University of Strathclyde
Strathclyde Institute of Pharmacy and Biomedical Sciences

Immunological Control of *Toxoplasma gondii Infection*

By

Stuart Woods

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This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination, which has lead to the award of a degree.

Signed:

Date:

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Abstract

Prevalent worldwide, the protozoan parasite, *Toxoplasma gondii*, is an important cause of spontaneous abortion, ocular disease, mental retardation and encephalitis. Currently there are no human vaccines available. The first major aim of this study was to test potential HLA restricted peptide vaccines, previously shown to be protective in HLA-transgenic mice, against oocyst infection. The ability of entrapment within non-ionic surfactant vesicles to improve the efficacy of the HLA-B*0702 restricted vaccine was also studied. In parallel we tested the novel *T. gondii* ΔRPS13 live-attenuated vaccine against oocyst challenge. As determined by survival, only ΔRPS13 provided a measure of protection against oocyst challenge. We also demonstrated that the live vaccine induced a greater $CD8^+$ T cell effector response than the adjuvanted peptide vaccine. Successful vaccination is in large part dependent on inducing an appropriate response in the primary host cell populations that consequently influences the development of adaptive immunity. Parasite induced macrophage arginase-1 expression, for example, has been shown to be influential during *T. gondii* infection. Arginase-1 expression is negatively regulated by Map Kinase Phosphatase-2 (MKP-2), the second major aim of the project was to study the effect of MKP-2 deficiency on *T. gondii* infection. MKP-$2^{-/-}$ mice were found to be more susceptible to infection with increased parasite growth and increased mortality compared with wild type mice. Increased susceptibility was associated with reduced serum nitrite levels and enhanced tissue arginase-1 expression although the Th1 response was unaltered. *In vivo* inhibition of iNOS and arginase-1 revealed that while NO production is of paramount importance in controlling parasite growth arginase-1 could also limit parasite growth independently. *In vitro* studies utilising macrophages confirmed a role for arginase-1 in parasite control. Results highlight a complex interaction between iNOS and arginase-1 and *T. gondii* in L-arginine metabolism but indicate that manipulation of early infection events influence disease outcome.

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Abbreviations

AMA	Apical membrane antigen
AP-1	Activator protein 1
APC	Antigen presenting cell
BAG-1	Bradyzoite antigen 1
CCR	Chemokine receptor
CD	Cluster of differentiation
CMI	Cell mediated immunity
CNS	Central nervous system
DC	Dendritic cell
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
FCS	Foetal calf serum
GPI	Glycosylphosphatidylinositol
HIV	Human immunodeficiency virus
HSP	Heat shock protein
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	inducible nitric oxide synthase
LPS	Lipopolysaccharide
MΦ	Macrophage
MCP	Monocyte chemotactic protein
MHC	Major histocompatibility complex
Mic	Microneme
MIP	Macrophage inflammatory protein
MJ	Moving junction
MKP	MAPK phosphatase
MyD88	Myeloid differentiation primary response gene 88
NISV	Non-Ionic Surfactant Vesicles
NK	Natural killer

NO	Nitric oxide
OT	Ocular toxoplasmosis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PV	Parasitophorous vacuole
RFLP	Restriction fragment length polymorphisms
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediates
ROP	Rhoptry
RPM	Revolutions per minute
SAG-2a	Surface antigen 2a
SDS	PAGE Sodium dodecyl sulphate polyacrylamide gel electrophoresis
S.E.M	Standard error of the mean
STAT	Signal transducer and activator of transcription
TE	Toxoplasmic encephalitis
TGF	Tumor growth factor
TgHSP70	*T. gondii* heat shock protein 70
Th	T helper
TLA	*Toxoplasma* lysate antigen
TLR	Toll like receptor
TNF	Tumor necrosis factor
Treg	T regulatory
YFP	Yellow fluorescent protein

Chapter 1

Introduction

1.1. Discovery of *Toxoplasma gondii*

Toxoplasma gondii was first described by Nicolle and Manceaux in 1908 (Nicolle & Manceaux, 1908), when it was found in the spleen of a small African rodent called the *Ctenodactylus gundi* and given the name 'Toxoplasma' derived from its arched shape (toxo, meaning arc or bow and plasma, meaning life). *T. gondii* was simultaneously discovered by Splendore, in rabbit tissues (Splendore 1908). *T. gondii* is described as a ubiquitous, obligate intracellular protozoan parasite from the Apicomplexa subphyla. *T. gondii* is capable of infecting any nucleated cell from warm blooded animals, resulting in a huge array of potential intermediate hosts. However, the definitive host for the parasite is the *Felidae* family, in which sexual reproduction occurs. The sexual reproductive cycle was identified in 1969 by Hutchison and colleagues at the University of Strathclyde (Hutchison *et al.*, 1969). Infection of humans is found worldwide and it is thought that about one third of the global population is infected with the parasite.

1.2. Epidemiology

T. gondii is a highly prevalent and ubiquitous parasite with an estimated 500 million individuals infected worldwide (Montoya & Liesenfeld 2004; Weiss & Dubey 2009). Through genotypic analysis utilising multi-locus restriction fragment length polymorphisms (RFLP) at 6 loci, across 106 isolates, *T. gondii* was reported to have evolved into 3 distinct clonal lineages differing by less than 1% at a genetic level (Su *et al.*, 2003). This indicated that these three strains arose from 2 common predominant strains and have since undergone limited recombination (Su *et al.,* 2003; Grigg *et al.* 2001). These 3 archetypal lineages were defined as Type I, II and III (Howe & Sibley 1995).

Type I strains such as RH have been identified as the most virulent of the *T. gondii* strains causing rapid death in Laboratory animals (Saeij *et al.*, 2005) and linked with severe disease in humans (Sibley & Boothroyd 1992). Types II and III are classed as non-virulent strains (Howe & Sibley 1995). The rapid doubling time of type I strains and their demonstrated ability over the type II and III

strains, to cross physiological barriers (Barragan & Sibley 2002) such as the blood-brain barrier, intestinal epithelium and the placenta, is thought to be linked with their virulence.

There is a huge variance in the disease phenotypes of the various strains and their geographical locations and as a result it is difficult to definitively associate a parasite strain with a disease phenotype. For example Type I strains have been associated with congenital toxoplasmosis and ocular toxoplasmosis in immunocompetent and immunocompromised hosts in the USA (Silveira *et al*., 2003). However this contradicts studies in the UK where ocular toxoplasmosis has been associated with type II strains. These studies also contradict work done by Grigg *et al*. (2001), who demonstrated that type I-III recombinant strains were responsible for ocular toxoplasmosis in immunocompetent hosts.

Although type II strains are associated with a less virulent disease phenotype, this can vary depending on the genetic background of the host as demonstrated in laboratory models. For example BALB/c mice are resistant to *T. gondii* due to the presence of the H-2L^{d} haplotype (Brown & Mcleod 1990). In C57BL/6 mice the outcome of infection is dependent on the route of infection. Oral infection results in severe susceptibility to disease and mortality. However C57BL/6 mice are resistant to intraperitoneal infection (Mcleod *et al*., 1989). However, disease severity can also vary depending on the parasite load administered.

More recently two further types of *T. gondii* have also been described in the literature, types IV and V. These are also classified as atypical strains that are recombinant strains derived from types I, II and III that may have been formed by atypical allele combinations. These strains are most often found in patients from South America and Africa and thus the term ‘atypical’ has been created through initial sampling bias in which these geographical regions were largely ignored (Saeij *et al*., 2005).

1.3. Transmission

T. gondii is capable of successfully infecting the host by a number of routes and life cycle stages: ingestion of oocysts via the fecal-oral route, consumption of tissue cysts from undercooked meat, and congenital infection by tachyzoites (Figure 1.1).

Currently it is thought that around one third of the global population is infected with the organism (Dubey 1998). It has been suggested that the ability of *T. gondii* to infect via the oral route has led to it being such a prevalent infection (Su *et al*. 2003). The most common source identified for transmission of *T. gondii* is the consumption of raw or undercooked meats containing tissue cysts or food and water that have come into contact with oocysts derived from cat feces. It has been found that the prevalence of infection in a given population often correlates with eating habits (Montoya & Liesenfeld 2004). Epidemic outbreaks of toxoplasmosis in humans and sheep have been associated with infected cats, highlighting their importance in propagating infection (Teutsch *et al.*, 1979).

T. gondii is also transmitted vertically from mother to foetus when the mother is infected during pregnancy. The first recorded incidence of this occurring in humans was in 1939 (Wolf *et al*., 1939) when a newborn presented with seizures and eye lesions. The child died at one month old and post-mortem examination confirmed the presence of *T. gondii* tachyzoites. Since then, research has confirmed that during the acute stages of infection tachyzoites may cross the placenta and disseminate into the foetal circulation (Remington 1990). There have also been a number of reported incidents of reactivation of chronic *T. gondii* infection during pregnancy, resulting in primary infection of the foetus (Andrade *et al*., 2010).

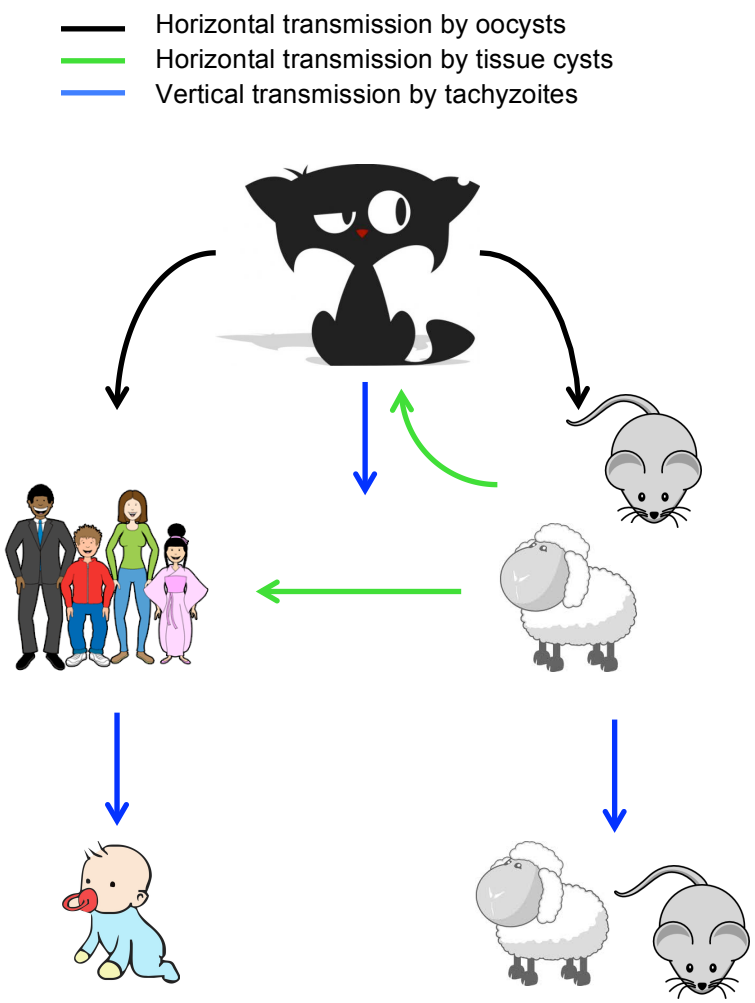

Figure 1.1. Transmission cycle of *T. gondii*.

T. gondii may be transmitted from the feline definitive host to any other warm blooded species by the ingestion of oocysts shed in cat feces. The parasite may then be disseminated within the intermediate host pool by the ingestion of tissue cysts, such as in undercooked or raw meats. In all hosts *Toxoplasma* may be transmitted vertically by infecting the foetus during pregnancy.

1.4. Life cycle

The lifecycle of *T. gondii* may be divided into two stages; the extra-intestinal stage (Figure 1.2), which can occur in any of the hosts, species or the enteroepithelial stage (Figure 1.3), which only occurs in the definitive host. Both of these cycles begin with the ingestion of either tissue cysts or oocysts.

1.4.1. Extraintestinal reproduction

This stage of the life cycle is also known as the asexual stage. Following ingestion, the oocysts or tissue cysts are digested by the host's gastric juices as they pass through the stomach and small intestine. The sporozoites (from oocysts) or bradyzoites (from tissue cysts) are released and infect the gut epithelial cells. By 12-18 hours the parasites have converted to tachyzoites (Dubey, 1998) within parasitophorous vacuoles (PV) in infected cells. The tachyzoite is a fast dividing stage of the life cycle. After multiple rounds of endodiogeny the parasites lyse the host cells and go on to infect more cells and disseminate within the host. New host cells infected include those from the immune system such as macrophages and dendritic cells, which the parasite has been reported as using as "Trojan horses" to aid dissemination (Lambert *et al.*, 2009).

The acute infection enters the chronic phase with the fast dividing tachyzoites converting into the slow dividing bradyzoites, existing within tissue cysts. This interconversion is thought to be dependent on environmental stress factors such as nutrient starvation or immune pressures such as IFN-γ, IL-12, TNF-α and nitric oxide (Lyons *et al.*, 2002). This conversion process has been shown to be dependent on parasite derived heat shock proteins (HSP) with inhibition of these resulting in reduced bradyzoite formation (Weiss *et al.*, 1998). The conversion of tachyzoites to bradyzoites my be monitored by changes in surface antigens (SAG), expression of bradyzoite antigens (BAG) or the transcription of stage specific enzymes such as P-type ATPase and glycolytic enzymes (Reviewed by Lyons *et al*. 2002).

Following conversion the bradyzoites exist within tissue cysts which can be found in neuronal tissues, skeletal and cardiac muscles and the eye (Dubey & Frenkel, 1976). Within the tissue cyst the parasite may exist for the duration of the host's life without causing overt illness. However, recent evidence suggests that chronic disease can be associated with an increased likelihood of neuropsychiatric disease (Henriquez *et al.,* 2009). It has been suggested, but not proven that the periodic rupturing of the tissue cysts and the subsequent conversion of the bradyzoites back into tachyzoites and reactivation of infection of the host is required for maintaining a lasting protective immunity as well as the persistence of the parasite (Hunter & Reichmann, 2001).

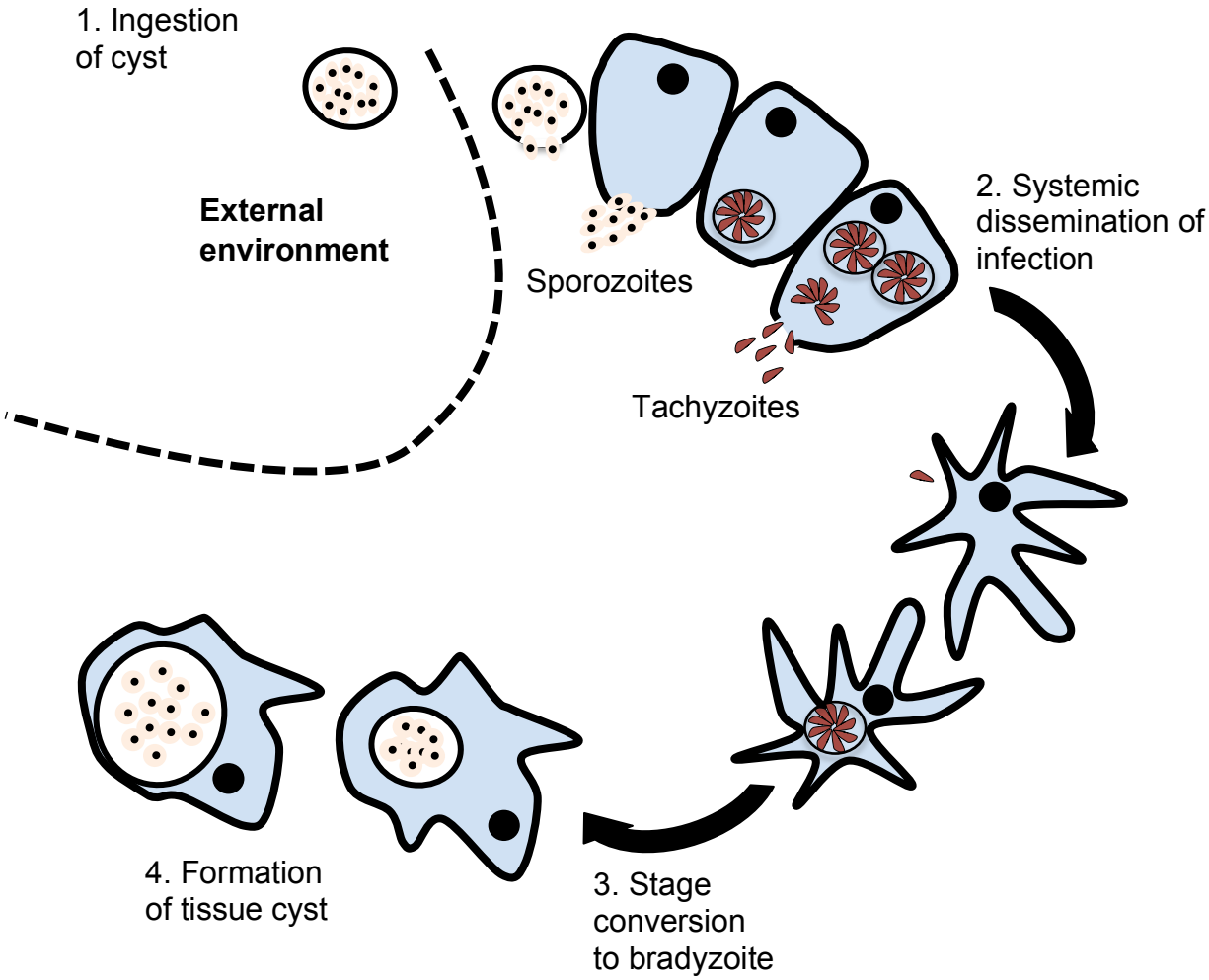

Figure 1.2. The extra intestinal stage of *Toxoplasma gondii* life cycle

The extra intestinal stage of the parasite's life cycle may occur in any host, following the ingestion of tissue cysts or oocysts (1). Bradyzoites or sporozoites, respectively, are released which convert into the fast dividing tachyzoite stage which disseminates within the host (2). The tachyzoites then convert to slow dividing bradyzoites (3) and form tissue cysts. In this stage the parasite may exist within the host for the remainder of the host's life (4).

1.4.2. Enteroepithelial reproduction

The enteroepithelial stage of the *T. gondii* life cycle is initiated in the same manner as the extra intestinal stage, with the ingestion of tissue cysts or oocysts. Importantly, this has only been described in the cat. The bradyzoites or sporozoites, are released from the cysts and oocysts respectively following digestion and infect the gut epithelial cells (Dubey *et al.*, 1997). Here the parasite forms schizonts, which produce merozoites. The merozoites eventually go on to form macrogametes and microgametes by gametogenesis, in the gut ileum. The microgamete fertilises the macrogamete, producing a tough cell wall around the fertilised gamete, which becomes a zygote and eventually forms an oocyst. The oocysts are then shed from the gut wall and excreted in the cat faeces. A cat may excrete oocysts for many weeks following infection. The oocysts are capable of surviving in the environment for many months while remaining infectious (Frenkel *et al.*, 1975).

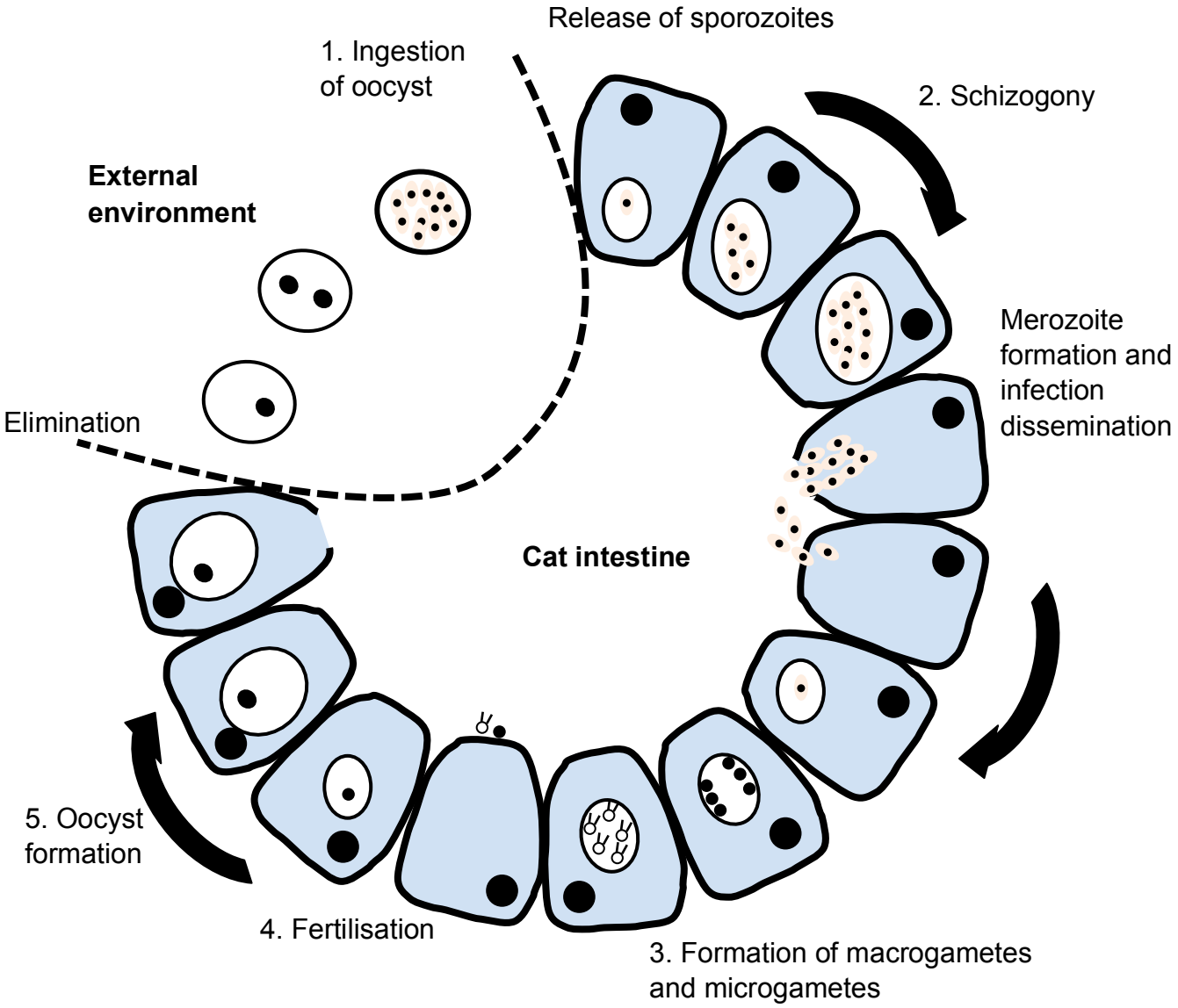

Figure 1.3. The intraepithelial lifecycle of *T. gondii*

Bradyzoites or sporozoites are released from ingested tissue cysts or oocysts (1). These undergo schizogony, forming merozoites (2), which differentiate into either macrogametes or microgametes (3). Following fertilisation of the macrogamete by the microgamete, the oocysts forms (4). The oocysts are then passed in the cat faeces (5), where they persist in the environment until picked up by a new host.

1.5. Disease outcome

1.5.1.Immunocompetent host

In adults and adolescents with a healthy immune system infection with *T. gondii* is typically asymptomatic, and generally goes undiagnosed. If the patient does suffer any symptoms they are usually non-specific, flu like symptoms, such as malaise, fever, and muscle ache. Typically the infection is self limiting, and dose not require any medical intervention (Montoya & Liesenfeld 2004). There is increasing evidence that even in immune competent individuals, chronic infection is associated with changes to behaviour and an increased likelihood of developing certain neuropsychiatric diseases (reviewed, Henriquez *et al*., 2009)

1.5.2. Immunocompromised

For those who are immunocompromised, such as HIV and transplant patients, infection with *T. gondii* can be life threatening. In such cases toxoplasmosis is usually as a result of reactivation of tissue cysts (Montoya & Liesenfeld 2004). Infection with *T. gondii* is common in AIDS patients with up to 25% being treated for the disease. *Toxoplasma* is also the most common opportunistic pathogen of the central nervous system in these patients (Porter & Sande 1992). Lesions may form in the brain causing the development of toxoplasmic encephalitis (TE). These patients can present with a range of clinical symptoms such as altered mental status, seizures and sensory abnormalities. The disease is often fatal if not treated promptly (Wijdicks *et al*., 1991). Toxoplasmosis may also present as chorioretinitis, or pneumonitis or indeed the disease may involve multiple organs in some patients.

Transplant patients are at severe risk of disease from the reactivation of chronic infection. The anti-rejection medications which are given to suppress the immune system can result in the patients struggling to deal with the infection resulting in severe disease (Derouin & Pelloux 2008).

1.5.3. Congenital Toxoplasmosis

Congenital toxoplasmosis results from the vertical transmission of the parasite, from mother to foetus during the acute stage of infection. Transmission generally will only occur if the mother was seronegative before the pregnancy. If chronic infection is established before the pregnancy, then it is highly unlikely that vertical transmission will occur (Silveira *et al*., 2003). The incidence of congenital infection varies greatly world wide, varying with the seropositivity of the population (Allain *et al*., 1998). In the UK the incidence is approximately 1 per 10,000 women (Allain *et al*., 1998). The stage of pregnancy when the mother becomes infected is critical in determining whether the foetus becomes infected and the severity of the disease. Infection of the foetus is more likely to occur as gestation progresses. However, the later into the pregnancy infection occurs, generally the less severe the disease outcome (Allain *et al*., 1998). Infection during the first trimester has severe consequences and may result in mental retardation, and or hydrocephalus and also carries a high risk of abortion. Infection during the third trimester invariably results in less severe, often asymptomatic disease and carries a low risk of abortion (Allain *et al*., 1998). However, essentially all congenitally infected individuals will develop ocular lesions requiring treatment later in life.

1.5.4. Ocular Toxoplasmosis

Ocular toxoplasmosis (OT) can cause loss of eyesight and may be a result of vertical transmission of the parasite or it can be acquired post-natally (Montoya & Remington 1966; Holland 1999). The route of acquisition of OT would appear to be dependent on geographical location. In Brazil it has been shown that most cases of OT are acquired post-natally, whereas in the United States it is generally as a result of congenital infection (Vallochi *et al*., 2002). In the United Kingdom, the incidence of congenitally derived symptomatic OT is around 0.4/100,000/year. Ocular toxoplasmosis is the most common cause of retinochoroiditis in immune-competent patients and is the most common manifestation of the disease (Holland 2004).

1.6. Treatment

Infection of immunocompetent individuals is often self-limiting, if symptoms present. Therapeutic treatment is therefore not routinely given unless the symptoms persist or become more severe. Typically pyrimethamine and sulphadiazine are given, in combination with corticosteroids to reduce inflammation, for OT (Montoya & Liesenfeld 2004). However, the additional benefits of corticosteroids have not been proven and corticosteroids given without anti-parasitic compounds (due to misdiagnosis) can result in exacerbation of infection and destruction of the retina ((Montoya & Liesenfeld 2004; Stanford & Gilbert 2009)

Treatment of an expectant mother with an acute infection typically consists of spiramycin or pyrimethamine and sulphadiazine to prevent congenital infection. Work by Ricci *et al.,* (2003), indicated that mothers who where not treated were 4 times more likely to transmit the infection to the foetus.

1.7. Ultrastructure of *Toxoplasma gondii*

T. gondii is a eukaryote, as such it possesses organelles common to all eukaryotic cells (nucleus, ER, Golgi; Figure 1.5). However, it is important to note that it only possesses one single mitochondrion and an apicoplast (the non-photosynthetic reduced relic of a once 'red algae like' endosymbiont). Furthermore, the parasite possesses three distinct secretory organelles: dense granules, rhoptries and micronemes. These are specialised organelles essential for invasion of the host cell, establishment of the parasitophorous vacuole membrane and maintenance of the parasite. The micronemes and rhoptries are located at the apical end of the parasite, while the dense granules are dispersed throughout the cytosol of the parasite. Each organelle has a specific role and complement of proteins. Many of these secreted proteins are potential vaccine candidates (Reviewed by Boothroyd 2009; Dubremetz and Ferguson 2009; McFadden 2011)

1.7.1. Dense granules

Dense granules are distributed throughout the tachyzoites and are responsible for the secretion of specific dense granule proteins (GRA). Currently 16 different GRA proteins have been identified, ranging in mass from 21-41KDa and are of little similarity to other identified proteins (Braun *et al*., 2008). Further to GRA proteins 2 NTP-ases and 2 protease inhibitors have also been identified (Sun *et al*., 2011). The dense granule proteins are secreted from the apical tip of the parasite, through fusion of the dense granule with the plasma membrane. The proteins then either associate with the parasitophorous vacuole membrane (PVM) or form part of the tubular network that exists between the parasite and the PVM. Secretion via the dense granules forms the default pathway for the secretion pathway of many soluble proteins.

