experiments are detailed in **(Table 3.1)** and demonstrate that female mice had significantly less parasites ($p=0.021$) using a ratio paired t test.



D

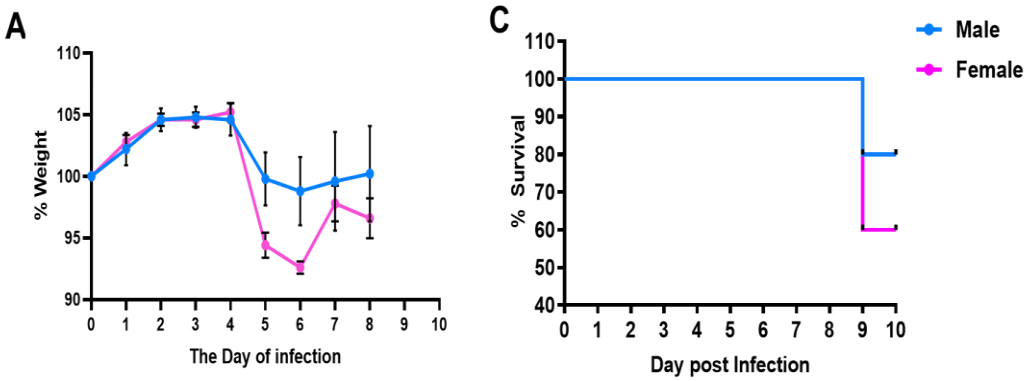
Runs	1	2	3	4
Male	1.1763×10^9	105.2211×10^6	31×10^6	119.6×10^6
Female	84.6×10^6	51.08×10^6	13.77×10^6	110.95×10^6

Table 3.1 Male mice have increased *T. gondii* multiplication compared with female mice

(A) Five male and five female mice were infected with $2x10^4$ (ip) type II *Prugniaud* tachyzoites, expressing firefly luciferase. Mice were imaged at 4, 6, 8 and 10 days post-infections. This signal increased during the infection course and its peak was at day 6. By day 10, the signal was reduced or no longer detectable in some of mice. **(B)** Quantitative analysis of parasite burdens was significantly higher among males in comparison to females in particularly at 6-day post infection when parasite numbers peaked. **(C)** The result of the mean of total Area Under Curve (AUC) of the parasite burden during infection course between male and female groups was significantly higher in male mice than female mice. Each value represents the mean of 5 animals per experimental group were analysed using one-tailed nonparametric Mann–Whitney analyses ± SEM. *p<0.05. The results shown a representative of 4 independent experiments with similar results. **(D)** The means of AUC from the 4 individual experiments had also been analysed using a ratio paired t test (p=0.021).

3.2.2 Female mice increased mortality following *T. gondii* infection compared to male mice

Following infection, male and female mice gained a small quantity of weight, until day 4 post-infection. However, a decline in weight was observed in males and females by day 6. Data from a single representative experiment is shown **(Figure 3.2 A)**. When the data from the 4 experiments were analysed using a ratio paired t test, female mice had lost significantly more weight than male mice (p=0.043) **(Table 3.2 B)**. Female mice had reduced survival compared with male mice. Data from a single representative experiment is shown **(Figure 3.2 C).** When the data from the 4 experiments were analysed using a ratio paired t test (p=0.002) **(Table 3.2 D)**.



B

Runs	1	2	3	4
Male	99	96	105	103
Female	93	96	102	99

D

Runs	1	2	3	4
Male	80	60	80	50
Female	60	40	50	30

Figure 3.1 Female mice are more susceptible to *T. gondii* infection compared to male mice

(A) Weight differences from a single representative experiment. **(B)** The table of weight percentage of day 6 post-infection of multiple runs analysed using ratio paired t test (p=0.043) **(C)** Survival percentage from a single representative experiment. **(D)** The table of survival percentage of multiple runs analysed using ratio paired t test (p=0.002). Each value represents the mean of 5 animals per experimental group were analysed.

3.2.3 Quantitative analysis of cytokines detected in the plasma of male and female mice infected with *T.gondii*

A cytometric bead array (CBA) was used to simultaneously determine the level of cytokines IL-6, IL-10, MCP-1, IFN-γ, TNF-α and IL-12p70 in the serum of uninfected mice and mice infected with *T. gondii* at 6 day postinfection. Generally, there were significant increases in most cytokines levels within the infected group compared to the uninfected group in both sexes. However, MCP-1, IFN-γ and TNF-α cytokine levels were greater in infected females compared with infected males ($p=0.05$, $p=0.05$ and $p=0.03$, respectively) **(Figure 3.3).** Each value represents the mean of 5 animals per experimental group and were analysed using one-tailed nonparametric Mann–Whitney analyses ± SEM *$p < 0.05$.

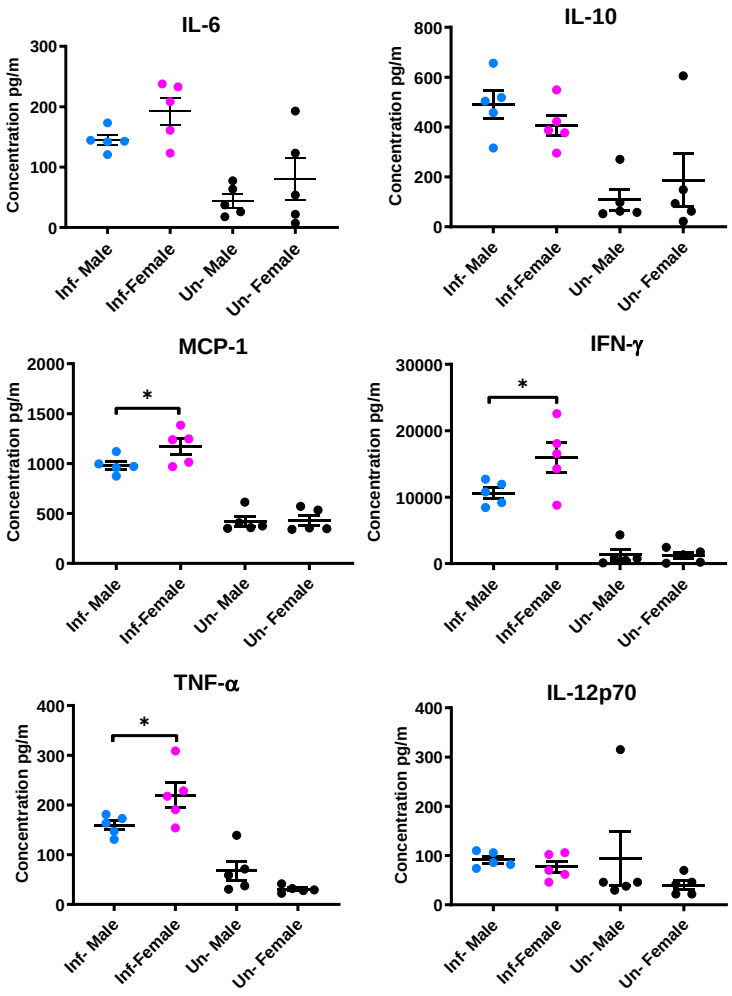


Figure 3.2 Cytokines concentration in the plasma of male and female mice infected with *T.gondii*

IL-6, IL-10, MCP-1, IFN-γ, TNF-α and IL-12p70 were detected. The levels of these cytokines significantly increased in infected mice compared to uninfected mice. However, in female group MCP-1, IFN-γ and TNF-α cytokines significantly produced in comparison to the male group with (p=0.05, p=0.05, and p=0.03, respectively). Each value represents the mean of 5 animals per experimental group and were analysed using one-tailed nonparametric Mann–Whitney analyses ± SEM. *p< 0.05.

3.3 Discussion

Studies of a wide variety of inbred mice of diverse genetic backgrounds and major histocompatibility complex haplotypes found a difference in the susceptibilities of males and females to *T. gondii* infection (Roberts et al., 1995). Roberts et al.,*1995a* demonstrated that female mice from a wide variety of strains exhibited greater levels of mortality and weight loss than their male counterparts (Roberts et al., 1995). However, enumeration of parasite burdens during the acute phase of infection was not easy to achieve and thus not reported in these early studies. To overcome this, this study used *T. gondii* genetically engineered to express the luciferase gene to allow facile quantification using an In Vivo Imaging System (IVIS). Female BALB/c mice infected with these genetically altered parasites were confirmed to be more susceptible to acute infection, as determined by higher mortality rates and weight loss. However, importantly and contrary to expectations, the current studies demonstrate that male mice had higher parasite burdens during the acute infection despite their lower mortality. The reasons for this and the contribution of the immune response was therefore examined in more depth.

Plasma collected from experimental animals at 6 day postinfection was used to determine cytokine production during *T. gondii* infection. Results showed that infected male and female mice exhibited a significant increase in levels of most of the examined cytokines namely, IL-6, IL-10, MCP-1, IFN-γ, TNF-α and IL-12p70 compared to uninfected group. However, pertinent to the sex differences found in *T. gondii* immunity, MCP-1, IFN-γ and TNF-α concentrations were found to be significantly higher in plasma from infected female mice than male mice. It is well known that these cytokines play a key role in the pathogenesis of toxoplasmosis. The induction of a type 1 inflammatory response with IFN-γ and TNF-α is known to be

important during the early stages of immunity to *T. gondii* (Jones et al., 2006). IFN-γ is the main mediator of resistance to *T. gondii* and stimulates multiple intracellular mechanisms to kill the parasite and inhibit its replication. Many studies of mice deficient in IFN-γ demonstrate that they are susceptible to *T. gondii* infection and fail to control parasite burden (Dupont et al., 2012). Consistent with the higher levels of IFN-γ in female mice infected with *T. gondii,* female C57BL/6 mice when inoculated with *Plasmodium chabaudi* exhibited higher expression of IFN-γ compared to infected male mice (Cernetich et al., 2006).

TNF-α is also critical for controlling disease and often acts in concert with IFN-γ to restrict parasite replication. Furthermore, TNF-α and IFN-γ, through stimulation of nitric oxide have been shown to contribute to induction of stage conversion and the formation of tissue cysts (Donahoe et al., 2017). TNF-α is also an important factor for control of *T. gondii* in vivo and survival of acute and chronic murine toxoplasmosis. Since, TNF knockout mice failed to control intracerebral *T. gondii* and succumbed to an acute necrotizing toxoplasma encephalitis as well as early after infection–splenic NO levels were reduced (Schlüter et al., 2003). Notably, mice deficient in the TNF receptor subunit (TNFRp55) are more susceptible to intrapertoneal infection with *T. gondii* and develop increase parasie numbers in their liver, lung and spleen (Silva et al., 2009). In keeping with the increased levels of TNF-α in female mice infected with *T. gondii.* recent study found women respond to bacterial endotoxin with greater pro-inflammatory responses including significantly higher increases levels of TNF-α (Wegner et al., 2017).

Monocyte chemoattractant protein 1 MCP-1/CCL2 is is known to be produced during the early phase of *T. gondii* infection. Mice deficient in the CCR2 chemokine receptor (its ligand), fail to recruit monocytes and succumb to infection. Both CCR2-/- and

MCP-1-/- mice show amplified mortality and pathology in response to oral *T. gondii* challenge (Gov et al., 2013). Sex differences in MCP-1/CCL2 have been found in macaques with females having significantly higher levels of MCP-1/CCL2 in their in plasma compared to their male counterparts during HIV infection. The elevated levels of MCP-1 in female macaques was surprising given the lower viral loads in HIV infected women reported in earlier studies (George et al., 2019).

Overall the studies described in this chapter suggests that female mice respond to *T. gondii* infection with a greater inflammatory cytokines than male mice. The increased level of these cytokines in the female mice may explain why they had less parasite burden during the acute infection of *T. gondii* than the male group. However, the raised levels of cytokines might also explain why they also suffered more in terms of higher mortality rates and severe disease outcomes. There is now abundant evidence about the major role of cytokine production in the pathogenesis of *T. gondii* and it is also well known that these cytokines play a double-edged sword role during infection. Therefore, the balance in their production is required to maintain their beneficial role in controlling infection while minimising their pathological impact on the host, since, excessive production of proinflammatory cytokines may cause host pathology and tissue damage (Prell and Tarrant, 2018). Pro-inflammatory and anti-inflammatory cytokine responses must be strongly regulated for ideal control of infection and cytokine imbalances due to loss of control can play a key in the pathological alterations linked with toxoplasmosis (Denkers and Gazzinelli, 1998). Similar observation have been observed in mice infected with influenza viruses where females have reduced viral load and increased inflammation (Robinson et al., 2011). The mechanisms of sex differences can be difficult to evaluate and it is sometimes difficult to determien cause and effect. There is overwhelming evidence that sex-associated hormones can also modulate immune responses and it is suggested that

sex hormones may modulate cytokine production in vivo and contribute to gender-related differences in normal and pathological immune responses (Verthelyi and Klinman, 2000). Therefore, the following chapter studies the influnced of sex hormones in MCP-1 production In vitro.

Chapter 4 . Sex hormones influenced MCP-1/ CCL2 production by BMM

1.4 Introduction

Monocyte Chemoattractant Protein MCP-1, also known as Chemokine (C-C motif) ligand 2 (CCL2) is a potent monocyte attractant and is a member of the CC chemokine subfamily (Yadav et al., 2010). CCL2, is a proinflammatory chemokine that stimulates the accumulation of monocytes-macrophages at sites of inflammation (Nakatsumi et al., 2017). MCP-1/CCL2 is a chemoattractant which binds to the CCR2 receptor on monocytes, macrophages and lymphocytes. The importance of CCL2/CCR2 interaction has been observed in acute inflammatory responses by using CCR2 knockout animals which have a reduction of monocytes recruitment to the site of inflammation. MCP-1/CCL2 is a key element in the polarization of Th0 cells toward a Th2 phenotype as CCR2 knockout mice demonstrate impaired Th2 immunity (Zhang et al., 2010). MCP-1/CCL2 is produced by many cell types, including endothelial, fibroblasts, epithelial, smooth muscle and microglial cells (Deshmane et al., 2009), however, monocytes/macrophages are found to be the main source of MCP-1/CCL2 (Yoshimura et al., 1989).

Sex hormones, like estrogen and androgens , have been shown to effect macrophage biology and function via either immunopotentiating or immunosuppressive action depending on concentration of hormone and conditions (Svensson et al., 2015). For instance, estrogen and androgen influence the physiological functions of macrophage via estrogen receptors (ERs) and androgen receptor (AR) respectively, that are expressed in immune cells including macrophages (Klein, 2004, Kadel and Kovats, 2018).

As mentioned in chapter 3, the level of MCP-1/CCL2 was significantly greater in the serum of female mice infected with *T. gondii* in comparison to male at day six post-infection. Therefore, here it is investigated whether estrogen and testosterone affect

the secretion of MCP-1/CCL2 from bone marrow macrophages (BMM) that were infected with *T. gondii*.

4.2 Results

4.2.1 BMMs Characterisation

BMMs were obtained from bone marrow of 6-8 week-old BALB/c male mice. Cells were then cultured in complete DMEM medium supplemented with 20% L929 cells for 10 days to produce BMMs. The cells then were analysed to confirm their macrophages phenotype by flow cytometry. For the in vitro experiments, cells at a minimum were 87% CD11b+ and 91 % of F4/80+. Cells were typically 80% or greater double positive for both markers **(Figure 4.1).**

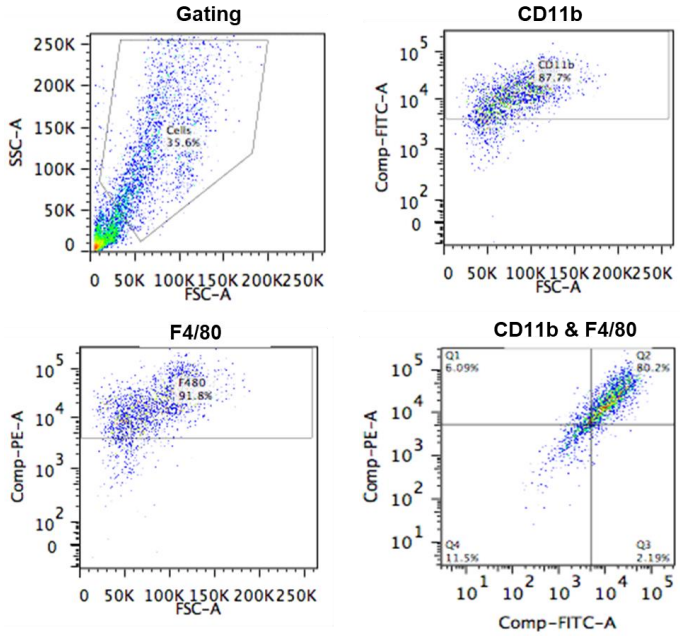


Figure 4.1 Characterisation of BMMs

BMMs were phenotypically characterised by flow cytometry. Live cells were gated by their forward and side scatter. 91.8% of cells were F4/80 positive and 87.7% of cells were CD11b positive. 80.2% of cells were double positive for F4/80 and CD11b.

Standardisation of T. gondii multiplicity of infection (MOI) and LPS concentration used to stimulate macrophages

The effect of multiplicity of infection or MOI (represents the ratio of the numbers of parasite to the numbers of the host cells) was found to determine MCP-1/CCL2 production by macrophages. *T. gondii* induced MCP-1/CCL2 by macrophages in a dose dependent fashion after incubation period of 24 hours. While an MOI of 1:1 and 1:2 induced significant amounts of MCP-1/CCL2 an MOI of 1:4 induced measurably more. LPS was used as positive control and found to induce MCP-1/CCL2 production at all concentrations used after incubation period of 24 hours. All subsequent infections used an MOI of 1:4 for *T. gondii* infection or a concentration of 2µg/ml for LPS stimulation **(Figure 4.2).**

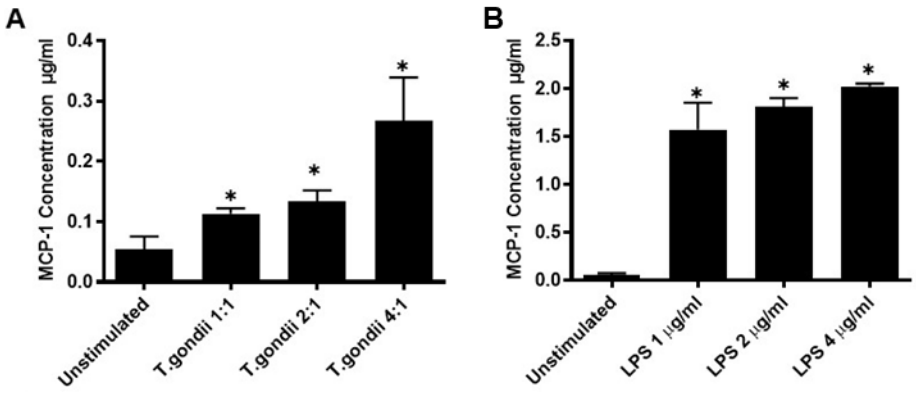


Figure 4.2 Standardisation of *T. gondii* multiplicity of infection (MOI) and LPS concentration used to stimulate macrophages

(A) *T. gondii* induced MCP-1/CCL2 in a dose dependent fashion. **(B)** LPS induced MCP-1/CCL2 at concentrations above 2µg/ml. Each value represents the mean of 3 replicates and were analysed using one-tailed nonparametric Mann–Whitney test ± SEM *p<0.05.