1.7.2. Rhoptries

These are club-shaped organelles and the largest of the three secretory bodies that form part of the apical complex. The size of the individual rhoptry varies

with the size of the stage of the life cycle (Hajj *et al*., 2006). These membrane bound organelles are acidic and possess two structurally distinct regions: a basal bulbous region and narrow apical duct. They manufacture and secrete a large variety of proteins and enzymes (ROPs), released during invasion of a nucleated host cell. They are synthesised as pre-proteins and become active when being packaged for secretion (Sadak *et al*., 1988). Currently there have been over thirty rhoptry proteins identified (Dlugonska 2008). The proteins are associated with the establishment and the repair of the parasitophorous vacuole membrane. This aids the survival of the parasite within the host cell, protecting it from cellular endocytotic pathways. Rhoptry neck proteins are also secreted from the rhoptry bodies. These are involved in the invasion of the host cell (Boothroyd & Dubremetz 2008).

1.7.3. Micronemes

Micronemes (MIC) are small structures that are present in large numbers at the anterior of the parasite with the rhoptries. They are membrane bound, with a densely packed interior, containing a large variety of proteins. Currently there have been at least thirteen distinct proteins characterised (Fritz *et al.*, 2012). The proteins play an important role in the movement of the parasite, functioning as ligands (Soldati *et al.,* 2001). MIC proteins are secreted onto the surface of the parasite, mediated by Ca^{2+} release, facilitating a gliding movement (Blackman & Bannister 2001) They are also important in the initial recognition of the host cell and are involved in host cell attachment and invasion (Nishi *et al.*, 2008).

1.7.4. Apicoplast

This organelle is a vestigial plastid, similar to a chloroplast, and is thought to have initially been acquired through secondary endosymbiosis of an algae (Köhler *et al*., 1997) and has become essential for the survival of the parasite. One of its many essential roles includes fatty acid metabolism and biosynthesis (Ramakrishnan *et al*., 2011)

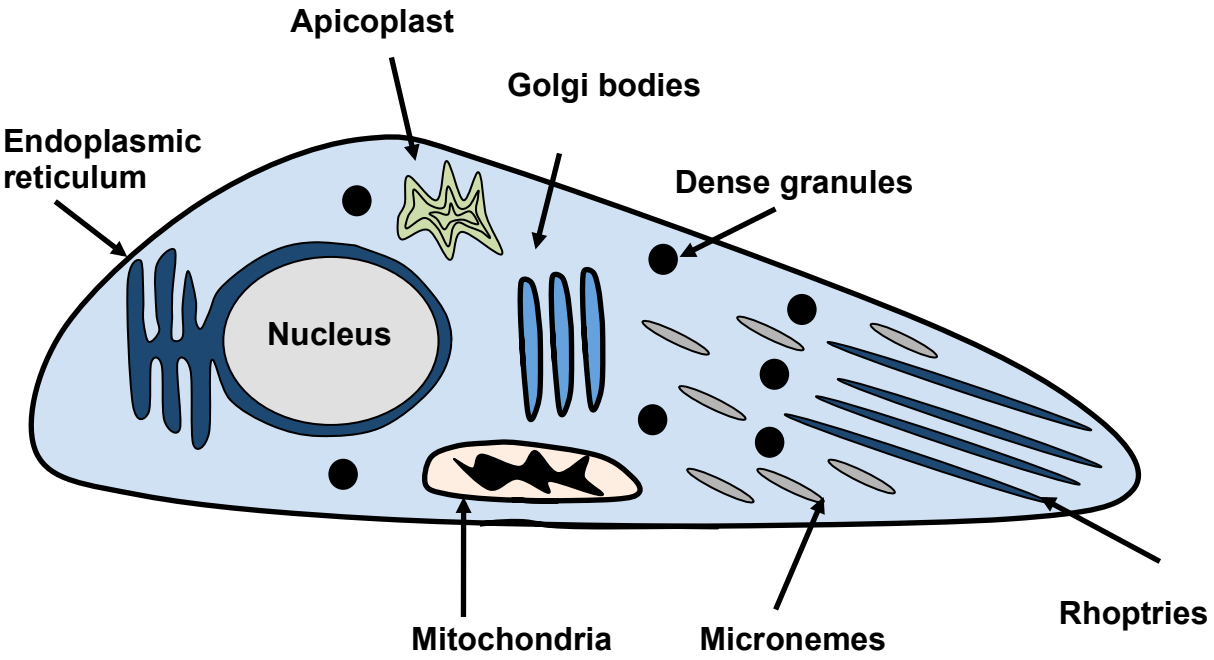

Figure 1.4. Ultrastructure of the *T. gondii* tachyzoite

This is a schematic representation of the tachyzoite stage of *T. gondii.* The rhoptries and micronemes are localised to the anterior end of the parasite, while the dense granules are dispersed throughout. The nucleus is located at the posterior end of the parasite with the endoplasmic reticulum located behind (Ricci *et al.,* 2003).

1.8. Host cell infection by *Toxoplasma gondii*

After the exit from an infected cell or the rupture of a cyst the parasite migrates to other host cells by a gliding motility (Keeley & Soldati 2004). This involves the interaction of actin filaments and myosin proteins, embedded in the parasite's inner membrane complex (IMC) by two gliding associated proteins (Skillman *et al*., 2011). The actin then binds the enzyme, aldose, which acts to link the complex to the cytosolic regions of five different transmembrane proteins that span the plasma membrane. When these linkages are released they do so from the apical end down the length of the parasite. This results in a forward motion of the parasite in the direction of the apical tip (Wetzel *et al*. 2003).

Recognition of potential new host cells is achieved by the interaction of GPI-anchored surface antigens (SAG) on the parasite with the host cell membrane. Although the *T. gondii* genome contains a large number of these surface proteins (Jung *et al*,. 2004), only 6 have been identified as being important in the identification of new host cells and initiation of the attachment and invasion process. These comprise SAG 1-3 and SAG related sequences 1-3. The importance of SAG proteins in attachment and invasion was highlighted in studies utilising SAG specific antibodies to inhibit their function (Mineo & Kasper 1994) and gene knockout models. In one example a SAG 3 deficient parasite displayed a two fold attenuation in host cell infectivity (Dzierszinski *et al*. 2000). While important for host cell attachment, SAG proteins have also been described as being immunogenic and have been the target of some partially successful vaccine strategies, including whole protein (Letscher-bru *et al*., 2003) and DNA vaccination (Couper *et al*., 2003) regimens.

Attachment of the apical end of the parasite is the next stage in the invasion process that is mediated by the Ca^{2+} dependent secretion of microneme proteins. The role of the MIC proteins has been exemplified by using Ca^{2+} antagonists (Carruthers *et al*., 1999) as well as deletion of the different MIC proteins, which result in reduced attachment and a reduced lethality of infection

(Cérède *et al*., 2005). The final stage in host cell attachment is the release of apical membrane antigen 1 (AMA1), which associates with RON proteins to form a moving junction (MJ) in the cell membrane (Giovannini *et al*., 2011). The MJ is a very close interaction of the host cell and the parasite. This small ring like structure moves along the length of the parasite as it penetrates the host cell by pulling on the tight attachments it has created with the host cell in the form of MIC proteins. Just prior to this the parasite injects the contents of the rhoptry bodies into the host cell cytoplasm to aid formation of the PVM. As the parasite moves it pinches and invaginates the host cell membrane resulting in the formation of the PVM. As this is occurring it is thought that the MJ acts to sieve out host cell transmembrane proteins (Charron & Sibley 2004). The invasion process and formation of the PVM from the host cell membrane does not damage the integrity of the host cell membrane. Ultimately the PV is composed of vacuolar space and extracellular medium. The parasite exists within this vacuole where it undergoes replication. *Toxoplasma* may exit the host cell either by rupture of the host cell membrane through increased numbers of parasites from endogeny, or by the action of perforin like protein 1 which helps to form pores in the cell membrane (Kafsack *et al*., 2009).

1.9. Immune response

The immune response to *T. gondii* is complex, involving every facet of innate and adaptive immunity. Underpinning the response is the delicate balance between the immune mechanisms utilised by the host to control the parasite and the immune evasions and modulation mechanisms utilised by *T. gondii* in order to persist within the host. Another important dimension that epitomises *T. gondii* infection is that there is a delicate balance between the host immune response limiting parasite growth and the production of inflammatory mediators that results in immunopathology (Leiberman and Hunter 2002). Dissecting the immune response is a complex process as it is partially dependent on the parasite strain, species of host or genetic background of the experimental host being utilised and the route of infection. However, there are many common mechanisms, mostly established in murine models, some of which have been confirmed in human studies (Figure 1.4).

1.9.1. Protective Immunity

In the immunocompetent host the immune response develops rapidly against the infection. However, this does not result in sterile immunity and chronic infection is eventually established. It has been suggested that protective immunity is maintained by the occasional rupturing of tissue cysts, releasing the bradyzoites, and re-stimulating the specific effector responses (Hunter & Reichmann 2001).

1.9.2. Innate Immunity

Neutrophils

Neutrophils play an important role in the acute phase of infection with *T. gondii*. Upon release from the bone marrow, they circulate in the blood where they only survive for a few hours unless recruited to a site of infection. They are the earliest cells recruited to the infection site and their rapid migration is required for successful control of *T. gondii* infection (Bliss *et al.*, 2000). Recruited neutrophils phagocytose the invading tachyzoites and kill these by the release

of reactive oxygen and nitrogen species. Recently it was demonstrated that neutrophils could control *T. gondii* growth through the release of neutrophil extracellular traps (NET's), which are composed of chromatin and seeded with antimicrobial (Brinkman *et al*., 2004). It was shown that the NETs could directly kill tachyzoites from all 3 clonal lineages as well as interfering with infection of the host cells (Abi Abdallah *et al*., 2012) Neutrophils are also an important early source of pro-inflammatory cytokines such as IL-12 and TNF-α, either preformed within the granules or synthesised *de novo* (Bliss *et al*., 2000). These cytokines are not produced in large concentrations, but it is the number of circulating neutrophils and the speed at which they migrate into the site of infection that is important. Macrophage inflammatory proteins MIP-1α and MIP-1β, that serve to attract macrophages, immature dendritic cells and T cells, are also secreted by the neutrophils (Denkers *et al*., 2004).

Migration of neutrophils into the site of infection has been shown to be dependent on CXCR2 and CCR1 chemokine receptors (Del Rio *et al*., 2001; Khan *et al*., 2001). Deficiencies in these receptors in murine models result in decreased trafficking of neutrophils into the site of infection, and an increased parasite burden irrespective of an effective adaptive immune response (Del Rio *et a*l., 2001; Khan *et al*., 2001). Deletion of the neutrophils in early murine infection also results in increased mortality (Denkers *et al.,* 2004). This same phenotype is not seen if the neutrophils are neutralised during the later stages of infection, highlighting their importance during the early acute stage. Neutrophils also possess a number of Th-1 specific receptors which are thought to help with the development and maintenance of a Th-1 response (van Gisbergen *et al*., 2005). Neutrophil recruitment and development are IL-17 dependent. As such, it has been demonstrated that IL-$17^{-/-}$ mice have an increased parasite burden with reduced neutrophil recruitment and induction (Kelly *et al*., 2005).

Macrophages

The key role for macrophages in response to infection with *T. gondii* was first highlighted in 1972 (Remington *et al*., 1972), when it was demonstrated that macrophages from mice chronically infected with *T. gondii* were capable of limiting parasite growth in *ex vivo* culture. Together with DCs and neutrophils they provide the key initial innate response to infection. As well as being key producers of IL-12, their functions include phagocytosis and phagolysosomal degradation, antigen presentation and the production of reactive oxygen and nitrogen species (Stafford *et al*., 2002). The production of nitric oxide (NO) through the conversion of L-arginine to citrulline by iNOS, following stimulation by IFN-γ, endogenous TNF-α and TLR signaling, is an important mediator in controlling infection with *T. gondii*. However, overproduction of NO can also result in severe immune pathology in the host. This has been demonstrated in iNOS deficient mice, which although possessing a greater parasite burden when infected with *T. gondii*, displayed reduced tissue necrosis compared with the C57BL/6 parental strain (Khan *et al*., 1997).

Within infected macrophages the tachyzoites exist in a parasitophorous vacuole. In the murine model the macrophage may clear the infection through autophagy if the macrophage is activated. This mechanism requires the IFN-γ induced, GTP-ases that can degrade the PV, exposing the tachyzoites which are then entrapped within an autophagosome (Zhao *et al*., 2007). Importantly, humans lack these immune related GTP-ases (Howard *et al*., 2011) and rely on other mechanisms including the induction of tryptophan inducing enzymes to control parasite growth as discussed below.

Macrophages are also thought to limit the availability of key nutrients such as iron and tryptophan (Reviewed by Stafford *et al*., 2002). Iron stored within macrophages, represents a large portion of metabolically available iron in mammals. Tryptophan is an essential amino acid for *T. gondii*. The induction of indolamine-2-3 dioxygnase by IFN-γ degrades tryptophan, so limiting its availability to the parasite. It has also been suggested that there is an arginase

dependent mechanism for the regulation of parasite growth (Butcher *et al.*, 2011). This may operate through restricting the availability of L-arginine, the substrate for arginase as it has been shown that *T. gondii* is auxotrophic for this amino acid (Fox *et al.*, 2004).

Dendritic cells

The activation and maturation of DC's during infection with *T. gondii* are key events responsible for the development of an effective immune response. DCs have been demonstrated to be one of the most important sources of IL-12. Depletion of DCs reduced IL-12 and increased the susceptibility to acute infection (Liu *et al.*, 2006). Adoptive transfer of DCs into DC depleted mice restored IL-12 and IFN-γ production and improved resistance to *T. gondii* infection (Liu *et al.*, 2006). In response to signals from infected cells and neutrophils such as CCR5 ligands, DCs mobilise to the site of infection (Aliberti *et al.*, 2000). CCR5 induced deficiencies in mice, produced either by gene-deletion or treatment with CCR5 antagonist, result in altered DC trafficking and reduced IL-12 production. After trafficking to the infection site, the DCs acquire antigen by phagocytosis, leading to maturation, associated with an up-regulation in MHC expression, activation, and enhanced pro-inflammatory cytokine production (Kobayashi *et al*., 2003). This is followed by migration to the T cell rich regions of the spleen and lymph nodes. The secretion of pro-inflammatory cytokines, including IL-12, by DCs has been shown to be TLR dependent, resulting in signaling via MyD88 (Scanga *et al.,* 2002). Murine models lacking MyD88 have been demonstrated to have significant retardation of IL-12 production when infected with *T. gondii* (Scanga *et al.,* 2002). DCs have been demonstrated to be able to differentiate between live and dead tachyzoites. Infection with viable parasites resulted in greater IL-12 production and CD40L expression by DCs than those pulsed with inactive tachyzoites, suggesting the ability of the parasite to influence DC function (Subauste & Wessendarp 2000). This is possibly through the actions of cyclophilin-18, a protein secreted by *T. gondii* which induces DC IL-12 production through binding of CCR5 (Aliberti *et al.,* 2003). IL-12 production by DC's is important

during the acute stage of infection but it has also been demonstrated that it is required to control reactivation of chronic infection (Fischer *et al.,* 2000). DCs also have an important role in the dissemination of *T. gondii* within the host (Lambert *et al.*, 2009) as they can be found in the liver and spleen within hours of oral infection.

Natural Killer Cells

NK cells have an important role early in the response during the acute phase of *T. gondii* infection, being the third most abundant lymphocyte population (Backstrom *et al*., 2004). As part of the innate immune response NK cells have a major role in the production of IFN-γ before specific $CD4^+$ and $CD8^+$ T cells are activated, and consequently aid in the polarisation of the $CD4^+$ T cell response (Goldszmid *et al*., 2007) through the enhancement of classical activation of macrophages, the upregulation of macrophage expression of MHC class II and co-stimulatory molecules and enhancing their antigen presentation potential. This IFN-γ production is induced through IL-12 production by activated macrophages, dendritic cells and neutrophils (French *et al.*, 2006). There are also direct interactions between NK cells and DCs, mediated by NKG2D on the NK cell, that alter NK cell IFN-γ production and up regulate IL-12 production by the DCs (Backstrom *et al*., 2004).

NK cells are also directly cytotoxic to infected cells, through modification of MHC class I on the surface of infected cells by the binding of *T. gondii* antigen. Apoptosis of the target cell can be induced via Ca^{2+}-dependent granule exocytosis and release of cytotoxic proteins, binding of the Fas ligand (FasL), and membrane-bound or secreted cytokines, such as TNF-α (Zamai *et al*., 1998). During infection NK cells migrate to the infection site. To achieve this they have a variety of chemokine receptors and of these chemokine receptor 5 (CCR5) appears to be key. A study using CCR5 deficient mice indicated a decrease in NK migration during *T. gondii* infection. This resulted in the death of the host due to an elevated parasite burden (Khan *et al*., 2006). NK cells have also been shown to aid the development of a $CD8^+$

T-cell mediated immune response (Combe *et al*., 2005). These responses typically require the help of $CD4^+$ T-cells, through cytokine production. However, a $CD4^+$ T cell independent system has been identified in which NK cells play a role. The exact mechanism is not well understood but is characterised by an extended NK response and prolonged IL-12 production (Combe *et al*., 2005).

1.9.3. Cell mediated immunity (CMI) and adaptive immunity

Protective immunity to *T. gondii* requires the development of an effective type-1 response involving both innate and adaptive immunity. IL-12 secretion from neutrophils, macrophages and DCs is instigated by the interaction of pathogen associated molecular patterns (PAMPs) from *T. gondii* with pathogen recognition receptors. Toll-like receptor (TLR) 11 is one of the important receptors and a potent inducer of IL-12 from dendritic cells in the murine model (Pifer *et al.*, 2010). It is bound by the *T. gondii* protein profilin. This protein is thought to be one of the most important inducers of DC IL-12. Deficiency in either TLR11 or profilin results in attenuation of the IL-12 response and increased susceptibility to infection (Yarovinsky *et al.,* 2005, Pifer *et al*., 2010). TLRs 2 and 4 are activated by glycosylphosphatidylinositol (GPI) anchors, although these do not play as significant a role in initiating the IL-12 response in mice as TLR11 ligation (Debierre-Grockiego *et al*., 2007). During *T. gondii* infection signaling through TLR molecules, via the MyD88 signalling cascade has been shown to be essential. $MyD88^{-/-}$ mice are unable to survive infection with *T. gondii* (Sukhumavasi *et al.*, 2008). However it has been shown that while deletions of individual TLRs, such as TLR 2, 4, or 11 increase the parasite burden, over all the mice are able to survive infection.

Ultimately the actions of the innate cells culminate in the development of an adaptive T cell response that is required for successful control of the pathogen. The presentation of antigen by professional antigen presenting cells and the release of IL-12 activates specific type-1 $CD4^+$ and $CD8^+$ T cells, leading to their proliferation, differentiation and development of their effector functions.

Interaction of the T cells with DCs and macrophages through CD40 also acts to increase the activation state of the cells and further enhances their secretion of IL-12 (Kobayashi *et al*., 2003; Zhao *et al*., 2007). IL-12 secretion drives the polarisation of $CD4^+$ T cells towards that of a Th1 phenotype (Reis e Sousa *et al*., 1997). This results in IFN-γ and TNF-α production by the $CD4^+$ T cells, which further activates macrophages and dendritic cells. IL-2 is also secreted, by Th1 cells which enhances proliferation of both $CD4^+$ and $CD8^+$ T cells. Activated antigen specific $CD8^+$ effector T cells secrete IFN-γ and TNF-α and are cytotoxic against infected cells. IFN-γ, along with IL-12, has been demonstrated to be the key mediator in response to *T. gondii* infection using anti-IFN-γ antibodies (Suzuki *et al*., 1988). It has also been shown that IFN-γ production is required during both the acute and chronic stages of infection to promote macrophage activation and polarize immunity towards a type-1 response (Gazzinelli *et al*., 1992). Both $CD4^+$ and $CD8^+$ T cells are important for controlling infection (Suzuki & Remington 1988). Adoptive transfer experiments have, however, demonstrated the $CD8^+$ T cells play the primary role (Parker *et al*., 1991).

The ultimate goal of an effective immune response is to not only control the active infection, but to develop a robust $CD8^+$ T cell memory response. The $CD8^+$ T cells, raised during the T cell expansion phase, can be classified into 2 phenotypes. About 90% of $CD8^+$ T cells are selective effector cells (SLEC), defined by being $KLRG1^{hi}$ $CD127^{lo}$ and about 10% are memory precursor cells (MPEC), which are defined as $KLRG1^{lo}$ $CD127^{hi}$ (Joshi *et al* 2007; Wilson *et al*., 2008). Although differing in phenotype both populations possess similar effector functions in terms of their cytotoxicity and cytokine production. However, during the contraction phase of the CD8 T cell response, the majority of the SLECs go through apoptosis, while the MPECs fully differentiate into memory cells (Weiguo & Kaech 2010). It has been demonstrated that IL-12 is an important mediator in the development of a $CD8^+$ T cell memory response. Low levels of IL-12 have been shown to increase the differentiation of $CD8^+$ T cells into memory cells in a *Listeria monocytogenes* model (Pearce & Shen 2007).

Work by Joshi *et al,* (2007) showed that IL-12 levels and the Th1 associated transcription factor T-bet directly correlated and that increased expression of T-bet correlated with increased SLEC and inversely correlated with MPEC $CD8^+$ T cells (Joshi *et a*l., 2007).

In the *T. gondii* murine model of infection it has been demonstrated that IL-12 is required for the generation of SLEC $CD8^+$ T cells and this is independent of $CD4^+$ T cell help (Wilson *et al.*, 2008). It has also been found that SLECs were more frequent than MPECs in the $CD8^+$ T cell population (Wilson *et al.*, 2008). The virulence of the parasite strain used in an infection model can alter the quantity of IL-12 produced. It has been demonstrated that RH can limit the IL-12p40 and IL-12p70 produced by innate effector cells (Robben *et al.*, 2004; Tait *et al.*, 2010) so limiting IL-12 signaling to $CD8^+$ T cells. One hypothesis proposed is that by inducing IL-12, *T. gondii* is restricting the development of MPECs and thereby limiting the development of an effective memory response, and aiding the persistence of the parasite within the host (Tait *et al*., 2010; Gigley *et al*., 2011).

1.9.4. Humoral immune response

The antibody response to *T. gondii* infection is not generally considered to be as important as cell-mediated immunity for controlling *T. gondii* infection. However the humoral response may play a role in helping to limit dissemination within the host (Kaneko *et al*., 2004). Within the first 7 days of infection IgM is produced. IgM acts to opsonise the parasites, thus enhancing neutrophil and macrophage function, activating the complement pathway and preventing invasion of the host cell (Kaneko *et al*., 2004). The importance of IgM has been demonstrated in $IgM^{-/-}$ BALB/c mouse models, which display an increased susceptibility to infection (Couper *et al*., 2005). C57BL/6 mice, which are susceptible to infection have a decreased IgM response in comparison to the resistant A/J mice (McLeod *et al.*, 1989). As infection progresses, IgG is secreted which is a potent activator of antibody dependent cytotoxicity (ADCC). IgG

antibodies have also been demonstrated to confer protection to the foetus during infection, due to their ability to cross the placenta (Nimri *et al.,* 2004).

Natural, nonspecific immunoglobulin has also been demonstrated to have a protective effect during *T. gondii* infection (Vercammen *et al*., 1999) by reducing parasite proliferation and enhancing phagocytosis. The intracellular nature of *T. gondii*, and its ability to rapidly invade new cells means they are less susceptible to the actions of antibodies. However in cases of persistent active infection by tachyzoites, antibodies have been shown to be essential in survival of the host (Kang *et al*., 2000). IgA, which plays a critical role in mucosal immunity, has also shown to be protective during *T. gondii* infection in the murine model. Intranasal vaccination, which has resulted in high titres of IgA, has been associated with increased resistance upon infection (Igarashi *et al*., 2008). Antibodies further aid the response against *T. gondii* by helping to enhance phagosome-lysosome fusion, following phagocytosis (Joiner *et al*., 1990).

1.9.5. Immune regulatory mechanisms

An effective immune response against infection with *T. gondii* requires a delicate balance between the pro-inflammatory response needed to control parasite multiplication and the immune pathology which it also generates. IL-10 is an important cytokine in the regulation of the pro-inflammatory response (Reviewed by O'Garra & Vieira 2007). IL-10 acts to down regulate DC and macrophage activation and consequently inhibits their production of inflammatory cytokines and other effector functions. Due to the ability of IL-10 to down regulate IL-12 production and therefore IFN-γ production, it also acts to down regulate MHC class II and co-stimulatory molecule expression (Ding *et al*., 1993, Florentino *et al*., 1991, de Waal Malefyt *et al*., 1991). IL-10 is therefore a key mediator in the suppression of the Th1 driven inflammatory response. Originally identified as a Th2 cytokine, IL-10 has since been found to also be produced by macrophages, DCs, Tregs and antigen specific $CD4^+$ and $CD8^+$ T cells (Couper *et al*., 2008). The importance of IL-10 in regulating the immune

response during *T. gondii* is demonstrated in IL-10 deficient mice that succumb to infection, with excessive production of IL-12, IFN-γ and nitric oxide (Gazzinelli *et al*., 1996). During the chronic stages of infection it has been shown that IL-10 is not essential in controlling an over reactive immune response. While IL-10 deficient mice succumb to primary infection with *T. gondii* (Jankovic *et al*., 2010), if treated with sulphadiazine they survive even if re-infected and control parasite load (Wille *et al.,* 2004). In addition IL-10 deficient mice were able to generate a robust memory response following vaccination with TS-4 and were able to survive challenge with RH strain parasites (Wille *et al.,* 2004).

IL-27 is an important regulatory cytokine during a highly polarised Th1 response, as is seen during infection with *T. gondii* (Hunter *et al*., 2004). It possesses similar structural and sequential homology to IL-12 (Trinchieri *et al*., 2003). Initially IL-27 was identified as a promoter of the Th1 response, in conjunction with IL-12, during infection with *Leishmania* infection (Yoshida *et al*., 2001). The role of IL-27 during infection with *T. gondii* was elucidated using IL-27R$^{-/-}$ mice (Hunter *et al*., 2004). During the acute stages of infection with *T. gondii* the IL-27R$^{-/-}$ mice developed a Th1 response as determined by IFN-γ production independently of IL-12. However, 2 weeks post-infection the IL-27R$^{-/-}$ mice displayed lethal inflammatory disease with increased IFN-γ and greater proliferation of highly activated CD4^{+} and CD8^{+} T cells (Villarino *et al*., 2003). This had led to the suggestion that IL-27 only plays a role in promoting IFNγ response in circumstances where IL-12 is a limiting factor and that its role in limiting the pro-inflammatory response is its primary function (Hunter *et al*., 2004). Nevertheless it has been recently demonstrated that in mice overexpressing IL-27, Treg populations are diminished resulting in cases of spontaneous inflammation (Wojno *et al*., 2011). IL-27 is currently thought to exert its regulatory role through inhibiting the secretion of IL-2, a potent cytokine required for T cell proliferation (Wojno *et al*., 2011; Villarino *et al*., 2006). IL-27 has also been shown to exert an effect through the suppression of

Th17 T cell development (Stumhofer *et al.*, 2006). These cells through their secretion of IL-17 enhance the activity of neutrophils as previously discussed.

TGFβ is also described as in important cytokine in regulating immunopathology. Secreted by intra-epithelial lymphocytes and Th2 cells. As well as promoting the development of Th17 cells, in conjunction with IL-6 and IL-23 from macrophages, it induces the development of Treg cells (Reviewed by Gaddi & Yap 2007). TGF-β is also thought to limit the gut immunopathology often associated with *T. gondii* infection by down regulating IFN-γ and TNF-α production of lymphocytes found in the lamina propria (Mennechet *et al*., 2004).

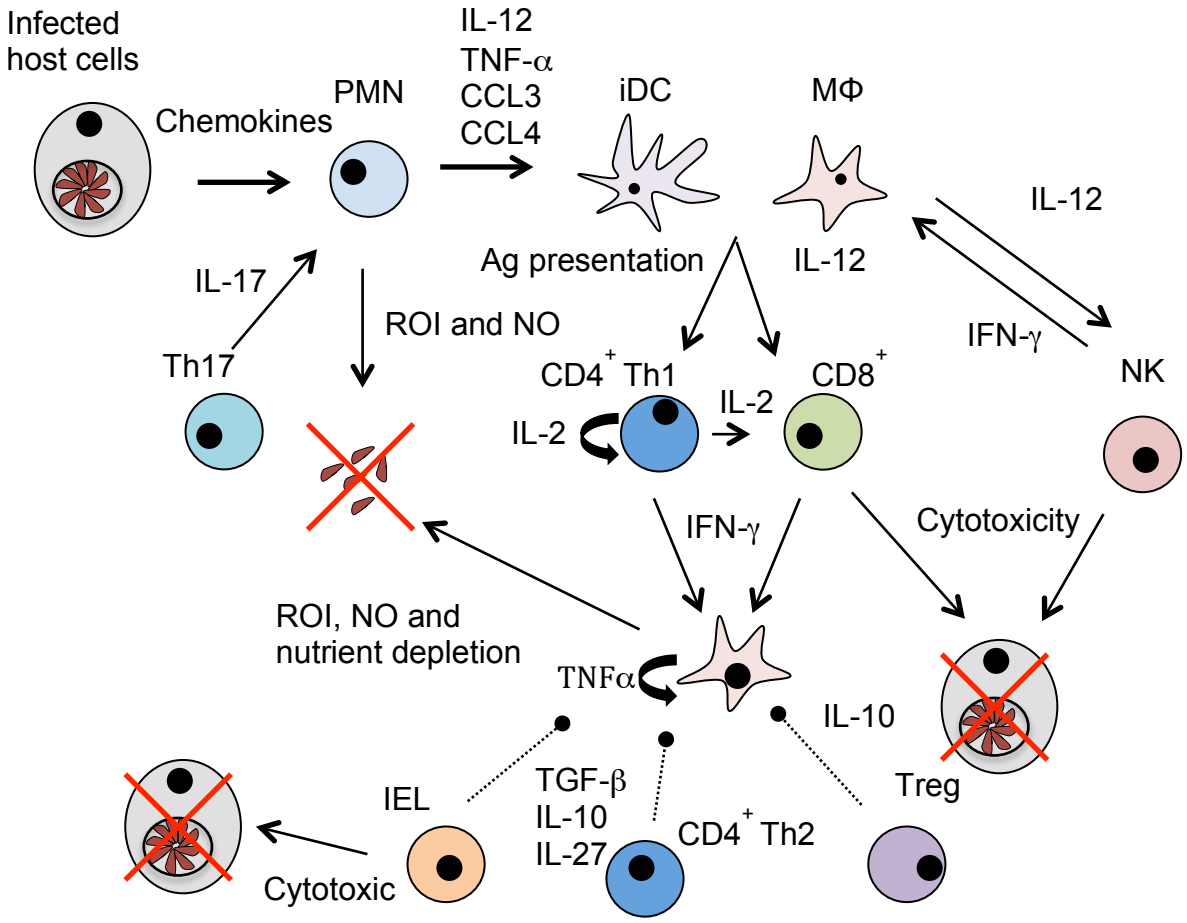

Figure 1.5. The immune response to *T. gondii*

Both the innate and adaptive components of the immune response are involved in control of *T. gondii* infection. Infected cells secrete chemokines, which attract neutrophils (PMN) to the site of infection. These in turn secrete chemokines and cytokines that attract immature DC (iDC), and macrophages (MΦ). IL-12 secreted by these cells is a key mediator and required for inducing NK cells to produce IFN-γ and exert their cytotoxic properties. IL-12 secretion also results in the activation of specific $CD4^+$ and $CD8^+$ T cell responses. DC's play a key role in this activation, being the main source of IL-12, presenting antigen to the T cells and polarisng the response towards a type-1 phenotype. Macrophages are the most important cell type in limiting dissemination of the parasite. IFN-γ together with endogenous TNF-α induces the secretion of reactive oxygen and nitrogen species that directly kills the parasite. This inflammatory response is regulated by the secretion of TGF-β, IL-10 and IL-27 by Th2 polarised $CD4^+$ T cells, T regulatory cells and intraepithelial lymphocytes (IEL). Regulation is required to prevent immune related pathology often associated with a vigorous type-1 response.

1.10 Vaccination

Toxoplasma gondii is a ubiquitous pathogen and all humans are at risk of infection. This can have potential life threatening consequences for the immune compromised or those congenitally infected. Therefore all humans would potentially benefit from vaccination. Currently there are no vaccines available for human use. Medications used for treating infection such as, sulphadiazine and pyrimethamine are associated with allergy and bone marrow toxicity, respectively (McLeod *et al*., 2006; Caumes *et al*., 1995; Santin *et al*., 1990) and therefore not ideal for long term use. Furthermore as the symptoms of *T. gondii* infection are non-specific, they are not normally administered soon enough to prevent infection and are incapable of killing bradyzoite forms. Consequently, drugs neither prevent nor eliminate infection and therefore a vaccine would represent the best method of successfully preventing infection.

Research into an effective vaccine for *T. gondii* has been ongoing for a number of years (Reviewed by Jongert *et al*., 2009). There is, however, no established standard method for assessing *T. gondii* vaccines; the animal models, strain of *T. gondii,* dose and route of infection used for challenge varies between laboratories. This makes comparing different vaccines challenging.

1.10.1 Killed, whole lysate and purified antigen vaccines

Early *T. gondii* vaccine studies used killed parasites, crude whole tachyzoite lysates, soluble fractions, and particulate fractions as vaccines with varying degrees of success (Krahenbuhl *et al*., 1972, Waldeland and Frenkel, 1983). These studies also assessed different adjuvants in the vaccines such as use of Freunds incomplete and complete adjuvant and liposomes with varying degrees of success. A number of studies using killed parasites or crude antigens as vaccines also demonstrated that although enhanced survival following challenge infection could be induced parasite numbers and or cyst burdens were not controlled (McLeod *et al.,* 1985, Lunden *et al*., 1993).

Highly purified subunits of *T. gondii* such as GRA, ROP and MIC proteins have also been used for vaccination with varying degrees of success (Reviewed by Jongert *et al*., 2009). Surface antigen 1 (SAG1) is a stage specific protein found on tachyzoites, which has been extensively trialed as a vaccine candidate. In one particularly successful study intranasal delivery of SAG1 formulated with Quil A as adjuvant resulted in 90% survival upon challenge and no evidence of tissue cysts in the surviving members of the vaccinated group (Khan *et al.,* 1991).

Recently, subunit vaccines have been rationally designed by utilising bioinformatics to identify peptide sequences, derived from *T. gondii* antigens, which bind to known HLA alleles. This allows targeted vaccination with known immunogenic or immunodominant antigens, which are capable of eliciting a response from specific components of the immune system. An example of this is the use of the GRA6 derived peptide HF10 (HPGSVNEFDF). This peptide is an immunodominant MHC class 1, L^d restricted peptide. Vaccination of BALB/c mice with this peptide resulted in protection against challenge (Blanchard *et al.,* 2008). However, the more refined the subunit vaccine the less immunogenic it becomes. Thus the use of appropriate potent adjuvants is very important. In addition, by using epitopes from a number of different antigens the breadth of protection and effectiveness of the vaccine is likely to be enhanced (reviewed Jongert *et al*., 2009; De Temmerman *et al*., 2011).

1.10.2 Live attenuated vaccines

Live attenuated *T. gondii* parasites by mimicking natural primary infection have the potential of inducing similar life long immunity to natural infection, without the associated pathology (Jongert *et al*., 2009). So far such vaccines have offered the strongest protection against infection (reviewed by Innes & Vermeulen 2006 Jongert *et al*., 2009; Innes *et al*., 2011). The TS-4 strain, derived by the chemical mutation of the RH strain, is a temperature sensitive mutant that displays retarded growth at 37°C yet remains immunogenic (Pfefferkorn and Pfefferkorn, 1976). Vaccination of mice with TS-4 resulted in protection against

lethal RH challenge and reduced congenital transmission. However, vaccination with TS-4 failed to prevent the formation of tissue cysts upon challenge with an avirulent strain (McLeod *et al*., 1988).

The S48 strain of *T. gondii* was isolated in 1988 from an aborted ovine foetus. Following continual passages in culture, the parasite lost its virulence and became tissue cyst deficient. Sheep vaccinated with S48 developed a strong immune response to the pathogen, which resulted in a reduced incidence of abortion and neonatal mortality although vertical transmission was still apparent (Buxton *et al*., 1991; Innes *et al*., 1995a and b). The S48 strain has since been developed into a commercially available vaccine for veterinary use under the name 'Toxovax".

More recently the live attenuated ΔRPS13 strain has been derived from the RH strain. The parasite lacks an essential ribosomal protein under the tetO system that results in arrest of the parasite in the G1 phase of the division cycle. Mice vaccinated with this strain were protected against challenge with their wild-type RH counter parts (Hutson *et al.,* 2010).

Ultimately, the use of live attenuated vaccines in humans may be limited due to safety concerns that the organism may revert back to its pathogenic state. Although the risk of this happening may be eliminated through the use of gene deletion mutants, the general adoption of such a vaccine is unlikely to be accepted by the public.

1.10.3 DNA vaccines

With their capability of inducing both $CD4^+$ and $CD8^+$ T cell immunity, DNA vaccines have been the focus of a large number of vaccines studies against *T. gondii* (Coombes & Mahony 2001). A large number of plasmids and live vectors have been used with a variety of success, though comparison of the different vectors has not been carried out. While DNA vaccines encoding for SAG1 have

received the greatest focus of study (reviewed by Jongert *et al.,* 2009), the vaccine potential of a large number of dense granule, rhoptry and microneme proteins have also been assessed. Vaccination with SAG1 has been shown to be moderately protective against adult acquired infections and has been correlated with inducing a cytotoxic T cell response, which has been shown to be one of the key mediators of immunity during infection with *T. gondii* (Couper *et al.,* 2003). However, this vaccine did not prevent vertical disease transmission. It has also been noted that the success of a particular DNA vaccine candidate is dependent on the animal model used. One study demonstrated that a GRA1 DNA vaccine was protective in C3H mice but not in BALB/C or C57BL/6 mice (Vercammen *et al.,* 2000). It has, however, been shown that the efficacy of a DNA vaccine may be enhanced through combining multiple DNA vaccines in the same vaccine formulation (Vercammen *et al*., 2000). Delivering ROP2 DNA in combination with SAG1 has been shown to enhance the CMI response and protection from infection in BALB/c mice challenged with RH strain parasites (Frachado *et al.,* 2003)

1.10.4 Oocyst vaccines and oocyst challenge

Following primary infection, cats will shed oocysts in their faeces that can survive for months in the environment and are an important cause of infection in grazing animals and humans (Tenter *et al*., 2000; Boyer *et al*., 2011). Nevertheless despite a multitude of vaccine projects against *T. gondii* (reviewed by Jongert *et al*., 2009) the use of oocysts in parasite challenge infections is extremely limited. However, veterinary vaccine strategies have been developed with the aim of preventing or reducing oocyst shedding from cats. Originally this was trialed using the live attenuated T-263 strain of *T. gondii* (Frenkel *et al*., 1991; Freye *et al*., 1993), which resulted in reduced oocyst shedding upon challenge. This vaccine was trialed on commercial pig farms through vaccination of stray cats, resulting in reduced contamination of the surrounding environment with oocysts and a reduction in infection of intermediate hosts (Mateus-Pinilla *et al*., 1999). However, due to the *in vivo* method used to

maintain this parasite strain, the vaccine has not been developed for large-scale commercial use. Subsequently, more refined vaccination methods such as the use of crude protein extracts, viral vectors and plasmid DNA vaccination have been used with some success (Angus *et al*., 2000; Mishima *et al*., 2002; Zulpo *et al*., 2012).

1.11. Aims

There are a number of drug therapies available for treating the acute stages of infection with *T. gondii* including the synergistic use of the anti-folates pyrimethamine and sulphadiazine or the antimicrobial agent spiramycin. However these treatments are accompanied by unpleasant side effects and are ineffective against the latent bradyzoite stage of infection (Lyons *et al*., 2002). Due to the generally asymptomatic nature of primary infection in the immunocompetent host, by the time the infection is detected the available treatments are redundant. An effective vaccine would therefore offer considerable advantages over current chemotherapeutic treatments. The feasibility of developing a protective vaccine has been demonstrated using live attenuated strains of *T. gondii* (Innes *et* al., 1995, Mcleoad *et al*., 1988) Hutson *et al*., 2010). Other prophylactic therapeutic strategies that have been experimentally tested have included the use of subunit vaccines (Cong *et al*., 2010; Cong *et al* 2011; Cong *et al*., 2012) and DNA vaccines (Couper *et al*. 2003).

Recently Cong *et al* (2010, 2011, 2012) demonstrated that protective immunity against *T. gondii* tachyzoite challenge could be induced by HLA class one restricted peptide vaccines in HLA transgenic mice. Together the 3 HLA supertypes utilised in the transgenic mice (HLA-A*0201, HLA-A*1101 and HLA-b*0702) represent 90% of the human population. The first aim of the present project was to test the efficacy of HLA restricted subunit peptide vaccines against oral oocyst challenge, one of the major natural routes of infection with *T. gondii*. Vaccination studies were carried out in mice expressing HLA-A*0201 or A*1101 or B*0702 with peptide pools known to afford protection against tachyzoites challenge. Adjuvants used to enhance the protective effectiveness of the peptides included non-ionic surfactant vesicle (NISV) and GLA-SE. The vaccine potential of the RH ΔRPS13 gene deletion mutant that has already been demonstrated to induce protection against tachyzoite challenge was also assessed against oocyst challenge.

Integral to the success of any vaccine must be the suitable manipulation of immune regulatory pathways in order to direct an appropriate response that is protective. In order to achieve this, it is important that we understand the early events in the host pathogen interplay that result in healing/cure. Key to this is the early interaction with cells of the innate immune response such as neutrophils, macrophages and DC's that ultimately direct the resulting adaptive response (reviewed by Pifer *et al*., 2001 & Coffman *et al*., 2010).

It has been well established that NO from myeloid cells plays a significant role in controlling the growth of *T. gondii* (Scharton-Kersten *et al*., 1997). More recently arginase-1 which competes with iNOS for their common substrate – L-Arginine (El-Kasmi *et al*., 2008) has been indicated as being a major susceptibility factor for *T. gondii*. Paradoxically as *T. gondii* is an arginine auxotroph (Fox *et al.,* 2004) it has also been suggested that arginase-1 could enhance protection against the parasite by starving the parasite of this essential metabolite (Butcher *et al*., 2011) . Consequently, the role of arginase-1 during infection with *T. gondii* is controversial but the indications suggest that it is likely to a play a significant role in the outcome of infection. Interestingly the dual specific phosphatase, MKP-2 has recently been identified as not only being a negative regulator of arginase-1 production but also a positive regulator of iNOS expression (Al-Mutairi *et al*., 2010). As such MKP-2 is likely to be influential in deciding the outcome of *T. gondii* infection as well as providing a potential target for therapeutic intervention and vaccine strategies. Consequently, the second aim of this thesis was to study the role of MKP-2 in *T. gondii* infection using recently available MKP-2 gene-deficient mice on a C57BL/6 background.

Chapter 2

HLA restricted vaccine for *Toxoplasma gondii*

2.1 Abstract

Previous work carried out over a number of years has highlighted the key role played by major histocompatibility complex class I through the interaction with $CD8^+$ cytotoxic T cells in controlling T. gondii infection. This suggests that a vaccine capable of inducing a strong CD8+ T cell response might afford protection against this parasite. Vaccines containing pooled, HLA allele-specific class I-binding peptides from T. gondii tachyzoites have been found to be protective against tachyzoite challenge in previous work related to this study. In the present study we tested the vaccine potential of these pooled peptides against the more natural oocyst challenge and attempted to improve their efficacy by using non-ionic surfactant vesicles (NISV) as an adjuvant. It has also been well established that the most effective protective vaccines against *T. gondii* so far are live attenuated vaccines. So in parallel we tested the novel RH ΔRPS13 gene deletion mutant in the oocyst challenge model, as it has already been demonstrated to be protective during tachyzoite challenge. Mice expressing HLA-A*0201 or HLA-A*1101 or HLA-B*0702 supertype alleles which were vaccinated with their respective peptide pools were not protected against oocyst challenge, as demonstrated by mortality. While the vaccines were capable of inducing IFN-γ production no protection was observed. The HLA-B*0702 restricted peptide vaccine which entrapped within NISVs also failed to induce protective immunity against oocyst challenge. Vaccination with ΔRPS13 in all HLA transgenic mice successfully induced a level of protective immunity, as demonstrated by an enhanced IFN-γ specific response and increased survival rates upon oocyst challenge. Together these results demonstrate that peptides derived from tachyzoites and known to specifically induce a $CD8^+$ mediated response that is protective against tachyzoite challenge, does not protect against sporozoite-induced infection. Future studies should examine whether sporozoite expressed peptides confer protection in this model.

2.2. Introduction

The challenge in vaccine development is to develop a vaccine that induces a protective immune response and possesses a high efficacy across as large a population as possible. Typically, identification of potential vaccine candidates has comprised the empirical testing of crude antigenic components such as killed *T. gondii or* lysates or sub-units formulated within traditional or experimental adjuvants. Overall this approach has met with limited success (Reviewed by Jongert *et al*., 2009), More recently a rational immunosense approach has been employed to predict vaccine efficacy through the use of MHC binding algorithms to identify specific peptides. However this refinement in identification of potentially protective epitopes has reduced the number of immunogenic components comprising the potential vaccine (Jongert *et al*., 2009). This in turn has lead to the necessity of utilising more effective immunological adjuvants.

2.2.1. Current vaccine status

The development of successful vaccine strategies would be of huge benefit in tackling toxoplasmosis. Currently, there are a number of live attenuated *T. gondii* strains that have been used to successfully vaccinate mice including S48, TS-4 and T-263 against infection (Mcleod *et al.*, 1988, Lu *et al*., 2009). In addition the S48 strain is currently licensed for veterinary use in sheep, under the brand name "Toxovax". However safety concerns have limited the progression of these live vaccines for use in humans.

In experimental studies live vaccines have proved to be much more successful than many subunit vaccines in the context of *T. gondii*. The S48 strain would appear to have been attenuated through repeated *in vitro* culture, whereas the TS-4 strain was attenuated though selection for temperature sensitivity (Pfefferkorn & Pfefferkorn 1976; Buxton *et al* 1991). Nowadays, attenuated mutants can be created by deleting key components of the parasite's metabolism, making auxotrophic mutants or by deleting virulence factors. A

cps1-1 knockout is auxotrophic for uracil so fails to replicate *in vivo* but vaccinating mice with this strain induces long-term immunity to virulent challenge (Gigley *et al*., 2009). Another example is a recently attenuated RH strain of *T. gondii* lacking the gene encoding ΔRPS13, a key ribosomal protein that results in cell cycle arrest (Hutson *et al*., 2010). Vaccination with this strain is able to induce complete protection against tachyzoite challenge in HLA transgenic mice which express either the A*0201, A*1101 or B*0702 supertype alleles (Hutson *et al.,* 2010). The array of antigens presented by the live attenuated strains will be very similar to that of a naturally occurring infection, and so will be much more likely to induce robust T cell mediated responses. In addition, they are effectively 'self- adjuvating' through natural PAMPs. A live attenuated strain of *T. gondii* (S48) has been wildly used as a vaccine for sheep and pigs, illustrating the potential of such an approach.

Many subunit vaccines comprising of either recombinant proteins or DNA vaccines have been comparatively successful under laboratory conditions against *T. gondii* (Sun *et al*., 2011, Lau *et al*., 2011, Golkar *et al*., 2007). One of the main drawbacks with restricting a vaccine to a limited number of antigens, compared to the large array that may be found in a live attenuated strain, is their potentially reduced ability to interact with as many MHC molecules. As a consequence there is a strong possibility of limiting the percentage of the host population that is capable of being protected by the vaccine. For example the GRA6 immunodominant peptide is protective in H-2L^{d} mice such as the BALB/c but not in HLA-B*0702 transgenic mice, in spite of there being similarities in the Ld and B07 binding motifs (Blanchard *et al*., 2008). Further complications using this method of vaccine design, are the polymorphisms between the different strains of *T. gondii* meaning that some vaccine candidates might only protect against some strains of *T. gondii.* However, this potential limitation in vaccine effectivness could possibly be circumvented by focusing vaccine composition on conserved antigenic sequences, or by adding variants of epitopes to cover all strains of *T. gondii.*

There have however been a number of recent studies showing some vaccine success with peptides comprising epitopes predicted to bind human class I HLA molecules (Cong *et al*., 2010, Cong *et al*., 2011, Cong *et al*., 2012). The authors used the online epitope databases and resources tool (www.iedb.org) to screen a number of *T. gondii* secreted proteins for peptides that were predicted to bind one of the 3 major HLA supertypes. In an attempt to further increase the range of protection, proteins selected were conserved across the Type-I and Type-II strains of *Toxoplasma.* Those that had the ability to elicit IFN-γ production by PBLs isolated from chronically infected HLA typed-humans were then used to vaccinate the appropriate HLA transgenic mice. Following tachyzoite challenge protection was measured by *in vivo* bioluminescent imaging (Cong *et al*., 2010, Cong *et al*., 2011, Cong *et al*., 2012). Reduced parasite burdens following tachyzoite challenge was observed across all three strains of HLA-transgenic mice.

2.2.2. Class 1 HLA supertypes

In some studies, epitopes for vaccines have been based on Class I HLA supertype families to over-come the highly polymorphic nature of MHC (Cong *et al*., 2011, Cong *et al*., 2012). HLA supertypes are families of HLAs that possess an overlapping binding specificity. They were first identified when it was found that HLA*0301 and HLA*1101 bound peptides with similar motifs (Kubo *et al.,* 1994). Since then a number of different HLA supermotifs have been identified, which allow peptide ligands to bind a number of different HLA molecules (Sette & Sidney 1998). This subject has been reviewed extensively by Sette & Sidney (1999). There are a number of HLA supertype families including HLA-A2, HLA-A3 and HLA-B7. The families are defined by common residues in their binding domains and the commonality in the supermotifs present in the peptides that they bind.

Alleles belonging to the HLA-A2 supertype includes the HLA-A*0201 allele that recognise peptides of about 10 amino acids in length. They bind peptides

containing small and aliphatic hydrophobic residues in position 2 or in the C terminus. HLA-A3 supertypes include HLA-A*1101 and display an affinity for a broad range of motifs in position 2 and at the C terminus. HLA-A3 alleles bind peptides of 9-10 residues in length. B7 supertypes display specificity for proline residues in position 2 and hydrophobic aliphatic or aromatic residues in the C terminus. The supermotifs bound by each superfamily can be found in Table 1.

Table 1. Class I HLA Supermotif residues

HLA-A2		HLA-A3		HLA-B7	
Position 2	C -terminus	Position 2	C -terminus	Position 2	C -terminus
L	L	A	R	P	A
I	I	V	K		L
V	V	I			I
M	M	L			M
A	A	M			V
T	T	S			F
Q		T			M
					Y

Laboratory studies utilised transgenic mice, which expressed transgenic constructs of either HLA-A*0201, HLA-A*1101 or HLA-B*0702 alleles, of the corresponding HLA-A2, HLA-A3 or HLA-B7 superfamilies (Sette & Sidney 1999). If the antigen is protective in the HLA transgenic mice, then the vaccine should have similar efficacy in humans possessing the HLA supertypes. If each allele within the superfamilies is taken individually on its own, then population coverage of between 35 and 55% can be achieved regardless of ethnicity. If it is assumed that each allele within a superfamily displays the same binding affinities then taken together the population coverage of the supertypes and their epitopes is around 90% (Sette & Sidney 1999). Similar approaches to vaccine design have been undertaken against influenza (Alexander *et al*., 2010) and hepatitis (Livingston *et al*. 1999). The population coverage of the HLA-A*0201, HLA-A*1101 and HLA-B*0702 restricted epitopes can be found in Table 2.

Table 2. Combined population coverage.			
Population/Area	MHC Class 1 HLA-A*0201, HLA-A*1101, HLA-B*0702		
	Coverage[1]	Average Hit[2]	PC90[3]
Australia	74.03%	7.06	1.54
Europe	86.93%	11.10	3.06
North Africa	33.03%	2.99	0.6
North America	72.80%	9.06	1.47
North-East Asia	70.94%	8.52	1.38
Oceania	70.49%	7.92	1.36
Other	82.02%	10.68	2.23
South America	91.35%	11.76	6.05
South-East Asia	78.25%	9.47	1.84
South-West Asia	71.68%	8.36	1.41
Sub-Saharan Africa	68.59%	8.52	1.27
Average (Standard Deviation)	72.74% (14.36%)	8.68 (2.25)	2.02 (1.40)

The table was created using the Immune Epitope Database (www.iedb.org) Supertype alleles HLA-A2 (HLA A*0201, HLA A*0202, HLA A*0203, HLA A*0204, HLA A*0205, HLA A*020601, HLA A*0207, HLA A*6802, HLA A*6901), HLA-A3 (HLA A*0301, HLA A*1101, HLA A*310102, HLA A*3301, HLA A*6801) and HLA-B7 (HLA B*0702, HLA B*0704, HLA B*0705, HLA B*1508, HLA B*5101, HLA B*530101, HLA B*5401, HLA B*550101, HLA B*5502, HLA B*5601, HLA B*5602, HLA B*7801) alleles identified by Sette and Sidney (1998) and the vaccination peptides in Table 3 were used to predict the population coverage if the peptides were combined into one inoculation (23 peptides in total) for human use. [1]The predicted population coverage of the HLA supertype alleles and the peptide epitopes. [2]The predicted average number of HLA and epitope combinations recognised in each population. [3]The predicted minimum number of HLA and epitope combinations recognised by 90% of the population.

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2.2.3 Adjuvants

Adjuvants are a key consideration in vaccine design. They can ultimately help shape the immune phenotype induced by vaccination. Many subunit vaccines are poorly immunogenic in nature and require additional components to aid in the development of a protective T cell response. They often act by providing a signal inducing components of the innate immune response, such as inducing TLR signaling (reviewed by Coffman *et al*., 2010). Until recently, ALUM was the only adjuvant approved for use in humans. ALUM acts through enhancing the antigen half-life, enhance phagocytosis and inducing the inflammasome via NALP3. Although ALUM is a good inducer of antibody responses, it is a poor inducer of T cell mediated immunity (Coffman *et al*., 2010) and perhaps not appropriate for vaccines against *T. gondii*.

Amongst the adjuvants used comparatively successfully in anti-*T. gondii* vaccines is GLA-SE, an oil in water emulsion, which contains a synthetic TLR4 ligand as an adjuvant. (Cong *et al*., 2010; Cong *et al*., 2011; Cong *et al*., 2012) Previously this was shown to be a potent inducer of a Th1 response (Coler *et al*., 2010). PADRE, a highly immunogenic, non-natural pan $CD4^+$ T cell epitope was also used (Cong *et al*., 2010; Cong *et al*., 2011; Cong *et al*., 2012). It was specifically designed to have a promiscuous binding specificity for Class II HLA-DR. PADRE is capable of inducing a CD4 T cell response (Alexander *et al*., 2010), essential for developing protective immunity. PADRE was used in this study as the epitopes selected were HLA class I restricted so had an extremely limited capacity to induce a $CD4^+$ T cell response that would be capable of supporting the $CD8^+$ cytotoxic T cell response.

Other potential adjuvants, which have been demonstrated to induce a strong Th1 response and are also capable of inducing a CTL response, are non-ionic surfactant vesicles (NISV, Brewer and Alexander 1992). The potential of NISVs as an adjuvant has been examined in this study. NISVs are liposome-like substances comprised of non-polar amphiphiles, which are molecules that are composed of both lipophilic and hydrophilic regions. They form an enclosed

bilayer structure with hydrophilic heads enclosing the hydrophilic tails of the lipid molecules. The structures have an aqueous core into which the antigen of interest is entrapped (Baillie *et al*., 1985; Brewer & Alexander 1992). It has already been demonstrated that NISVs are potent adjuvants. It has been shown using BSA entrapped within NISV that they where capable of inducing similar antibody titres to that of Freunds Complete Adjuvant (FCA, Brewer & Alexander 1992). Studies found that mice inoculated with BSA entrapped within NISV, produced increased levels of IgG2a (indicative of a Th1 type response) than mice treated with FCA and BSA. It has also been demonstrated that NISV are capable of inducing a cytotoxic T cell response (Brewer *et al*., 1996) making them a very favorable option in a vaccine against *T. gondii.* Indeed they have been used successfully in previous studies (Brewer & Alexander 1992; Roberts *et al*., 1994: Walker *et al*., 1995).

2.2.4. Vaccines and *Toxoplasma gondii*

Toxoplasma gondii is widely transmitted throughout the mammalian population. It has the ability to exist within a host even though protective immunity has been established. The parasite has a far-reaching impact in terms of human health, being a major problem in the immune compromised and it has the potential to cause severe complications during pregnancy. As the current drug regimes are not entirely effective, prophylactic vaccination is potentially a more effective method for controlling *T. gondii* infection. As already stated, protection against *T. gondii* infection is mediated by an IFN-γ mediated, $CD8^+$ T cell response (Gazzinelli *et al*., 1993; Suzuki and Remington, 1988; Parker *et al*., 1991; Subauste *et al*., 1991). Therefore utilising a vaccine, which will specifically induce a $CD8^+$ T cell response, could be the best approach for successful vaccination.

To this end, this study aimed to examine the ability of peptide vaccines, previously shown to be protective against tachyzoite challenge, to induce protection against oocyst infection, in transgenic class I HLA transgenic mice. Three transgenic strains possessing either human supertype HLA-A*0201, HLA-A*1101, or HLA-B*0702 alleles, were vaccinated with their respective class I restricted peptide pools along with adjuvants PADRE and GLA-SE. Optimal $CD8^+$ T cell priming is known to be reliant on $CD4^+$ T cell help during *T. gondii* infection (Jordan *et al*., 2009; Casciotti *et al*., 2002). The peptides used in the vaccine are HLA class I restricted so there would be little induction of $CD4^+$ T cells following vaccination. PADRE is a pan HLA-DR epitope and can induce a non-specific $CD4^+$ T cell response to vaccination. GLA-SE is a synthetic TLR4 agonist, which can induce the activation of antigen presenting cells and has previously been shown to bolster the induction of Th1 pro-inflammatory cytokine production (Coler *et al*., 2010).