4.2.3 Estradiol 17beta (E2) down-regulates MCP-1/CCL2 production in a dose-dependent manner

BMM were treated with a range of concentrations of E2, starting with 62μM to 3.8μM and then stimulated with *T. gondii* or LPS, after 24hrs incubation. MCP-1/CCL2 levels were measured by ELISA. E2-treated cells had significantly reduced MCP-1/CCL2 production **(Figure 4.3).**

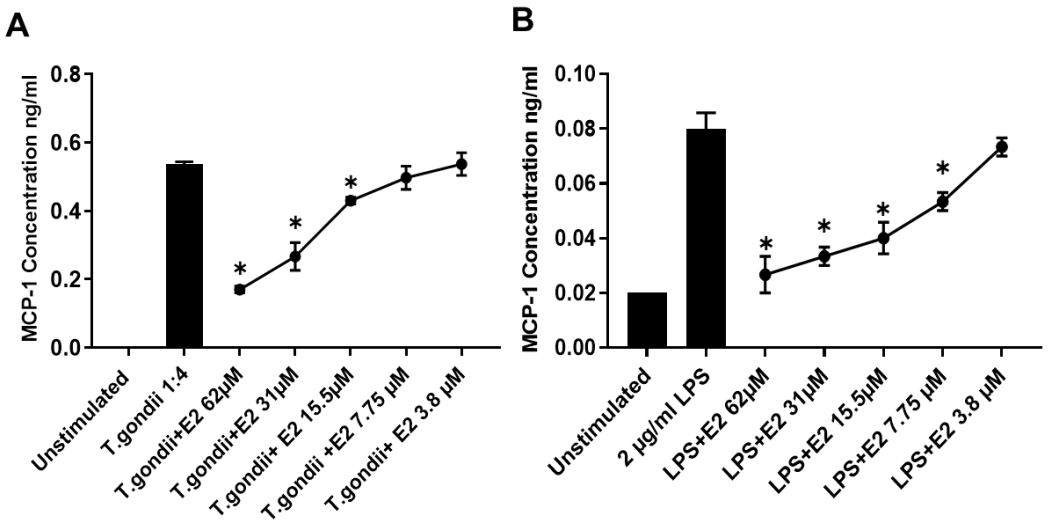


Figure 4.3 Estradiol 17beta (E2) down-regulates MCP-1/CCL2 production in a dose-dependent manner

BMM were treated with a range of concentrations of E2 and then stimulated with **(A)** *T. gondii* **(B)** LPS. Treatment of cells with 62μM to 7.75μM of E2 and stimulated with *T. gondii* or LPS significantly reduced MCP-1/CCL2 production**.** Each value represents the mean of 3 replicates and were analysed using one-tailed nonparametric Mann–Whitney test ± SEM *p<0.05.

4.2.4 Testosterone down-regulates MCP-1/CCL2 production in a dose-dependent manner

BMM were treated with a range of concentrations of testosterone from 62μM to 3.8μM and then stimulated with either *T. gondii* or LPS after 24hrs incubation, MCP-1/CCL2 level were measured by ELISA. Treatment of cells with testosterone prior to stimulation significantly reduced MCP-1/CCL2 production **(Figure 4.4).**

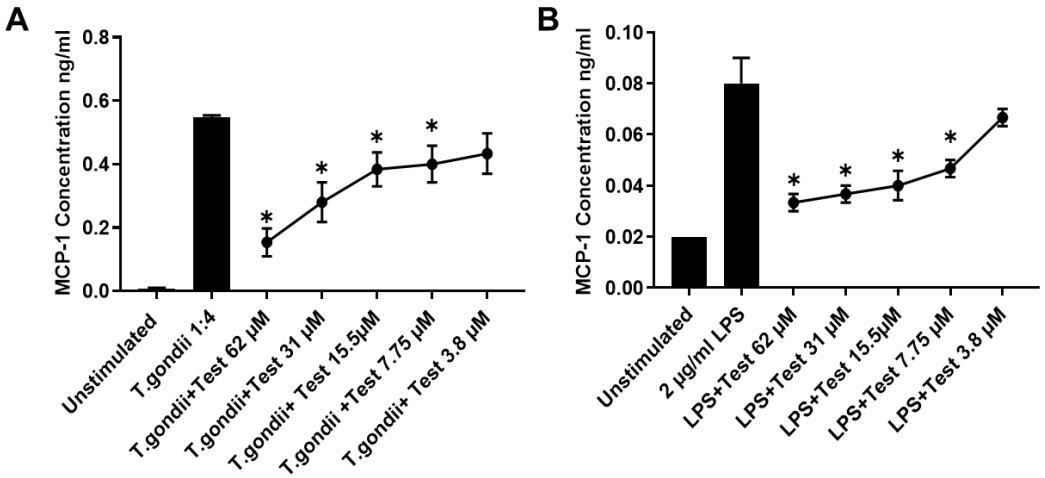


Figure 4.4 Testosterone down-regulates MCP-1/CCL2 production in a dose-dependent manner

BMM were treated with a range of concentrations of testosterone and then stimulated with **(A)** *T. gondii* **(B)** LPS. Treatment of cells with 62μM to 7.75μM of testosterone and stimulated with *T. gondii* or LPS significantly reduced MCP-1/CCL2 production**.** Each value represents the mean of 3 replicates and were analysed using one-tailed nonparametric Mann–Whitney test ± SEM *p<0.05.

Ethanol used as a solvent for E2 and Testosterone does not affect MCP-1 production at concentrations used

Concurrent with the above experiments we determined the effect of ethanol (which was used as a solvent) on MCP-1 production. The concentrations of ethanol examined cover the range used in the E2 and testosterone experiments starting from 0.1% and 0.14% respectively **(Figure 4.5).**

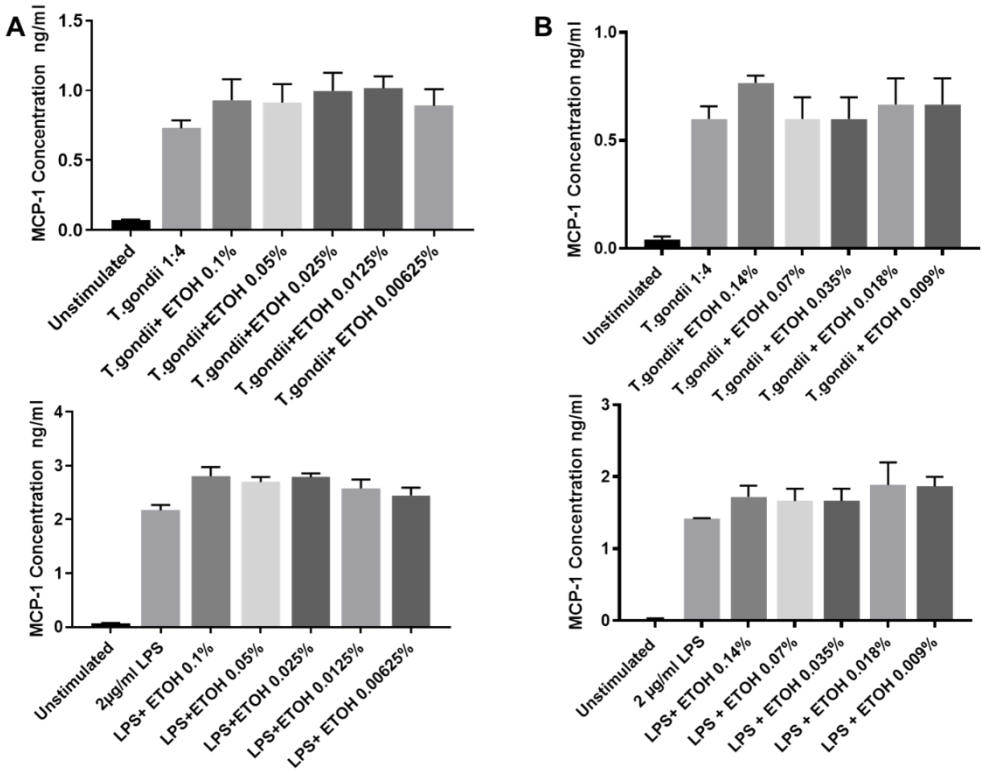


Figure 4.5 Ethanol used as a solvent for E2 and Testosterone does not affect MCP-1 production at concentrations used

(A) Concentration used in dissolving E2 **(B)** Concentration used in dissolving Testosterone. Upper panel for BMM stimulated with *T. gondii*. Lower panel BMM stimulated with LPS.

4.3 Discussion

In the previous chapter MCP-1/CCL2 was found to be present in female mice at higher concentrations than observed in males. It is well known that sex hormones modulate cytokine production within the immune system (Bhatia et al., 2014). Therefore, we studied the influence of the sex hormones estradiol 17beta (E2) and testosterone on MCP-1/CCL2 production by BMMs stimulated with LPS or *T. gondii*. The results show that BMMs stimulated with *T. gondii* tachyzoites produced MCP-1/CCL2. Although this is the first demonstration that macrophages respond in this way to *T. gondii* infection, it is in agreement with a study that found that infection of human fibroblasts with tachyzoites of RH and *Prugniaud* strains of *T. gondii*, significantly increased MCP-1/CCL2 secretion (Brenier-Pinchart et al., 2002). In murine studies, the role of MCP-1/CCL2 during *T. gondii* infection seems to be in enrolling a novel population of Gr-1^{+} monocytes that are able to induce nitric oxide (NO) and TNF-α which have microbicidal activity against *T. gondii*. The importance of MCP-1 and these cells is evident by the finding that mice deficient in CCR2 (the receptor for CCL2), fail to recruit monocytes and succumb to infection (Sukhumavasi et al., 2007, Neal and Knoll, 2014). Gr1+ monocytes have also been shown to mediate mucosal immunity to *T. gondii*, as CCR2−/− and MCP-1−/− mice both exhibit increased mortality and pathology in response to oral *T. gondii* challenge, but are rescued by the adoptive transfer of Gr1+ monocytes (Dunay et al., 2008). However, the influence of sex on MCP-1/CCL2 production during *T. gondii* infection has not until now been studied. The finding that MCP-1/CCL2 is increased in female mice compared with male mice infected with *T. gondii* could be due to differences in the biology of the sexes or alternatively as a consequence of their different susceptibilities to *T. gondii* infection. Therefore, the studies carried out here were to determine if E2 or testosterone influenced MCP-1 production by macrophages exposed to *T. gondii.* The results

revealed that treatment of BMMs with E2 suppressed MCP-1/CCL2 production in a concentration-dependent manner following stimulation with either *T. gondii* or LPS. These results are consistent with a previous study that demonstrated E2 downregulated MCP-1/CCL2 release by monocytes and suggested that this could be related to regulation of MAPK activity (Lee et al., 2012). In addition, E2 was found to prevents MCP-1/CCL2 expression in endometrial stromal cells, suggesting that E2 may regulate endometrial macrophage migration by regulating MCP-1/CCL2 expression (Arici et al., 1999). Moreover, ERαKO mice show increased levels of proinflammatory chemokines (Ccl2, Ccl3, Ccl5, and Cxcl1) during neuroinflammation, implying a role of E2 in regulation of chemokines (Brown et al., 2010). The anti-inflammatory role of high E2 has been largely attributed to regulation of signalling through estrogen receptors (ERs), that suppress activation of NF-κB-mediated inflammatory responses (Robinson et al., 2011). E2 has also been noted to mediate an anti-inflammatory role in cardioprotection and protection of the central nervous system (Yang et al., 2016). Therefore, studies appear to support the observation that E2 ameliorates inflammation and our results appear to be consistent with the opinion that E2 inhibits inflammation. However, the ability of E2 to down-modulate MCP-1/CCL2 does not support the in vivo observation that female mice produce more of this mediator than male mice when infected with *T. gondii.* One possible explanation is that the increased levels of MCP-1/CCL2 observed is female mice, relative to male mice is due to increased immune activation. Alternatively, testosterone might be responsible for limiting MCP-1/CCL2 in male mice.

Treatment of BMMs with testosterone was found to suppress MCP-1/CCL2 production in a concentration-dependent manner following stimulation with either *T. gondii* or LPS. It is known that testosterone has a negative effect on circulating levels of multiple proinflammatory cytokines, including, IL-6, IL-10, and MCP-1/CCL2 and

macrophage activation (Ruige et al., 2012). Furthermore, in vitro studies demonstrate that macrophages and monocytes treated with testosterone have reduced production of proinflammatory cytokines (Kadel and Kovats, 2018, Trigunaite et al., 2015). Removal of testosterone by gonadectomy of young male mice increased inflammation associated morbidity and pathology upon influenza A virus (IAV) infection. This effect could be reversed by the addition of testosterone, which resulted in reduced morbidity, mortality, and inflammation (vom Steeg et al., 2016). Morooka *et al., (2016)* found that MCP-1/CCL2 promoter activity was predominantly suppressed by dihydrotestosterone DHT (the physiologically active metabolite of testosterone) interactions through functional nuclear factor-kappa B (NF-κB) in 3T3-L1 adipocytes co-cultured with RAW264.7 macrophages (Morooka et al., 2016). Our results support the previous findings suggesting a role for testosterone in the inhibition of MCP-1/CCL2 secretion by BMMs. The ability of testosterone to ablate MCP-1/CCL2 production could explain our previous observation that male mice produce less MCP-1/CCL2 during *T. gondii* infection.

In summary, male and female sex hormones appear to have very distinctive and selective roles in remodelling the immune system and immune responses (Trigunaite et al., 2015). The results here propose that sex hormones can reduce inflammation as testosterone and estrogen may decrease immune responses via reducing MCP-1/CCL2 secretion by BMMs, therefore controlling monocytes emigration to the infection site leading to less inflammation during *T. gondii* infection. However, the evolutionary advantage of this different influence of female and male sex hormones on immunity remains uncertain.

Chapter 5 . Temporal progression of histopathological changes in male and female mice infected orally with *T. gondii* tissue cysts

5.1 Introduction

It is well known that *T. gondii* infection can cause histopathological changes in several tissues. Early studies in mice intraperitoneally infected with the ME49 strain of *T. gondii* shows development of inflammation in multiple tissues including lymphoid, hepatic, splenic and brain (McLeod et al., 1984, Conley and Jenkins, 1981). However, sex differences in histopathological changes during *T. gondii* infection have not been studied yet. Therefore, this chapter will compare between male and female BALB/c mice infected orally with *T. gondii* at histopathological level in tissues were collected from spleen, lung, liver, and brain at different days post-infection. Some early studies that examined brain tissues of mice found sex differences in histopathological changes during *T. gondii* infection. These sex differences reflect more severe lesions in female than in male experimental animals (Kittas et al., 1984).

In chapter 3 of this thesis female mice were found to have increased inflammation, but reduced parasite burdens relative to male mice during the early stages of *T. gondii* infection. However, the studies performed in chapter 3 used tachyzoites to initiate infection intraperitoneally and had to be terminated before mice passed humane endpoints. This meant that it was not possible to study the later stages of disease. Therefore, the studies described in this chapter compare the progression of disease in male and female BALB/c mice infected orally with *T. gondii* tissue cysts at the histopathological level. Tissues examined are spleen, lung, liver, and brain at different days post-infection. It is hypothesised that female mice mount a stronger inflammatory response than male mice and might contribute to greater histopathological changes in infected organs. To cross validate this model of infection survival rate and weight loss are also determined throughout the course of infection.

5.2 Results

Mouse spleens, lungs, livers and brains were isolated at days 4, 7, 16, 22, 30 and 42 post-infection and placed in 10% buffered formalin. Tissues were paraffin embedded, sectioned and stained with haematoxylin and eosin (H&E). The samples that exhibited inflammation and abnormal architectures were scored from 0 indicating no changes to 5 indicating high severity of abnormality.

5.2.1 Female mice lost more weight and had poorer survival following *T. gondii* infection compared to male mice

Following infection, male and female mice gained weight until day 8 before starting to lose weight. Female mice generally lost more weight than male mice and this difference was statistically significant (p=0.004) by day 9 post infection **(Figure 5.1 A)**. The survival rate of female mice over the course of the experiment was 60% while 90% of male mice survived (p=0.048) **(Figure 5.1 B)**.

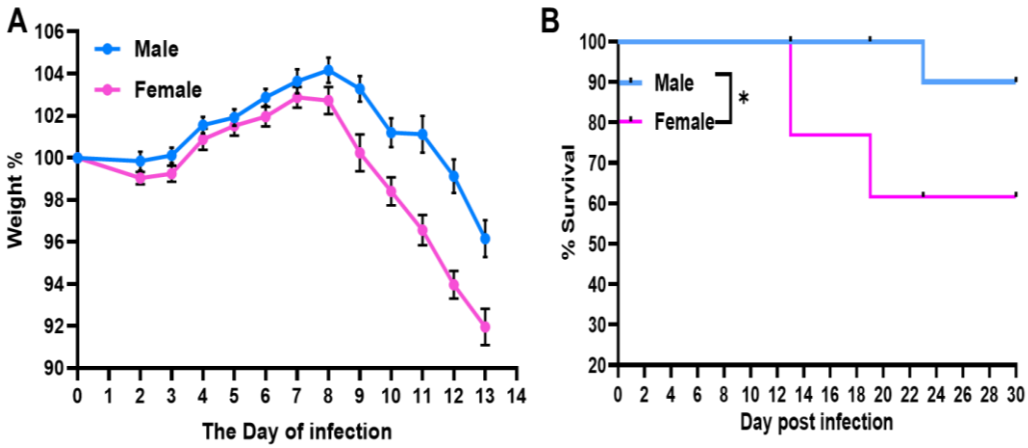


Figure 5.1 Female mice are more susceptible to *T. gondii* infection compared to male mice

(A) Weight differences and the weight at day 9 post infection (p=0.004) was analysed using one-tailed nonparametric Mann–Whitney test ± SEM. **(B)** Survival rate of male and female mice infected with *T. gondii*. Survival was compared using the Gehan-Breslow Wilcoxon Test (p=0.048). Each value represents the mean of 25 animals per experimental group.

5.2.3 Splenic inflammation

Splenic organization was found to be rapidly disrupted during *T. gondii* infection. Examined spleen sections examined from day 7 onwards exhibited abnormal architectures of white pulp **(Figure 5.2).** The severity of these changes was observed to generally increase from day 7 to 42 post-infection. No significant sex difference was observed between males and females **(Table 5.1).**

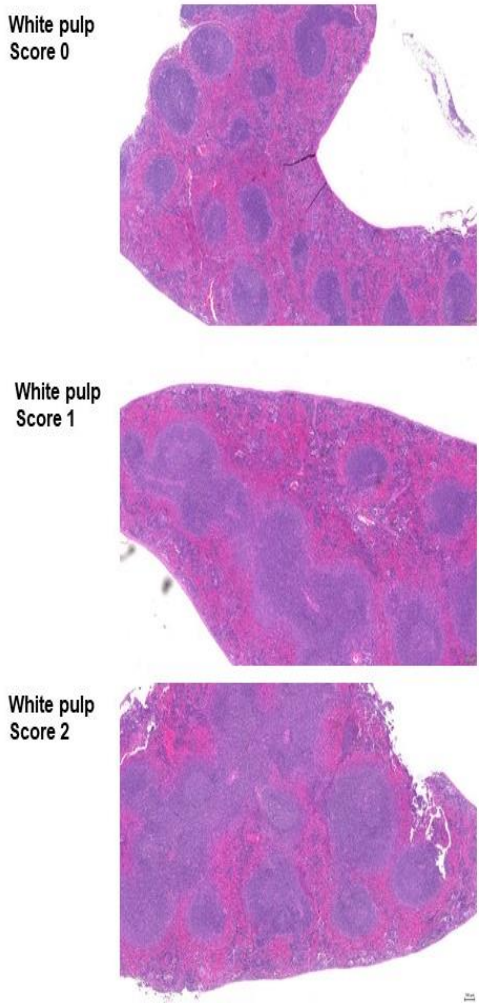


Figure 5.2 Representative images for splenic inflammation during *T. gondii* infection

Scored from 0 no changes to 3 indicating high severity of inflammation distribution. Magnification: 4×H&E. Sections were examined under 4x magnification.

Table 5.1 Splenic inflammation scoring of white pulp distribution following *T. gondii* infection [a]

Day Postinfection	Mouse sex	White pulp
4	M	0 0 0 1 0
	F	0 0 0 0 0
7	M	0 1 1 0 1
	F	0 1 0 1 1
16	M	0 0 1 0 0
	F	0 1 1 1 1
22	M	1 1 1 2 1
	F	1 1 1 1 1
30	M	1 2 2 1 1
	F	2 2 2
42	M	2 2 2 2
	F	2 1 2

[a] Splenic inflammation scoring of white pulp distribution and its development during the course of infection from 0 no changes to 3 high severities. White pulp inflammation developed from day 4 post-infection and increased during the infection. The data represent 5 animals per experimental group.