The HLA-B*0702 restricted peptide vaccine was also entrapped within non-ionic surfactant vesicles (NISV), Original studies utilising NISVs with *Toxoplasma* lysate antigen, demonstrated NISVs to be robust adjuvants (Roberts

et al., 1994). While PADRE and GLA-SE had been used previously by Cong *et al*., (2010, 2011a, 2011b), this is the first report where NISVs are included with an HLA class I restricted vaccine.

In addition, herein we also report the use of ΔRPS13, a live attenuated *T. gondii* RH strain for its potential to protect against oocyst challenge in HLA-A*0201, HLA-A*1101 and HLA-B*0702 restricted transgenic mice strains. Previously ΔRPS13 was shown to induce protection in Swiss Webster mice (Hutson *et al*., 2010). Current commercially available live attenuated vaccines, such as for Polio and Tuberculosis are thought to rely on $CD8^+$ T cell mediated immunity (Rappuoli *et al*., 2007). Several live attenuated strains of *T. gondii* such as TS-4 and CPS-1 are already known to induce protection in a T cell mediated manner, as determined by IFN-γ production and protection against lethal challenge.

2.3. Methods

2.3.1. Ethical approval

All studies were carried out with AALAC and IACUC guidelines in accordance with US laws and institutional policies at the University of Chicago and the U.S. Department of Agriculture. Group sizes were determined on the basis of our previous experience and all experiments were conducted with sufficient sample sizes to have at least an 80% power to detect differences of key parameters at the 5% level of significance. Experimental groups consisted of 10 mice per group for challenge infection and 6 mice per group for immunological study. This allowed for group sizes large enough to perform meaningful statistical analysis by Kruskal Wallis multiple comparisons test and the Mann Whitney U tests where appropriate on the Prism 5 (GraphPad Software, USA) statisitical analysis software package.

2.3.2. Experimental design

Two experimental designs were used in this vaccine study, utilising two different adjuvants; GLA-SE and non-ionic surfactant vesicles. Both studies also include vaccination with ΔRPS13 RH *T. gondii*. The outcome of vaccination in both experiments was assessed by oocyst challenge and splenocyte stimulation assays. In each strategy, eight mice per group were vaccinated. Five mice were used for a challenge infection and three utilised for immunological analysis by flow cytometry and serum IFN-γ ELISA. The experimental mice were housed in the US Department of Agriculture (USDA), Maryland and all vaccinations, challenge infections blood sampling and dissections were conducted here. Tissue samples were then transported on ice to the George Washington University, Washington DC for the splenocyte stimulations and flow cytometery analysis. Serum samples were frozen and transported to University of Kentucky, Kentucky for cytokine analysis. Each experiment was carried out only once, with a total of 16 mice per vaccination group, for each experiment.

In the first experiment (Table 3) pools of HLA-A*0201, HLA-A*1101 or HLA-B*0207 restricted peptides (Table 4) were used to vaccinate their respective HLA transgenic mouse. The peptide vaccinations include GLA-SE and PADRE as adjuvants.

Table 3. Peptide specific vaccination experimental design

Vaccination groups	Mouse Strains	No. Mice	Vaccination regime	Analysis
PBS/DMSO Control	HLA-A*0201 HLA-A*1101 HLA-B*0702	16 16 16	Prime ↓ 2wks Boost ↓ 2wks Boost ↓ 1wk Challenge or Sacrifice	**Challenge:** 10 mice per strain / vaccination group infected orally with 100 Me49 oocysts (See 2.3.8). **Immunology:** 6 mice per strain / vaccination group sacrificed for immunological analysis Serum: IFNγ ELISA Spleen: *ex vivo* splenocyte stimulation with TLA, oocyst lysate and specific peptides. T cell populations analysed by flow cytometery.
T. gondii ΔRPS13 (100,000 tachyzoites)	HLA-A*0201 HLA-A*1101 HLA-B*0702	16 16 16		
PADRE (50μg) GLA-SE (20μg) adjuvants alone	HLA-A*0201 HLA-A*1101 HLA-B*0702	16 16 16		
HLA-A*0201, HLA-A*1101 or HLA-B*0702 restricted peptides (50μg) with PADRE GLA-SE	HLA-A*0201 HLA-A*1101 HLA-B*0702	16 16 16		

Previous studies (Cong *et al*., 2010 Cong *et al*., 2011, Cong *et al*., 2012) indicated that pooling the peptides into groups by their respective HLA specificity resulted in greater immunogenicity. The peptide amino acid sequences and the three-peptide pools used can be seen in Table 4.

Table 4. Peptide pools		
HLA-A*0201 Specific	HLA-A*1101 Specific	HLA-B*0702
(GRA6) VVFVVFMGV	(SAG1) KSFKDILPK	(GRA10) SSRLKRLPPE
(GRA6)FMGVLVNSL	(GRA6) AMLTAFFLR	(GRA3) VPFVVFLVA
(GRA3)FLVPFVVFL	(GRA7) RSFKDLLKK	(GRA7) LPQFATAAT
(SAG2C) FLSLSLLVI	(SAG2C) STFWPCLLR	(SAG3)SRMASVALAF
(SAG2C) FMIAFISCFA	(SRS52A) SSAYVFSVK	
(SA2X) FVIFACNFV	(GRA5) AVVSLLRLLK	
(SAG2X)FMIVSISLV		
(SAG3) FLLGLLVHV		
(SAG3) FLTDYIPGA		
(SRS52A) ITMGSLFFV		
(GRA5) GLAAAVVAV		
(MIC1)VLLPVLFGV		
(MIC2)FAAAFFPAV		

The mice were given 50μg of each peptide from their respective peptide pool, with 50μg of the PADRE peptide (AKFVAAWTLKAAA) and 20μg of the Glucopyranosyl Lipid Adjuvant-Stable Emulsion (GLA-SE), made to a total volume of 50μl in sterile PBS. The vaccine was given subcutaneously into the base of the tail. ΔRPS13, a live attenuated strain of RH *T. gondii* (Hutson *et al*., 2010) was also given to each of the strains. 100,000 tachyzoites where given intraperitoneally in 200μl of sterile PBS. After the prime, two further vaccine boosts were given at two-week intervals.

The second vaccination protocol used non-ionic surfactant vesicles in conjunction with the HLA-B*0702 restricted, GRA7 derived, LP9 (LPQFATAAT) peptide (Table 5). Only this peptide was utilised in the vaccine study with NISVs.

Table 5. Peptide specific vaccination with NISV experimental design				
Vaccination groups	Mouse Strains	No. Mice	Vaccination regime	Analysis
PBS Control	HLA-B*0702	16	Prime ↓ 2wks Boost ↓ 1wks Challenge or Sacrifice	**Challenge:** 10 mice per strain / vaccination group infected orally with 100 Me49 oocysts (See 2.3.8). **Immunology:** 6 mice per strain / vaccination group sacrificed for immunological analysis Serum: IFNγ ELISA Spleen: *ex vivo* splenocyte stimulation with TLA, oocyst lysate and specific peptide. T cell populations analysed by flow cytometery.
T. gondii ΔRPS13 (100,000 tachyzoites)	HLA-B*0702	16		
Empty NISV	HLA-B*0702	16		
NISV entrapped LP9 (5μg) peptide and PADRE(5μg)	HLA-B*0702	16		
PADRE (50μg) GLA-SE (20μg) LP9 peptide (50μg)	HLA-B*0702	16		

LP9 (LPQFATAAT) was used in conjunction with the PADRE peptide epitope. HLA-B*0702 mice were given 5μg of LP9 and PADRE subcutaneously, into the base of the tail. LP9 and PADRE were entrapped in separate vesicle preparations and then combined for inoculation; 5μg of each was administered to each mouse subcutaneously at the base of the tail. The GLA-SE adjuvant with PADRE and LP9 was also used in parallel. LP9/PADRE alone controls were given along with empty vesicles and PBS only as vehicle controls. A further experimental group given 100,000 tachyzoites of the ΔRPS13 attenuated RH *T. gondii* strain via intraperitoneal injection. In this experiment groups were given a prime and one vaccination boost with a two-week interval.

2.3.3. Mice

HLA-A*0201, HLA-A*1101 and HLA-B*0702 transgenic mice, expressing human MHC class I alleles were maintained in breeding colonies in the US Department of Agriculture, Maryland, USA. All vaccinations and challenges were carried out in the same location. *Ex vivo* stimulations were carried out at George Washington University, Washington DC, USA. For experiments male and female mice aged between 6 and 20 weeks were used.

2.3.4. Peptide selection

The peptides selected for use in this vaccination study had previously been shown to be successful immunogens (Cong *et al*., 2010 Cong *et al*., 2011, Cong *et al*., 2012). The McLeod laboratory at the University of Chicago carried out the peptide screening. Briefly, the peptides were initially selected by screening secretory and excretory proteins from *T. gondii* against the HLA-A*0201, HLA-A*1101 and HLA-B*0702 supertype alleles using the Immune Epitope Database and Analyses Resource (http://iedb.org). Peptides that produced a binding high score were synthesised by Synthetic Biomolecules, San Diego. The peptides were then screened against human peripheral blood lymphocytes from patients who were sero positive for *T. gondii* and also possessed the HLA-A*0201, HLA-A*1101 or HLA-B*0702 alleles. This screening was achieved by ELISpot assay for IFNγ. Peptides that proved to induce a strong IFN-γ response were taken forward as vaccine candidates.

2.3.5. Maintenance and harvesting of ΔRPS13 RH *T. gondii in vitro* culture

ΔRPS13 RH *T. gondii* were routinely maintained in confluent human foreskin fibroblasts (HFFs) grown in 75cm^2 tissue culture flasks (TPP, Switzerland) in 10ml Dulbecco's Modified Eagle Medium (DMEM) complete medium (DMEM, 1% L-glutamine, 10% foetal calf serum, 100U/ml penicillin (Cambrex Bioscience, Veniers, Belgium), 100mg/ml streptomycin (Cambrex Bioscience, Veniers, Belgium) 50U amphotericin B (Cambrex Bioscience, Veniers, Belgium)

and supplemented with tetracycline. The cultures were incubated at 37°C in 5% CO_2. Once the majority of the HFFs were infected with replicating tachyzoites, the flask was scraped using a 30cm cell scraper (TPP, Trasadingen, Switzerland). The parasite solution was then carefully passaged at least 10 times though a 25G needle (BD, Drogheda, Ireland). A 10-fold dilution was made of the parasite solution in complete DMEM and passed into a confluent flask of HFFs. For infection the tachyzoites were enumerated with a haemocytometer and the appropriate dilution made in sterile PBS.

2.3.6. Non-ionic surfactant vesicles

Non-ionic surfactant vesicles were prepared based on the protocols established by Brewer & Alexander, 1992. Glassware used was autoclaved and heated at 180°C for 6 hours to remove any endotoxin. 150μmol vesicles were prepared, as previously described (Brewer & Alexander 1992) from 1-monopalmitoyl glycerol (24.8mg or 75μmole, Larodan, Sweden), cholesterol (23.2mg or 60μmole, Sigma, UK) and dicetyl phosphate (8.2mg or 15μmole Sigma, UK).

The powders were mixed in 15ml Pyrex test tubes and heated to 130°C in a dry block (Grant Instruments, Cambridge, U.K.). Spontaneous formation of the vesicles occurred after the addition of 2.5ml sterile PBS (Sigma, UK) and vigorous vortexing for 1 minute. The newly formed vesicles were then incubated at 60°C for 2 hours and then allowed to cool to 20°C

The peptides were entrapped in the vesicles by adding 0.5mg of each to separate vesicle preparations. These vesicle/peptide mixes were placed into a sonicating water bath at 20°C for 6 minutes. Following this the preparations were flash frozen in liquid nitrogen, then thawed to 30°C in a water bath, a minimum of 12 times. The mix was then incubated for a further 2 hours at 30°C. The vesicles underwent ultra-centrifugation at 100,000g for 1 hour at 4°C to separate un-entrapped antigen. To prevent the density of the lipids present

hindering the formation of a stable pellet, 5mls of sterile 0.9% NaCl was added to each of the vesicle preparations prior to centrifugation.

The pellet was re-suspended in 1ml of sterile 0.9% NaCl. The NISVs were then stored at -80°C until required. The quantity of peptide entrapped within the vesicles was determined by the modified ninhydrin assay of the vesicle pellet.

2.3.7. Modified ninhydrin assay

10μl of the peptide/vesicles, empty vesicles and the peptide specific standards were dried in 1.5ml microcentrifuge tubes in a heat block at 110°C. Once dried, 150μl of 13.5M NaOH was added to all samples and standards and thoroughly vortexed. Holes were pierced in the tubes before being wrapped in Nesco film and autoclaved in a bench top pressure cooker for 25 minutes to hydrolyse the peptides and destroy the vesicles.

Once cooled to room temperature the 13.5M NaOH was neutralised with the addition of 250μl glacial acetic acid. 500μl of ninhydrin reagent (Sigma, UK) was added to all samples and standards, thoroughly votexed and boiled for 20 minutes. After cooling, 250μl of all the samples and standards were added to fresh centrifuge tubes, containing 750μl of 50% propan-2-ol and thoroughly votexed. 200μl aliquots of the samples and standards were placed in a 96 well ELISA plate and the absorbance measured at 570nm on a microplate reader (Spectramax 190, Molecular Devices, USA).

2.3.8. Challenge infections

Oocyst batches for use in challenge infectinos were previously isolated and titrated by Dubey Laboratory at the US Department of Agriculture, Maryland in BALB/c mice. Lethal challenge dose was found to be 1000 oocysts. One week after the final vaccination, mice for challenge from each group were infected with 100 Me49 oocysts by oral gavage. Challenge with *T.* gondii infections were

carried out by the technicians in the Dubey Laboratory at the USDA. Subsequently it was determined that 100 oocysts was infact a lethal challenge dose for the HLA transgenic mice strains used in this study.

2.3.9. Serum IFN-γ

One week after the last boost 3 mice from each group were sacrificed for immunological analysis. Venous blood was collected into BD Vacutainer blood collection tubes with a clot activator and serum separation additive (BD Bioscience, USA). Following 30-minute incubation at room temperature the samples were centrifuged at 4000g for 10 minutes and the serum collected and placed into a fresh 1.5ml centrifuge tube. The samples were stored at -20°C until analysis. Serum IFN-γ levels were determined by capture ELISA by the Suzuki laboratory, based at the University of Kentucky, USA.

2.3.10. Flow cytometry

Flow cytometry was used to determine the phenotype of the immune response raised by the vaccination regimes. The same protocol was used for each of the experiments. In each experiment there was a period of approximately 10 hours between the spleens being harvested and the stimulations being applied *ex vivo*, due to the experiments being based at two different institutes and number of groups being assessed. IFN-γ and TNF-α production by both $CD4^+$ and $CD8^+$ T cells was determined. Additionally the markers for selective effector (KLRG1) and memory precursor (CD127) $CD8^+$ T cells were also assayed along with Granzyme B levels.

Mice for immunological analysis were sacrificed by cervical dislocation and the spleens removed aseptically and placed into ice cold PBS in 1.5ml centrifuge tubes. Single cell suspensions were created by crushing the spleens though a 70μm cell strainer (BD Bioscience, UK), with ice-cold stain wash buffer (SWB: 10% FCS in sterile PBS). The cell suspension was centrifuged at 2000rpm for 7 minutes, the supernatant decanted and the pellet re-suspended in 3mls of pre-

warmed (37°C) RBS lysis buffer (Sigma, USA). After a three-minute incubation at room temperature, the samples were quenched to 40mls with cold SWB and centrifuged as before. The pellets were re-suspended in complete RPMI 1640 medium and counted using a haemocytometer.

Four stimulations were set up; medium alone, TLA, 10μg/ml, oocysts lysate antigen (OocystAg) 10μg/ml, or 10μg/ml/peptide of the specific peptide pools for each HLA strain. In the case of the NISV experiment only the LP9 peptide was used. $1x10^6$ splenocytes from each sample were plated out per re-stimulation, into round bottom, 96 well tissue culture plates and incubated for 6 hours at 37°C with 5% CO_2. Additional wells were set up for compensation and fluorescence minus one (FMO) controls. After 6 hours Monensin (BD Bioscience, USA) was added to a final concentration of 0.665μg/ml. The plates were further incubated for 6 hours. After this time the cells were transferred to 96 well V bottom plates for FACS staining, and centrifuged at 2000rpm for 7 minutes and washed twice with cold PBS.

All of the samples, FMO controls, and the Aqua compensation control, were then re-suspended in Aqua Live/Dead stain (Invitrogen, USA) diluted to 2μg/ml in PBS and incubated in the dark at 4°C for 30 minutes before being washed twice in cold PBS. The experimental samples and FMO controls were then re-suspended in 100μl of the surface stain mix, made up in SWB. At this time the surface single stains for compensation were also set up. Anti-mouse CD16/CD32 FC-block (BD Bioscience, USA) was added to the surface stain mix, to a concentration of 1μg/ml. Table 3 shows the surface stains and the working concentration.

Table 6. Surface stain mix.	
Anti CD4α-APC-Cy7	0.4μl per sample
Anti CD8β-FITC	0.6μl per sample
Anti KLRG1-PeCy7	0.6μl per sample
Anti CD127-Biotin	1μl per sample
Streptavidin e710	0.5μl per sample
SWB	96.9μl per sample

The samples were incubated for 20 minutes in the dark at 4°C and then washed once in SWB. The samples and controls were re-suspended in 100μl of Fixation/Permabilisation buffer (BD Bioscience, USA) and incubated for a further 45 minutes in the dark at 4°C.

The cells were then washed twice in Permeabilisation wash buffer (BD Bioscience) and the intracellular stain mix added to the samples. Table 4 shows the intracellular stains and their working concentrations. At this time, 100μl of the intracellular single colour stains for the compensation controls were added along with the FMO controls. The FMO combinations can be found in table 5. All of the stains were made up in the Permeabilisation wash buffer. The plates were incubated for 1 hour in the dark at 4°C.

Table 7. Intracellular stain mix.	
Anti IFNγ-PE	0.5μl per sample
Anti Granzyme B-APC	5μl per sample
Anti TNFα-e450	1.25μl per sample
SWB	93.25μl per sample

Table 8. FMO Combinations		
IFNγ	Granzyme B	TNFα
Anti CD4α-APC-Cy7	Anti CD4α-APC-Cy7	Anti CD4α-APC-Cy7
Anti CD8β-FITC	Anti CD8β-FITC	Anti CD8β-FITC
Anti KLRG1-PeCy7	Anti KLRG1-PeCy7	Anti KLRG1-PeCy7
Anti CD127-Strep-e710	Anti CD127-Strep-e710	Anti CD127-Strep-e710
	Anti INFγ-PE	Anti IFNγ-PE
Anti Granzyme B-APC		Anti Granzyme B-APC
Anti TNFα-e450	Anti TNFα-e450	

After incubation the samples were washed twice with SWB buffer and re-suspended in 200μl PBS. The samples were stored in the dark at 4°C until analysis.

500,000 events per sample were acquired on a FACSCalibur (BD Bioscience, USA) using Flowjo data acquisition software. The data was then analysed on Flowjo analysis software. Compensation controls were used to set up a compensation matrix. The gating strategy gated on the live cells on forward and side scatter. From here the cells were gated on side scatter verses the live dead stain. Cells with a low staining were sub gated on CD4 or CD8 and subsequently IFN-γ and TNF-α, To look at the selective effector cell and memory precursor cell response induced by the vaccine, the $CD8^+$ T cells were further sub gated on KLRG1 and CD127 respectfully. $CD8^+$ T cells were also sub gated on Granzyme B to assess the response upon re-stimulation.

2.3.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 5.0, GraphPad Software, California). All results shown are standard error of the mean (SEM). Statistically significant differences were determined by Kruskal Wallis multiple comparisons test followed using Prism 5 (GraphPad, USA) Software statisical analysis software. P values equal to or below 0.05 were considered significant.

2.4 Results

2.4.1. Vaccination of HLA transgenic mice with ΔRPS13 mutants protected against oocyst challenge

HLA-A*0201 (Figure 2.4.1 A), HLA-A*1101 (Figure 2.4.1 B) and HLA-B*0702 (Figure 2.4.1 C) mice that were vaccinated as described with either PBS, ΔRPS13 tachyzoites, PADRE peptide and GLA-SE adjuvant or 50μg of HLA specific peptide pool with PADRE and GLA-SE, were challenged with 100 Me49 oocysts. All of the PBS and adjuvant alone controls had died by day 8 post-infection. The HLA-A*1101 (B) and HLA-B*0702 (C) peptide specific vaccinated animals survived until day 10 post-infection when they succumbed to infection. The HLA-A*0201 peptide specific vaccine (C) appeared to have no effect on survival over the vehicle and adjuvant controls and mice vaccinated with this formulation also succumbed to infection by day 8 post infection.

Mice vaccinated with ΔRPS13 tachyzoites offered the best protection when challenged with the Me49 oocysts. By day 8 post infection, 80% of the HLA-A*0201 vaccinated mice had succumbed to infection. This increased to 90% on day 9 although all remaining animals survived until the experiment was terminated on day 12. The HLA-A*1101 vaccinated mice started to succumb to infection from day 8 onwards, with the group size dropping by 10% each day until the end of the experiment on day 12. HLA-B*0702 vaccinated group size dropped to 90% on day 8 and then to 70% by day 10 post infection. Seventy percent of the mice survived until the end of the experiment on day 12 (Figure 2.4.1). Statistical analysis of time to death for HLA-A*1101 and HLA-B*0702 mice shows statistically significant protection (P=0.0001) in mice vaccinated with Δ RPS13 compated with the PBS control.

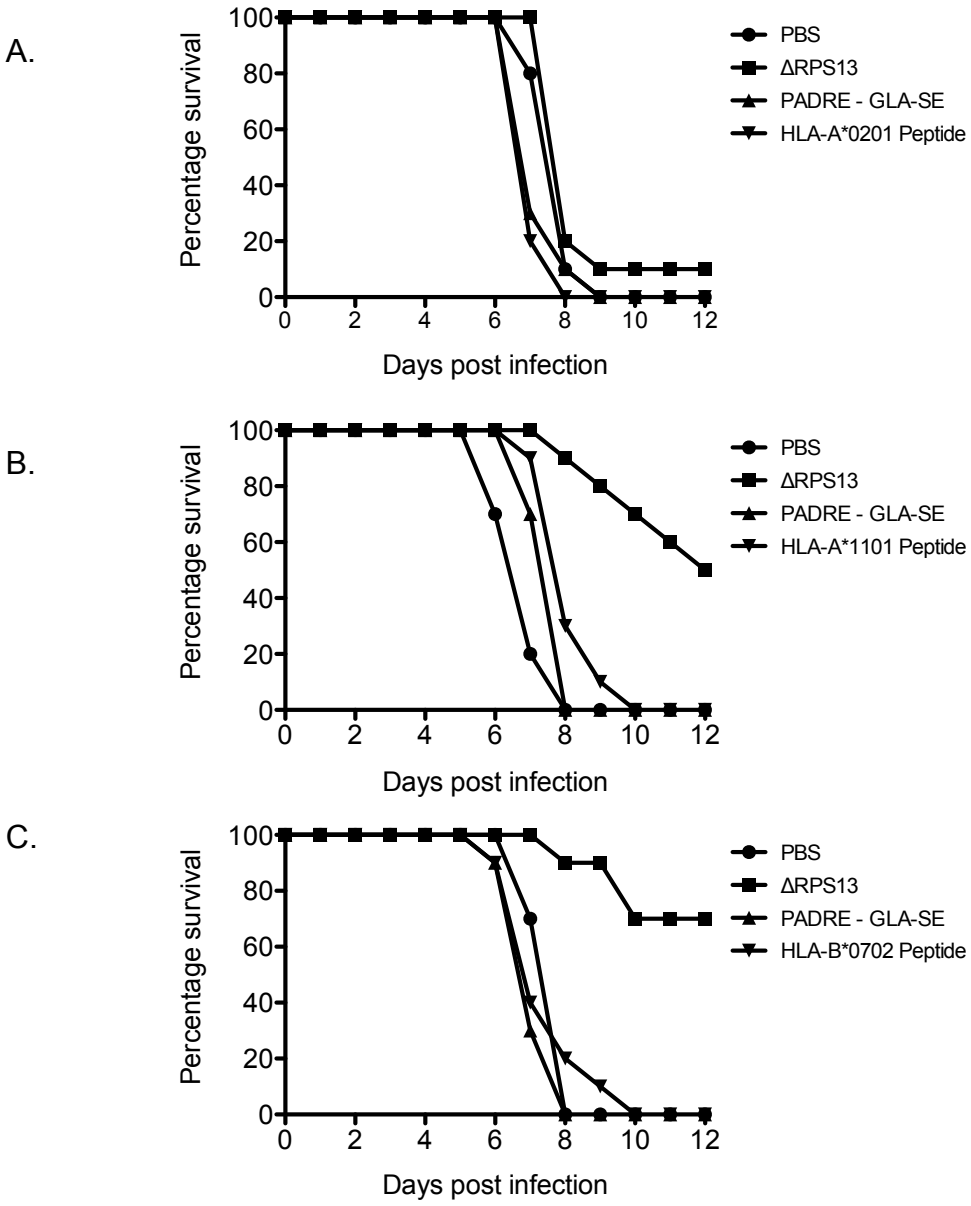
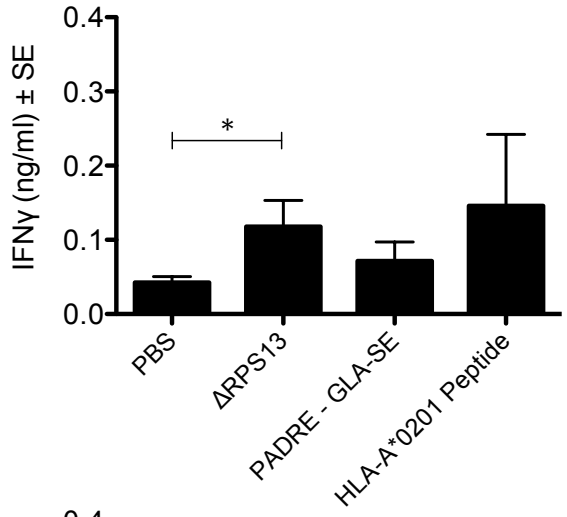


Figure 2.4.1. Survival curves of vaccinated (A) HLA-A*0201, (B) HLA-A*1101 and (C) HLA-B*0702 transgenic mice after oocyst challenge.
The three strains of HLA transgenic mice were challenged with 100 Me49 oocysts following vaccination with either; PBS, ΔRPS13, PADRE and GLA-SE or HLA specific peptide pool with PADRE and GLA-SE. Mice were monitored daily until a terminal endpoint was reached. The experiment was ended on day 12 post-infection. For each group n=10. The experiment was carried out on only one occassion. Time to death in HLA-A*1101 and HLA-B*0702 for PBS vaccinated vs ΔRPS13 vaccinated was statistically significant with P = 0.0001.

2.4.2. Vaccination had little effect on serum IFN-γ concentrations pre-challenge.

Vaccinated mice were sacrificed one week after the last vaccination booster. The blood was collected and the serum isolated. Serum were analysed for IFN-γ levels using an ELISA. HLA-A*0201 mice, but not HLA-A*1101 or HLA-B*0702 mice vaccinated with ΔRPS13 had increased levels of IFN-γ in their serum compared with that of PBS vaccinated control mice (p=0.0159) (Figure 2.4.2 A-C). None of the other vaccine formulations induced significantly greater serum IFN-γ levels in any of the 3 transgenic mouse strains examined (Figure 2.4.2 A-C).

A.



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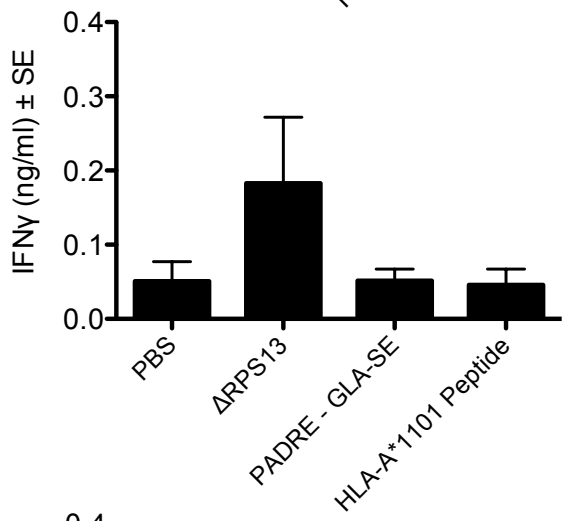


C.