5.2.4 Toxoplasmosis pneumonia

Pneumonia characterises by thickening of the alveolar wall as well as infiltrating of inflammatory cells during *T. gondii* infection. Lung tissue sections from male and female mice were scored for pneumonitis from day 4 to 42 post-infection **(Figure 5.3)**. While 3 out of the 4 female mice examined on day 4 post infection had signs of pneumonitis, none of the males had any noticeable pneumonitis. All mice exhibited pneumonitis by day 7 post infection and the vast majority of mice examined at the alter timepoints also had a degree of pneumonitis. No notable sex difference was found in terms of pneumonia severity **(Table 5.2).**

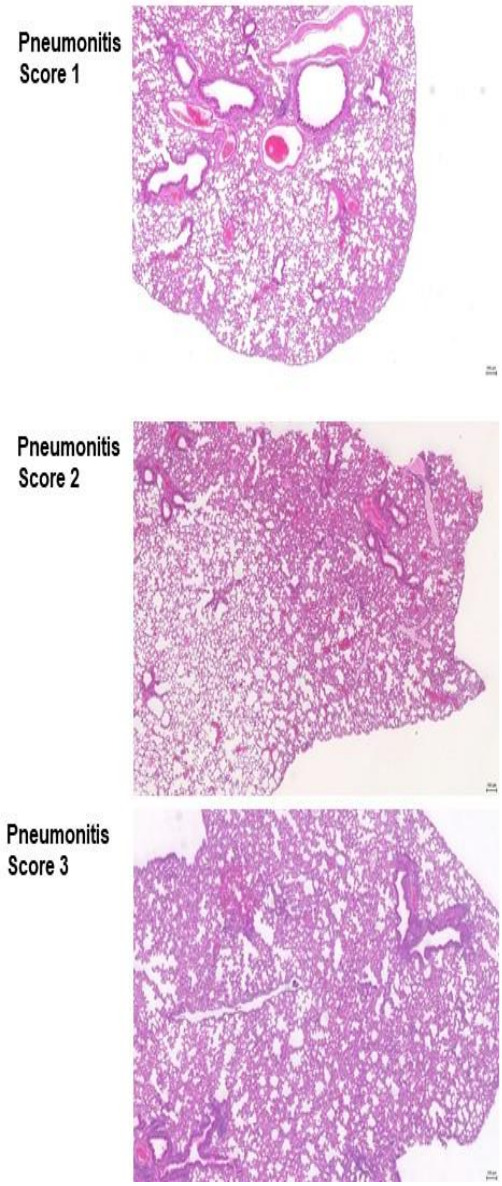


Figure 5.3 Representative images for pneumonitis during *T. gondii* infection

Scored from 0 no changes to 3 indicating high severity of inflammation distribution. Sections were examined under 4x magnification.

Table 5.2 Toxoplasmosis pneumonia scoring [a]

Day Postinfection	Mouse sex	Histological score of : Pneumonitis
4	M	0 0 0 0 0
	F	1 1 0 1
7	M	1 1 2 1 1
	F	2 2 1 1 2
16	M	1 1 1 1 1
	F	1 1 1 1 1
22	M	0 2 1 1 2
	F	1 2 2 2 2
30	M	1 1 1 1 1
	F	2 2 1
42	M	1 1 2 1 1
	F	0 3 2

[a] Toxoplasmosis pneumonia scoring development during the course of infection from 0 no changes to 3 high severities. Pneumonia was noted in female mice from day 4 post-infection and male mice from day 7 post infection and increased during infection. The data represent 5 animals per experimental group.

5.2.5 Toxoplasmosis hepatitis

Liver sections showed histopathological changes such as acinar and portal triad (bile duct, portal vein, and arteriole) inflammation that scored from 0 no changes up to 3 indicating high severity **(Figure 5.4).** The greatest severity of hepatitis was observed during the acute stage of the infection. Acinar inflammation was significantly greater in female mice compared with male mice on day 4 post-infection (p=0.043). Females had greater portal triad inflammation than male mice (Day 7, p=0.024; Day 16, p=0.5, Day 22, p=0.2; Day 30, p=0.048) **(Table 5.3).** Due to the sex differences observed a further independent experiments were performed to verify these results and liver sections examined from male and female mice infected with *T. gondii* at 22 and 30 days post-infection. Female mice had increased severities of both acinar and portal triad inflammation compared with male mice at 22 days post-infection (p=0.007and p=0.019, respectively) **(Table 5.4)**. At 30 days post-infection female mice also had increased severities of both acinar and portal triad inflammation compared with male mice (p=0.02 and p=0.046, respectively) **(Table 5.5)**.

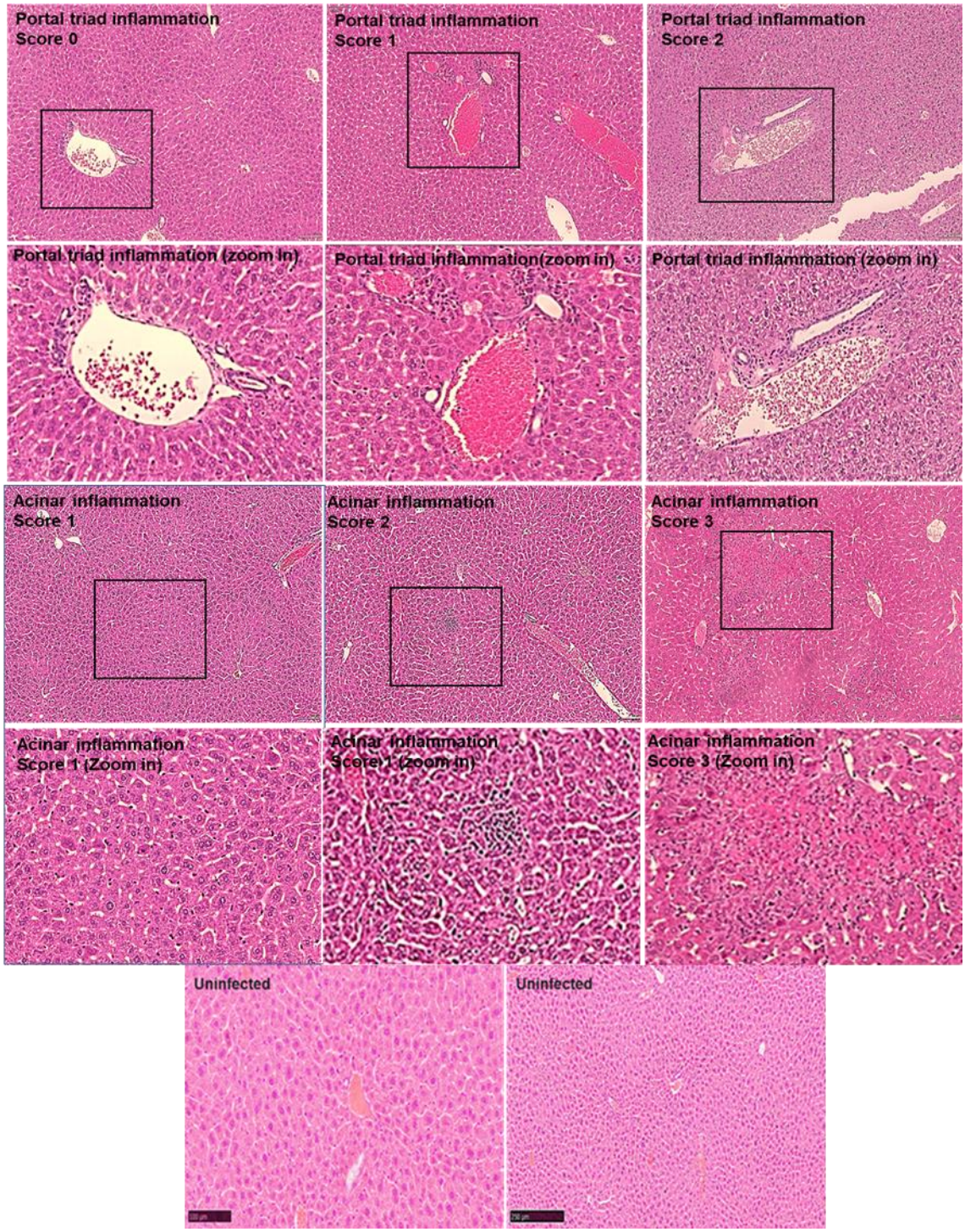


Figure 5.4 Representative images for toxoplasmosis hepatitis

Abnormal architectures including acinar and portal triad inflammation that scored from 0 no changes up to 3 indicates high severity. Magnification: 4 for acinar inflammation and x10 portal triad inflammation.

Table 5.3 Toxoplasmosis hepatitis scoring (experiment 1) [a]

Day Postinfection	Mouse sex	Histological score of : Portal triad inflammation	Acinar inflammation
4	M	1	1
		0	0
		0	1
		0	2
		1	1
	F	1	0
		1	1
		2	0
		2	1
7	M	2	1
		1	1
		2	1
		2	1
		2	1
	F	2	1
		2	1
		3	2
		3	0
		2	1
16	M	1	2
		1	1
		2	2
		2	2
		1	3
	F	2	3
		1	2
		1	2
		1	1
		1	1
22	M	2	1
		2	1
		1	2
		1	2
		1	1
	F	1	2
		2	1
		1	2
		2	2
		1	0
30	M	3	3
		3	2
		2	2
		2	2
		2	3
	F	2	2
		2	2
		1	1
42	M	2	1
		1	1
		2	1
		3	2
		1	1
	F	2	2
		1	2
		2	3

[a] Acinar and portal triad inflammation was scored from a range of 0 for no changes to 3 as severe. There were significant differences between male and female in acinar inflammation at day 4 post-infection (p=0.043). Also, females had greater portal triad inflammation at 7 post-infection (p=0.024) and 30-day post-infection (p=0.048). The data represent 5 animals per experimental group and were analysed using a one-tailed nonparametric Mann–Whitney analyses.

Table 5.4 Toxoplasmosis hepatitis scoring (experiment 2) [a]

Day Postinfection	Mouse Sex	Histological score of : Portal triad inflammation	Acinar inflammation
22	M	0	1
		1	1
		1	1
		0	0
		0	1
		0	2
		0	1
		1	1
		1	2
		1	2
	F	1	2
		2	3
		2	3
		1	2
		1	2

[a] Acinar and portal triad inflammation was scored from a range of 0 for no changes to 3 as severe. There were significant differences between acinar and portal triad inflammation with females having more severe symptoms (p=0.007 and p=0.019, respectively). The data are representative of 10 male and 5 for female mice. Data were analysed using one-tailed nonparametric Mann–Whitney test.

Table 5.5 Toxoplasmosis hepatitis scoring (experiment 3) [a]

Day Postinfection	Mouse Sex	Histological score of : Portal triad inflammation	Acinar inflammation
30	M	0	2
		1	1
		0	1
		0	1
		0	1
		1	1
		1	1
	F	0	3
		1	1
		1	3
		1	2
		0	1
		1	1
		1	2
		1	2
		2	2
		1	2
		1	2
		0	3

[a] Acinar and portal triad inflammation was scored from a range of 0 for no changes to 3 as severe. There were significant differences between acinar inflammation and portal triad with females having more severe symptoms (p=0.020 and p=0.046, respectively). The data are representative of 7 male and 11 for female mice. Data were analysed using one-tailed nonparametric Mann–Whitney test.

5.2.6 Toxoplasmosis Encephalitis(TE)

Brain sections exhibited abnormal architectures including meningitis, perivascular cuffing with mononuclear cell infiltration, small and large lesions, and necrosis **(Figure 5.5)**. These abnormalities increased in severity as disease progressed to 42 days post-infection. The brains of female mice had more lesions in comparison to those of male mice at day 22 and 42 post infection (p=0.020 and p=0.018) respectively. Tissue cysts were observed at the later stage of infection in the brain of some female mice, but not in the brain of any male mice examined. This difference was statistically significant at day 42 post infection (p=0.018) **(Table 5.6).**

Due to the sex differences observed a further two independent experiments were performed with male and female mice infected with *T. gondii.* As these mice developed more severe disease than those in the first experiment, tissue was collected at 22 and 30-day post-infection. At day 22 post-infection perivascular cuffing, lesions and necrosis significantly greater in the brains of female mice (p=0.040, p=0.014 and p=0.003) respectively **(Table 5.6)**. Females at 30-day post-infection had significantly higher scoring in meningitis and perivascular cuffing than males (p=0.015 and p=0.041) respectively. Small and large lesions, as well as necrosis developed during infection in both male and female mice, no sex difference was observed. The tissue cysts were not observed in males while females significantly had more cysts in their brains (p=0.041) **(Table 5.7).**

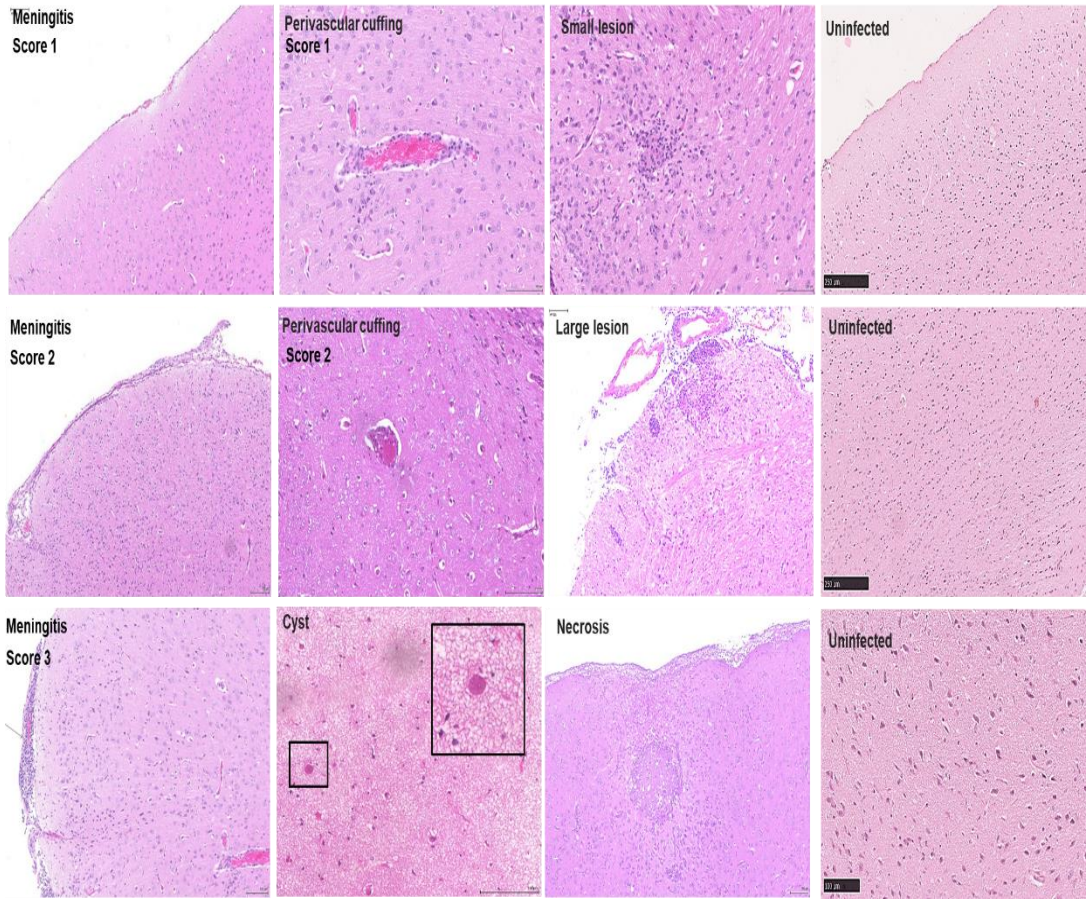


Figure 5.5 Representative images for toxoplasmosis encephalitis

The abnormal architectures including meningitis, perivascular cuffing, small and large lesions, necrosis and tissue cyst. Magnification x4 for meningitis, large lesions and necrosis. x 20 for small lesions and perivascular cuffing and tissue cyst.

Table 5.6 Toxoplasmosis encephalitis scoring (experiment 1) [a]

Day Postinfection	Mouse sex	Histological score of :				Number of:	
		Meningitis	Perivascular cuffing	Small lesions	Large lesions	Necrosis	Tissue cyst
4	M	1	0	0	0	0	0
		0	0	0	0	0	0
		1	0	0	0	0	0
		0	0	0	0	0	0
		0	0	0	0	0	0
	F	0	0	0	0	0	0
		0	0	0	0	0	0
		1	1	0	0	0	0
		0	0	0	0	0	0
		1	0	0	0	0	0
7	M	0	0	0	0	0	0
		1	0	0	1	0	0
		2	1	0	0	0	0
		1	0	0	0	0	0
		1	0	0	0	0	0
	F	1	0	0	0	0	0
		1	0	0	0	0	0
		0	0	0	0	0	0
		1	0	0	0	0	0
		0	0	0	0	0	0
16	M	1	1	0	1	0	0
		2	0	1	1	2	0
		1	1	2	1	0	0
		1	1	6	1	0	0
		2	1	2	0	0	0
	F	1	0	2	0	0	0
		2	1	2	0	0	0
		1	2	8	0	0	0
		2	2	6	0	0	0
22	M	1	0	0	1	0	0
		2	1	0	0	0	0
		1	0	0	0	0	0
		1	0	0	0	0	0
		2	1	2	1	1	1
	F	1	2	2	2	2	0
		1	1	5	0	0	0
		2	3	7	1	0	0
		4	2	5	4	2	0
		1	1	0	1	0	0
30	M	3	1	3	0	0	0
		3	1	3	0	0	0
		2	0	0	0	0	0
		3	3	1	3	0	0
		3	2	6	0	0	0
	F	2	1	6	0	0	0
		2	1	6	0	0	0
		3	2	5	4	0	0
42	M	2	0	1	0	0	0
		1	2	1	1	1	0
		2	1	1	0	0	0
		1	1	0	0	0	0
		2	1	0	0	0	0
	F	2	1	5	0	0	2
		3	1	3	0	0	1
		2	1	2	2	2	1

[a] Meningitis and perivascular cuffing was scored from a range from 0 for no changes to 5 as severe developed from day 4 post infection and increased during the infection. While small and large lesions, as well as necrosis developed at 16-day post infection and their severity, increased during infection. Female mice had more lesions in comparison to those of male mice at day 22 and 42 post infection (p=0.020, p=0.018) respectively. The tissue cysts were not observed in males while female had a number of cysts at day 42 post infection (p=0.018). The data represents approximately 5 animals per experimental group and were analyzed using one-tailed nonparametric Mann–Whitney test.

Table 5.7 Toxoplasmosis encephalitis scoring (experiment 2) [a]

Day Postinfection	Mouse Sex	Histological score of :				Number of:	
		Meningitis	Perivascular cuffing	Small lesions	Large lesions	Necrosis	Tissue cyst
22	M	2	0	2	0	0	0
		2	0	5	1	0	0
		2	0	3	0	0	0
		1	0	5	0	0	0
		2	0	3	1	0	0
		2	1	6	1	0	0
		1	0	1	0	0	0
		2	0	2	1	0	0
		2	0	7	0	0	0
		1	0	4	0	0	0
	F	2	0	5	0	0	0
		2	1	8	0	0	0
		2	0	4	1	1	0
		3	2	6	2	1	0
		2	2	9	2	1	0

[a] Perivascular cuffing, lesions and necrosis range from 0 for no changes to 5 indicating high severities and are significantly more pronounce in female mice (p=0.040, p=0.014 and p=0.003) respectively. The data represents of 10 for males and 5 for females per experimental group and were analyzed using one-tailed nonparametric Mann–Whitney test.

Table 5.8 Toxoplasmosis encephalitis scoring (experiment 3) [a]

Day Postinfection	Mouse Sex	Histological score of :				Number of:	
		Meningitis	Perivascular cuffing	Small lesions	Large lesions	Necrosis	Tissue cyst
30	M	1	0	0	0	0	0
		2	0	2	0	0	0
		1	0	2	0	0	0
		1	0	4	0	0	0
		2	0	1	0	0	0
		1	0	5	0	0	0
		2	0	5	0	0	0
	F	2	0	1	0	0	0
		2	1	5	1	1	1
		2	0	3	0	0	0
		2	0	2	0	0	0
		2	0	1	0	0	0
		2	0	2	0	0	0
		2	1	2	2	1	1
		2	1	8	1	1	2
		2	0	2	0	0	0
		2	1	3	0	0	2
		2	1	6	0	0	1

[a] Meningitis and perivascular cuffing range from 0 for no changes to 5 indicating high severity and are significantly more pronounce in females (p=0.015 and p=0.041) respectively. Small and large lesions, as well as necrosis developed during infection in both male and female mice, no significant sex difference was observed. The tissue cysts were not observed in males while females significantly had more cysts in their brains (p=0.041). The data represents of 7 for males and 11 for females per experimental group and were analyzed using one-tailed nonparametric Mann–Whitney test.

5.3 Discussion

In this study we use histopathological analyses to expand upon the knowledge of sex differences in the immune response to *T. gondii* infection using an oral cyst infection. The results show that using this model, infected females were more susceptible to the infection than male mice with increased severity of disease including lower survival rates and increased weight loss. This is similar to reported in chapter 3 for mice infected intraperitoneally with tachyzoites. This study however reports the histopathological abnormalities observed from day 4 to 42 in BALB/c male and female mice orally infected with *T. gondii* Beverley (RRA) strain.