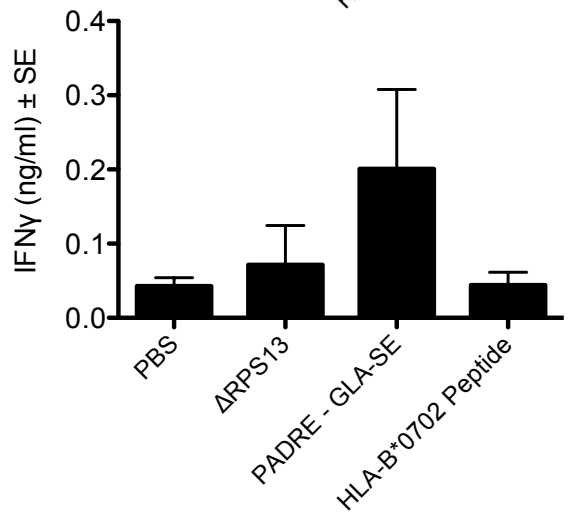


Figure 2.4.2. Serum IFN-γ concentrations of the HLA transgenic mice (A) HLA-A*0201, (B) HLA-A*1101 and (C) HLA-B*0702.

Serum from vaccinated HLA-A*0201, HLA-A*1101 and HLA-B*0702 transgenic mice was collected 1 week after the final boost and analysed by ELISA to determine IFN-γ content. For each group n=6. The experiment was carried out once. *P <0.05

2.4.3. Vaccination with ΔRPS13 induces $CD4^+$ IFN-γ upon stimulation

Spleens were collected from vaccinated HLA-A*0201, HLA-A*1101 and HLA-B*0702 mice 1 week after the final vaccine boost. The spleens were processed to produce a splenocyte suspension that was re-stimulated *ex vivo* with either PBS alone, oocysts lysate antigen (OocystAG), the HLA specific peptide pool or *Toxoplasma* lysate antigen (TLA). The splenocytes were stimulated for 12 hours and stained with fluorescently labeled anti-CD4 and anti-IFN-γ antibodies and processed by flow cytometery

Irrespective of mouse strain examined, vaccination with ΔRPS13 induced more robust and consistent levels of $CD4^+$ splenocytes capable of IFN-γ production than any other vaccine protocol examined. This was evident following *ex vivo* stimulation of splenocytes with either oocysAg or TLA and significantly greater than in splenocytes derived from mice given PBS as a vehicle control (OocystAg: HLA-A*0201, p=0.015; HLA-A*1101, p=0.002; HLA-A*0702, p=0.0411 and TLA: HLA-A*0201, p=0.005; HLA-A*1101, p=0.002; HLA-A*0702, p=0.002). Vaccination with ΔRPS13, GLA-SE/PADRE or GLA-SE/PADRE/specific peptide generally increased levels of $CD4^+$ splenocytes capable of IFN-γ production. This was evident even in cells receiving no antigenic stimulation or stimulated with the appropriate pool of HLA-matched peptides and most pronounced in HLA-A*1101 mice (p=0.002) (Figure 2.4.3 A-C).

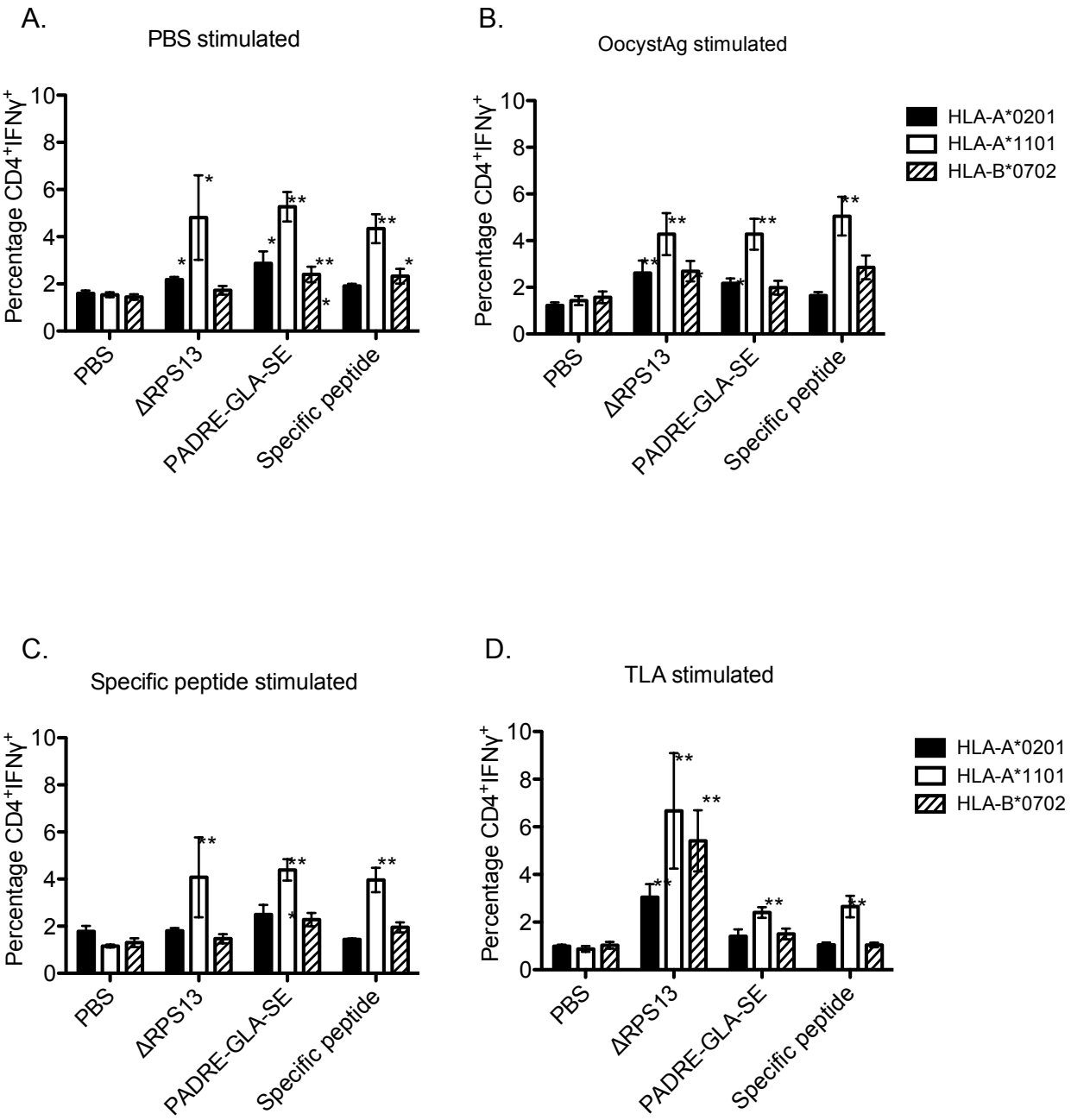


Figure 2.4.3. CD4$^+$ IFN-γ$^+$ T cells induced by vaccination.

Spleens from PBS, ΔRPS13, Adjuvant or peptide pool vaccinated HLA transgenic mice were removed and splenocytes stimulated with either (A) PBS, (B) oocyst lysate antigen, (C) Specific peptide pools, or (D) TLA. CD4$^+$ IFN-γ$^+$ T cell populations were then determined by FACS analysis. Statistical analysis was determined in comparison to the relevant PBS vaccinated controls. For each group n=6. The experiment was repeated once * $P < 0.05$, ** $P < 0.005$.

2.4.4. Vaccination with HLA-specific peptides can induce specific $CD8^+$ T cell IFN-γ

Following stimulation of the splenocytes from vaccinated mice with PBS alone, oocystAg, the HLA specific peptides or TLA, the cells were stained with fluorescently labeled antibodies for CD8 and IFN-γ. These were then analysed by FACS.

Vaccination of HLA-A*1101 and HLA-B*0702 mice with the appropriate HLA-matched peptide pools in GLA-SE/PADRE successfully induced.$CD8^+$ T cells capable of inducing IFN-γ as assessed *ex vivo* following stimulation with the matched peptide pools (HLA-A*1101, p =0.003 and HLA-B*0702 p =0.002). In contrast vaccination of HLA-A*0201 mice using the same system failed to induce $CD8^+$ T cells capable of inducing IFN-γ as assessed *ex vivo* following stimulation with the matched peptide pool. Vaccination with ΔRPS13 despite reducing mortality in each of the three HLA-transgenic mice, did not efficiently induce CD8+ T cells capable of producing IFN-γ following *ex vivo* stimulation with the appropriate HLA-matched specific peptide pool, TLA or oocystAg.

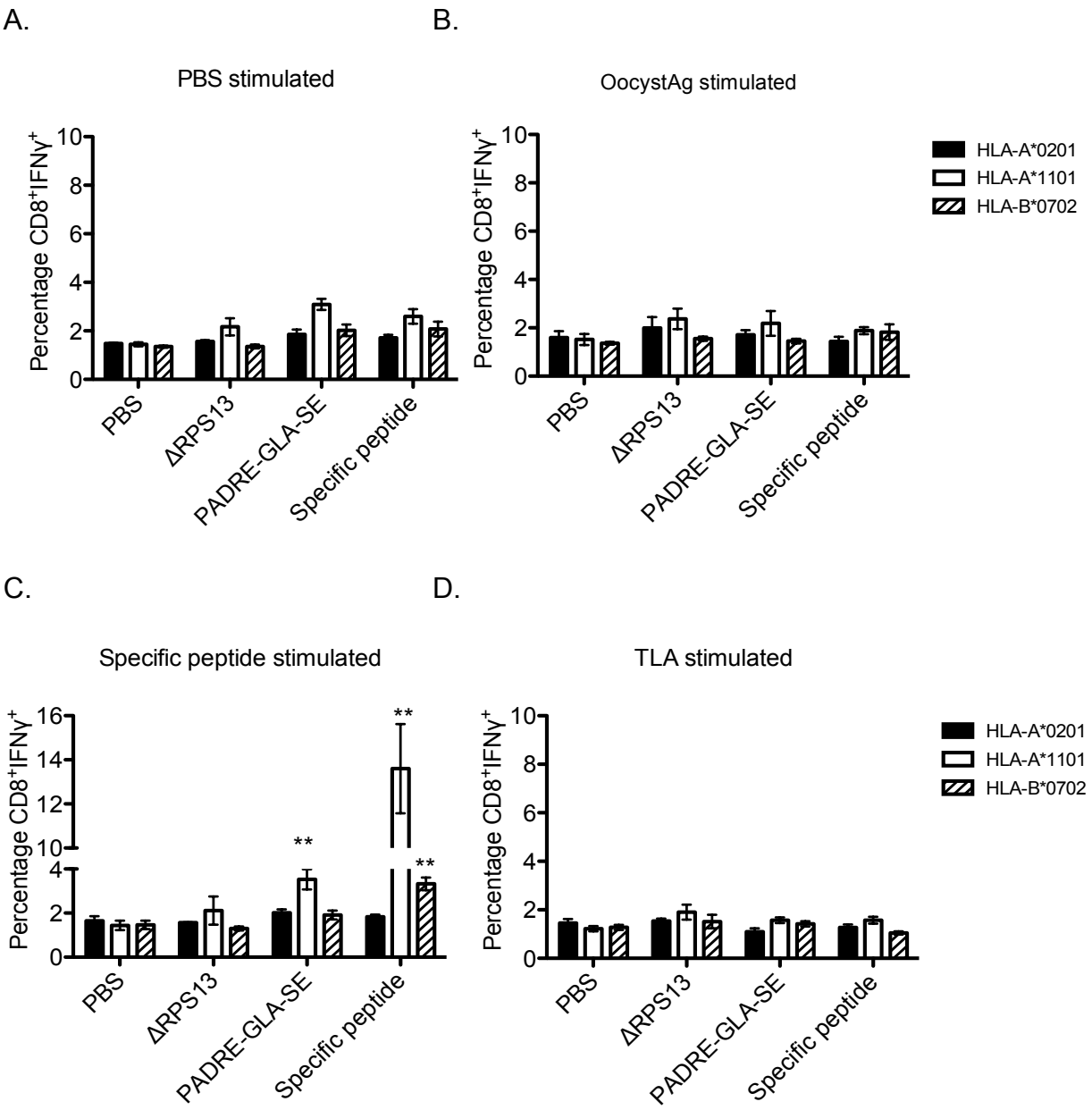


Figure 2.4.4. CD8$^+$ IFN–γ$^+$ T cells elicited by vaccination.

Spleens from PBS, ΔRPS13, Adjuvant or peptide pool vaccinated HLA transgenic mice were removed and splenocytes stimulated with either (A) PBS, (B) oocystAg, (C) Specific peptide pools, or (D) TLA. CD8$^+$ IFNγ$^+$ T cell populations were then determined by FACS analysis. Statistical analysis was determined in comparison to the PBS vaccinated controls. For each group n=6. Significance of p=<0.05 indicated by (*) and p=<0.005 (**). The experiment was carried out once.

2.4.5. Vesicle formulations and entrapment efficiencies with selected peptides.

In order improve the efficacy of the peptide vaccines non-ionic surfactant vesicles were investigated as potential vaccine adjuvants. However their use was limited due to problems formulating the vesicles with the peptides. Table 9 shows the entrapment results for each peptide.

None of the HLA-A*0201 restricted peptides were successfully entrapped into vesicles. The peptides were only soluble in neat DMSO, which interfered with ultracentrifugation so as it was impossible to pellet the vesicles and remove the unentrapped peptides. The same was true of the HLA-A*1101 peptides with the exception of the GRA7 derived peptide; RSFKDLLKK. However this peptide only gave an entrapment efficiency of 5%.

Two of the HLA-B*0702 restricted peptides; GRA3 derived VPFVVFLVA and GRA7 derived LPQFATAAT, were soluble in DMSO and successfully entrapped within vesicles with entrapment efficiencies of 9 and 19 percent respectively. The GRA7 derived LPSFATAAT peptide, entrapped within NISVs was selected and taken forward for use in the HLA-B*0702 transgenic mice as this peptide has already been demonstrated to be immunogenic and protect against tachyzoite challenge.

Table 9. Peptide/NISV Formulation				
HLA Restriction	Peptide sequence	Solubility	Stable pellet following ulta-centrifugation?	Entrapment efficiency
HLA-A*0201	(GRA6) VVFVVFMGV	DMSO	No pellet	-
	(GRA6)FMGVLVNSL	DMSO	No pellet	-
	(GRA3)FLVPFVVFL	DMSO	No pellet	-
	(SAG2C) FLSLSLLVI	DMSO	No pellet	-
	(SAG2C) FMIAFISCFA	DMSO	No pellet	-
	(SA2X) FVIFACNFV	DMSO	No pellet	-
	(SAG2X)FMIVSISLV	DMSO	No pellet	-
	(SAG3) FLLGLLVHV	DMSO	No pellet	-
	(SAG3) FLTDYIPGA	DMSO	No pellet	-
	(SRS52A) ITMGSLFFV	DMSO	No pellet	-
	(GRA5) GLAAAVVAV	DSMO	No pellet	-
	(MIC1) VLLPVLFGV	DMSO	No pellet	-
	(MIC2)FAAAFFPAV	DMSO	No pellet	-
HLA-A*1101	(SAG1) KSFKDILPK	DSMO	No pellet	-
	(GRA6) AMLTAFFLR	DMSO	No pellet	-
	(GRA7) RSFKDLLKK	PBS	Loose pellet	5%
	(SAG2C) STFWPCLLR	DMSO	No pellet	-
	(SRS52A) SSAYVFSVK	DMSO	No pellet	-
	(GRA5) AVVSLLRLLK	DMSO	No pellet	-
HLA-B*0702	(GRA10) SSRLKRLPPE	PBS	Loose pellet	9%
	(GRA3) VPFVVFLVA	DMSO	No pellet	-
	(GRA7) LPQFATAAT	PBS	Loose pellet	19%
	(SAG3)SRMASVALAF	DMSO	No pellet	-

Table 9. Entrapment efficiencies of the HLA restricted peptides-NISV formulations.

Table 9 shows the maximum entrapment efficiencies achieved for each HLA restricted peptide. Entrapment of each peptide was attempted on at least two occasions and repeated until enough entrapped peptide was produced for vaccine studies.

2.4.6. Vaccination with HLA-B*0702 specific GRA6 peptide in NISV failed to protect against oocyst challenge.

In an attempt to improve the efficacy of the HLA-B*0702 restricted peptide vaccine, the GRA6 and PADRE peptides were formulated with NISVs as an adjuvant.

HLA-B*0702 mice were vaccinated with either PBS or 100,000 ΔRPS13 RH *T. gondii* tachyzoites or empty NISV or 5μg of the HLA-B*0702 specific peptide, LP9 and 5μg of PADRE peptide entrapped within NISVs, or 5μg of the LP9 and PADRE peptides mixed with 20μg of the GLA-SE adjuvant. The mice were given a prime and one vaccine boost, 2 weeks apart. One week after the boost, the mice were challenged with 100 oocysts from the Me49 strain. By day 8 all of the groups, apart from those vaccinated with ΔRPS13 had died. All mice vaccinated with the ΔRPS13 survived until day 10-post infection with 70% surviving until the experiment ended on day 12-post infection.

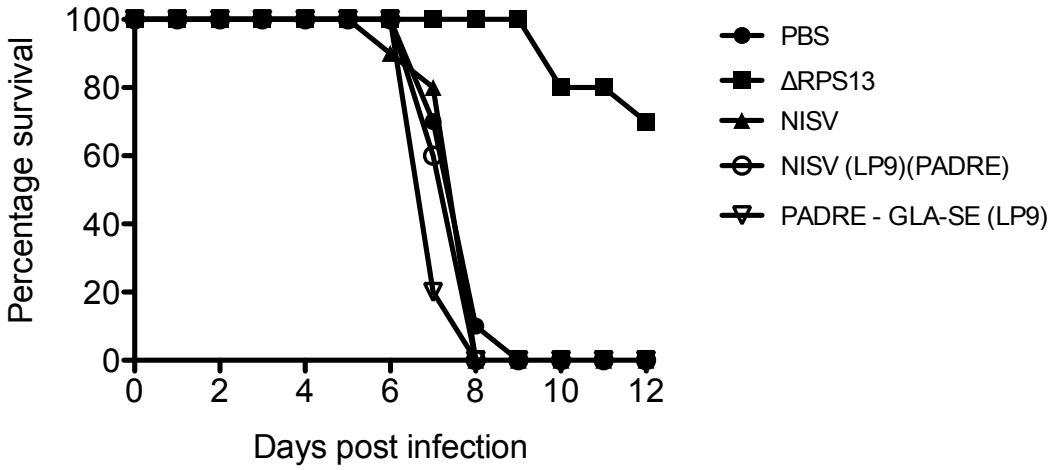


Figure 2.4.5. Survival curves of vaccinated HLA-B*0702 transgenic mice following oral challenge.

Mice were vaccinated with PBS, ΔRPS13, empty NISV, NISV containing LP9 peptide and PADRE, or LP9 peptide with GLA-SE and PADRE adjuvants. Mice were challenged perorally with Me49 oocysts and monitored daily until a terminal endpoint was reached. For each group n=10. The experiment was carried out once.

2.4.7. Vaccination with Δ RPS13 or LP9 and PADRE entrapped in NISVs enhances serum IFN-γ

Serum was collected and the IFN-γ levels assayed by ELISA from HLA-B*0702 mice vaccinated with either PBS, ΔRPS13 tachyzoites, empty NISVs, NISV's with entrapped LP9 peptide, NISV's with entrapped LP9 peptide and PADRE or PADRE, GLA-SE and LP9 peptide combination. IFN-γ levels in the ΔRPS13-vaccinated animals were increased over the PBS vehicle control, however this was not statistically significant.. Mice treated with NISVs with LP9 peptide and PADRE demonstrated a slight increase in IFN-γ levels however this was not statistically significant over the PBS vehicle control.

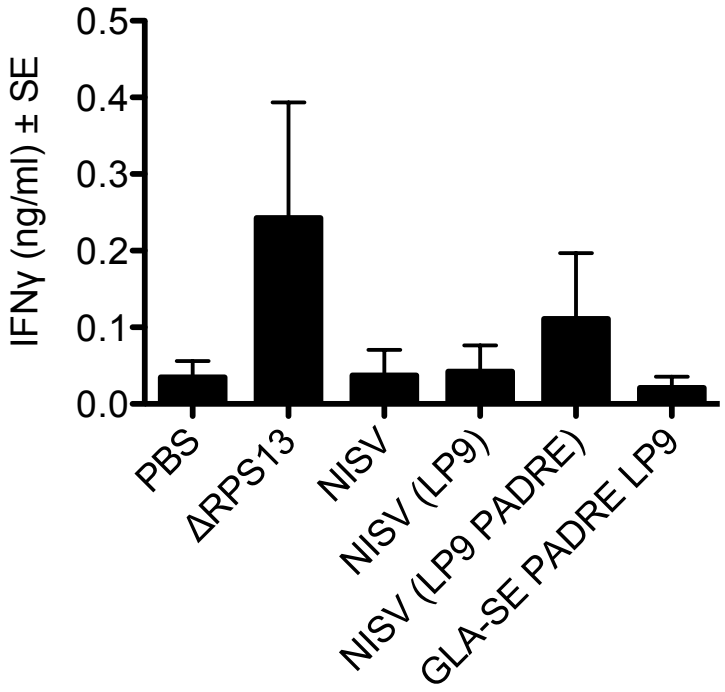


Figure 2.4.6. Serum IFN-γ elicited by NISV vaccination in HLA-B*0702 mice

Following vaccination with PBS, ΔRPS13, empty NISV, NISV containing LP9 peptide and PADRE, or LP9 peptide with GLA-SE and PADRE adjuvants, serum was harvested and IFN-γ content determined by ELISA. Although both the ΔRPS13 and NISV with LP9 peptide and PADRE have increased levels of serum IFN-γ, this is not statistically significant. For each group n=6. The experiment was carried out once.

2.4.8. Splenocytes from ΔRPS13-vaccinated mice produce increased IFN-γ levels compared with splenocytes from mice vaccinated with NISV entrapped peptide.

The spleens were collected and the splenocytes stimulated with either PBS alone, oocystAg, LP9 peptide or TLA for 12 hours. The supernatants were collected and IFN-γ levels established by ELISA. Stimulation of splenocytes with oocystAg elicited a strong IFN-γ response from the ΔRPS13-vaccinated group, statistically significant over all other groups with a ($p = 0.004$). Stimulation of splenocytes from the ΔRPS13 vaccinated mice with the LP9 peptide elicited a small IFN-γ response that was not significant greater than other vaccinated groups of mice. Stimulation with TLA elicited IFN-γ production in splenocytes from all of the vaccinated groups. However, this was significantly greater in ΔRPS13 vaccinated group ($p = 0.001$)

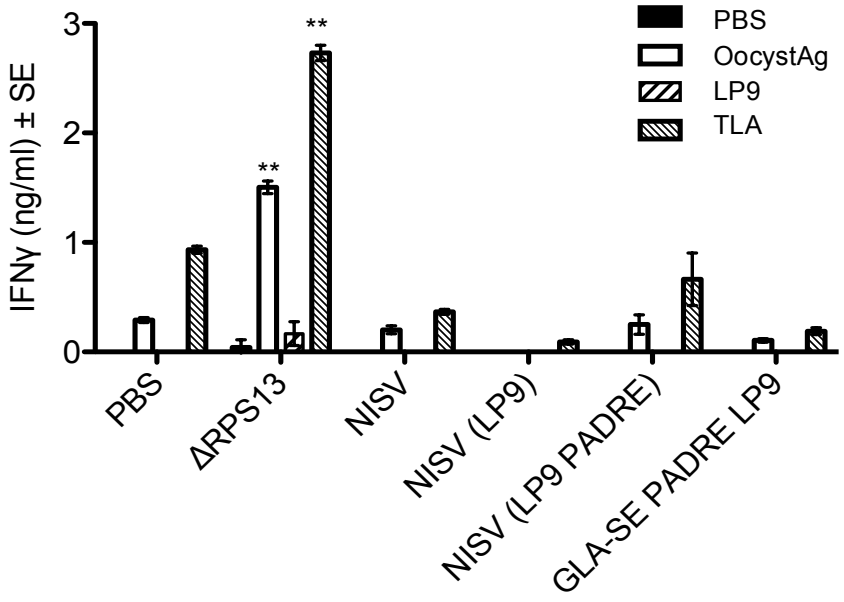


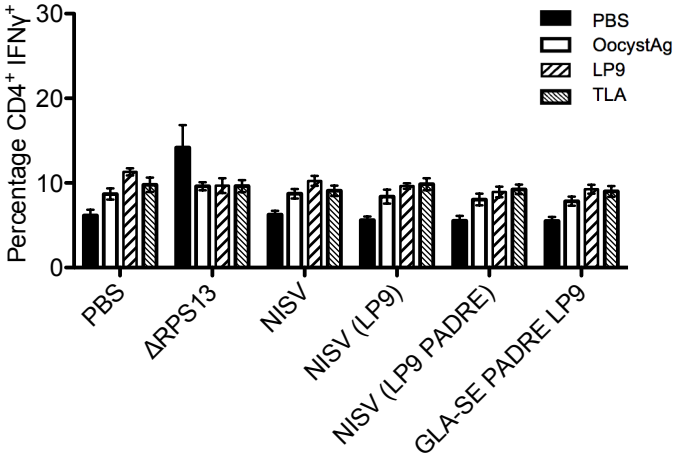
Figure 2.4.7. IFN-γ secretion following splenocyte stimulation.

Splenocytes from HLA-B*0702 mice vaccinated with PBS, ΔRPS13, empty NISV, NISV containing LP9 and PADRE, or LP9 peptide with GLA-SE and PADRE and stimulated with PBS, oocystAg, LP9 peptide or TLA. IFN-γ production was assed by ELISA. Statistical analysis was determined in comparison to PBS vaccinated control. For each group n=6. The experiment was carried out once. ** P <0.005

2.4.9. $CD4^+$ IFN-γ^+ and $CD4^+$ TNF-α^+ T cells induced by vaccination with NISV containing HLA-B*0702 restricted LP9 peptide

Following stimulation, splenocytes from vaccinated HLA-B*0702 mice were stained with fluorescently labeled antibodies to determine $CD4^+$ T cells that were positive for IFN-γ and TNF-α. None of the vaccination protocols resulted in a statistically significant increase in $CD4^+$ IFN-γ^+ T cells over the PBS stimulated controls, irrespective of antigen (oocystAgLP9 peptide or TLA) used *ex vivo* for stimulation (Figure 2.4.8 A). Differences in the percentage of $CD4^+$ TNF-α^+ cells (Figure 2.4.8 B) following stimulation with PBS, oocystAg, LP9 peptide, or TLA are not statistically significant between any of the vaccine treated groups

A.



B.

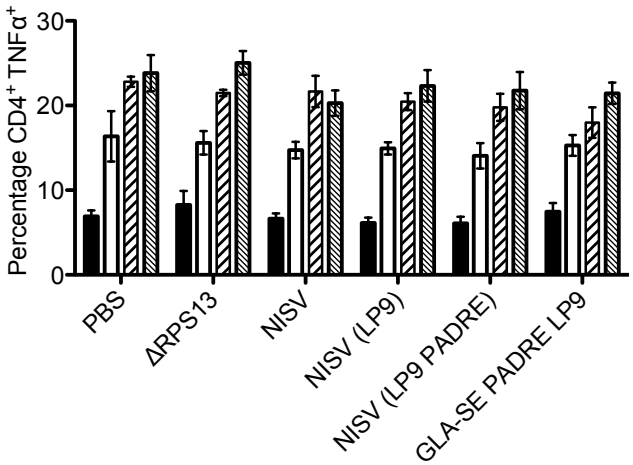
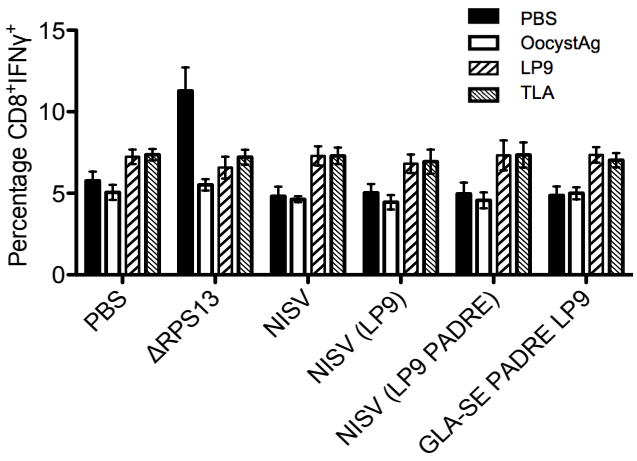


Figure 2.4.8. CD4$^+$ IFN-γ^+ (A) and CD4$^+$ TNF-α^+ (B) T cells induced by vaccination. Splenocytes were harvested from HLA-B*0702 mice vaccinated with PBS, ΔRPS13, empty NISV, NISV containing LP9 peptide and PADRE, or LP9 peptide with GLA-SE and PADRE, and restimulated with PBS, oocystAg, LP9 peptide, or TLA. Percentage of (A) CD4$^+$IFN-γ^+ and (B) CD4$^+$TNFα^+ was determined by FACS analysis**.** For each group n=6. The experiment was carried out once.

2.4.10. $CD8^+$ IFN-γ^+ and $CD8^+$ TNF-α^+ T cells induced by vaccination with NISV containing HLA-B*0702 restricted LP9 peptide.

Splenocytes from vaccinated HLA-B*0702 mice were stained with anti-CD8 to determine the populations of $CD8^+$ cells also positive for IFN-γ or TNF-α. (Figure 2.4.9 A and B). $CD8^+$ $IFN\gamma^+$ T cell populations in splenocytes stimulated with oocystAg, LP9 peptide or TLA were not significantly different between any of the experimental groups. PBS and oocyst antigen stimulation gave comparable percentages of $CD8^+$ $IFN\gamma^+$ cells within all of the groups except in those given ΔRPS13 where PBS stimulation appears to have given rise to more $CD8^+$ IFN-γ^+ cells. However, this is not statistically significant. Stimulation with LP9 peptide and TLA induced comparable numbers of $CD8^+$ IFN-γ^+ cells, which was greater than PBS and oocyst lysate antigen stimulation. (Figure 2.4.9 B) Stimulation of the splenocytes with the antigens and PBS did not yield any significant differences in the $CD8^+$ $TNF\alpha^+$ populations between any of the experimental groups

A.



B.

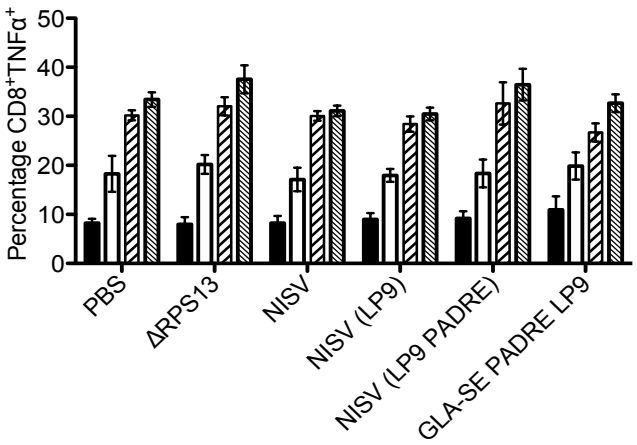


Figure 2.4.9. (A) CD8$^+$ IFN-γ^+ and (B) CD8$^+$ TNF-α^+ T cells induced by vaccination. Splenocytes were harvested from HLA-B*0702 mice vaccinated with PBS, ΔRPS13, empty NISV, NISV containing LP9 peptide and PADRE, or LP9 peptide with GLA-SE and PADRE, and stimulated with PBS, oocystAg, LP9 peptide, or TLA. Percentage of (A) CD8$^+$IFN-γ^+ and (B) CD8$^+$TNF-α^+ was determined by FACS analysis. For each group n=6. The experiment was carried out once.

2.4.11. Vaccination with ΔRPS13 induces a greater population of $CD8^+$ Granzyme B^+ T cells

Splenocytes were stained to determine the Granzyme B (GzB) content of the $CD8^+$ T cells from the vaccinated groups. Only the ΔRPS13-vaccinated group demonstrated an increased percentage of $CD8^+GzB^+$ cells compared to all other experimental groups. This increase was statistically significant over the PBS vehicle control groups when stimulated with PBS, LP9 peptide and TLA, with p values of 0.0043, 0.0087 and 0.0022 respectively (Figure 2.4.10).

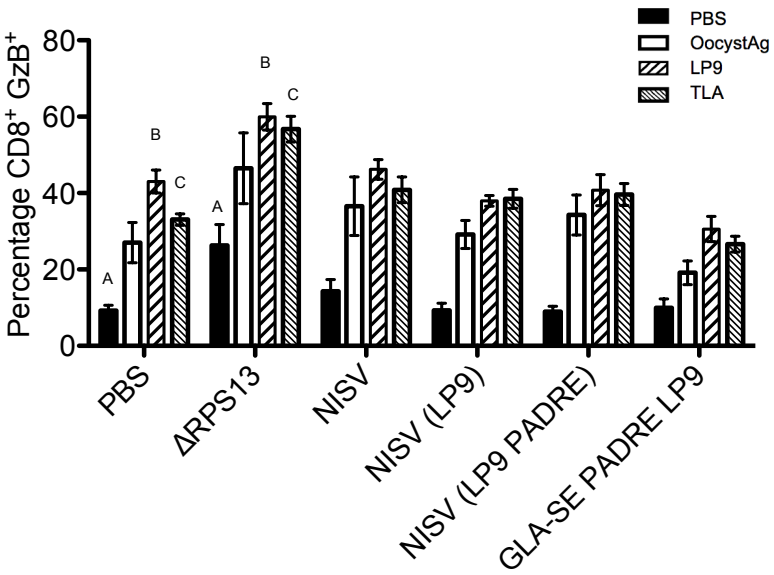
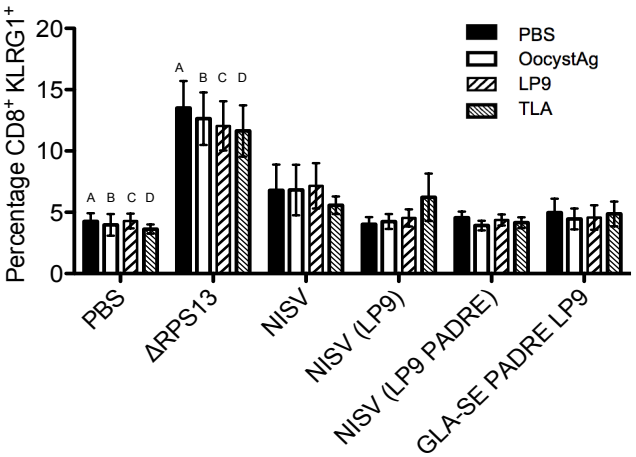


Figure 2.4.10. CD8$^+$ Granzyme B$^+$ T cells induced by vaccination. Splenocytes were harvested from HLA-B*0702 mice vaccinated with PBS, ΔRPS13, empty NISV, NISV containing LP9 peptide and PADRE, or LP9 peptide with GLA-SE and PADRE, and stimulated with PBS, oocystAg, LP9 peptide, or TLA. Percentage of CD8$^+$ Granzyme B$^+$ cells was determined by FACS analysis. Statistical comparisons (lablled A, B and C) are significant to ** (P=<0.005). For each group n=6. The experiment was carried out once.

2.4.12. Vaccination with ΔRPS13 induces a population of selective effector $CD8^+$ T cells

Splenocytes were stained to quantify the populations of CD8 T cells expressing either KLRG1 or CD127. The percentage of $CD8^+$ $KLRG1^+$ T cells was only significantly increased in splenocytes from the ΔRPS13 vaccinated group. (Figure 2.4.11 A) This was statistically significant, over the PBS vehicle control splenocytes with p values of 0.0022, 0.0043, 0.0022 and 0.0022 in the PBS, oocyst lysate antigen, LP9 peptide and TLA stimulated groups respectfully. $CD8^+CD127^+$ splenocyte populations in the all of the experimental groups were comparable populations with the exception of ΔRPS13, which had a statistically significant decease in the $CD8^+$ $CD127^+$ population across all of the stimulated groups with p values of 0.0304, 0.0450 and 0.0022 respectively. Splenocytes from the GLA-SE/PADRE/LP9 peptide vaccinated groups had a significant increase in the PBS and TLA stimulated groups with p values of 0.0129 and 0.049. (Figure 2.4.11 B).

A.



B.

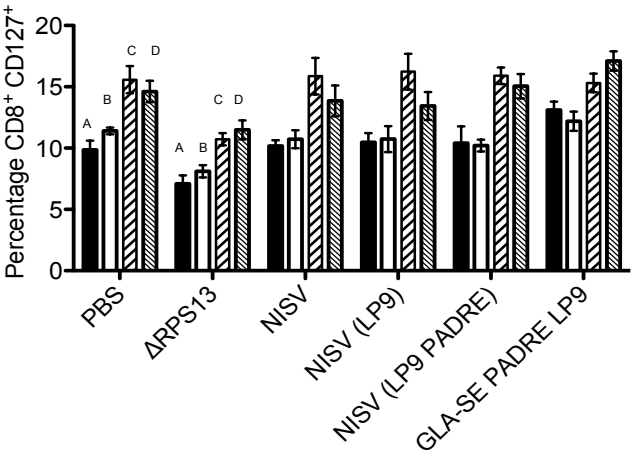


Figure 2.4.11. (A) CD8$^+$ KLRG1$^+$ and (B) CD8$^+$ CD127$^+$ T cells induced by vaccination.

Splenocytes were harvested from HLA-B*0702 mice vaccinated with PBS, ΔRPS13, empty NISV, NISV containing LP9 peptide and PADRE, or LP9 peptide with GLA-SE and PADRE, and re-stimulated with PBS, oocystAg, LP9 peptide, or TLA. FACS was used to determine the percentage of CD8$^+$ cells also positive for either CD127 or KLRG1. Statistical comparisons (lablled A, B, C and D) are significant to ** (P=<0.005). For each group N=6. The experiment was only carried out once.

2.5 Discussion

The present study examined the efficacy of class 1 HLA-restricted peptide vaccines in HLA class 1 transgenic mice during peroral oocyst challenge. Previous work had described these peptide vaccines as being protective against IP challenge with tachyzoites, as demonstrated by reduced parasite burdens as measured by *in vivo* bioluminescent imaging (Cong *et al.,* 2010; Cong *et al*., 2011; Cong *et al*., 2012). We also studied for the first time the use of the live attenuated ΔRPS13 RH *T. gondii* strain as a vaccine against oocyst challenge. Previously this strain has been shown to be protective against tachyzoite challenge (Hutson *et al*., 2010) in a similar manner to the peptide vaccines. This study also set out to examine the type of immune response generated by vaccination and looked for markers that indicated whether the vaccines could induce a long-term memory response.

Upon oocyst challenge, none of the HLA-A*0201, HLA-A*1101 or HLA-B*0702 transgenic mice vaccinated with their respective peptide pools formulated with GLA-SE and PADRE peptide were protected against oocyst challenge. Similarly no protection was induced in HLA-B*0702 mice vaccinated with LP9 peptide entrapped within non-ionic surfactant vesicles. All mice in both control and vaccinated peptide vaccinated groups succumbed to infection on days 8 to 10 post infection. The only mice afforded any protection were those vaccinated with the live attenuated ΔRPS13 RH *T. gondii* strain. However, this vaccine was only protective against oocyst challenge for the HLA-A*1101 and HLA-B*0702 mice. ΔRPS13 vaccinated HLA-A*0201 mice still succumbed to challenge infection by day 8 post-infection.

Oral infection with oocysts is not only the natural route of infection for *T. gondii*, but it has also been indicated to be the most common source of infection of mothers of congenitally infected children in North America (Boyer *et al*., 2011). There is a great deal of literature on vaccine strategies against *T. gondii* in murine models that are known to provide a degree of protection against tachyzoite or tissue cyst challenge. However, none of these studies utilised

oocyst or sporozoite challenge to determine vaccine efficacy. Those studies, which have used oocyst challenge, have primarily involved feline or ovine infection models (Angus *et al*., 2000; Falón *et al*., 2009; Zulpo *et al*., 2012). The vaccine candidates tested in these models have been either live-attenuated organisms (Buxton *et al*., 1991; Buxton *et al*., 1993) or crude protein vaccines, such as crude rhoptry extracts (Zulpo *et al*., 2012), or vaccination with a virus vector expressing ROP2 (Mishima *et al*., 2002) or DNA vaccine encoding SAG1 (Angus *et al*., 2000). So far there have been no studies that have carried out oocyst challenge following peptide vaccination.

Although IFN-γ independent mechanisms of parasite control have been described (Khan *et al.,* 1996) it is the general consensus of opinion that IFN-γ is the key cytokine mediating the protective immune response against *T. gondii* (Gazzinelli *et al*., 1993; Jun *et al*., 1993). Consequently establishing whether or not a vaccine candidate is stimulating IFN-γ production is an important part of indicating a vaccine's potential efficacy (Angus *et* al., 200; Cong *et* al., 2010; Cong *et al*., 2011; Cong *et al*., 2012; Min *et al* 2012). Systemic IFN-γ concentrations in the serum were therefore determined to establish if the HLA restricted peptides or live attenuated vaccine were inducing this indicator of protective immunity. With the exception of the live attenuated ΔRPS13 in the HLA-A*0201 mice all of the vaccination protocols failed to elevate serum IFN-γ in comparison to the PBS controls. However, the ΔRPS13 vaccinated HLA-A*0201 mice were not protected upon oocyst challenge. This might indicate that IFN-γ is not critical for protection against oocyst challenge in mice or even that IFN-γ might be responsible for pathology following oocyst challenge. Notably in some murine models using oral challenge with tissue cysts, IFN-γ has been demonstrated to be detrimental and associated with increased intestinal pathology (Liesenfeld *et al*., 1996).

Splenocytes from vaccinated HLA transgenic mice were analysed ex vivo for antigen specific IFN-γ production induced by vaccination. Overall, HLA class 1

restricted peptide vaccination only produced a significant increase in $CD4^+IFN\text{-}\gamma^+$ cells over the PBS vaccinated control mice in the HLA-A*1101 mice. The HLA-A*0201 and HLA-B*1101 mice demonstrated no increase in the number of $CD4^+IFN\text{-}\gamma^+$ cells over their respective PBS vaccinated control groups. The relatively poor CD4+ T cell response is not entirely unexpected given the HLA class 1 restricted nature of the peptide vaccines.

An increase in $CD8^+IFN\text{-}\gamma^+$ cells as a result of vaccination with HLA class 1 restricted peptides, was only apparent in HLA-A*1101 and HLA-A*0201 mice. However, this response was only apparent upon stimulation with their specific peptide pool and not tachyzoite or oocyst derived antigen. This is most likely as a result of the lysate antigens requiring processing and presentation by APCs present in the splenlocyte culture. As a result there will be a reduced quantity of the specific peptides for restimulating the cells in comparison to those cells directly stimulated with the peptide pools. The previous studies undertaken by Cong *et al* (2010, 2011 and 2012) to elucidate the protective abilities of these peptides only used re-stimulation with the respective peptide pools and not lysate antigen. In addition, these previous studies also did not determine the specific $CD4^+$ or $CD8^+$ T cell responses to vaccination, and only utilised ELISPOT assays for IFN-γ derived as a result of peptide stimulation.

The lack of $CD4^+$ T cell response highlighted in the present study could pose a problem for vaccination using the HLA class I restricted peptides. Work by Casciotti *et al* (2002) determined that although $CD8^+$ T-cell responses can be induced without $CD4^+$ T cell help during *T. gondii* infection, it is not maintained during chronic infection. However it was also shown by Jordan *et al*., (2009) that an optimal $CD8^+$ T cell response to vaccination required a $CD4^+$ T cell response on challenge. The lack of a *T. gondii* specific response following the HLA Class I restricted peptide vaccine could ultimately hinder the response upon challenge, particularly long term. Although work by Cong *et al* (2010, 2011 and 2012) did establish protection with the vaccination regimes used in the

present work, protection was only assessed one week following the final vaccination boost. To facilitate CD4 T cell activation and assist with CD8 T cell development mice were also given PADRE a universal CD4 epitope in this study (Alexander *et al*., 2000). Future studies should address whether the inclusion of a T. gondii CD4 T cell epitope offers any advantage over PADRE. Potentially such an epitope could not only assist in priming the immune response, but might also provide better protection upon challenge as CD4 T cells would be activated by infection.

Vaccination with ΔRPS13 was demonstrated to induce an increased $CD4^{+}IFN\text{-}\gamma^{+}$ cell response, compared with the PBS vaccinated groups. However, while no increase in $CD8^{+}IFN\text{-}\gamma^{+}$ cells was induced in comparison to the PBS vaccination controls the ΔRPS13-vaccinated mice were protected upon challenge. This is an unusual observation as vaccination with other live attenuated *T. gondii* strains, such as CPS-1-1, are associated with $CD4^{+}$ and $CD8^{+}$ T cell IFN-γ induction (Gigley *et al*., 2009). However, following vaccination with CPS1-1, upon challenge with RH *T. gondii* it was noted that the protective response, although $CD4^{+}$ and $CD8^{+}$ T cell mediated, was associated with reduced IFN-γ but enhanced IL-12p70 activity compared with non-vaccinated controls. $CD8^{+}$ T cell dependent, $CD8^{+}IFN\text{-}\gamma^{+}$ independent, induced protection has been described previously following vaccination in a *Plasmodium yeolii* model (Trimnell *et al.,* 2009). Thus while IFN-γ was required for the initial successful priming by vaccination, protection remained effective when this cytokine was neutralized upon challenge infection and was demonstrated to be dependent upon $CD8^{+}$ T cells that were cytolytic (Trimnell *et al.,* 2009). Similarly ΔRPS13 may be inducing IFN-γ independent $CD8^{+}$ T cell immunity towards *T. gondii* (Jordan *et al*., 2009; Jongert *et al*., 2010; Suzuki *et al* 2010; Gigley *et al*., 2011).

The expression of CD127 and killer-cell lectin like receptor G1 (KLRG1) on the $CD8^{+}$ T cells post-vaccination was also examined. These markers are associated with defining selective effector (SLEC; $CD127^{lo}$ $KLRG1^{hi}$) and memory precursor

(MPEC; $CD127^{hi}$ $KLRG1^{lo}$) populations (Araki *et al.,* 2010). KLRG1 is expressed on primed T cells (Henson & Akbar 2009) while CD127 is highly expressed on naive T cells. CD127 is lost once the T cell is primed and differentiated into an effector cell (Paiardini et al. 2005). Cells that are then able to re-express CD127 are those, which can go on to differentiate into memory cells.

In this study, mice vaccinated with the ΔRPS13 live attenuated strain as opposed to those vaccinated with peptides displayed a significant increase in $CD8^+$ T cell KLRG1 expression compared with mice receiving PBS as a control. This indicates that this regimen has induced a higher population of antigen primed $CD8^+$ T cells over control mice and other vaccine candidates. In mice vaccinated with the ΔRPS13 live attenuated strain, but not other vaccinated groups CD127 expression was reduced significantly below that of the controls. This indicated an increased population of selective effector cells, as this reduction in CD127 was associated with an increased in KLRG1 expression over the other vaccine groups.

In addition the $CD8^+$ T cells were examined for their granzyme B content, as a marker of their potential cytotoxic ability (Macleod *et al*., 2011, Smyth *et al*., 2012, Jordan *et al*., 2009) as a result of vaccination. Only those mice vaccinated with ΔRPS13 null mutants demonstrated an up regulation in $CD8^+$ T cell granzyme B content. Significantly this was the vaccinated group giving the protection from vaccination. Increased granzyme B content, paired with a decreased in IFN-γ has been previously observed and is associated with a protective response in a number of vaccination models.(Smyth *et al*., 2012; Jorndan *et al*., 2009). Smyth *et al*., (2012) demonstrated protection following repeated peptide vaccination using an OT-1 murine model with a viral vector expressing OVA peptide. This study demonstrated a protective response being associated with inducing memory CTL's with enhanced granzyme B and reduced IFN-γ phenotype. Jordan *et al*., (2009) demonstrated the same profile in mice vaccinated with a live attenuated *T. gondii* that expressed OVA peptide. OVA antigen specific $CD8^+$ T cells where shown to have enhanced granzyme B

but limited IFN-γ following vaccination. Mice displaying this phenotype where shown to be protected following lethal challenge with RH *T. gondii* expressing OVA.

Taken as a whole the class I HLA restricted subunit vaccines, which proved protective against tachyzoite challenge and capable of eliciting an IFN-γ response by Cong *et al*, (2010, 2011 and 2012) failed to protect against oocyst challenge in this study. The differences we encountered in the efficacy of the peptide vaccine, in particular the complete lack of an increase in IFN-γ production upon re-stimulation in the HLA-A*0201 mice may be due to a number of practical problems encountered when carrying out the study. The HLA-A*0201 restricted peptides proved to be very insoluble and began to precipitate out of solution when diluted for use. This may have resulted in a reduced accuracy of dosing. The candidate vaccine peptides were selected through bioinformatics analysis from a range of *Toxoplasma* proteins. However, only a limited number of the peptides used are secreted by the sporozoite stage of the *T. gondii* life cycle. Peptides derived from GRA3, GRA5, SAG1, SAG2C, SAG2X (Table 2) were likely not protective against oocyst challenge because they are not produced by, or have limited production by the sporozoites stage of the life cycle (Tilley *et al*.,. 1997; Saeij *et al*., 2008; Manger *et al*., 1998). The only peptides selected for use in this vaccine study readily expressed by sporozoites, were those derived from GRA6, GRA7, SRS52A, MIC1 and MIC2. Therefore the peptide vaccine may be enhanced by analyzing sporozoite specific proteins, such as TgERP (Hill *et al*., 2011) for peptides specific for the HLA-A*0201, HLA-A*1101 and HLA-B*0702 alleles and including them in future vaccine formulations against this life cycle stage.

Non-ionic surfactant vesicles (NISVs) have proved to be very effective adjuvants in vaccination studies against *T. gondii* infections using entrapped soluble tachyzoite antigen (STAG) (Roberts *et al*., 1994: Walker *et al*., 1995). These studies demonstrated that STAG entrapped NISV could not only induce successful protection against murine congenital toxoplasmosis (Brewer *et al*.,

1994) but also effectively stimulate a human antibody response in SCID mice reconstituted with human PBLs (Walker *et al.,* 1995). In addition as NISV have previously been shown to be effective vaccine adjuvants incorporating entrapped peptides we believed these adjuvants could improve the effectiveness of our anti-toxoplasma peptide vaccines (Brewer & Alexander 1992). However a number of issues were encountered during formulation of the HLA peptides in NISV that limited their use. The insoluble nature of the HLA-A*0201 and HLA-A*1101 peptides severely limited the ability to successfully entrap them within the vesicles, and during the ultracentrifugation step of the vesicle preparation, the vesicles failed to pellet but separated into two phases. As a result only one of the HLA-B*0702 peptides, (GRA6 peptide LP9; LPQFATAAT) could be entrapped. No enhanced protection was observed following vaccination of this peptide within NISVs.

Overall these studies demonstrated that the HLA class 1 restricted peptide antigens failed to protect HLA transgenic mice against oocyst challenge. However we have demonstrated for the first time, that vaccination with the live attenuated ΔRPS13 strain of *T. gondii* provides protection against oocyst challenge. With protection being associated with decreased IFN-γ production and granzyme B positive $CD8^+$ T cell phenotype.

2.6 Critical discussion

The work carried out in this chapter was aimed at testing the efficacy of HLA restricted peptide vaccines and a live attenuated vaccine, at protecting against *Toxoplasma* oocyst challenge. There were a number of technical and practical issues in conducting the work, which may have had a critical impact on some of the analysis conducted.

Antigen processing differences between the murine and human immune systems were not fully taken into consideration at the start of the project, when the peptides where being selected.

As previously mentioned the peptides where identified by running *T. gondii* proteins through databases which identified peptide epitopes with a strong binding efficiency for the HLA-A*0201, HLA-A*1101 or HLA-B*0702 supertype alleles (Work not carried out as part of this thesis). The identified peptides where then screened against HLA matched T cells from chronically infected humans, and peptides which induced a significant IFN-γ response where taken forward for use as potential vaccines in HLA transgenic mice. Successful positive outcomes were intended to demonstrate the potential efficacy of the vaccines in humans. This system of peptide identification relies on the antigen processing pathways of both the human and HLA-transgenic murine model being the same so as the same peptides are derived from the parent protein in each model. However work by Sesma *et al.,* (2003) determined that murine cells expressing the HLA-B27 supertype, used in a spondyloarthritis model, presented a different array of peptides from human cells expressing HLA-B27. Subsequently they determined that this difference was due to proteosomal and tapsin mediated, species-specific, differences in the way the antigens were processed. Thus while we have identified peptides that are antigenic and are capable of binding MHC class I in the transgenic mouse as a result of potential processing differences they may not be presented in our current *in vivo* murine system in significant abundance so as to elicit a protective immune response. This could explain why peptides shown to elict an IFN-γ response from human PBL's failed to protect the appropriate HLA transgenic mice.

Similar bioinformatics tools now exists which allow the identification of antigens from full protein sequences and take into account the cleavage sites of the immune proteasome and the specifities of the tapsin binding sites (http://tools.immuneepitope.org/analyze/html/mhc_processing.html). This modeling, however, still does not take into account any species differences

between the murine models being used for challenge infections following vaccination and the antigen processing in humans, the ultimate targets for any potential vaccine.

Another potential flaw in this murine model was highlighted by Dubey *et al.,* (2012) when he demonstrated that these transgenic strains possessed an enhanced susceptibility to *T. gondii* infection over their parental strains. This could significantly impinge in determining whether any protection is gained from vaccination in the HLA-A*0201 and HLA-A*1101 transgenic mice, particularly during oral challenge was significant as these mice are on a C57BL/6 background, a strain already extremely susceptible to oral infection. This enhanced susceptibility to oral infection may have masked any protective effect derived from the peptide vaccines.

Other factors that may have had an impact on this work and the results generated were related to situations around the harvesting of tissues for the splenocyte restimulation. As previously stated all vaccinations were carried out at the US Department of Agriculture, MD. For immunological analyses following vaccination spleens were harvested at the USDA and placed into chilled PBS and stored on ice before transported to George Washington University, DC by Metro with a journey time of approximately one hour. Due to the large number of samples in each experiment and the need to change location the samples were not processed, counted and placed into *in vitro* culture until approximately 10 hours after they were initially harvested from the vaccinated animals. This time period would have undoubtly had an effect on the viability of the splenocytes and their ability to respond to their appropriate stimulations. Viability staining was carried out when the cell samples where being prepared for analysis by flow cytometery in order to gate out dead cells, which in some samples represented a very high proportion of the acquired material (data not shown). This situation was unavoidable in this project due to the nature of the funding allocations and the multi-center locations of the collaborators on the project. However, utlising facilities already present in the USDA instead of those that

required transport to another institute would have likely increased the viability of the work by ensuring that the splenocytes were in culture in best possible time.

The data presented in this chapter is representative of one experimental run with group sizes of 10 for the infective challenge studies and 6 for the pre-challenge immunological analysis following vaccination. This, however, was not how the experiment was set up at the start of the study. It was originally intended that the work would consist of two experiments run back to back, with a group size of 5 for the challenge infections and 3 for the pre-challenge immunology post-vaccination, per run. However, due to time constraints and the large numbers involved it was difficult to keep both runs truly independent of each other, particularly on the cull days for immunological analysis. Also with only three in a group for the immunological studies it was difficult to obtain meaningful statistics. As a result the decision was made to combine both runs for analysis and for the study to be repeated at some future date.

Work in this chapter demonstrated the live attenuated ΔRPS13 strain as a potential vaccine candidate against *Toxoplasma gondii*. However, the goal of the study was primarily to develop and HLA restricted vaccine for ultimate use in humans. No experiments were carried out in this thesis to determine if the protection was indeed mediated by the HLA transgenes or whether it was mediated by the H2 repertoire of the transgenic mouse. It is unlikely that the HLA transgenes were solely responsible for the protection gained from vaccination with ΔRPS13 as this live attenuated strain has already been shown to protect outbred mice against tachyzoite challenge (Hutson *et al*., 2010). While studies utilizing ΔRPS13 are interesting and useful in developing an understanding of a protective immune response towards *T. gondii* any role which ΔRPS13 may play in moving forwards to the ultimate goal of a vaccine for use in humans is likely to be very limited due to safety concerns of the attenuated strain reverting to its natural wild type.

For the work contained in this chapter to be taken forward these studies need to be repeated and it would be prudent to rescreen the peptides selected and take into account the proteasome and tapsin specificities. Ideally looking for peptides, which will be present in both human and murine systems, so that the HLA transgenic mouse model can still be utilised for immunological and challenges.

Chapter 3

The role of the dual specific phosphatase MAP Kinase Phosphatase-2 during infection with *Toxoplasma gondii*

3.1 Abstract

The dual specific phosphatase, MAP kinase phosphatase-2 (MKP-2) has recently been demonstrated to negatively regulate macrophage arginase-1 expression, while at the same time to positively regulate iNOS expression. Consequently, MKP-2 is likely to play a significant role in the host interplay with intracellular pathogens. Here we demonstrate that MKP-$2^{-/-}$ mice on the C57BL/6 background have enhanced susceptibility compared with wild-type counterparts following infection with type-2 strains of *Toxoplasma gondii* as measured by increased parasite multiplication during acute infection, increased mortality from day 12 post-infection onwards and increased parasite burdens in the brain, day 30 post-infection. MKP-$2^{-/-}$ mice did not, however, demonstrate defective type-1 responses compared with MKP-$2^{+/+}$ mice following infection although they did display significantly reduced serum nitrite levels and enhanced tissue arginase-1 expression. Early resistance to *T. gondii* in MKP-$2^{+/+}$, but not MKP-$2^{-/-}$, mice was NO dependent as infected MKP-$2^{+/+}$, but not MKP-$2^{-/-}$ mice succumbed within 10 days post–infection with increased parasite burdens following treatment with the iNOS inhibitor L-NAME. Conversely, treatment of infected MKP-$2^{-/-}$ but not MKP-$2^{+/+}$ mice with nor-NOHA increased parasite burdens indicating a protective role for arginase-1 in MKP-$2^{-/-}$ mice. *In vitro* studies using tachyzoite-infected bone marrow derived macrophages and selective inhibition of arginase-1 and iNOS activities confirmed that both iNOS and arginase-1 could mediate anti-parasite growth. However, the effects of arginase-1 were transient and ultimately the role of iNOS was paramount in facilitating long-term inhibition of parasite multiplication within macrophages.