Splenic tissue exhibited abnormal architectures of white pulp as results of *T. gondii* infection. This alteration was observed from the acute stage of infection and continues to the chronic stage of infection. *T. gondii* initially spreads from intestine, to multiple organs such as the spleen before establishing the chronic infection in the brain of the host (Petit-Jentreau et al., 2018). *T. gondii* employs various immunoregulatory mechanisms to avoid host defenses in order to persist a latent stage. These mechanisms have been demonstrated or suggested to dysregulation of cytokines through various parasite derived molecules and pertinent to the results reported here, alteration in the splenic architecture (Glatman Zaretsky et al., 2012). No significant sex bias was observed in the histopathology of the spleen during toxoplasmosis. However, the differential response to infection between males and females observed here in tissues discussed are likely to be influenced by immunological events in the spleen that are not visualized with histopathology alone.

Inflammatory cells were found to be present in the lungs of mice early during *T. gondii* infection. The severity of pneumonia increased during the course of infection. Toxoplasmosis pneumonia is a common symptom in immunocompromised humans,

but has also been reported in immunocompetent people (Shen et al., 2015). Pneumonia is known to occur in mice infected with *T. gondii* and has been associated with increased levels of transcript for various cytokines including IFN-γ, IL-4 and IL-10 (Filice et al., 1999). The balance of these cytokines is important as uncontrolled inflammatory Th1 cell responses, including IFN-γ which can mediate killing of *T. gondii*, frequently causes immunopathology and consequently regulatory cytokines such as IL-4, and IL-10 control this deleterious side effect (Fenoy et al., 2012). No notable sex differences were found in our data about toxoplasmosis pneumonia severity. However, women and female mice have been reported to be more susceptible to inflammatory lung disease (Cabello et al., 2015, Mikerov et al., 2011). The results suggest that lung inflammation during *T. gondii* infection is not a major determinant of sex mediated differences in disease outcome.

Female mice had greater liver inflammation than male mice following *T. gondii* infection, indicated by immune cell infiltration within acinar and portal triad areas. Similar liver inflammation has been previously reported in BALB/c mice and has been linked to CD4+ T cell infiltration (He et al., 2016, Heimesaat et al., 2006). The inflammation at the acute stage was moderate, however, in the chronic stage the inflammation was severe in both sexes. Although both males and females developed hepatitis this was more severe at the later stage of infection in females. The literature indicates that at least some types of hepatitis are more prevalent in females. Autoimmune hepatitis AIH, a disease of the hepatic parenchyma defined by progressive inflammatory destruction is highly prevalent in women which represent 70–90% of affected patients (Lohse AW, 2015, Alvarez et al., 1999). In addition, women have higher rates of spontaneous hepatitis C virus (HCV) clearance than men, but increased incidence of symptomatic disease associated with increased IFN-γ and IFN-γ responses (Bulteel et al., 2016, Buzzetti et al., 2017).

Female mice developed more severe Toxoplasmic Encephalitis (TE) than male mice following *T. gondii* infection. This manifested as increased immune cell infiltration and these abnormalities characterised by meningitis, perivascular cuffing, lesions, as well as necrosis. A sex difference in TE was previously reported in SCID mice infected *T. gondii*. Female mice had a higher mortality rate, a higher parasite burden, and larger necrotic lesions in their brain than in male mice (Walker et al., 1997). The development and pathogenesis of TE are characteristically associated with the host's immune response including activation of resident immune cells, immune cell infiltration and cytokines production (Sukhumavasi et al., 2008, Wilson and Hunter, 2004). However, the balance of cytokine production is critical and can sometimes have a negative effect on the host leading to severe irreversible tissue damage (Denkers and Gazzinelli, 1998). Thus, although type 1 cytokines including IFN-γ are essential for protective immunity to *T. gondii*, under certain conditions in other tissues IFN-γ over production can result in pathology and death (Sarciron and Gherardi, 2000, Gavrilescu and Denkers, 2001). The literature consistently demonstrates a requirement for both CD4+ T and CD8+ T cells for control of cysts and prevention of TE (Gazzinelli et al., 1991, Parker et al., 1991).

The data here also found that of tissue cysts were observed only within the brain of female, but not in the brains of male mice. This data is also consistent with previous studies in a number of different strains of mice (Roberts et al., 1995). The greater number of cysts observed in female mice relative to male mice could be explained be a number of potentially conflicting hypotheses: (i) *T. gondii* differentiation from tachyzoite to bradyzoite is dependent on the immune response and consequently increased levels of cytokines such as IFN-γ during the acute response may induce and maintain parasite differentiation. (ii) The increased parasite number in the brain

is due to a less efficient immune response in their brains and the inflammation is a secondary effect (Skariah et al., 2010).

Collectively, these data present a developing picture of the sex differences in histopathological alterations within several tissues during *T. gondii* infection. Notably female mice tended to have increased pathology in all tissues examined which is consistent with them having a stronger immune response to *T. gondii* infection. However, unlike the results reported in chapter 3 where female mice had reduced parasite numbers, while in this model of infection females have more parasite in their brains. For a deep understanding the next chapter will examine the mechanism behind the increased inflammation within brains of females during *T. gondii* chronic stage using transcriptomics of brain samples collected from infected male and female BALB/c mice with *T. gondii*.

Chapter 6 . Transcriptomics analysis of brain tissue during *T. gondii* chronic infection and functional validation

6.1 Introduction

In the previous chapter, infection of female mice with *T. gondii* results in increased parasite number, more severe inflammation and pathology in their brains compared with infected male mice. RNA-seq analysis has previously been used to determine the host response in the brain during the chronic stage of *T. gondii* infection (Garfoot et al., 2019).This methodology can provide insight into processes that are altered following *T. gondii* infection and has the potential to highlight differences between the sexes in response to infection. These differences can then be further validated through functional assays.

Therefore, the aim of this chapter is to explore deeply the mechanism that might influence the sex differences observed in the brains of mice following *T. gondii* infection by analysing the transcriptional profile of the brains of male and female BALB/c mice and where practical to validate key changes through alternative means.

6.2 Results

6.2.1 Non targeted approach

6.2.1.1 *T. gondii* induces global transcriptomic alteration in both sexes

Principal Component Analysis (PCA) was performed to discriminate the RNA transcriptomic profile of brain tissues collected from infected and uninfected mice in both sexes at day 30 post-infection. The results demonstrate a clear separation between the four groups examined **(Figure 6.1).** Therefore, orthogonal projections to latent structures discriminant analysis (OPLS-DA) was applied to generate Variable Importance Projection (VIP) scores that account for separation of infected male and female mice **(Figure 6.1)** (supplementary table is provided).

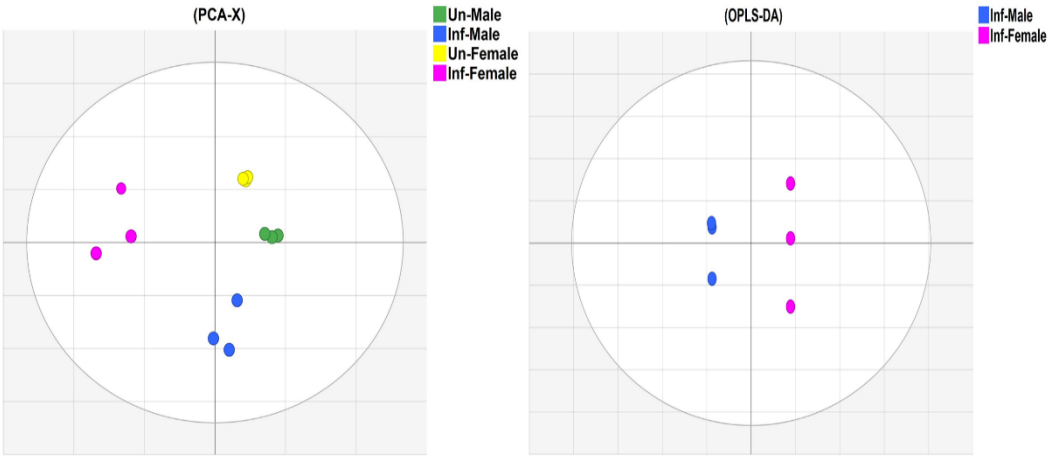


Figure 6.1 The PCA plot and OPLS-DA

Show that obvious discrimination between the four groups. The shows male and female infected achieving a clear separation.

6.2.1.2 Pathway analysis

A common approach to interpreting gene expression data is pathway analysis based on annotation of the differentially expressed (DE) genes to a dictionary of annotated pathways. To define DE genes, filters were applied as VIP values greater or equal to 1 and the significantly different ($q<0.05$) between infected males and infected females with a cut off log 2 of fold change > 2 or<0.5 (comparing infected to uninfected mouse for each sex) **(Figure 6.2)**. A combined list of these genes representing transcripts that are DE expressed in males and females was then uploaded to the Panther online tool for pathway analyses (Thomas et al., 2006). **Figure 6.3** displays the pathways identified through this analysis. Heatmaps for the top pathways as well pathways previously implicated in the pathogenesis of toxoplasmosis were created to demonstrate the genes that are involved in each pathway **(Figure 6.4-10).**

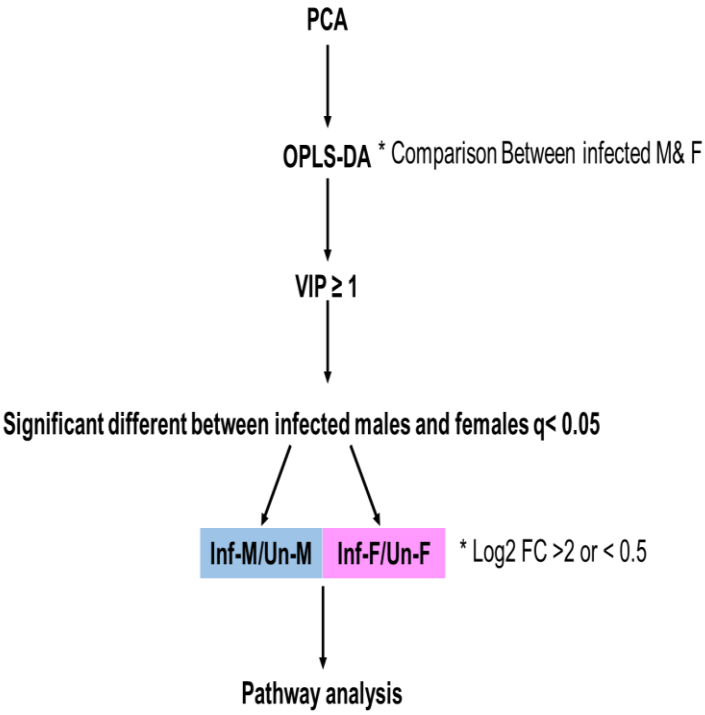


Figure 6.2 Flow chart for filtration criteria of DE

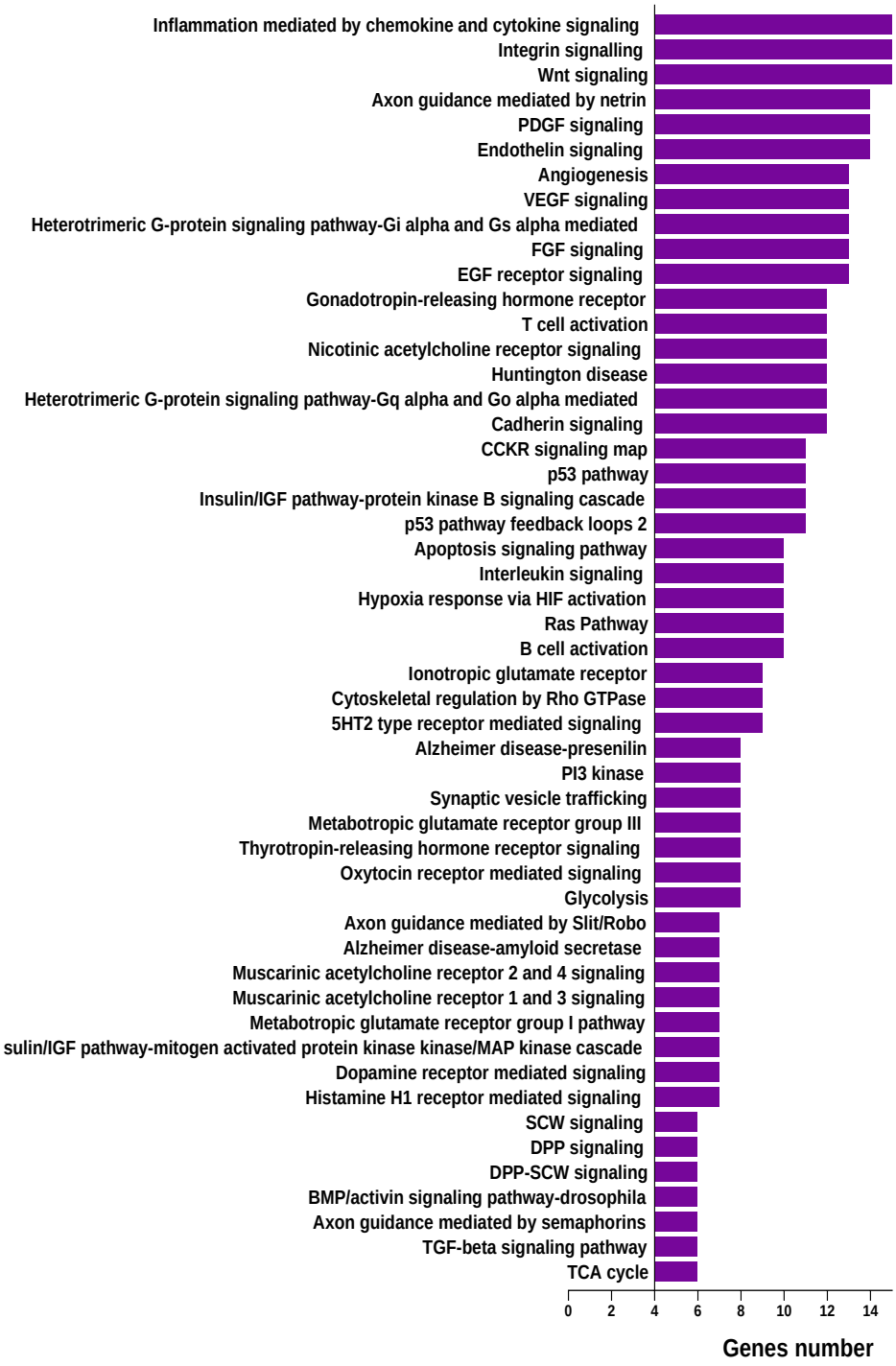


Figure 6.3 The top 50 pathways of DE genes as computed by Panther

6.2.1.2.1 Sex differences in mRNA expression of PIK3 classes genes during *T. gondii* infection

A number of pathways were identified (including: inflammation mediated by chemokine and cytokine, integrin, axon guidance mediated by netrin, T cells activation and apoptosis) that have PI3 kinases involvement. PI3 kinases are involved in a large number of intracellular signalling pathways and have been classified into 3 classes. Each class is characterised by one or more catalytic subunits which in the case of Class I (A and B) and Class III are known to form heterodimers with one or more regulatory units. *T. gondii* infection was found to alter levels of the catalytic subunit transcripts in the brains of mice and some of these were affected by the sex of the mice. Notably, *Pik3ca* (PIK3α) was downregulated in male and female brains following *T. gondii* infection, but to a greater extent in male mice than female mice (q=0.002). *Pik3cb* (PIKβ) was significantly upregulated in females, but not males following infection (q=0.039). *Pik3cd* (PIK3δ) was upregulated in both sexes but to a greater extent in females than in males (q=0.007). *Pik3cg* (PIK3γ) was upregulated in the brains of male mice but not female mice following infection (q=0.007). Both class II (*Pik3c2a, Pik3c2b)* and Class III (*Pik3r4)* were downregulated in the brains of female mice, but not in the brains of male mice (q=0.0003, q=0.0003 and q=0.035, respectively). The individual pathways where PIK3 catalytic subunits are known to play a role are discussed below or (in the case of T cell activation and apoptosis) in the next section of the chapter that deals with a targeted approach for analysis of transcript differences **(Figure 6.4)**.

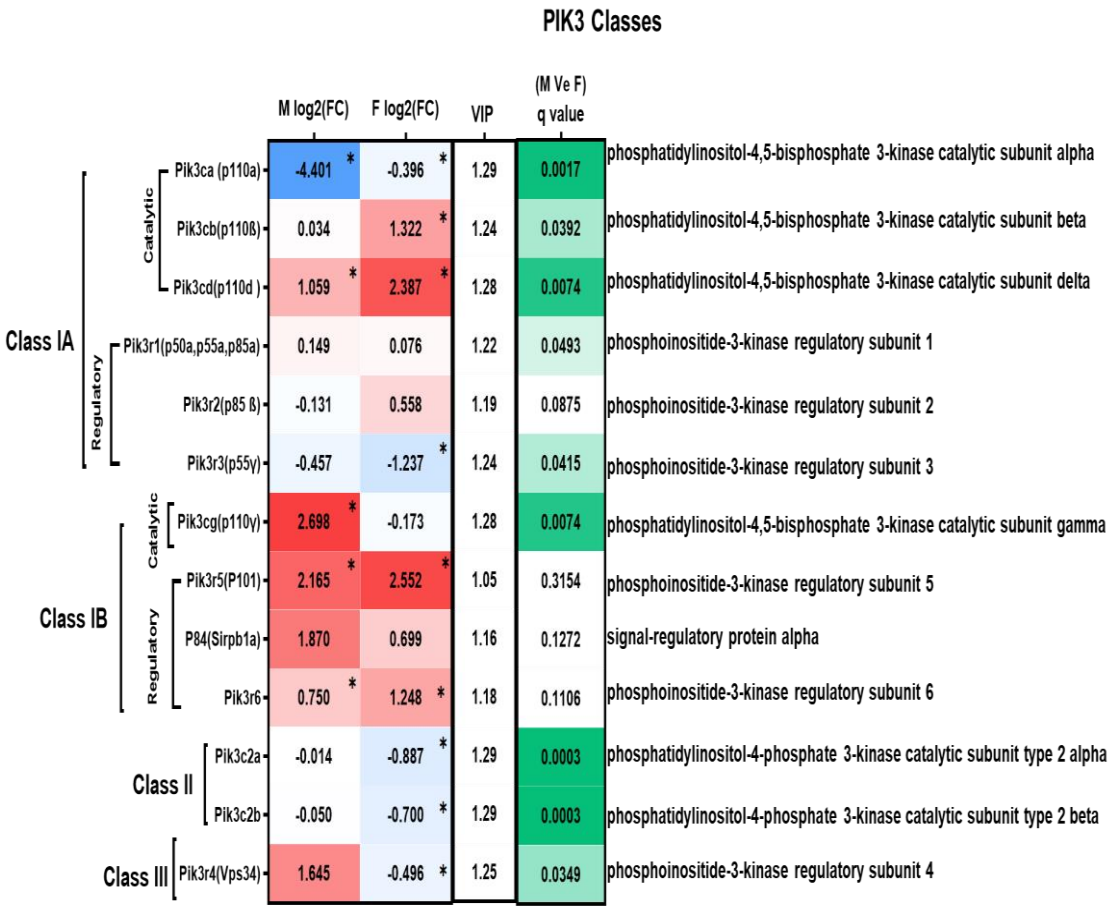


Figure 6.4 Heatmap of mRNA transcripts of PIK3 classes genes

Log2 fold change obtained through comparing the mean of infected to uninfected mice of each sex. The red colour shows upregulated mRNA expression, while the blue shows downregulated mRNA expression. Also VIP values are shown. The green colour shows corrected P value (q value) from comparison of the mean of infected males and infected female mice. The asterisks denote significant differences ($q<0.05$) of the mean of uninfected and infected mice of the same sex. The corrected p value (q value) was obtained using the Benjamini–Hochberg test.

6.2.1.2.2 Sex differences in mRNA transcripts involved in inflammation mediated by chemokine and cytokine signalling during *T. gondii* infection

In addition to the catalytic subunits, PIK3α , PIKβ and PIK3γ. *T. gondii* infection was found to alter levels of transcripts of other genes that are part of inflammation mediated by chemokine and cytokine signalling pathways **(Figure 6.5).** The transcript levels of these were affected by the sex of the mice. *Rac1* was significantly upregulated in male mice, but downregulated in female mice (q=0.010). Notably, *ifngr2*, was significantly upregulated in male and female brains following *T. gondii* infection (q=0.05), but this upregulation was considerable greater in the brains of male mice (male, 5.527 versus female 0.592 log2FC). *Raf1* was significantly upregulated in females, but not males following infection (q=0.009). *Ccl12* was upregulated in both sexes, but to a greater extent in males than in females (q=0.049). Many other genes in this pathway were downregulated in the brains of female mice, but not in the brains of male mice, such as *Myth14*, *Plcl1* and *Plcb4* **(Figure 6.5)**.