3.2 Introduction

Cellular processes are controlled by both intra and extracellular signals. These signals often lead to post translational modifications of proteins within the cell, causing an alteration of the effector functions of the proteins. In the case of mammalian cells phosphorylation is the most common modification with approximately one third of proteins undergoing the process. This phosphorylation is often reversible and alters the characteristics of the proteins by altering bonds within the protein, such as addition of ionic and hydrogen bonds. The protein may also be subject to conformational modifications such as concealing binding sites, so altering any enzymatic properties of the protein and possibly the intra-cellular location (reviewed by Kryiakis & Avruch 2012).

3.2.1 Mitogen-activated protein kinase

Mitogen-activated protein (MAP) kinases are the proteins responsible for these modifications and the genes that encode them account for 2% of mammalian genes. MAP kinase enzymes exert their effect by catalysing the transfer of γ-phosphoryl groups to a hydroxyl on amino acid residues on their substrates. Which residues the kinases modify determines what family the MAP kinase belongs to. The largest family of MAP kinases phosphorylate tyrosine and threonine residues (Camps *et al.,* 2000).

Protein kinases have a highly conserved structure. They possess two domains, one either side of the active site. The N-terminal is comprised of 5 stranded anti-parallel β-pleated sheets and an α-helix. This is responsible for the orientation of nucleotide sequence being phosphorylated. The C-terminal consists of α-helices responsible for binding the substrates (Krupa *et al*., 2004).

The activation of MAP kinase molecules results in the initiation of a cascade of signaling events, which ultimately play key roles in the activities of the cell such as proliferation and differentiation. They are critical in the activation of the immune response, being involved during the activation of the T cell. After

presentation of antigen by APCs, leukocyte-specific protein tyrosine kinase (lck, phosphorylates tyrosine residues on the intracellular regions of the CD3 and ζ-chains which form part of the T-cell receptor complex. This results in the formation of docking sites for SH2 domains on ZAP-70, another tyrosine kinase. This molecule then phosphorylates LAT, a protein which is then bound by further signaling proteins. The outcome of this cascade of events results in activation of transcription factors, such as NFAT and transcription of gene products involved in activation of the T-cell.

Kinases play a key role in the activation of the innate immune response via MyD88 signaling, which has a central role in signaling through TLRs (Egan *et al.,* 2009). Activation of the TLRs results in recruitment of MyD88. Down stream of this IRAK-1 and 4, TRAF-6 and TAK-1 kinases, which trigger MAP kinases, such as JNK and p38, and NFκB signaling pathways (Egan et al. 2009). There have been five distinct classes of MAP kinases identified in mammalian cells, these include ERK (extracellular signal-related kinase) 1/2, JNK(c-Jun N-terminal kinase) 1/2/3, p38 α/β2/γ/δ, ERK 3/4 and ERK5. While the ERKs are mainly associated with proliferation and differentiation, JNK and p38 are primarily involved in stress and immune responses (Camps *et al*., 2000).

3.2.2 Mitogen-activated protein kinase phosphatases

The activity of MAP kinases is regulated by a dual specific phosphatase (DUSP), called MAP kinase phosphatase (MKP), which acts through tyrosine and threonine dephosphorylation (Keyse *et al*., 2008). Currently it is known that eleven isoforms of the protein exist, each varying in structure, subcellular location, regulatory mechanisms and substrate specificity. MKPs have been implicated in the regulation of disease, and an alteration in their function or expression has been identified in a number of cancers (Keyse *et al*., 2008).

A number of recent studies have established a role for MKPs in the regulation of the immune response, with MKP-1 (DUSP-1) playing a key role in regulating expression

of genes induced by stimulation with LPS, which can protect against endotoxic shock (Hammer *et al*., 2006; Li *et al*., 2009). This is thought to occur through the dephosphorylation of p38 and JNK (Li *et al*., 2009). A role for MKP-5 (DUSP-10) has been implicated in the regulation of both the innate and adaptive immune responses. Increased levels of pro-inflammatory cytokines have been observed in MKP-$5^{-/-}$ intraperitoneal macrophages stimulated with LPS. An infection model using lymphocytic choriomeningitis virus found the adaptive $CD4^{+}$ and $CD8^{+}$ T cells responses produced more inflammatory cytokines over the wild types resulting in immunopathology and death. (Zhang *et al.,* 2004). Additionally, the immuno-modularity properties of dexamethasone have been linked to its ability to induce MKP-1, leading to the dephosphorylation of p38 (Fürst *et al*., 2007).

3.2.3 MAP Kinase Phosphatase -2

MAP Kinase phosphatase-2 (MKP-2) is a nuclear located class one DUSP (Sloss *et al*., 2005). *In vitro,* MKP-2 has been found to dephosphorylate ERK and JNK (Chu *et al*., 1996). However, it has no effect on p38 function despite its high binding affinity for the molecule. MKP-2 has been shown to be induced by stress factors, growth factors and hormones.

MKP-$2^{-/-}$ deficiency on a C57/BL6 background, was utilised by Al-Mutairi *et al*., (2010) to dissect the role of MKP-2 during the immune response towards *Leishmania mexicana*. In this study MKP-2 was found to play a key role in protective immunity. MKP-2 deficient mice, infected with *L. mexicana,* displayed an increased susceptibility to infection that was manifest as a reduced ability to control lesion size and parasite burden than their wild-type counterparts. MKP-2 deficient mice also displayed a modified T helper 1 / T helper 2 response, with a diminished Th1 response and an enhanced Th2 response. The same study examined the effect of the MKP-2 deletion on bone-marrow derived macrophages, identifying reduced NO production, enhanced arginase activity and an increased susceptibility of the macrophage to infection with *L. mexicana*.

The macrophages susceptibility to infection was reversed with addition of the arginase inhibitor nor-NOHA to the culture medium.

3.2.4 MKP-2 and *Toxoplasma gondii*

The early acute stage of *T. gondii* infection is characterized by widespread tachyzoite dissemination and tissue damage. The rapid onset of immunity, initiated in large part by the parasite driving innate type-1 immune responses via a number of well characterized pathogen associated molecular patterns (PAMPS) controls parasite replication (Bhatnagar *et al.,* 2007; Denkers 2010; Erridge *et al*., 2010; Pifer & Yarrovinsky 2011), results in the life-long chronic stage of infection associated with encystment of the parasites in skeletal muscle and the central nervous system (reviewed in Lieberman & Hunter 2002). Protection against both acute and chronic disease is mediated primarily by type-1 responses, with NK cells as part of the innate response but primarily $CD8^+$ T cells playing the most significant roles (Parker *et al*., 1991; Suzuki *et al*., 1985; Subauste *et al.,* 1991) via IFN-γ production.

The mechanisms by which IFN-γ, the major effector cytokine mediating resistance during *T. gondii* infection, promotes anti-*Toxoplasma* activity are not yet fully clear. Several IFN-γ-regulated genes iNOS, IDO, and more recently, p47 GTPases, have been implicated in playing significant roles in mediating these protective responses. IFN-γ acts with TNF-α to activate macrophages and induces inducible nitric oxide synthase (iNOS) expression that catalyzes the formation of nitric oxide (NO) from L-Arginine. NO is capable of directly killing the parasites (Gazzinelli *et al.*, 1993; Jun *et al*., 1993). Although NO production appears to be the predominant pathway used by classically activated macrophages to control *T. gondii* proliferation in tissue culture, the role of NO during *in vivo* infection is less clear. iNOS-deficient murine models have shown that mice lacking iNOS are capable of surviving and controlling tachyzoite growth during the acute stage of infection and only succumb during infection during the chronic stage of the life cycle (Scharton-Kersten *et al*., 1997). Death

was associated with uncontrolled proliferation of tachyzoites in the brain, suggesting that the protective anti-toxoplasmic effect in the brain may be iNOS-dependent (Scharton-Kersten et al., 1997; Khan *et al*., 1997). Nevertheless, the observation that iNOS deficient mice are able to survive acute infection in an IFN-γ mediated manner suggest that there are alternative pathways other than NO production mediating anti-*Toxoplasma* resistance *in vivo* (Khan et al. 1998). Induction of the IFN-γ inducible gene Indoleamine 2,3 dioxygenase (IDO) has also been implicated in mediating some of the IFN-γ dependent anti-*Toxoplasma* activity (Pfefferkorn & Guyre 1984). IDO catalyzes the degradation of the essential amino acid L-tryptophan through the kynurenine pathway, thereby compromising metabolic processes of the parasite (Fujigaki *et al*., 2002). Interestingly the relative contributions of iNOS and IDO to parasite control appear to be tissue specific (Fujigaki *et al*., 2002). More recently immunity-related GTPases (IRGs) have emerged as potent effectors of *T gondii* killing in mice (Taylor *et al*., 2000; Collazo *et al*., 2001). Thus, murine astrocytes have been shown to have the ability to kill intracellular *T. gondii* independently of iNOS and IDO via IFN-γ inducible IRGs (Halonen *et al*., 1998; Halonen *et al*., 2001; Melzer *et al*., 2008). Different IRGs have been shown to play roles in controlling acute and chronic infection although the mechanisms through which p47 GTPases confer resistance to *T. gondii* infection have not been determined (Howard *et al*., 2011).

While a type-1 response is required for parasite immunity overproduction of IFN-γ, TNF-α and NO can result in immune pathology and death. Consequently, protective immunity to *T. gondii* must rely on a delicate balance of type-1 responses by type-2 and Treg cells (Denkers and Gazzinelli, 1998; Gazzinelli *et al*., 1996, Roberts *et al.*, 1996) in order to effectively control parasite proliferation without excessive inflammation. The classic indicator of alternative macrophage activation, arginase-1, can be induced innately by *T. gondii* apparently via both STAT-6 dependent (Butcher *et al*., 2011; Marshall *et al*., 2011) and independent mechanisms (El-Kasmi *et al*., 2008; Marshall *et al*.,

2011). In contrast, arginase-1 has been associated with both enhancing infection with *T. gondii* by competing with iNOS for their common substrate L-arginine (El-Kasmi *et al*., 2008) and also with limiting parasite growth by starving the parasite of the L-arginine and polyamines it requires for cell division (Butcher *et al.,* 2011; Pfaff *et al*., 2005).

The recent study into the role of MKP-2 during infection with *L. mexicana* infection demonstrates it is a negative regulator of macrophage arginase-1 and also a positive regulator of iNOS expression (Al-Mutairi *et al*., 2010). Furthermore MKP-$2^{-/-}$ C57BL/6 mice have been found to have an enhanced susceptibility to the intracellular parasite *L. mexicana*. Susceptibility in MKP-$2^{-/-}$ mice was in large part due to enhanced parasite growth that could be reversed by inhibiting arginase-1 activity. Given the importance of NO production as well as the apparent contradictory roles of arginase-1 in controlling murine *T. gondii* infections it is likely that the role of MKP-2 in *T. gondii* infection would be one of major importance. We consequently studied the course of *T. gondii* infection in MKP-$2^{-/-}$ and MKP-$2^{+/+}$ C57BL/6 mice. We identified that MKP-2 deficiency results in increased susceptibility to *T. gondii* infection and that this correlated strongly with impaired iNOS activity. In addition we identified an arginase-1 dependent control of parasite growth that functioned to protect the host both in conjunction with and independently of iNOS mediated effects.

3.3 Methods

3.3.1. Ethical approval

All animal procedures conformed to guidelines from The Home Office of the UK Government. All work was covered by two Home Office licenses: PPL60/3929, "mechanism of control of parasite infection" and PPL60/3439,"genetic models of cancer and inflammation" with approval by the University of Strathclyde ethical review panel and following consultation with the University statistician Group sizes were determined on the basis of our previous experience and all experiments were conducted with sufficient sample sizes to have at least an 80% power to detect differences of key parameters at the 5% level of significance. Experimental groups consisted of 5 mice per group allowing for group sizes large enough to perform meaningful statistical analysis by the, Students t test, Kruskal Wallis multiple comparison analysis and the Mann Whitney U tests where appropriate using Prims 5 statisical analysis software (Graphpad Prism, USA).

3.3.2 Experimental design

The work contained in this chapter followed the experimental plan layed out in Table 10.

Table 10. MKP-2 experimental designs			
Experiment	Experiment groups	Mouse numbers	Analysis
Survival following infection with Beverley *T. gondii*	Uninfected MKP-$2^{+/+}$ & MKP-$2^{-/-}$ Infected MKP-$2^{+/+}$ & MKP-$2^{-/-}$	10 mice per group	Mice infected with 10 Beverely strain tissue cysts via intraperitoneal infection. Mice monitored daily, including weight checks. 5 mice from each group euthanised on day 10 or 20 for splenocyte stimulation.
Parasite burden following infection with Pru-FLUC *T. gondii*	Infected MKP-$2^{+/+}$ & MKP-$2^{-/-}$	5 mice per group	Mice infected with 20,000 Pru-FLUC tachyzoites and imaged every second day until day 14. Mice continued to be monitored to day 30 where brains removed for *ex vivo* imaging and spleens for splenocyte stimulations.
In vivo inhibition of iNOS and nor-NOHA	MKP-$2^{+/+}$ untreated MKP-$2^{+/+}$ L-name MKP-$2^{+/+}$ nor-NOHA MKP-$2^{+/+}$ untreated MKP-$2^{+/+}$ L-name MKP-$2^{+/+}$ nor-NOHA	5 mice per group	Mice pre-treated with L-name (200mg/kg) and nor-NOHA (100μg/mouse). Mice then infected with 20,000 Pru-FLUC tachyzoites and treatment continued for duration of experiment. Mice monitored daily and imaged every second day.
Analysis of immune phenotype by FACS and serum NO & tissue arginase	Uninfected MKP-$2^{+/+}$ & MKP-$2^{-/-}$ Infected MKP-$2^{+/+}$ & MKP-$2^{-/-}$	5 mice per group	Mice infected with 20,000 tachyzoites and monitored until day 10 post infection then euthanised for splenocyte restimulation and FACS analysis. Tissues and serum harvested for assessment of tissue arginase by western blot and serum NO by griesse assay.
In vitro parasite growth	MKP-2+/+ and MKP2-/- BMD MΦ stimulated with: Medium, INF-γ, LPS, INF-γ /LPS, INF-γ /LPS & L-name, INF-γ /LPS & nor-NOHA	2 mice from each strain for BMD MΦ generation. 3 replicates for each group.	MKP-$2^{+/+}$ or MKP-$2^{-/-}$BMD MΦ treated as described and infected with Pru-YFP tachyzoites and imaged every 24 hours. Supernatants sampled for analysis of NO content
In vitro arginase production	Infected MKP-$2^{+/+}$ and MKP-$2^{-/-}$ BMD MΦ	2 mice from each strain for BMD MΦ generation. 3 replicates for each group.	MKP-2+/+ and MKP-2-/- BMD MΦ infected with Pru-FLUC tachyzoites, cells harvested for western blot analysis of Arginase 1 expression at 0, 10, 30, 60 minutes, 2, 4, 8 and 24hrs following infection.

3.3.3 Mice

MKP-$2^{+/+}$ and MKP-$2^{-/-}$ mice were provided by Robin Plevin, and, bred and maintained in house at the Strathclyde Institute of Pharmacy and Biomedical Sciences, Glasgow, UK. Male mice, aged 6-8 weeks were used for experiments.

3.3.4 MKP-2 Genotyping by polymerase chain reaction

MKP-$2^{+/+}$ and MKP-$2^{-/-}$ mice were genotyped by polymerase chain reaction. Tail tips were taken at the end of the experiments for DNA extraction. They were incubated overnight at 65°C, with shaking, in 1.5ml centrifuge tubes with 0.5ml lysis buffer (Tris pH 8.8 100mM, EDTA pH 8 5mM, NaCl 200mM, SDS 0.2% and Proteinase K 200μg/ml).

Following digestion the insoluble material was removed by centrifugation at 13,000rpm for 20 minutes. The supernatant was retained and placed in a fresh tube together with 1/10 volume (3M pH5.5) sodium acetate and 1/2 volume isopropanol, and the tube inverted several times to precipitate the DNA. The tubes were centrifuged at 13,000rpm for 5 minutes, supernatants discarded and 0.5ml of ice-cold 70% ethanol added to wash the pellet. The ethanol was removed by centrifugation at 13,000rpm for 5 minutes after which the supernatant was decanted. The pellet was allowed to air dry for 30 minutes at room temperature. 100μl of molecular grade water was added to each sample and then heated to 65°C for 5 minutes to dissolve the pellet prior to storage at -20°C.

For PCR, two sets of primers were used for each sample. See Table 5. The wild type primers designed for the wild-type MKP-2 gene and the knockout primers, designed over the mutated form of the MKP-2 gene, which occurs as a result of the knockout.

Table 11. Primers	
MKP-$2^{+/+}$ primer set	
Forward primer	5'- CCT CAG ACT GTC CCA ATC AC -3'
Reverse primer	5'- GAC TCT GGA TTT GGG GTC C -3'
MKP-$2^{-/-}$ primer set	
Forward primer	5'- TGA CTA GGG GAG GAG TAG AAG GTG GC -3'
Reverse primer	5'- ATA GTG ACG CAA TGG CAT CTC CAG G -3'

The PCR was set up in a designated PCR safety cabinet in 0.2ml PCR reaction tubes. Table 6 shows the PCR reagents used, their working volume per PCR reaction and the order in which they were added.

Table 12. PCR Components	
Molecular grade water (Invitrogen, UK)	3.25µl
ReddyMix (Thermo Scientific, UK)	5.25µl
Forward primer:	1µl (25pmol final concentration)
Reverse primer:	1µl (25pmol final concentration)
Genomic DNA Template:	2µl

The PCR reaction was carried out on the Techne TC-3000 Thermo Cycler using the thermo profile in table 7.

Table 13. Thermo profile			
Denaturation	95°C	5 minutes	1 cycle
Denaturation	95°C	30 seconds	40 cycles
Annealing	58°C	30 seconds	
Extension	72°C	5 minutes	
Extension	72°C	10 minutes	1 cycle

Samples were visualised on an 0.8% agarose gel stained with ethidum bromide. The product size expected for the wild type was approximately 1.3kb and 2.4kb for the knockout.

3.3.5 Maintenance of *Toxoplasma gondii* Beverley (RRA) strain

Beverley cysts were maintained *in vivo* at the Strathclyde Institute of Pharmacy and Biomedical Sciences, by passage of infective brain tissue homogenates through outbred CD1 albino mice. Briefly, infected mice were sacrificed by CO_2 inhalation and the brain removed prior to homogenisation by repeated passing through a 21-gauge needle, containing 2ml of sterile PBS (Lonza, UK), to obtain an even suspension. 15μl of brain suspension was placed on a microscope slide and the cysts enumerated by light microscopy.

3.3.6 Maintenance of transfected *Toxoplasma gondii* Prugniaud strains

Tachyzoites were routinely maintained in confluent human foreskin fibroblasts (HFFs) grown in 10ml DMEM complete medium comprising; Dulbecco's Modified Eagle Medium (DMEM) containing L-glutamine (Invitrogen, UK), 10% foetal calf serum (PAA, UK), 100U/ml penicillin (Cambrex Bioscience, Veniers, Belgium), 100mg/ml streptomycin (Cambrex Bioscience, Veniers, Belgium) and 50U amphotericin B (Cambrex Bioscience, Veniers, Belgium), in 75cm^2 tissue culture flasks (TPP, Switzerland) at 37°C in 5% CO_2. Once the majority of the HFFs were infected with replicating tachyzoites, the flask was scraped using a 30cm cell scraper (TPP, Trasadingen, Switzerland). The parasite solution was then carefully passaged at least 10 times though a 25G needle (BD, Drogheda, Ireland) and a 10-fold dilution, made in complete DMEM was passed into a confluent flask of HFFs.

3.3.7 Maintenance of *Toxoplasma gondii* RH strain *in vivo*

In vivo stocks of RH *T. gondii* were maintained in BALB/c mice. Mice were infected intraperitoneally with RH tachyzoites. Approximately 4 days later the mice were sacrificed by CO_2 inhalation and the tachyzoites harvested by intraperitoneal

washout, with 1ml of ice cold PBS. The tachyzoites were counted and diluted, and a dose of 2000 tachyzoites was given to another group of BALB/c mice.

3.3.8. Purification of *Toxoplasma* lysate antigen

RH tachyzoites where harvested from acutely infected BALB /c mice by intra-peritoneal washout as previously described. Peritoneal exudates were centrifuged at 12000rpm for 5 minutes and the pellet re-suspended in 1ml of fresh PBS prior to snap freezing in liquid nitrogen and placing into a water bath at 60°C until thawed. The sample was then initially passed through a 21G needle and then a 25G needle 10 times lysing the tachyzoites. The freeze/thaw and passage process was repeated a further 11 times. The sample was then centrifuged at 13000rpm for 10 minutes and the supernatant kept and stored at -20°C. The protein concentration was determined via the Bio-Rad protein assay (Bio-Rad, USA), as per the manufacturer's instructions using serial dilutions of BSA, starting at 2mg/ml as a standard.

3.3.9. *In vivo* bioluminescent imaging

In vivo parasite burden was assessed using bioluminescent imaging using type II Prugniaud *T. gondii* transfected with the firefly luciferase gene (given to us by Dr Rima McLeod, University of Chicago). The light data was quantified using Living Image software (Caliper Lifescience)

Intracellular FLUC *T. gondii* was harvested from *in vitro* culture for use in infection. Culture media was poured off and the HFF monolayer, infected with Pru FLUC *T. gondii*, and washed once with sterile PBS (Lonza, UK) prior to disruption with a cell scraper in 10mls sterile PBS. The intracellular parasites where then released the host cells by passage through a 21 gauge needle at least 10 times and centrifuged at 1200rpm for 10 minutes. The pellet was re-suspended in 1ml of PBS (Lonza, UK) and counted with a haemocytometer. The bioluminescent activity of the FLUC *T. gondii* was checked prior to infection using a standard curve generated by *in vitro* imaging. $1x10^6$ parasites were

seeded, in duplicate, in a black 24 well plate (Greiner Bio-one, UK). Doubling dilutions were made down the plate and 4 wells were set up containing only the PBS dilutant for a background control. The D-luciferin potassium salt, 15mg/ml solution in DPBS, (Caliper Lifesciences) substrate was added 1:100 to the wells and incubated at 37°C for 5 minutes prior to imaging, with 1-minute exposures using the IVIS Spectrum (Caliper Lifesciences). The data gathered was analysed using Living Image software (Caliper Life Sciences). The total flux (photons/second) was determined at each dilution and the total flux from the background wells subtracted.

For infection experiments, MKP-$2^{+/+}$ and MKP-$2^{-/-}$ mice were given a dose of 20,000 tachyzoites was delivered intraperitoneally in 400μl of PBS. Mice infected with the FLUC *T. gondii* were imaged using the IVIS Spectrum (Caliper Life Sciences).

In initial imaging experiments, to determine the optimum imaging parameters MKP-$2^{+/+}$ mice for imaging were given a dose of 150mg/kg of D-Luciferin potassium salt solution intraperitoneally, and immediately put under anesthesia using a 2.5% - 3.5% isoflorane/oxygen mix. Mice were imaged every 5 minutes, with a 1-minute exposure on a medium binning. This was conducted to establish the peak of the bioluminescent signal following administration of D-luciferin potassium salt. During imaging anesthesia was maintained with 2% isoflurane/oxygen mix. Optimal imaging signal was determined to be at 20 minutes post luciferin injection so this time was chosen for all further imaging in the MKP-$2^{+/+}$ and MKP-$2^{-/-}$ mice. To eliminate spurious background signals, uninfected mice were imaged in the same manner and the signal from these mice subtracted from the infected groups.

For imaging of the brains, surviving mice were injected with the D-Luciferin potassium salt (150mg/kg) and after 10 minutes mice were sacrificed by CO_2 inhalation. The brains were removed and soaked in 15mg/ml D-Luciferin potassium salt, dissolved in warmed RMPI 1640, for a further 10 minutes. They

were removed from the solution and imaged for 1 minute in a petri dish using the IVIS Spectrum.

3.3.10 Infection with Beverley strain

Tissue cysts were harvested from chronically infected CD1 mice as previously described. MKP-$2^{+/+}$ and MKP-$2^{-/-}$ mice were infected intraperitoneally with 10 tissue cysts of *T. gondii* Beverley stain in 200μl

3.3.11 Assaying serum nitric oxide

Serum nitric oxide levels were determined by Griess assay. Following euthanasia blood was collected by cardiac puncture, and red blood cells removed by centrifugation at 13,000rpm for 10 minutes. Protein was removed from the serum by adding $ZnSO_4$ to a final concentration of 15mg/ml, vortexing thoroughly and centrifugation at 13,000 rpm for 10 minutes. The supernatant was retained for the Greiss assay.

Standards were prepared using serial dilutions of $NaNO_2$, starting at 200μM in phenol red free RPMI 1640. 50μl of each sample and standard were plated, in duplicate, in a 96 well microtiter plate. Equal volumes of Greiss reagent (1:1 mix of 2% sulphanilamide in 5% H_3PO_4 and 0.2% Napthylene diamine HCL in ddH_20) was added and left in the dark for 10 minutes. The plate was read at a wavelength of 540nm in a spectrophotometer (Spectramax 190, Molecular Devices, USA).

3.3.10 *In vivo* inhibition of iNOS and Arginase 1To inhibit arginase or iNOS *in vivo,* 100μg per mouse and 200mg/kg of nor-NOHA (Iniesta *et al*., 2005) and L-NAME (Roberts *et al*., 2000), respectively, was delivered by daily intraperitoneal injection. The mice were dosed one day prior to infection and imaged as before.

3.3.12 Splenocyte Stimulation Assay

At the end of the experimental period the mice were sacrificed, the spleens harvested and a splenocyte stimulation assay set up. A single cell suspension was prepared by passing the spleens through a cell strainer with complete medium RPMI 1640 supplemented with 1% 2mM L-glutamine, 1% 100U/ml Penicillin-100μg Streptomycin and 10% Foetal calf serum. The suspension was then centrifuged at 1200rpm for 5 minutes and the pellet re-suspended in 3ml Boyles solution (10% Tris 0.17M in Ammonium Chloride 0.16M) and centrifuged at 1000rpm for 5 minutes. The resulting pellets were washed in 5ml complete medium by centrifuging at 1000rpm for 5 minutes prior to re-suspension in 2ml complete medium.

The cell count was then determined by Trypan Blue exclusion and single cell suspensions diluted to a concentration of $5x10^6$ cells/ml. 100μl aliquots were added to each well of the 96 well tissue culture plate and the cells were re-stimulated with either; complete medium alone, *Toxoplasma* lysate antigen (5μg/ml and 1μg/ml) or Concanavalin A (5μg/ml) or LPS (100ng/ml). Incubation for 72 hours at 37°C in 5% CO_2 the plates were stored at -20°C prior to analysis.

3.3.13 Cytokine ELISA

IFN-γ, Interleukin-5, Interleukin-6 and Interleukin-10 levels were assessed in the supernatants from the splenocyte stimulation by enzyme linked immunosorbent assay (ELISA). ELISA microplates were coated with 50μl per well of the appropriate capture antibody (Anti-IFN-γ at 2μg/ml, diluted in PBS pH7.4; Anti-IL-5 at 0.5μg/ml in PBS pH 9, Anti-IL-6 at 2μg/ml in pH9 and Anti-IL-10 at 2μg/ml in PBS pH6 (BD Bioscience, UK)) and incubated overnight at 4°C. The plates were then washed 3 times in wash buffer (1xPBS pH7.4 plus 0.5ml Tween per liter of PBS) and dried. The plates were subsequently blocked with blocking buffer, 200μl per well (10% heat-inactivated FCS in PBS pH7.4) and incubated at 37°C for 1 hour. Following blocking the plates were washed three times and dried. 30μl of each of the samples were then applied to the microplates together with the appropriate standards. After

a 2 hour incubation period the plates were washed 3 times as before. The samples and standards were then coated with 50μl per well of the appropriate Biotin labeled antibody diluted $^{1}/_{1000}$ in blocking buffer and incubated for 1 hour 37°C. After 3 washes the samples and standards were coated with 30μl per well Streptavidin-AKP (BD Bioscience, UK) diluted $^{1}/_{2000}$ and incubated for 45 minutes at 37°C. The plates were washed as before and 50μl per well of the 4-Nitrophenyl phosphate disodium salt hexahydrate (Sigma, UK) substrate was applied to the samples and standards. The substrate was made up in glycine buffer (Glycine, $MgCl_2$ and $ZnCl_2$ in distilled H_20 pH10.4) following the manufacturer's instructions. The microplates were incubated at room temperature, in the dark, until colour started to develop. The absorbance was the read at 405nm using a microplate reader (Spectramax 190, Molecular Devices, USA).