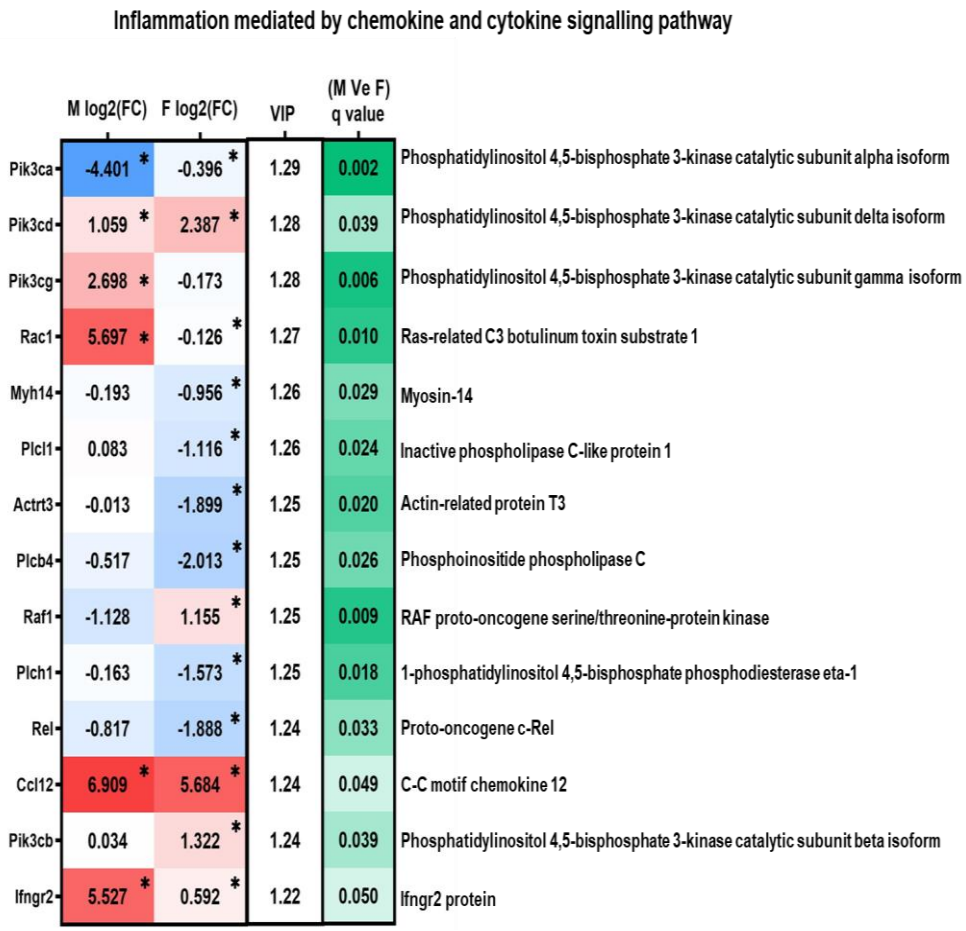


Figure 6.5 Heatmap of mRNA transcripts of genes involved in the inflammation mediated by chemokine and cytokine signalling pathway

Log2 fold change obtained through comparing the mean of infected to uninfected mice of each sex. The red colour shows upregulated mRNA expression, while the blue shows downregulated mRNA expression. Also VIP values are shown. The green colour shows corrected p value (q value) from comparison of the mean of infected males and infected female mice. The asterisks denote significant differences ($q<0.05$) of the mean of uninfected and infected mice of the same sex. The corrected p value (q value) was obtained using the Benjamini–Hochberg test.

6.2.1.2.3 Sex differences in mRNA expression of genes in integrin signalling pathway during *T. gondii* infection

In addition to the catalytic subunits PIK3α , PIKβ and PIK3γ. The signalling molecules *Rac1* and *Raf1*, mentioned previously, following *T. gondii* infection mRNA transcripts of other genes involved in the integrin signaling pathway were significantly different between males and females. Notably, *Itga3, Elmo1* and *Col9a2* were significantly downregulated in female mice (q=0.009, q=0.027 and q=0.036, respectively) **(Figure 6.6).**

Figure 6.6 Heatmap of mRNA transcripts of genes involved in the integrin signaling Pathway

Log2 fold change obtained through comparing the mean of infected to uninfected mice of each sex. The red colour shows upregulated mRNA expression, while the blue shows downregulated mRNA expression. Also VIP values are shown. The green colour shows corrected P value (q value) from comparison of the mean of infected males and infected female mice. The asterisks denote significant differences ($q<0.05$) of the mean of uninfected and infected mice of the same sex. The corrected P value (q value) was obtained using the Benjamini–Hochberg test.

6.2.1.2.4 Sex differences in mRNA expression of genes in axon guidance mediated by netrin pathway during *T. gondii* infection

In addition to the catalytic subunits PIK3α, PIKβ and PIK3γ and the signalling molecule *Rac1* previously mentioned, expression of genes that are involved in axon guidance were found to be sex dependently modulated following *T. gondii* infection. Female mouse brains had reduced levels of transcripts for netrin1 (Ntn1) and its receptors *Unc5c* and *Unc5b* (q=0.003, q=0.025 and q=0.028, respectively). Levels of these transcripts were not significantly altered in male brains following infection **(Figure 6.7).**

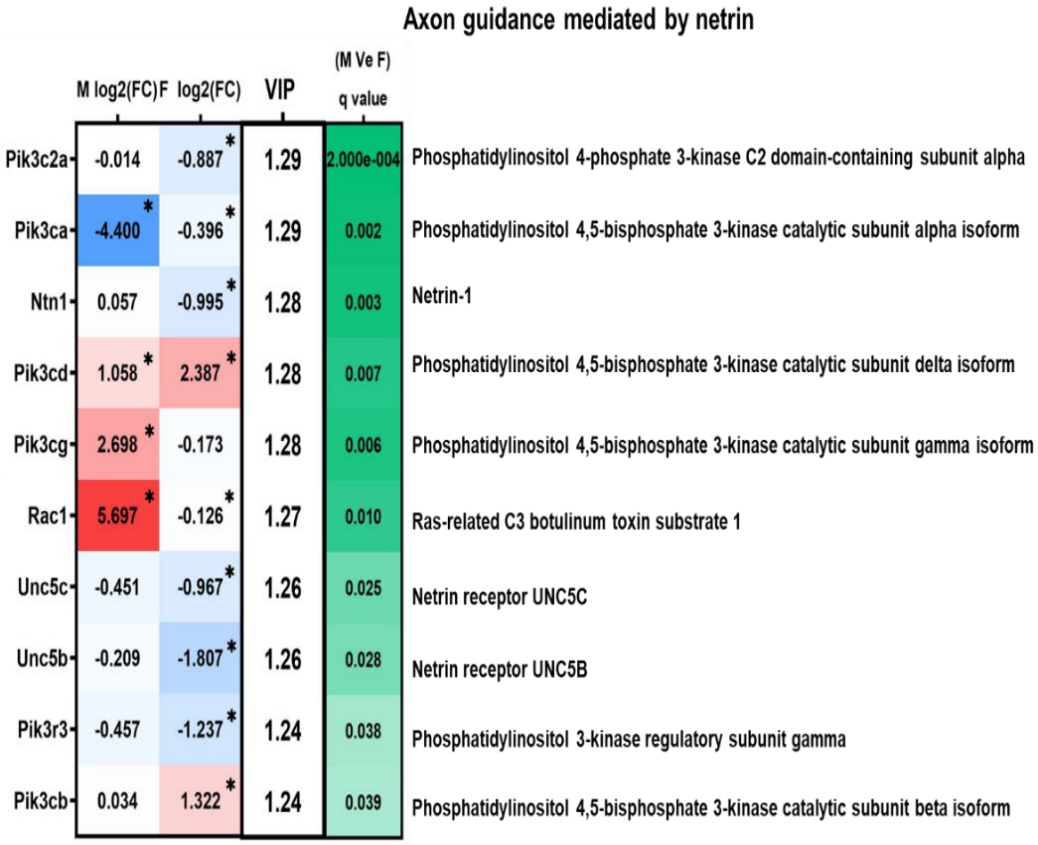


Figure 6.7 Heatmap of mRNA transcripts of genes involved in axon guidance mediated by netrin pathway

Log2 fold change obtained through comparing the mean of infected to uninfected mice of each sex. The red colour shows upregulated mRNA expression, while the blue shows downregulated mRNA expression. Also VIP values are shown. The green colour shows corrected P value (q value) from comparison of the mean of infected males and infected female mice. The asterisks denote significant differences ($q<0.05$) of the mean of uninfected and infected mice of the same sex. The corrected P value (q value) was obtained using the Benjamini–Hochberg test

6.2.1.2.5 Sex differences in mRNA expression of genes in Wnt signalling pathway during *T. gondii* infection

T. gondii infection caused alteration of mRNA transcripts of genes such as *Arid1a, Cdh18, Cdh20* and *En1* that involved in Wnt signalling pathway. Male mice had significantly reduced levels of transcripts for *Arid1a* (q=0.001), while levels of this transcript were unaffected in female mice. However, female mice had reduced levels of transcripts for the remaining genes involved in this pathway, but the levels were unaffected by infection in males **(Figure 6.8).**

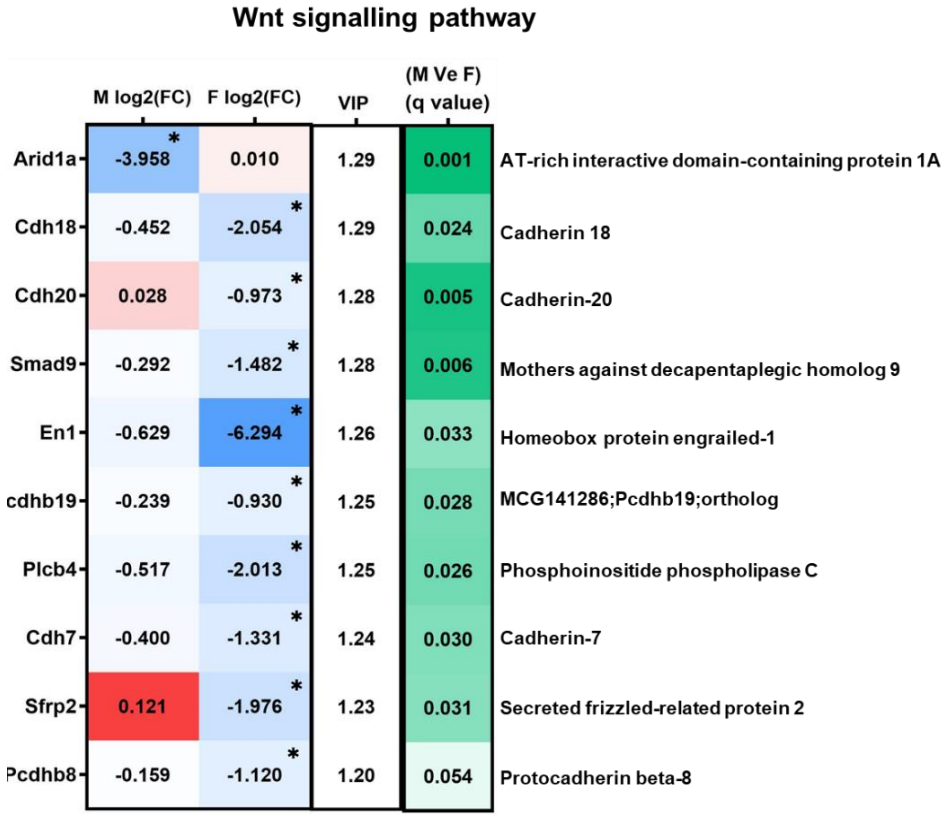


Figure 6.8 Heatmap of mRNA transcripts of genes involved in Wnt signalling pathway

Log2 fold change obtained through comparing the mean of infected to uninfected mice of each sex. The red colour shows upregulated mRNA expression, while the blue shows downregulated mRNA expression. Also VIP values are shown. The green colour shows corrected p value (q value) from comparison of the mean of infected males and infected female mice. The asterisks denote significant differences ($q<0.05$) of the mean of uninfected and infected mice of the same sex. The corrected p value (q value) was obtained using the Benjamini–Hochberg test.

6.2.2 Targeted approach for RNA seq analysis

A targeted approach for analyses of the mRNA transcripts was used. This relied on analysing processes that are known from the literature to be important in determining the outcome of *T. gondii* infection. These processes include: cytokines, T cell exhaustion and activation, apoptosis and pyroptosis.

6.2.2.1 Sex differences in mRNA expression of cytokine family proteins during *T. gondii*

There were sex differences in the levels of mRNA transcripts of cytokine genes during *T. gondii* infection. Female mice infected with *T. gondii* had increased transcripts for cytokines that are part of TNF superfamily including *FASL* (p=0.0123), *TNF* (p=0.050), and reduced level of *EDA* (p=0.0001), while male infected mice had increased transcript levels of *CD40IG* (p=0.0001) and *LTB* (p=0.050). The transcripts levels of some IL-1 family cytokines were upregulated in female infected mice including *IL18BP* and *IL18* (p=0.005 and p=0.009, respectively). In addition, female infected mice had increased transcript levels of *OSM* (p=0.05) that is part of IL-6 family, as well as *TGFB3* cytokines that is in TGFB family (p=0.04). The transcript level of some cytokines that are in the IL-12 family showed sex differences such as *IL12A* (p=0.03) and *IL27* (p=0.05) **(Figure 6.9) (Table 6.1).**

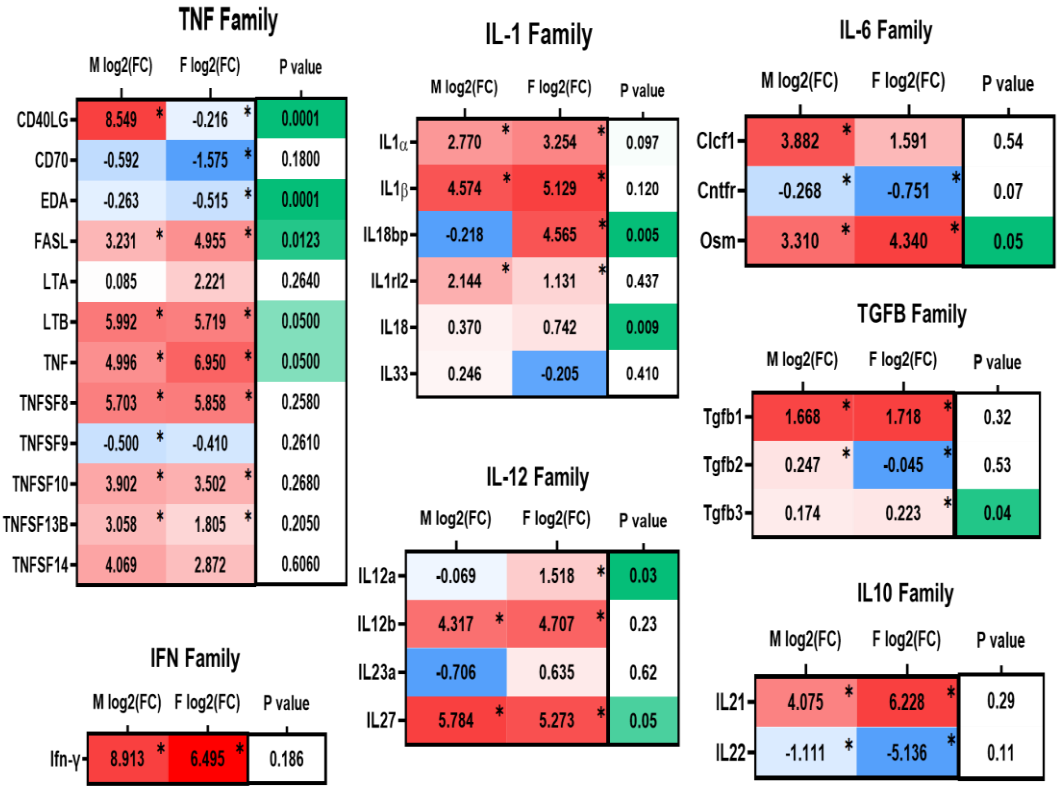


Figure 6.9 mRNA expression of cytokine family proteins during *T. gondii*

Log2 fold change obtained through comparing the mean of infected to uninfected mice of each sex. The red colour shows upregulated mRNA expression, while the blue shows downregulated mRNA expression. The green colour shows p value from comparison of the mean of infected males and infected female mice. The asterisks denote significant differences ($p<0.05$) of the mean of uninfected and infected mice of the same sex. Analysed using one-tailed nonparametric Mann–Whitney test ± SEM. *p.

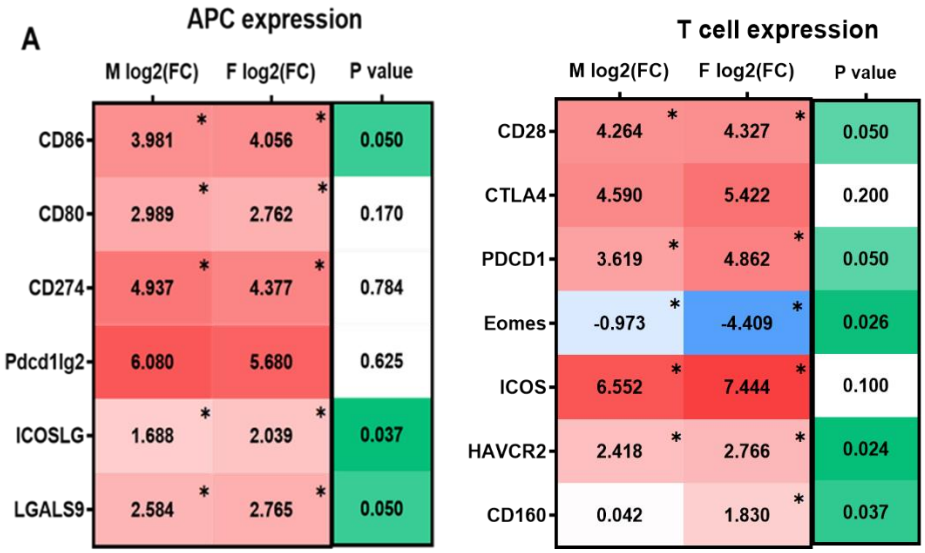
Table 6.1 Summary of cytokine family proteins genes that had sex differences in their expression in the brains of *T. gondii*-infected mice [a]

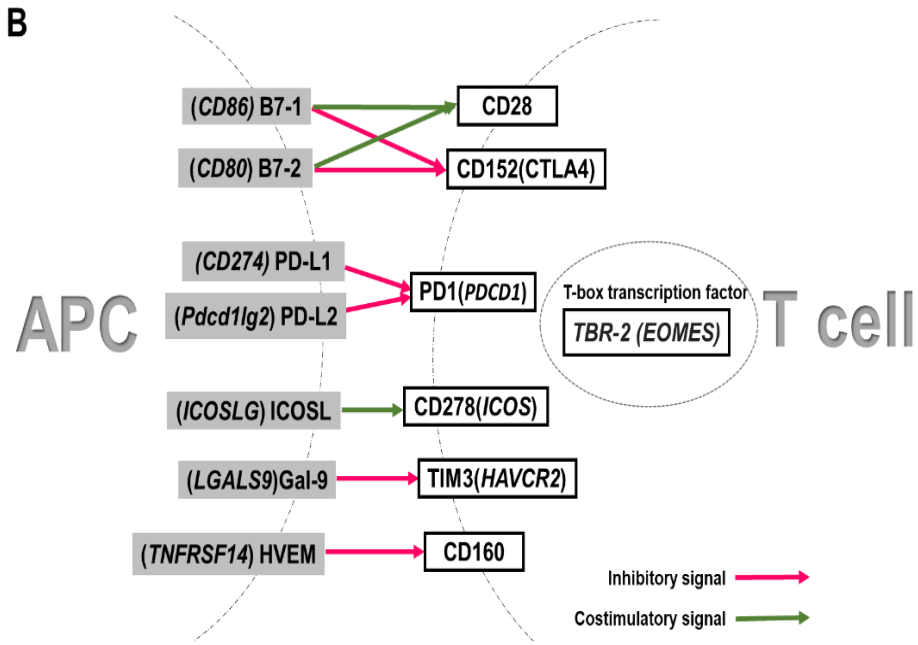
Gene	Cytokines	Family	Expression
CD40LG	CD40L		▲ Male
LTB	TNF-C		
EDA	EDA	**TNF**	▼ Female
FASLG	FasL		▲ Female
TNF	TNF-α		
IL18BP	IL-18BP	**IL-1**	▲ Female
IL18	IL-18		
IL12A	IL-12 p35	**IL-12**	▲ Female
IL27	IL-27		▲ Male
OSM	Osm	**IL-6**	▲ Female
TGFB3	TGF-β3	**Tgfb**	▲ Female

[a] Red arrows indicate upregulated mRNA expression while blue arrows denote downregulated mRNA expression.

6.2.2.2 Sex differences in mRNA expression of T cell exhaustion and activation markers during *T. gondii*

There were sex differences in the levels of mRNA transcripts of genes involved in the T cell/APC synapse including markers for T cell activation and exhaustion. The transcript levels for the catalytic subunits PIK3α , PIKβ and PIK3γ and the signalling molecule *Rac* 1 previously mentioned, and known to influence T cell activation and exhaustion were found to be differentially expressed in infected male and female mice. Female mice infected with *T. gondii* had increased transcripts for key T cell exhaustion markers including *PDCD1* (PD-1) (p=0.05), *HAVCR2* (TIM3) (p=0.024), *CD28* (p=0.05) and *CD160* (p=0.037) relative to infected males. Levels of transcripts for *EOMES* (*TB2-2*) a marker for T cell activation were reduced in females relative to males (p=0.026). In addition, ligands expressed on antigen presenting cells (APC) that play a role in T cell activation or deactivation such as *ICOSLG* (ICOSL), *CD86* (B7-1) and *LGALS9* (Gal-9) were significantly different between infected male and female mice **(Figure 6.10)**. Female infected mice had also downregulated levels of transcripts for *mTOR* (p=0.05), *GLUT1* (p=0.05) and *Rac*1(p=0.05) that are part of PI3K singling pathway. Transcripts for Rac1 were significantly upregulated in male mice (p=0.05). Transcripts for Foxo1 were upregulated in the brains of male mice (p=0.05), but not in females **(Figure 6.10)**.


A
APC expression
M log2(FC)
F log2(FC)
P value
CD86
3.981
4.056
0.050
CD80
2.989
2.762
0.170
CD274
4.937
4.377
0.784
Pdcd1lg2
6.080
5.680
0.625
ICOSLG
1.688
2.039
0.037
LGALS9
2.584
2.765
0.050
T cell expression
M log2(FC)
F log2(FC)
P value
CD28
4.264
4.327
0.050
CTLA4
4.590
5.422
0.200
PDCD1
3.619
4.862
0.050
Eomes
-0.973
-4.409
0.026
ICOS
6.552
7.444
0.100
HAVCR2
2.418
2.766
0.024
CD160
0.042
1.830
0.037


B
(CD86) B7-1
(CD80) B7-2
CD28
CD152(CTLA4)
(CD274) PD-L1
(Pdcd1lg2) PD-L2
PD1(PDCD1)
T-box transcription factor
TBR-2 (EOMES)
APC
T cell
(ICOSLG) ICOSL
CD278(ICOS)
(LGALS9)Gal-9
TIM3(HAVCR2)
(TNFRSF14) HVEM
CD160
Inhibitory signal
Costimulatory signal

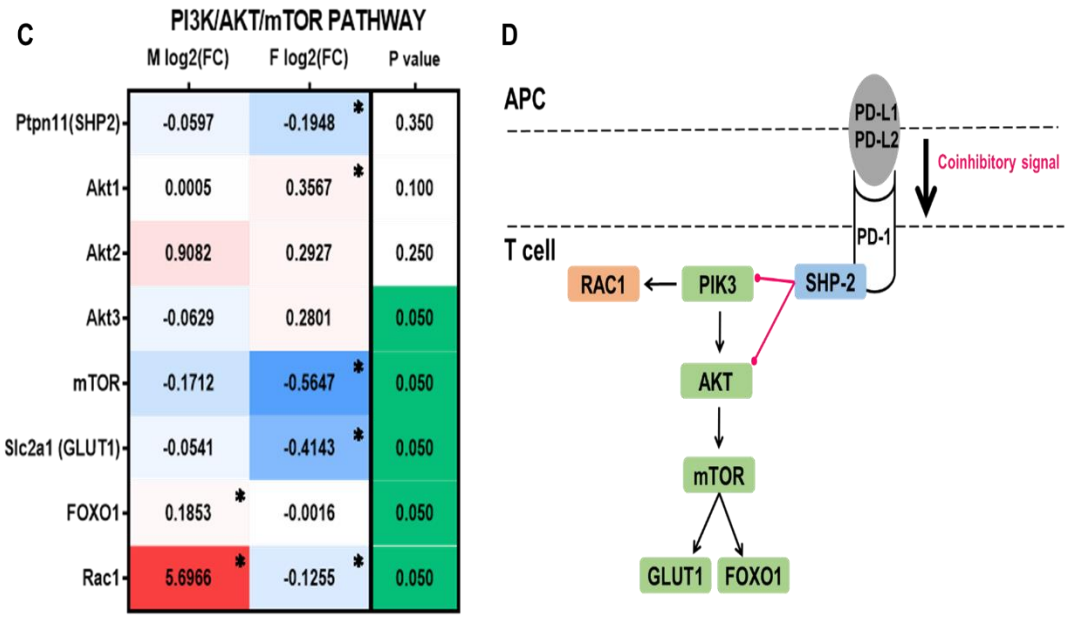
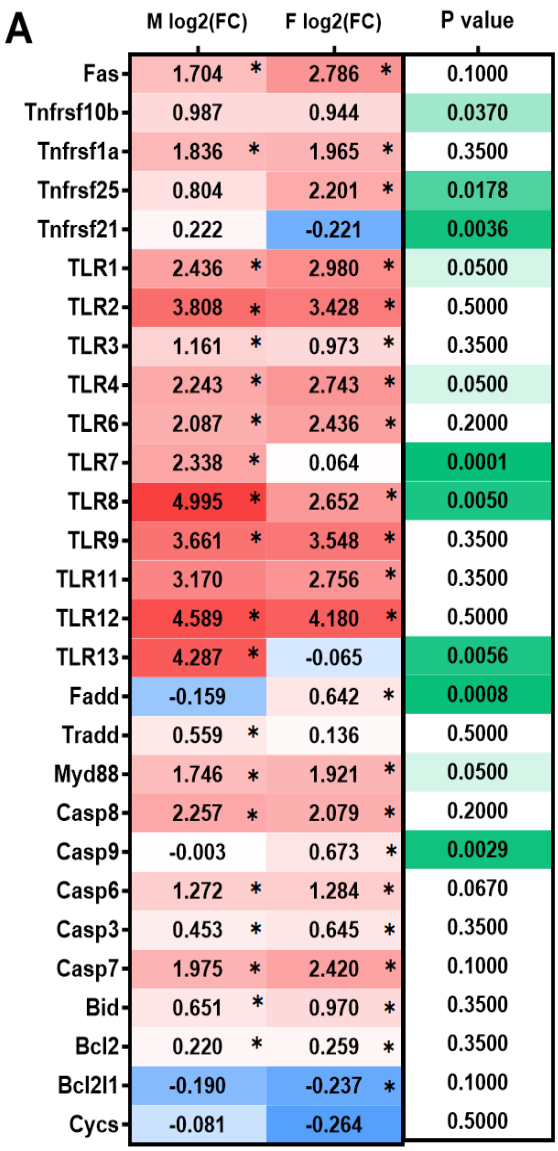


Figure 6.10 mRNA expression of T-cell exhaustion/activation receptors , their ligands and PI3K/AKT/mTOR singling pathway during *T. gondii* infection

(A) Log2 fold change heatmap of T-cell exhaustion/activation receptors and their ligands obtained through comparing the mean of infected to uninfected mice of each sex. The red colour shows upregulated mRNA expression, while the blue shows downregulated mRNA expression. The green colour shows p value from comparison of the mean of infected males and infected female mice. The asterisks denote significant differences ($p<0.05$) of the mean of uninfected and infected mice of the same sex. analysed using one-tailed nonparametric Mann–Whitney test ± SEM. *p. **(B)** T cell/APC synapse. **(C)** Log2 fold change heatmap of PI3K/AKT/mTOR singling pathway. **(D)** PI3K/AKT/mTOR singling pathway

6.2.2.3 Sex differences in mRNA expression of mediators involved in apoptosis pathway during *T. gondii*

There were sex differences in the levels of mRNA transcripts of genes that are involved in the apoptosis pathway including the catalytic subunits PIK3α, PIKβ and PIK3γ previously mentioned. In addition to female mice infected with *T. gondii* had increased transcripts for receptors that innate apoptosis including *TNFRSF10b* (p=0.037), *TNFRS25* (p=0.018), *TNFRS21* (p=0.004) and *TLR1* (p=0.05), *TLR4* (p=0.05) relative to infected males. Male infected mice had increased transcripts for TLRs receptors including *TLR7* (p=0.0001), *TLR8* (p=0.005) and *TLR13* (p=0.0056) relative to infected females. Levels of transcripts for some signalling molecules that play role in apoptosis such as *FADD* and *MYD88* (p=0.001 and p=0.05, respectively) were also increased in females relative to males. In addition, transcripts for *Casp 9* the functional mediator of apoptosis was increased in female mice compared with male mice (p=0.003) **(Figure 6.11)**.


A
M log2(FC)
F log2(FC)
P value
Fas 1.704 * 2.786 * 0.1000
Tnfrsf10b 0.987 0.944 0.0370
Tnfrsf1a 1.836 * 1.965 * 0.3500
Tnfrsf25 0.804 2.201 * 0.0178
Tnfrsf21 0.222 -0.221 0.0036
TLR1 2.436 * 2.980 * 0.0500
TLR2 3.808 * 3.428 * 0.5000
TLR3 1.161 * 0.973 * 0.3500
TLR4 2.243 * 2.743 * 0.0500
TLR6 2.087 * 2.436 * 0.2000
TLR7 2.338 * 0.064 0.0001
TLR8 4.995 * 2.652 * 0.0050
TLR9 3.661 * 3.548 * 0.3500
TLR11 3.170 2.756 * 0.3500
TLR12 4.589 * 4.180 * 0.5000
TLR13 4.287 * -0.065 0.0056
Fadd -0.159 0.642 * 0.0008
Tradd 0.559 * 0.136 0.5000
Myd88 1.746 * 1.921 * 0.0500
Casp8 2.257 * 2.079 * 0.2000
Casp9 -0.003 0.673 * 0.0029
Casp6 1.272 * 1.284 * 0.0670
Casp3 0.453 * 0.645 * 0.3500
Casp7 1.975 * 2.420 * 0.1000
Bid 0.651 * 0.970 * 0.3500
Bcl2 0.220 * 0.259 * 0.3500
Bcl2l1 -0.190 -0.237 * 0.1000
Cycs -0.081 -0.264 0.5000

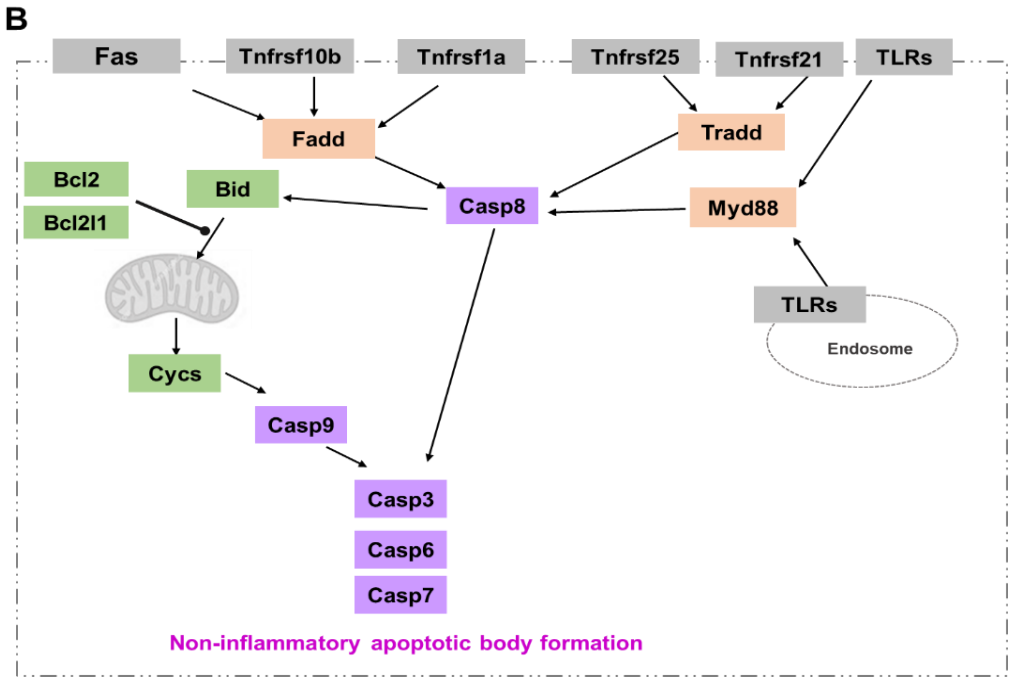
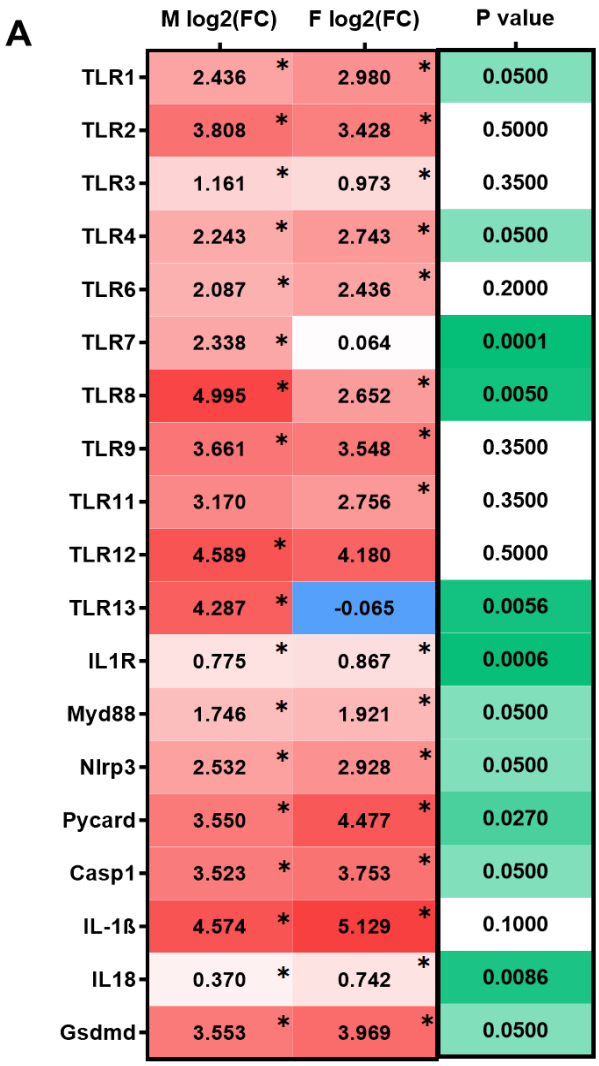


Figure 6.11 mRNA expression of apoptosis pathway

(A) Log2 fold change obtained through comparing the mean of infected to uninfected mice of each sex. The red colour shows upregulated mRNA expression, while the blue shows downregulated mRNA expression. The green colour shows p value from comparison of the mean of infected males and infected female mice. The asterisks denote significant differences ($p<0.05$) of the mean of uninfected and infected mice of the same sex. Analysis was by a one-tailed nonparametric Mann–Whitney test ± SEM. *p. **(B)** Apoptosis pathway.

6.2.2.4 Sex differences in mRNA expression of mediators involved pyroptosis pathway and NLRP3 inflammasome during *T. gondii*

There were sex differences between infected males and females in mRNA expression of many genes that are involved in the pyroptosis pathway such as those already mentioned above (*TLR1, TLR4, TLR8, TLR13 and MYD88*). In addition, female mice had increased level of transcripts for *IL1R* (p=0.001), *IL-18* (p=0.009) and *GSDMD* (p=0.05). The inflammasome components *NLRP3* (p=0.05), *PYCARD* (p=0.027) and *Casp1* (p=0.05) transcripts were also significantly raised in females relative to males (**Figure 6.12).**



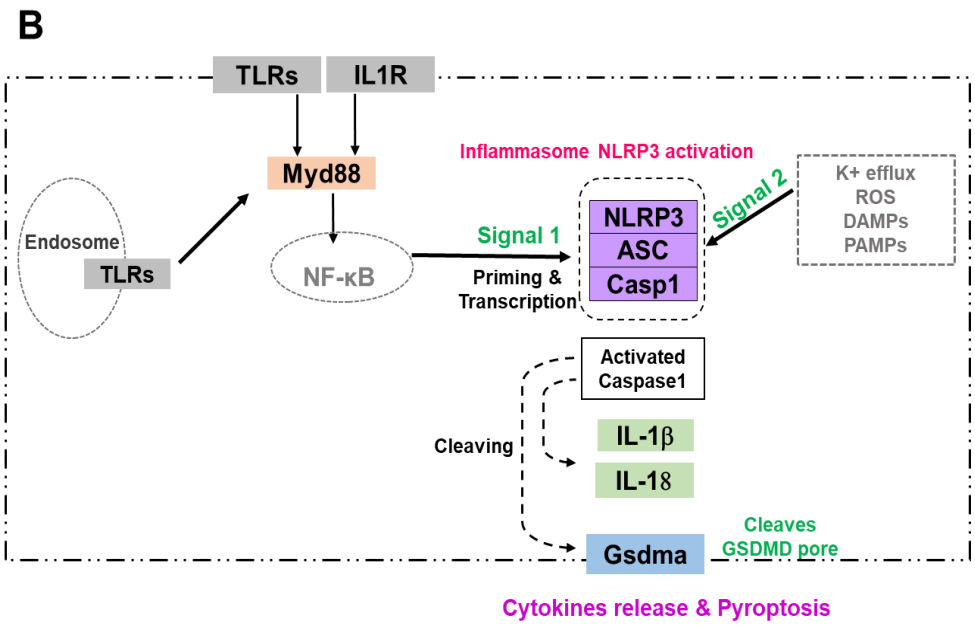


Figure 6.12 mRNA expression of pyroptosis pathway

(A) Log2 fold change obtained through comparing the mean of infected to uninfected mice of each sex. The red colour shows upregulated mRNA expression, while the blue shows downregulated mRNA expression. The green colour shows p value from comparison of the mean of infected males and infected female mice. The asterisks denote significant differences ($p<0.05$) of the mean of uninfected and infected mice of the same sex. Analyses were performed a one-tailed nonparametric Mann–Whitney test ± SEM. * p. **(B)** Pyroptosis pathway.

6.2.3 Immunohistochemistry analysis to validate transcriptomics results

Immunohistochemistry was used as a means to validate and confirm the functional significance of changes to transcript levels of genes involved in apoptosis and pyroptosis.

6.2.3.1 Detection of DNA fragmentation during *T. gondii* infection as a marker for apoptosis/pyroptosis

In situ DNA fragmentation was assessed by using the TUNEL assay in the brain tissues of both males and females infected and uninfected mice**.** The TUNEL assay method relies on the enzyme terminal deoxynucleotide transferase (TdT), which attaches deoxynucleotides to the 3'-hydroxyl terminus of DNA breaks. Quantitative analysis shows that infected females exhibited a higher number of DNA damaged cells in comparison to uninfected female mice ($p=0.028$) and their infected male counterparts ($p=0.048$) **(Figure 6.13).**

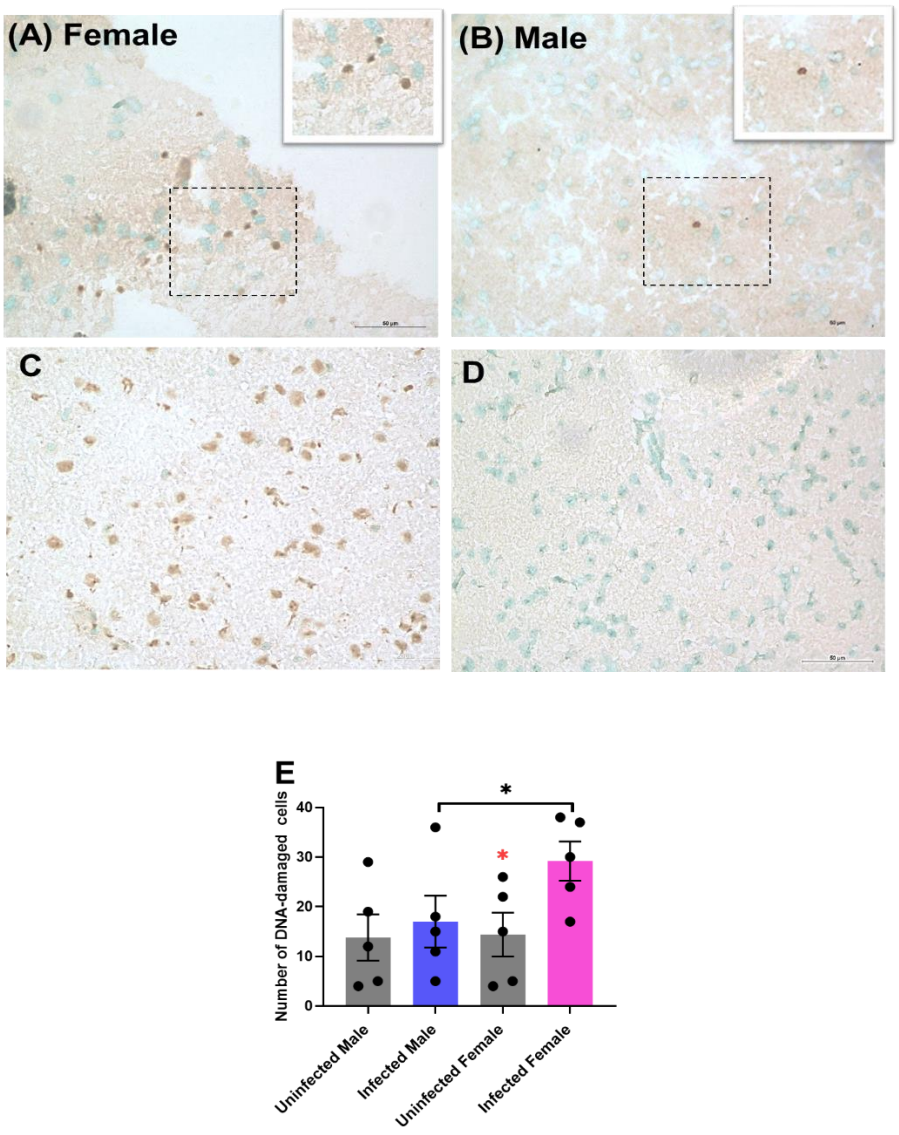


Figure 6.13 DNA fragmentation detection in the brain tissues of male and female infected and uninfected mice

(A&B) Positive staining is represented by a dark brown (DAB) signal of DNA fragmentation. Negative cells indicated by shades of blue-green. **(C)** Positive control cells were created by incubating sections with DNase I. **(D)** Negative control, sections were examined under 40x magnification. **(E)** Quantitative analysis shows infection increases DNA fragmentation in females, but not in males and that females had significantly increased numbers of cells with DNA fragmentation compared to infected males. Each value represents the mean of 5 animals per experimental group and were analysed using a one-tailed nonparametric Mann–Whitney analyses ± SEM. *p <0.05.

6.2.3.2 NLRP3 expression during *T. gondii* infection within brain tissue

Positive staining for NLRP3 was detected in the brain tissues of both males and females infected with *T. gondii*, but not in uninfected mice. NLRP3 expression was mostly associated with *T. gondii* tissue cysts and some clusters of parasites that were distributed in brain parenchyma. Quantitative analysis shows that infected females displayed a higher number NLRP3 positive tissue cysts ($p=0.020$) in comparisons to their male counterparts **(Figure 6.14).** Notably, not all tissue cysts were NLRP3 positive. In addition, BLAST (Basic Local Alignment Search Tool) was done to compare NLRP3 protein sequences to *T. gondii* protein sequence and the result indicted significant difference between NLRP3 sequences and *T. gondii* sequence.

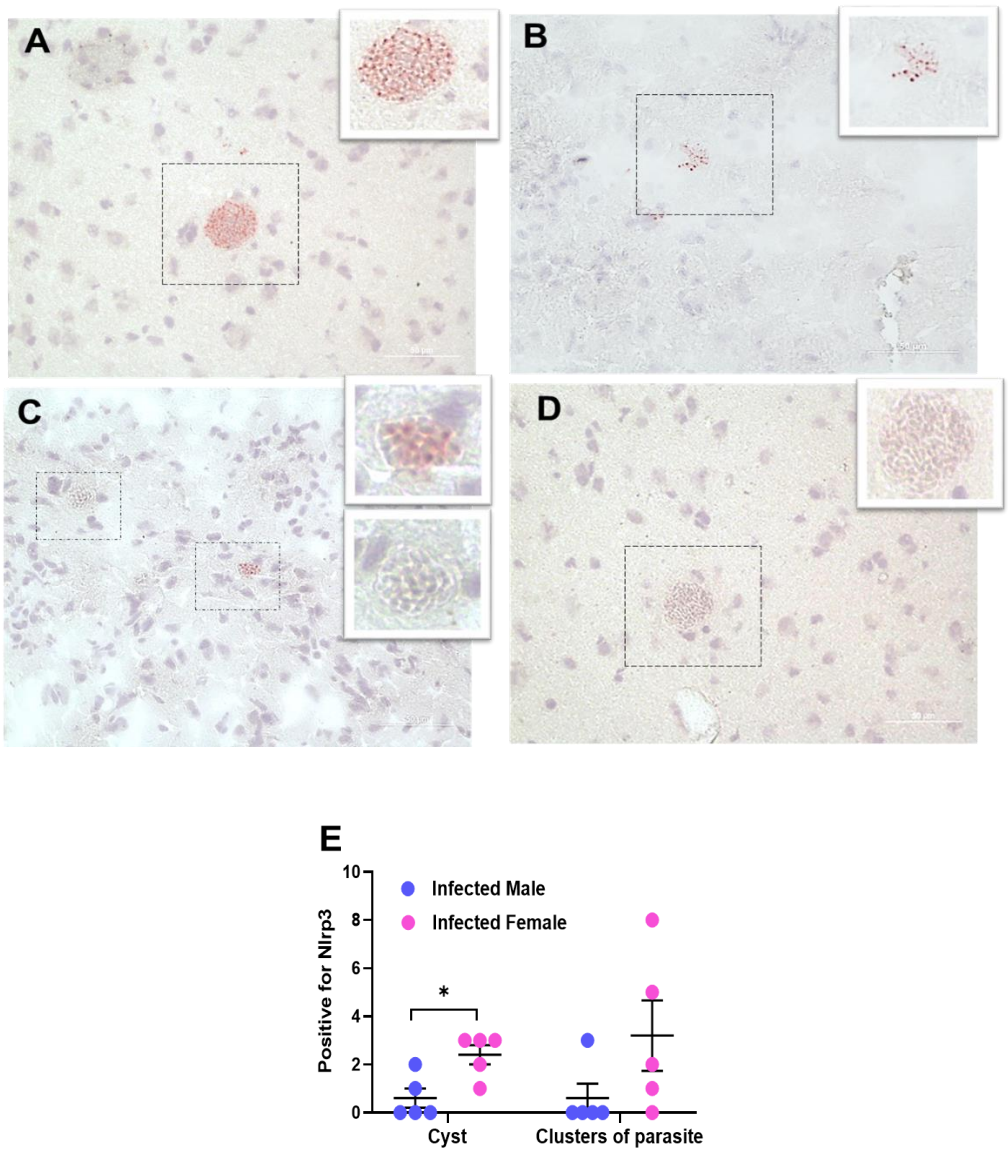


Figure 6.14 Positive staining of NLRP3 antibody detected in the brain tissues of both males and females infected mice

(A&B) Positive staining is represented by red (DAB) signal. **(C)** Positive staining for section has 2 cysts one with red (DAB) signal the second without signal (**D)** Negative control. Sections **(A&B&D)** were examined under 40x magnification, **(C)** under 20x magnification. **(E)** The Quantitative analysis of NLRP3 expression shows that females infected significantly expressed NLRP3 within tissue cyst of *T. gondii* compared to males. Each value represents the mean of 5 animals per experimental group and were analysed using a one-tailed nonparametric Mann–Whitney analyses ± SEM. * $p<0.05$.

6.2.3.3 Positive NLRP3 expression within bone marrow macrophages (BMMs) stimulated with LPS

LPS stimulated BMMs, but not unstimulated BMMs express NLRP3. This was conducted simultaneously with the brain tissue staining (as shown above) as positive control for NLRP3 expression staining **(Figure 6.15).**

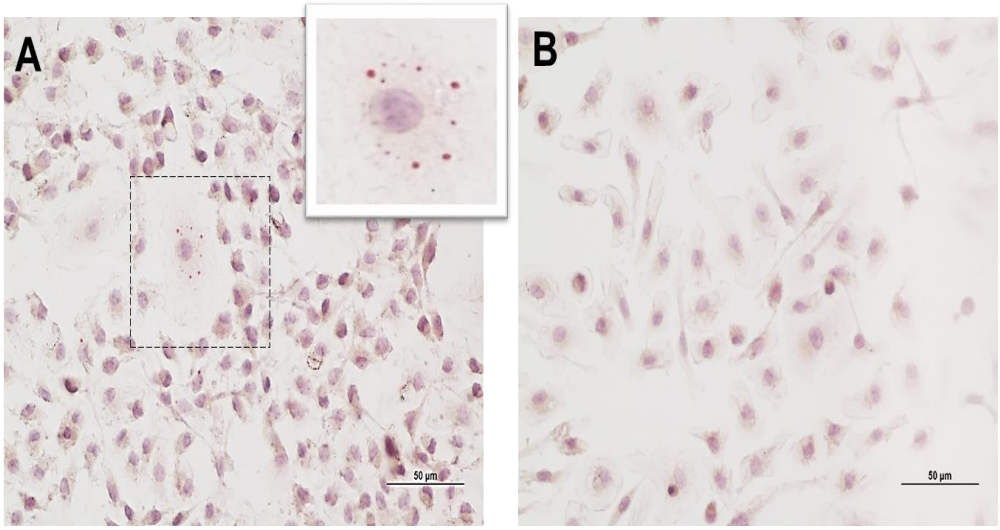


Figure 6.15 Stimulated BMMs expressed NLRP3 antibody

(A) BMM cells stimulated with LPS represented by red (DAB) signal. **(B)** Positive staining for unstimulated cells. Slides were examined under 40x magnification.

6.3 Discussion

The results in this chapter demonstrate sex differences in transcript levels in the brains of mice infected with *T. gondii*. The non-targeted pathway analysis approach highlighted sex differences in mRNA expression of multiple pathways such as 'inflammation mediated by chemokine', 'cytokine signaling pathway integrin signaling', 'Axon guidance mediated by netrin pathway', 'cytokines, T cell activation' and 'apoptosis signalling pathway'. All of these pathways depend on one or more catalytic PI3 kinase subunits. In addition, the pathway analysis approach identified the Wnt signalling pathway as being differentially affected in males and females infected with *T. gondii.*

There is relatively little known about the role of the PI3 kinases (PI3K) and the pathogenesis of *T. gondii* infection. However, it has been shown that *T. gondii* infection of macrophages activates PI3K, which in turn acts to inhibit apoptosis of the host cell (Kim and Denkers, 2006). Moreover, infection of the human retinal pigment epithelium cell line (ARPE-19) with *T. gondii* induces PI3K signaling leading to reduced ROS levels (Zhou et al., 2013). It has been found the production of IL-12 and IL-23 by *T. gondii*-infected Jurkat cells is positively regulated by PI3K (Ismail et al., 2017). Finally, *T. gondii* excreted-secreted antigens (ESA) are known to inhibit Foxp3 expression via the upregulation of PI3K signaling pathway in regulatory T cells (Tregs) (Chen et al., 2019).

Interestingly, there is convincing evidence that the PI3K and the ERα signaling pathways are interconnected and even that the two molecules might directly interact (Guo and Sonenshein, 2004). An in vitro study showed that stimulation with estrogen increases ERα-associated PI3K activity, leading to increased nitric oxide synthase (eNOS) (Simoncini et al., 2000). Estradiol has also been shown to act via PI3K to

protect rat neurons from ischemic-induced apoptosis (Jover-Mengual et al., 2010). There is also evidence that PI3K is affected by male androgens. For example, in a model of high fat diet induced vascular inflammation, castration of rats resulted in upregulation of PI3K expression and increased apoptosis. This was reversed by administration of low-doses of testosterone (Zhao et al., 2016). Taken together these results suggest that estrogens positively regulate, but androgens negatively regulate PI3K signaling. Looking at the transcriptomic data reveals that PI3K transcript levels following *T. gondii* infection are different between the sexes, with female mice having increased levels of Class 1A catalytic subunits, but reduced levels of the Class 1B catalytic subunit relative to male mice. However, transcripts for mTOR, GLUT1 and Rac1 were downregulated in female mice suggesting that overall the PI3K pathway was downregulated. In contrast, the PI3K pathway would appear to be active in male mice as they had increased levels of transcripts for Rac1 and Foxo1 which are positively regulated by PI3K.

The PI3K and the AKT/mTOR pathways are known to be critical in regulating many immunological pathways and processes (Weichhart and Säemann, 2008). The two processes that are strongly regulated in this manner and known to be important in the pathogenesis of *T. gondii* infection are T cell activation/exhaustion and apoptosis/pyroptosis. In keeping with downregulation of the PI3K in female mice, transcripts for T cell exhaustion were found to be greater in female mice than in male mice. Thus female mice had alteration in mRNA transcripts of multiple markers that are consistent with T cell exhaustion including PD-1, TIM3, CD160 and Eomes. T cell exhaustion has been previously described in C57BL/6 mice during the chronic toxoplasmosis. These mice exhibited progressive exhaustion, increased apoptosis, and poor recall responses along with elevated expression of PD-1. Consistent with this they had decreased transcripts for Eomes. Parallel with this CD8 exhaustion,

these mice had disease reactivation with increased parasites resulting in the mortality (Bhadra et al., 2011). PD-1 antagonizes T cell signalling by inhibiting PI3K/AKT/mTOR pathway and this would be consistent with the observation shown here that mTOR, GLUT1 and Rac1 were downregulated in female mice (Parry et al., 2005). Furthermore, PD-1 blockade has been shown to rescue CD8+ T cell exhaustion via the PI3K/Akt/mTOR signalling pathway (Zhao et al., 2019).

Female mice were found to have increased levels of transcripts involved in apoptosis and pyroptosis including TLR1, TLR 4, caspase 1 caspase 9, NLRP3, IL-1β, IL-18 and FasL. *T. gondii* has previously been shown to promote apoptosis and induce severe neurodegeneration in toxoplasmic encephalitis in mice and this is known to be accompanied by a high expression of apoptosis mediators, caspase 3, caspase 8, caspase 9, TNFR1, and NO (Dincel and Atmaca, 2015). Furthermore, *T. gondii* has been shown to promote apoptosis of mice CD4+ splenocytes via Fas-FasL interaction (Khan et al., 1996). To determine if the observed differences in transcripts associated with apoptosis/pyroptosis translated into differences in these events, immunohistochemistry staining was used. Female mice developed greater levels of DNA fragmentation as determined by TUNNEL and increased expression of NLRP3 within their brain tissue during infection with *T. gondii*. The NLRP3 expression was surprisingly mainly observed within the tissue cysts or around clusters of parasites and the histopathology results indicates that females have increased number of tissue cysts in their brain than males. Therefore, it might be suggested that the increased transcript levels of NLRP3 is due to the increased number of tissue cysts within the female brains. In addition, in vitro studies demonstrate that NLRP3 inflammasomes play a role in caspase-1-dependent release of IL-1β in response to *T. gondii* invasion. IL-18 which is also caspase-1 dependent plays a role in host resistance against *T. gondii* in vivo. However, the precise *T. gondii*-derived stimuli that can initiate

inflammasome activation remain undetermined (López-Yglesias et al., 2019). The PI3K pathway is known to promote cell survival and inhibit apoptosis (Gutiérrez-Kobeh et al., 2018). Inhibition of mTOR has been reported to induce apoptosis of epithelial cells of mice (Majumder et al., 2004). Thus the downregulation of this the PI3K/AKT/mTOR pathway in females is consistent with the increased levels of apoptosis/pyroptosis observed in female relative to male mice infected with *T. gondii.*

Sex dependent differences in the chemokine and cytokine signaling pathway are likely to play an important role in determining disease outcome as many of the processes dependent on this pathway are known to be induced by *T. gondii* infection. It is well known that *T. gondii* chronic infection induces neuroinflammation in mice, which is associated with increased microglia activation, recruitment of immune cells into the brain including Th1 and CD8 T cells (French et al., 2019). Inflammation mediated by chemokine and cytokine signaling pathway analysis indicates sex differences in mRNA expression in some genes that are part of phosphatidylinositol 3-kinase (PI3K) signaling pathway. PI3Ks is highly expressed in leukocytes and plays a particularly important role in chemokine-mediated recruitment and activation of innate immune cells at sites of inflammation. They also play a key role in antigen receptor and cytokine-mediated B and T cell development, differentiation and function (Hawkins and Stephens, 2015). Blocking the PI3K pathway with pharmacological inhibitors is known to suppress the production of proinflammatory cytokines such as IL-1 β, IL-6, IL-8, TNF- α (Xie et al., 2014). Thus the sex differences in PI3K transcripts could account for differences in cytokine levels observed between the sexes.

Taken together, it could be suggested that many of the sex differences observed here are linked to sex differences in the expression of components of the PI3K/AKT/mTOR pathway. This results in changes to ‘inflammation mediated by chemokine and

cytokine signaling pathway', 'integrin signaling', 'Axon guidance mediated by netrin pathway' and 'Wnt signalling pathway' (as defined by PANTHER) and T cell activation/exhaustion, apoptosis/pyroptosis pathways. Importantly, both estrogen and testosterone have been shown to influence the PI3K/AKT/mTOR pathway. However, the sex hormones also know to influence at least some of the events and pathways that are regulated by the PI3K/AKT/mTOR pathway. However further analysis is needed to complete our understanding of these complex interactions **(Figure 6.16)**.

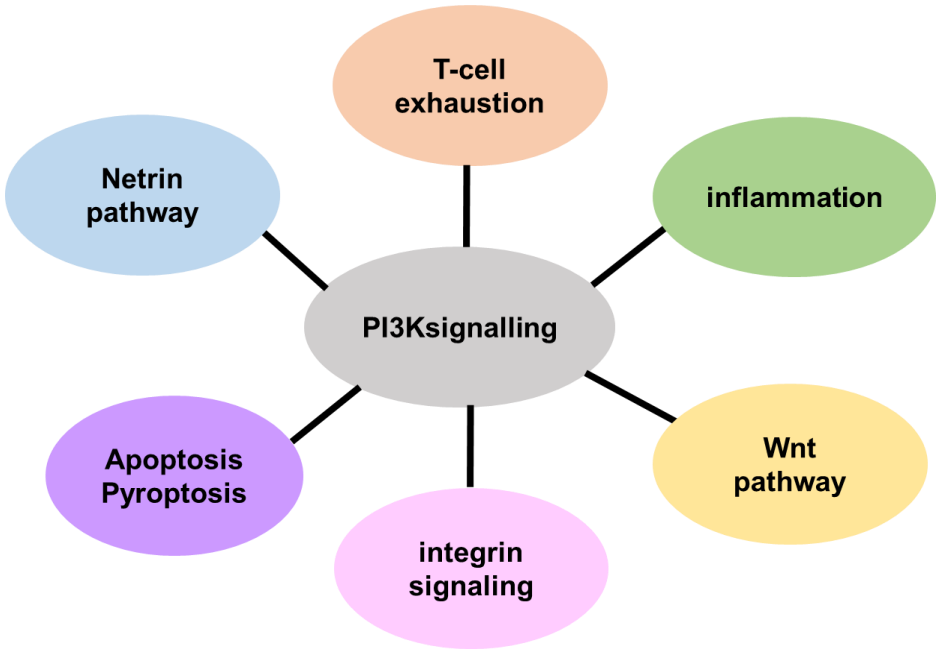


Figure 6.16 The relation of PI3K signalling pathway

Shows possible PI3K signalling relation with inflammation mediated by chemokine and cytokine signaling pathway, integrin signaling, Wnt pathway, Axon guidance mediated by netrin pathway, T cell activation and exhaustion, apoptosis and pyroptosis

Chapter 7 . The final discussion

7 Discussion

This thesis has explored the influence of sex on the pathogenesis of toxoplasmosis using 2 in vivo models in BALB/c mice. The first model was *in vivo* to examine *T. gondii*, multiplication at multiple timepoints in the same mice using an in vivo imaging system (IVIS) and luciferase expressing *T. gondii*. In addition, IL-6, IL-10, MCP-1, IFN-γ, TNFα and IL-12p70 levels were measured in small blood samples using a cytometric bead array. Female BALB/c mice were confirmed to be more susceptible to acute infection, as determined by higher mortality rates and weight loss. However male mice had higher parasite burdens during the acute infection despite their lower mortality. Plasma collected from experimental animals at 6 days post-tinfection showed that infected male and female mice exhibited a significant increase in levels of most of the examined cytokines compared to uninfected mice. However, MCP-1/CCL2, IFN-γ, TNFα concentrations were found to be significantly higher in plasma from infected female mice compared with infected male mice. Therefore this chapter suggests that increased inflammatory cytokines in female mice might be the reason for them having a reduced parasite burden relative to the male mice. However, the incresed levels of cytokines might be the cause for higher mortality rates and severe disease outcomes.

As mentioned in chapter 3, the concentration of MCP-1/CCL2 was significantly increased in the serum of female mice infected with *T. gondii* in comparison to male infected. Therefore, in vitro studies were used to explore the effect of estrogen and testosterone on the secretion of MCP-1/CCL2 from bone marrow macrophages (BMM) infected with *T. gondii*. This chapter demonstrates that estrogen and testosterone suppressed the production of MCP-1/CCL2 from *T. gondii* infected-BMM. Thus, it is proposed that sex hormones can decrease inflammation via

controlling monocytes emigration to the infection as estrogen and testosterone were shown to reduce MCP-1/CCL2 production from *T. gondii* infected-BMM. However, this hypothesis remains to be directly tested.

In the fifth chapter histopathological levels were compared in male and female mice infected orally with *T. gondii* tissue cysts. The data demonstrate that female mice had increased pathology in all examined tissues including spleen, lung, liver, and brain at different days post-infection suggesting that female infected mice develop a robust immune response to *T. gondii* infection. This is consistent with the results described in chapter 3 where female infected mice increased cytokine production with severe disease outcomes. However, the results reported in this model showed that infected females developed more parasite tissue cysts in their brains compared to infected male mice. This is consistent with previous work, but in contrast to the results in chapter 3 where female mice had lower parasite numbers (Roberts et al., 1995).

Additional to the in vivo model examining the histopathological progression during *T. gondii* infection, the final chapter of this thesis studied the mechanism that could influence the sex differences detected in the brains of infected male and female mice with *T. gondii* through analysing the transcriptional profile of their brains. Generally, sex differences in transcript levels in the brains of mice infected with *T. gondii* were detected. In particular, infected females had increased transcript levels of genes such as PD1 that is part of T cell exhaustion and NLRP3 is part of pyroptosis pathways. In addition, sex differences in transcript levels of components that are part of PI3K/AKT/mTOR pathway were also observed and could be linked to the observed differences noted in the above processes.

A recent study, published during the course of this work examined transcript changes during *T. gondii* infection reported few sex differences (Garfoot et al., 2019). However, consistent with the results of our studies, females had increased transcript levels of C-X-C motif chemokine ligand 13 and a C-C motif chemokine receptor 2 (CCR2) than in males during infection (Garfoot et al., 2019). However, in this study the ME49 strain of *T. gondii* was used and CBA/J male and female mice were infected intraperitoneally with 1×10^5 tachyzoites. Thus, differences in mouse strain, parasite strain or route of infection could account for different outcomes between studies. In addition, large variation between the replicate mice in the Garfoot study makes meaningful statistical analysis challenging.

Overall the results suggest the hypothesis that female mice mount a stronger immune response that control parasite levels, but this has immediate detrimental effects including increased weight loss and mortality. The increased inflammation in the early stages of infection in female mice induces T cell exhaustion that has detrimental effects in the chronic stages. It has been previously suggested that high antigen burden or inflammation (including anti-inflammatory) factors or both during the acute phase of infection may be responsible for development of CD8 exhaustion during the chronic stage of *T. gondii* infection (Bhadra and Khan, 2012). Therefore, future studies will test this hypothesis by measuring markers of T cell exhaustion including PD1 and EOMES in T cells in brains and spleens of male and female mice chronically infected with *T. gondii* by flow cytometry and immunohistochemistry. Moreover, increased NLRP3 expression and tissue damage in female mice brain might suggest the hypothesis that female mice infected with *T. gondii* suffer more from neurodegeneration diseases than male mice. It is well known that *T. gondii* infection of brain can cause neurodegeneration diseases (Li et al., 2019). Also, a number of studies have confirmed the pathogenic role of the inflammasome activation in

neurodegeneration diseases such as Alzheimer's disease (AD). Due to the increased activation of NLRP3 inflammasome that characterised by increased production of inflammatory cytokines leading to neurological injury (Geldhoff et al., 2013). In addition, there is large evidence of sex difference in the incidence, severity, and progression of several neurodegeneration diseases. Therefore, future studies will test this hypothesis by comparing markers of neurodegenerative diseases such as Alzheimer's disease (AD) in brains of male and female mice infected with *T. gondii.* That is characterised by the presence of activated microglia and reactive astrocytes by using immunohistochemical (IHC) labeling protocol to label beta amyloid (Aβ) proteins, as an AD neuropathological hallmark (Dandrea et al., 2001).

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Appendix

9.1.1 Table shows the top 100 of VIP results [a]

Symbol	Name	VIP	M	F	q value
Trf	transferrin	1.30	17.6	0	0.0001
Ube2g2	ubiquitin-conjugating enzyme E2G 2	1.30	48	0	0.0002
Dnajc14	DnaJ heat shock protein family (Hsp40) member C14	1.30	0.0	69.4	0.0002
Ovgp1	oviductal glycoprotein 1	1.30	19.6	0	0.0003
Vasn	vasorin	1.30	26.9	0	0.0004
Eif4g2	eukaryotic translation initiation factor 4, gamma 2	1.29	0.6	165	0.0001
Iqsec1	IQ motif and Sec7 domain 1	1.29	0.7	62.1	0.0001
Spg21	maspardin	1.29	0.8	91.7	0.0001
Ncoa7	nuclear receptor coactivator 7	1.29	15.6	0	0.0002
Nphp3	nephronophthisis 3 (adolescent)	1.29	8.3	0	0.0002
Tmc4	transmembrane channel-like gene family 4	1.29	3.5	0	0.0002
Paics	phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoribosylaminoimidazole, succinocarboxamide synthetase	1.29	66.2	49.4	0.0002
Pik3c3	phosphatidylinositol 3-kinase catalytic subunit type 3	1.29	10.9	0.2	0.0002
Mlxipl	MLX interacting protein-like	1.29	0.3	3.7	0.0002
Atraid	all-trans retinoic acid induced differentiation factor	1.29	5.8	101	0.0002
Tgfbr3l	transforming growth factor, beta receptor III-like	1.29	26	0	0.0003
Pik3ip1	phosphoinositide-3-kinase interacting protein 1	1.29	12.7	0	0.0003
Pik3c2b	phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 beta	1.29	2	6.4	0.0003
Pkd2l2	polycystic kidney disease 2-like 2	1.29	1.9	17.8	0.0003
Nim1k	NIM1 serine/threonine protein kinase	1.29	0.3	14.9	0.0003
Sfi1	Sfi1 homolog, spindle assembly associated (yeast)	1.29	34.3	0	0.0004
Trpc4ap	transient receptor potential cation channel, subfamily C, member 4 associated protein	1.29	54.2	33.4	0.0004
Vsx2	visual system homeobox 2	1.29	0.6	0.1	0.0004
Zfp574	zinc finger protein 574	1.29	0.7	9.4	0.0005
Sumo3	small ubiquitin-like modifier 3	1.29	1.2	79.4	0.0005
Arid1a	AT rich interactive domain 1A (SWI-like)	1.29	1	14.4	0.0006
Agl	amylo-1,6-glucosidase, 4-alpha-glucanotransferase	1.29	0.9	7.8	0.0007
Paxx	non-homologous end joining factor	1.29	1.8	32	0.0007
Pan3	PAN3 poly(A) specific ribonuclease	1.29	1.2	0	0.0008
Tbc1d24	TBC1 domain family, member 24	1.29	458	0	0.0009
Zhx1	zinc fingers and homeoboxes 1	1.29	20.4	0	0.0009
Nfs1	nitrogen fixation gene 1 (S. cerevisiae)	1.29	13.7	0	0.0009
Tsc22d4	TSC22 domain family, member 4	1.29	0.5	166	0.0009
Zfp512b	zinc finger protein 512B	1.29	9.6	0	0.0009

Symbol	Name	VIP	M	F	q value
Stt3b	STT3, subunit of the oligosaccharyltransferase complex, homolog B (S. cerevisiae)	1.29	23	0.2	0.0009
Psmd2	proteasome (prosome, macropain) 26S subunit, non-ATPase, 2	1.29	77	39	0.0009
Psmd4	proteasome (prosome, macropain) 26S subunit, non-ATPase, 4	1.29	77.5	1.4	0.0009
Zfpl1	zinc finger like protein 1	1.29	8	0	0.001
Psmd5	proteasome (prosome, macropain) 26S subunit, non-ATPase, 5	1.29	18	0	0.001
Emc8	ER membrane protein complex subunit 8	1.29	6.8	0	0.001
Pole2	polymerase (DNA directed), epsilon 2 (p59 subunit)	1.29	3.1	0	0.001
Mef2b	myocyte enhancer factor 2B	1.29	22.3	0	0.0011
Bhlhe41	basic helix-loop-helix family, member e41	1.29	0.4	19.7	0.0011
Gria2	glutamate receptor, ionotropic, AMPA2 (alpha 2)	1.29	1.4	91.6	0.0012
Sarnp	SAP domain containing ribonucleoprotein	1.29	26.1	0	0.0012
Mfsd11	major facilitator superfamily domain containing 11	1.29	8.4	0	0.0012
Numbl	numb-like	1.29	11.4	0	0.0012
Tcea1	transcription elongation factor A (SII) 1	1.29	26.3	0	0.0013
Tmx2	thioredoxin-related transmembrane protein 2	1.29	13.3	0	0.0013
Tm2d3	TM2 domain containing 3	1.29	12.5	0	0.0013
Gm37494	predicted gene, 37494	1.29	0.6	8.5	0.0013
Gja1	gap junction protein, alpha 1	1.29	114	1.9	0.0013
Scap	SREBF chaperone	1.29	12.1	32.9	0.0013
Fam69b	divergent protein kinase domain 1B	1.29	21.3	0	0.0014
Mamdc4	MAM domain containing 4	1.29	0.2	51.8	0.0014
Nup188	nucleoporin 188	1.29	8.5	0	0.0014
Ebpl	emopamil binding protein-like	1.29	0.4	13.3	0.0014
Myzap	myocardial zonula adherens protein	1.29	0	111	0.0015
Cyb5a	cytochrome b5 type A (microsomal)	1.29	0.2	67.1	0.0015
Nfx1	nuclear transcription factor, X-box binding 1	1.29	0.5	19.9	0.0015
Khdrbs1	KH domain containing, RNA binding, signal transduction associated 1	1.29	1.0	27.7	0.0017
Snapc1	small nuclear RNA activating complex, polypeptide 1	1.29	25.8	0	0.0019
Edc4	enhancer of mRNA decapping 4	1.29	0	10.8	0.0019
Abr	active BCR-related gene	1.29	1.8	96.3	0.0019
Itgad	integrin, alpha D	1.29	68.7	0	0.002
Lrrc71	leucine rich repeat containing 71	1.29	0	11.7	0.002
Aldh2	aldehyde dehydrogenase 2, mitochondrial	1.29	0.7	38.4	0.002
Myl6	myosin, light polypeptide 6, alkali, smooth muscle and non-muscle	1.29	16.7	391	0.0021
Fam160a1	family with sequence similarity 160, member A1	1.29	13.6	0	0.0022
Pcsk6	proprotein convertase subtilisin/kexin type 6	1.29	26.2	0	0.0023
Gm17108	predicted gene 17108	1.29	3.6	143	0.0023
Npb	neuropeptide B	1.29	16	0	0.0023
Rab8a	RAB8A, member RAS oncogene family	1.29	0.4	26.2	0.0023
Zbtb11	zinc finger and BTB domain containing 11	1.29	153	0	0.0024

Symbol	Name	VIP	M	F	q value
Msl1	male specific lethal 1	1.29	0	0	0.0024
Map2k7	mitogen-activated protein kinase kinase 7	1.29	0	27.2	0.0026
Vsig10l	V-set and immunoglobulin domain containing 10 like	1.29	49.7	0	0.0028
Rpl18	ribosomal protein L18	1.29	0.4	242	0.0028
Prpf19	pre-mRNA processing factor 19	1.29	0.8	35.9	0.0028
Chpt1	choline phosphotransferase 1	1.29	0.9	44	0.0028
Nrn1l	neuritin 1-like	1.29	8.6	0	0.0029
Gas2l1	growth arrest-specific 2 like 1	1.29	0.4	29.7	0.0033
Uckl1	uridine-cytidine kinase 1-like 1	1.29	0	17.5	0.0037
Ccdc62	coiled-coil domain containing 62	1.29	1.4	34.2	0.0038
H2-Q2	histocompatibility 2, Q region locus 2	1.29	138	2746	0.0038
Tldc1	MTOR associated protein , eak-7 homolog	1.29	12.1	0.3	0.004
Mir22hg	Mir22 host gene (non-protein coding)	1.29	0	19.8	0.0041
Ube2v1	ubiquitin-conjugating enzyme E2 variant 1	1.29	23.7	0	0.0048
Kdelc1	protein O-glucosyltransferase 2	1.29	0	9.9	0.0048
Mri1	methylthioribose-1-phosphate isomerase 1	1.29	25.4	0	0.0049
Rps28	ribosomal protein S28	1.29	0.9	1226	0.0064
Serp1	stress-associated endoplasmic reticulum protein 1	1.29	0.6	92.4	0.007
Chchd7	coiled-coil-helix-coiled-coil-helix domain containing 7	1.29	0.9	333	0.0085
Gng3	guanine nucleotide binding protein (G protein), gamma 3	1.29	2.7	241	0.0091
Hpcal4	hippocalcin-like 4	1.29	129	0.1	0.0096
Hypk	huntingtin interacting protein K	1.29	1.1	49.4	0.0098
Gipc3	GIPC PDZ domain containing family, member 3	1.29	0.5	139	0.0106

[a] The top 100 VIP scores results of comparing infected male to infected female mice, the mean of FPKM of males and females. q value < 0.05 of comparing the mean of 3 mice of infected males to infected females

9.1.2 Table shows the FPKM mean of mRNA expression of receptors involved in T-cell exhaustion [a]

		Un-Male	Inf- Male	Un-Female	Inf-Female	P value Inf(M vs F)	P value Un (M vs F)
APC Receptors	CD86	0.71	11.17	0.98	16.38	0.05	0.05
	CD80	0.23	1.86	0.35	2.37	0.20	0.20
	PD-L1	0.03	0.95	0.06	1.18	0.50	0.10
	PD-L2	0.73	49.69	1.06	54.19	0.35	0.05
	ICOSL	2.08	6.69	2.05	8.44	0.05	0.50
	Gal-9	4.56	27.32	5.29	35.93	0.05	0.05
	HVEM	0.19	3.70	0.24	6.54	0.10	0.50
T cell Receptors	CD28	0.05	0.93	0.16	3.22	0.05	0.05
	Ctla4	0.03	0.72	0.04	1.61	0.20	0.50
	PD-1	0.04	0.50	0.05	1.48	0.05	0.35
	Eomes	7.52	3.83	7.23	0.34	0.05	0.20
	ICOS	0.02	1.88	0.02	3.86	0.10	0.35
	TIM3	0.92	4.89	1.16	7.90	0.05	0.20
	CD160	1.02	1.05	0.70	2.48	0.05	0.05

[a] The FPKM mean of mRNA expression of receptors involved in T-cell exhaustion. Each value represents the mean FPKM of 3 mice and p value were analysed using one-tailed nonparametric Mann–Whitney test ± SEM $p < 0.05$.

9.1.3 Table shows the FPKM mean of mRNA expression of key factors involved in apoptosis [a]

	Un-Male	Inf- Male	Un-Female	Inf-Female	P value Inf(M vs F)	P value Un (M vs F)
Fas	1.51	4.90	1.13	7.79	0.10	0.10
Tnfrsf10b	0.31	0.61	0.93	1.79	0.05	0.05
Tnfrsf1a	6.14	21.94	6.49	25.32	0.35	0.50
Tnfrsf25	0.30	0.53	0.81	3.73	0.05	0.20
Tnfrsf21	25.14	29.33	23.14	19.85	0.05	0.20
TLR1	0.84	4.54	1.00	7.92	0.05	0.20
TLR2	0.81	11.39	1.14	12.22	0.50	0.05
TLR3	4.90	10.97	5.22	10.24	0.35	0.20
TLR4	0.64	3.01	0.66	4.41	0.05	0.50
TLR6	0.45	1.90	0.45	2.42	0.20	0.50
TLR7	0.82	4.17	8.87	9.27	0.05	0.05
TLR8	0.07	2.14	0.74	4.66	0.05	0.05
TLR9	0.22	2.78	0.23	2.67	0.35	0.50
TLR11	0	0.80	0	0.58	0.35	0.50
TLR12	0.17	4.11	0.25	4.62	0.50	0.05
TLR13	0.65	12.75	9.62	9.19	0.05	0.05
Fadd	0.78	0.70	2.21	3.45	0.05	0.05
Tradd	2.17	3.19	3.11	3.42	0.50	0.05
Myd88	2.63	8.81	2.95	11.19	0.05	0.05
Casp8	1.31	6.25	1.76	7.42	0.20	0.05
Casp9	18.80	18.77	39.15	62.43	0.05	0.05
Casp6	2.12	5.13	2.91	7.09	0.05	0.05
Casp3	6.55	8.96	6.30	9.85	0.35	0.50
Casp7	1.12	4.41	1.06	5.69	0.10	0.35
Bid	5.17	8.11	4.41	8.64	0.35	0.10
Bcl2	13.41	15.62	13.64	16.33	0.35	0.35
Bcl2l1	54.13	47.46	49.27	41.80	0.10	0.05
Cycs	25.26	23.89	28.35	23.60	0.50	0.05

[a] The FPKM mean of mRNA expression of factors involved in apoptosis pathway. Each value represents the mean FPKM of 3 mice and p value were analysed using one-tailed nonparametric Mann–Whitney test ± SEM $p < 0.05$.

9.1.4 Table shows the FPKM mean of mRNA expression of key factors involved in pyroptosis [a]

	Un-Male	Inf- Male	Un-Female	Inf-Female	P value Inf(M vs F)	P value Un (M vs F)
TLR1	0.84	4.54	1.00	7.92	0.05	0.20
TLR2	0.81	11.39	1.14	12.22	0.50	0.05
TLR3	4.90	10.97	5.22	10.24	0.35	0.20
TLR4	0.64	3.01	0.66	4.41	0.05	0.50
TLR6	0.45	1.90	0.45	2.42	0.20	0.50
TLR7	0.82	4.17	8.87	9.27	0.05	0.05
TLR8	0.07	2.14	0.74	4.66	0.05	0.05
TLR9	0.22	2.78	0.23	2.67	0.35	0.50
TLR11	0	0.80	0	0.58	0.35	0.50
TLR12	0.17	4.11	0.25	4.62	0.50	0.05
TLR13	0.65	12.75	9.62	9.19	0.05	0.05
IL1R	1.11	1.90	2.66	4.85	0.05	0.05
P2RX7	2.82	9.12	2.86	8.46	0.50	0.50
Myd88	2.63	8.81	2.95	11.19	0.05	0.05
Nlrp3	0.23	1.34	0.27	2.02	0.05	0.20
Pycard	1.66	19.39	2.02	44.98	0.05	0.20
Casp1	1.46	16.83	1.81	24.46	0.05	0.10
IL-1β	0.30	7.23	0.42	14.58	0.10	0.20
IL18	11.34	14.65	13.23	22.12	0.05	0.10
Gsdmd	0.72	8.46	0.78	12.17	0.05	0.20

[a] The FPKM mean of mRNA expression of factors involved in pyroptosis pathway. Each value represents the mean FPKM of 3 mice and p value were analysed using one-tailed nonparametric Mann–Whitney test ± SEM $p < 0.05$.