3.3.14 Flow cytometry for immune phenotype.

Flow cytometry was used to quantify the $CD8^+$ and $CD4^+$ T cells population and their TNF-α and IFN-γ cytokine response during acute infection with *T. gondii*. On day 10 post infection, the spleens from MKP-$2^{+/+}$ and MKP-$2^{-/-}$ mice infected with the Beverley strain of *T. gondii*, were harvested. Splenocyte cultures were set up as before with the inclusion of ionomycin and phorbol-13-myristate-12-acetate (*PMA*) stimulations (0.5μg/ml and 10ng/ml, respectfully) and an increased number of cells, $2x10^6$/500μl/per well in a 24 well plate (TPP, Switzerland). The plates were set up in duplicate. One set was incubated for 72 hours prior to cytokine ELISA. The second set was incubated for 3 hrs, after which Brefeldin A to a concentration of 10ng/ml was added prevent cytokine secretion from the cells.. The splenocyte cultures were incubated for a further 3 hrs after which the cells were harvested for staining. The plates also included wells of un-stimulated cells for use as compensation controls.

375μl from each sample well was transferred into separate FACS tubes and the final 125μl from each well was pooled, on a mouse per mouse basis, into a FACS tube for staining with isotype controls. The compensation controls were

harvested and collected in individual 5ml polystyrene round bottom tubes (BD Biosciences).

The FACS tubes were quenched with FACS-PBS (0.1% NaN_3 and 0.1% BSA in 1xPBS) and centrifuged for 6 minutes at 330g. The supernatant was discarded and the pellet re-suspended by briefly vortexing. 50μl of Fc-block (1μg/ml rat anti-mouse CD16/CD32 (BD Bioscience), 10% mouse serum in RPMI 1640) was added to each tube and incubated at room temperature for 10 minutes. 50μl of surface stain for CD3, CD4 and CD8 was then added to each sample and isotype control tube and incubated in the dark, at 4°C for 1 hour. Table 8 shows the stains and their concentrations.

Table 14. Surface stain mix	
Anti CD3-PerCP (BD Bioscience)	2μl per sample
Anit CD4α-APC-H7 (BD Bioscience)	1μl per sample
Anti CD8β-Alexa488 (BD Bioscience)	0.5μl per sample
FC-Block	46μl per sample

The compensation controls were set up using anti CD4 antibodies conjugated to the same flurochromes being used for the intracellular staining. Volumes and concentrations used in Table 9. One sample was left unstained but subjected to the same fixation and permeabilisation process.

Table 15.Compensation controls (Each sample made up to 50ml with FC block)	
Anti CD3-PerCP (BD Bioscience)	2μl per sample
Anti CD4α-APC-H7 (BD Bioscience)	1μl per sample
Anti CD8β-Alexa488 (BD Bioscience)	0.5μl per sample
Anti CD4α-PE (BD Bioscience)	1μl per sample
Anti CD4α-APC (BD Bioscience)	1μl per sample

Following incubation, the tubes were quenched with FACS-PBS and centrifuged at 330g for 6 minutes. The cells were then fixed and permeabilised using Fix/Perm kit (Invitrogen, UK). The pellets were re-suspended by vortexing in 100μl 'Reagent A' (Fixation medium) and incubated for 15 minutes, in the dark

at room temperature. The tubes were then quenched with FACS-PBS-Saponin (0.1% NaN_3, 0.1% BSA, 0.2% saponin in PBS) and centrifuged as before. The pellets were vortexed and re-suspended in 50μl 'Reagent B' (Permeabilisation medium) and 10μl of the intracellular stain mix was added to the samples. Intracellular isotype controls were prepared in the same manner and compensation controls were fixed and permeabilised but no further stains were added. The antibodies used for the intracellular staining and isotype controls are detailed in Table 10.

Table 16. Intracellular stain mix	
Intracellular stain mix	
Anti TNFα-PE (BD Bioscience)	1.25μl per sample
Anti IFNγ-APC (BD Bioscience)	0.5μl per sample
FC block	8.25μl per sample
Isotype control mix	
Anti IgG1-APC (BD Bioscience)	0.5μl per sample
Anti IgG1- PE (BD Bioscience)	1.25μl per sample
FC block	8.25μl per sample

Following incubation the samples were washed three times with FACS-PBS-Saponin and centrifugation at 330g for 6 minutes. All samples and stained controls were then re-suspended in 200μl PBS and stored in the dark at 4°C

500,000 events from each sample were collected in a FACSCanto flow cytometer (BD Bioscience). FACSDiva software was used to collect the data and analysis was carried out on Flowjo analysis software. Compensation controls were set up using the single stained and unstained controls to minimize bleed through of colours into other channels.

Live cells were gated on forward and side scatter. From this, the CD3 positive population was gated and further sub gated on $CD4^+$ or $CD8^+$ cell populations. Within each of these sub populations the percentage of cells that were positive for IFNγ, TNFα or IFNγ and TNFα were determined.

3.3.15 Generation of bone marrow derived macrophages

Bone marrow macrophages were cultured from 6 week old MKP2$^{+/+}$ or MKP-2$^{-/-}$ mice. The mice were euthanized by CO_2 inhalation and their femurs removed under aseptic conditions. Using a 25G needle, the bones were flushed with 5mls per bone of macrophage culture medium (DMEM supplemented with 20% FCS, 2% penicillin/streptomycin, 2% L-glutamine and 30% L-cell conditioned medium) to extract the bone marrow, which was then passed through a cell strainer with an additional 5mls per bone of macrophage medium, to generate a single cell suspension.

The cell suspension was plated out into a petri dish, 10mls per plate and incubated at 37°C in 5% CO_2. On day 3 10mls of fresh, pre-warmed macrophage medium was added to each plate. On day 7 the medium was removed and fresh macrophage medium added. If required the macrophages were split and fresh plates set up. On day 10 the macrophages were harvest for use.

The confluent macrophages were harvested first by decanting the medium and adding 10mls per plate of ice cold sterile PBS and left for a few minutes. The plates were then gently scrapped with a cell scraper to lift the cells off the base of the petri dish and the PBS containing the cells was transferred to a 50ml centrifuge tube. The cells were then centrifuged at 1200rpm for 10 minutes and the pellets re-suspended in 2mls of the medium required for the subsequent stimulation. The cells were counted by trypan blue exclusion, using a haemocytometer. Following the appropriate dilution, the cells were plated out and incubated overnight at 37°C in 5% CO_2 to allow the cells to adhere to the new plates. The next day the cells were then stimulated as required.

3.3.16 Assaying parasite growth by *in vitro* fluorescent imaging

To assay parasite growth in MKP-2 deficient and wild type bone marrow derived macrophages, a type II Prugniaud strain *T. gondii* previously transfected

with yellow fluorescing protein (YFP) donated by Marcus Meisnner, University of Glasgow was used.

50,000 bone-marrow derived MKP-$2^{+/+}$ or MKP-$2^{-/-}$ macrophages per well were plated out in complete phenol red free RPMI 1640 (10% FCS, 1% L-glutamine and 1% Penicillin/Streptomycin). Black 96 well tissue culture plates (Greiner Bio-One, UK) with clear bottoms and lids were used to minimise auto-fluorescence.

The macrophages were incubated at 37°C in 5% CO_2 overnight. To inhibit arginase or iNOS activity, macrophages were then pre-treated for 1 hour with either 50μM N^{ω}-hydroxy-nor-Arginine (nor-NOHA, Merk Chemicals, UK) or 5mM N (G)-nitro-L-arginine methyl ester (L-Name, Sigma, UK) respectively. Prugniaud YFP *T. gondii* parasites were harvested as previously described and added at a multiplicity of infection (MOI) of 1:1. After one hour the macrophages were further stimulated with LPS (100ng/ml) or IFN-γ (100U/ml) alone or in combination. The levels of YFP were assayed at 24, 48 and 72 hours using the transillumination feature of the IVIS Spectrum (Caliper Lifescience). An excitation wavelength of 500nm and emission wave of 540nm was used. The light data was quantified using Living Image software (Caliper Lifescience) using the uninfected macrophages as background controls.

3.3.17 Amidoblack assay

Protein concentrations of samples in sample buffer, for western blot analysis, were determined by amidoblack assay (Dieckmann-schuppert et al. 1997).

Cellulose acetate membranes (Sigma, UK) were divided into 1.25cm x 1.25cm squares and suspended over a tray with clips. Samples and standards were prepared by boiling in 2x sample buffer for 5 minutes. A BSA standard curve was prepared starting at 5mg/ml. 5μl of each sample and standard was applied to a square on the membrane, air dried for 10 minutes and covered in

amidoblack staining solution (0.5% Amidoblack, 45% Methanol, 45% ddH_2O and 10% glacier acetic acid) for 10 minutes with shaking.

The stain was discarded and excess rinsed off with water. The membrane was then washed 3 times, for 5 minutes each with de-staining solution (47.5% Methanol, 47.5% ddH_2O and 5% glacier acetic acid). The membrane was allowed to thoroughly dry and each square was cut and placed into a 1.5ml centrifuge tube with 500µl amidoblack dissolving solution (80% formic acid, 10% glacier acetic acid and 10% Trichloroacetic acid) and incubated with shaking at 50°C for 30 minutes. Once dissolved 250µl of each sample and standard was placed into a 96 well microtiter plate and the absorbance was read at 620nm.

3.3.18 Western blot for Arginase 1

Preparation of Samples for SDS-PAGE

Single cells suspensions for analysis were centrifuged in 1.5ml centrifuge tubes and re-suspended in of 200µl of SDS-PAGE of sample buffer (63mM Tris-HCl, pH 6.8, 2mM $Na_4P_2O_7$, 5mM EDTA, 10% (v/v) glycerol, 2% (w/v) SDS, 50mM DTT and 0.007% (w/v) bromophenol blue). The tube lids were pierced and protein denaturation was achieved by boiling the samples for 5 minutes prior to storage at -20°C.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

10% (w/v) acrylamide resolving gels were prepared (N'-methylenebis-acrlyamide, 0.375M Tris pH 8.8, 0.1% (w/v) SDS and 0.05% (w/v) ammonium persulfate (APS). The initiation of polymerisation occurred following the addition of 0.05% N'-tetramethylethylenediamine (TEMED)). The solution was thoroughly mixed and settled between two assembled glass plates allowing space for the stacking gel (10% (w/v) acrylamide, N'-methylenebisacrylamide,

125mM Tris pH 6.8, 0.1% (w/v) SDS, 0.05% APS and 0.05% (v/v) TEMED) was added to the top of the resolving gel and an appropriate comb inserted. The stacking gel polymerised after a period of 15 minutes before the comb was removed and the gel assembly placed in the electrophoresis tank (Bio-Rad Mini-PROTEAN II). Running buffer (24.8 mM Tris, 191.8 mM glycine and 0.1% SDS (w/v)) was added to the tank in both the outer and inner reservoirs. Protein samples were standardised by amidoblack assay and loaded with a Hamilton syringe. A protein marker of known molecular weights was also loaded. The gel was run at 70 volts until the samples had gathered at the base of the stacking gel, then the voltage was increased to 120 volts until the loading dye reached the bottom of the gel assembly.

Proteins resolved by SDS-PAGE were transferred to a nitrocellulose membrane by electrophoretic transfer. The transfer was constructed by placing the gel against a nitrocellulose membrane of the same size, assembled within a transfer cassette between 3mm blotting paper and 2 sponge pads. Prior to assembly the membrane, blotting paper and sponge pads were soaked in transblot buffer (25mM Tris, 19mM glycine, 20% (v/v) methanol) for at least 30 minutes. The cassette was immersed in transblot buffer in a Trans-Blot tank (Bio-Rad Mini Trans blot tank) where a current of 300mA was applied for 1 hour 45 minutes. The inclusion of an ice reservoir and magnetic stirrer ensured that the tank remained cool throughout the transfer and kept an even ion distribution.

Following protein transfer, the nitrocellulose membrane was removed and blocked for 2 hours at room temperature, with shaking, in 2% (w/v) BSA in NaTT buffer (150mM NaCl, 20mM Tris, 0.2% (v/v) Tween-20 pH 7.4). The membrane was placed in a 50ml centrifuge tube with 3mls of 0.2% (w/v) BSA in NaTT and Anti-Arginase I (Abcam, UK) at a 1/1000 dilution or Anti β-Tubulin at a 1/500 dilution, and incubated overnight at 4°C on a roller.

Incubated blots were washed six times at 15 minute intervals with NaTT buffer on a room temperature platform shaker, after which the secondary antibody conjugated to horseradish peroxidase (Jackson Immunoresearch Laboratories Inc, USA) was added at a concentration of 1/5000 in 0.2% (w/v) BSA in NaTT buffer for a period of 2 hours. Blots were then washed six times at 15 minute intervals with NaTT buffer.

Nitrocellulose membranes were exposed to enhanced chemiluminescence (ECL) reagent for 3 minutes and loosely blotted on paper towel in order to remove any excess liquid. The blots were placed in exposure cassette, covered with a clear plastic cover and subsequently exposed to Kodak X-OMAT LS film under darkroom conditions and developed with a Kodak M35-M X-OMAT processor. .

3.4 Results

3.4.1 Optomisation of *in vivo* and *in vitro* bioluminescent imaging

In this work luminescent and fluorescent strains were used as a means of assaying parasite growth throughout the course of the experiment. To establish the linear relationship between the light emitted and the number of parasites, serial dilutions of either Prugniaud-FLUC (luminescent imaging) or Prugniaud-YFP tachyzoites (fluorescent imaging) were plated out and imaged using the IVIS spectrum. For the Prugniaud-FLUC (Figure 3.4.1), following administration of D-luciferin potassium salt (150μg/ml) a linear relationship was established between the number of parasites per well and the total flux (photons per second) data. Following excitation at 430nm, Prugniaud-YFP (Figure 3.4.2) tachyzoites displayed a linear relationship between the number of tachyzoites per well and the total efficiency (photons/second/cm^2).

To establish the optimum time to image mice infected with Prugniaud-FLUC following intraperitoneal injection of D-luciferin potassium salt (150mg/kg). Wild type mice infected with Prugniaud-FLUC were treated with D-luciferin potassium salt and imaged sequentially, every 5 minutes for 35 minutes. The peak luciferase activity was demonstrated to be at 20 minutes post luciferin injection (Figure 3.4.3).

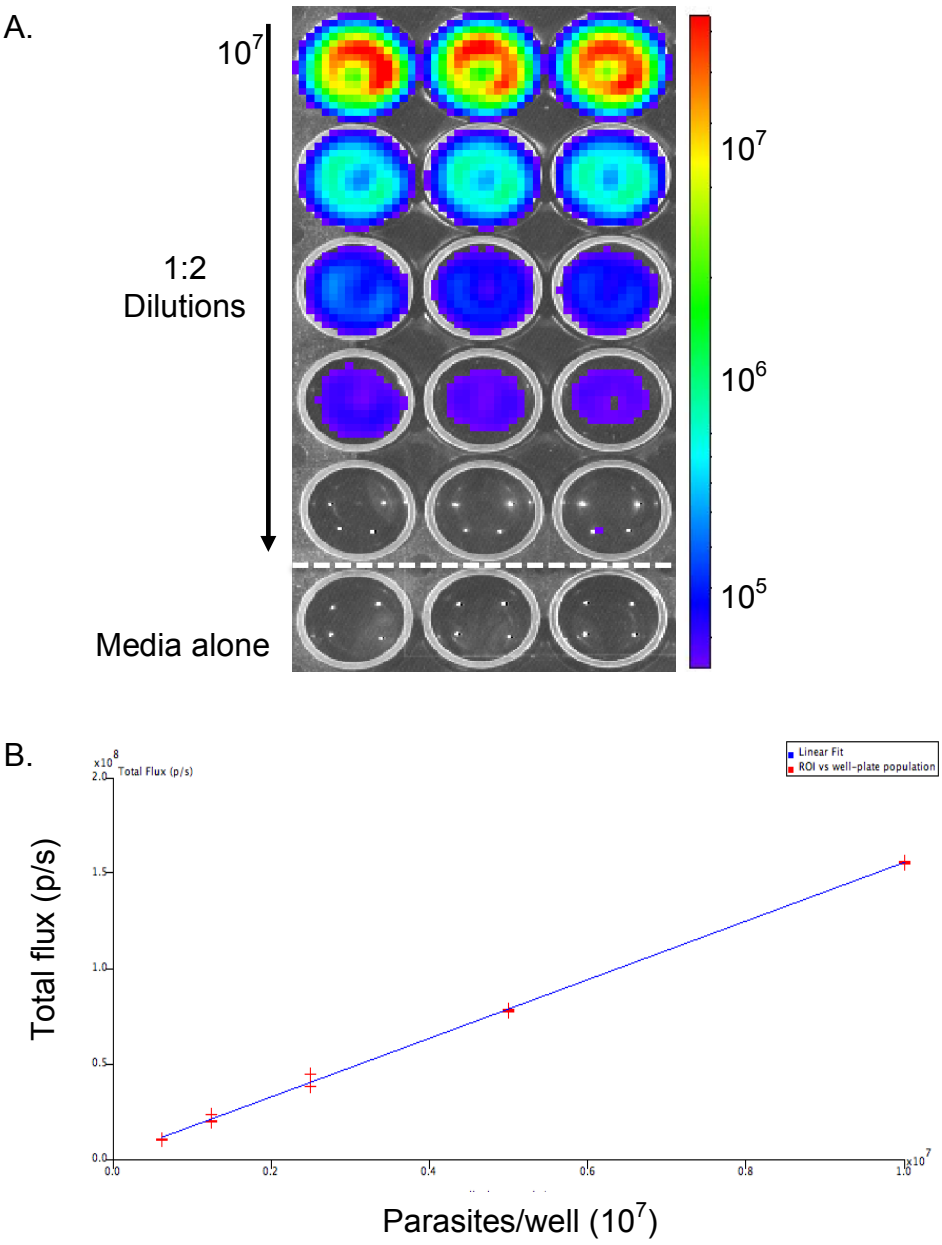


Figure 3.4.1. Linear relationship between parasite number and luminescent light data

1:2 serial dilutions of Prugniaud-FLUC starting at $1x10^7$ cells per well were treated with luciferin potassium salt (150μg/ml) and imaged in IVIS spectrum (A). Relationship between the number of parasites and the light data gathered was determined (B). Standardisation was repeated prior to all Prungniaud-FLUC infection experiments.

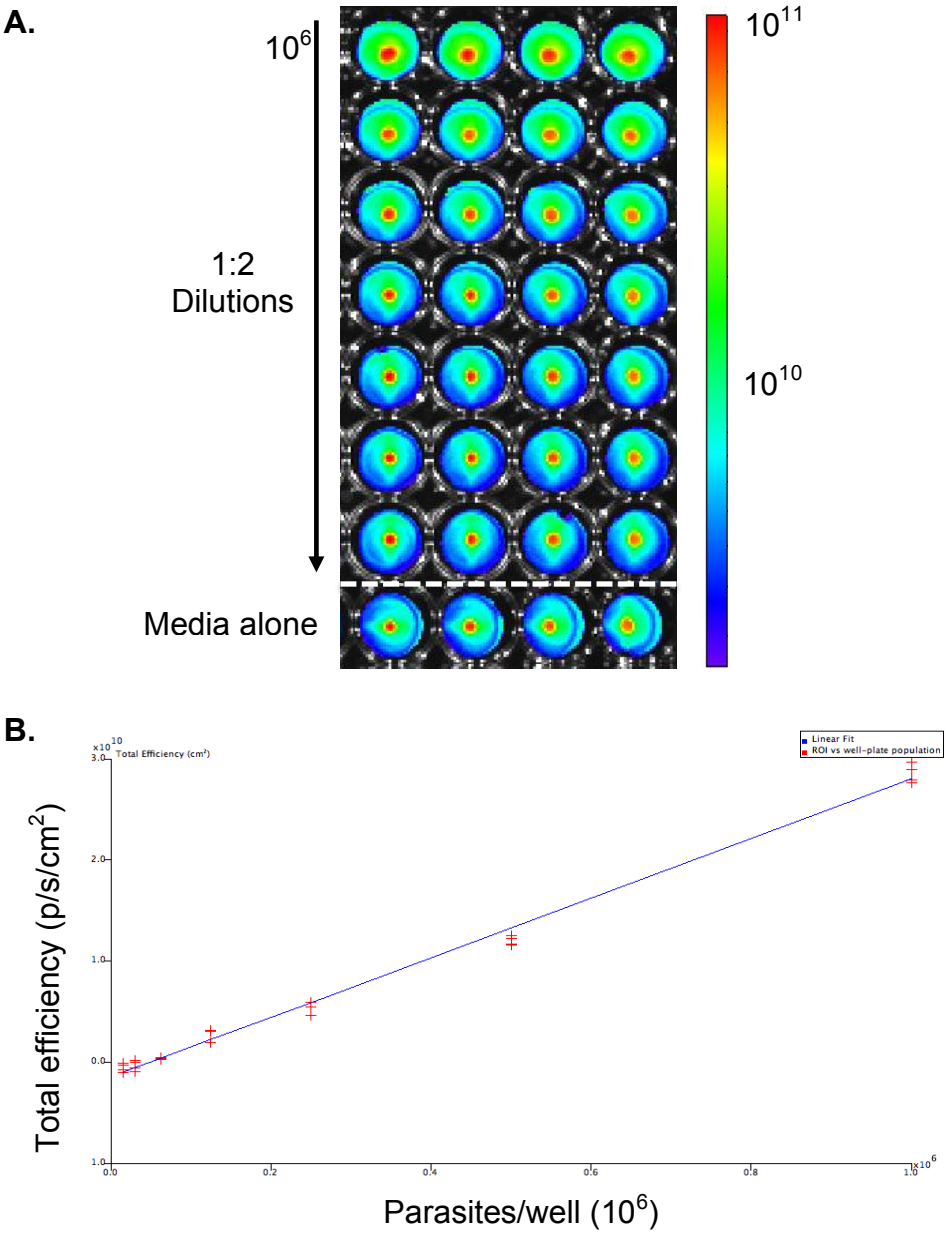
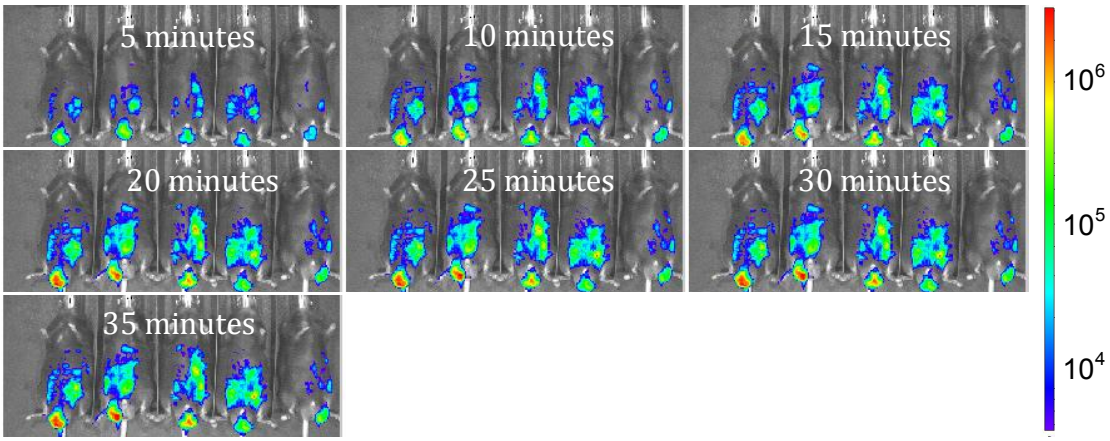


Figure 3.4.2. Linear relationship between Prunguid-YFP parasite number and fluorescent light data.

Serial dilutions of Prunguid-YFP tachyzoites were imaged (ex 430nm) in an IVIS spectrum (A). Relationship between parasite number and light data was determined (B). Experiment was repeated before every Prunguid-YFP tachyzoite infection experiment.

A.



B.

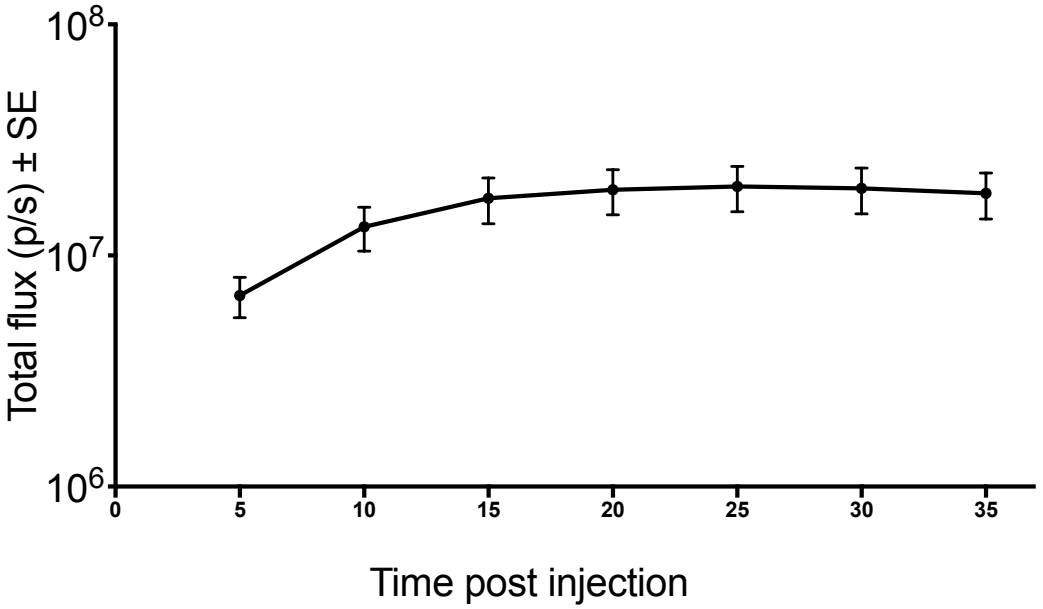


Figure 3.4.3. Optimal time for imaging Prunguid-FLUC infected mice, post luciferin injection.

Following i.p injection of D-luciferin potassium salt (150mg/kg) the Prunguid-FLUC infected mice were image sequentially, every 5 minutes (A). The peak total flux was established to be 20 minutes. This was repeated 3 times over the course of infection. N=5 mice per group.

3.4.2 MKP-2 deficiency results in increased susceptibility following infection with *T. gondii.*

Intraperitoneal infection with 10 cysts of the Beverley (type-II) strain of *T. gondii* resulted in significant mortality days15-25 post-infection in MKP-$2^{-/-}$ but not MKP-$2^{+/+}$ mice (Figure 3.4.4). In order to determine whether this was associated with any inability of the MKP-$2^{-/-}$ mice to control parasite growth MKP-$2^{-/-}$ and MKP-$2^{+/+}$ mice were infected intraperitoneally with 20,000 type-II strain Prugniaud-FLUC tachyzoites and parasite burdens measured every second day post-infection, by bioluminescent imaging. At days 8 (Figure 3.3.2.A) and 10 the bioluminescent intensity and consequently parasite burdens were significantly greater ($p<0.031$ and $p<0.0079$ respectively) in MKP-$2^{-/-}$ compared with MKP-$2^{+/+}$ mice (Figure 3.4.5.B). The increased susceptibility of MKP-$2^{-/-}$ mice compared with their wild-type counterparts was extended into the chronic phase of the disease and by day 30 post-infection the excised brains of MKP-$2^{-/-}$ mice were revealed by bioluminescence to have significantly higher parasite burdens ($p<0.02$) than similarly infected MKP-$2^{+/+}$ animals (Figure 3.4.6 A and B).

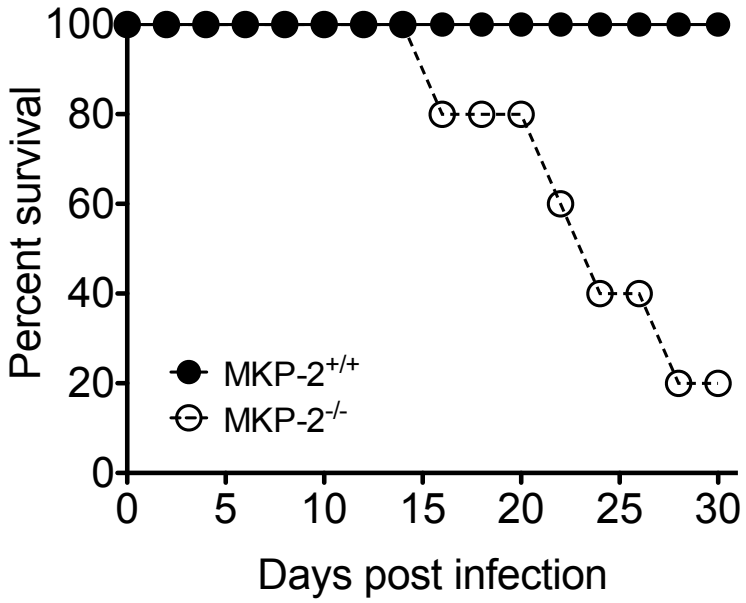


Figure 3.4.4. Survival curve for MKP-2$^{-/-}$ and MKP-2$^{+/+}$ mice infected with *T. gondii*.

Mice were infected with 10 Beverley tissue cysts intraperitoneally and mortality monitored. All experiments were repeated at least twice. N = 5 for both groups

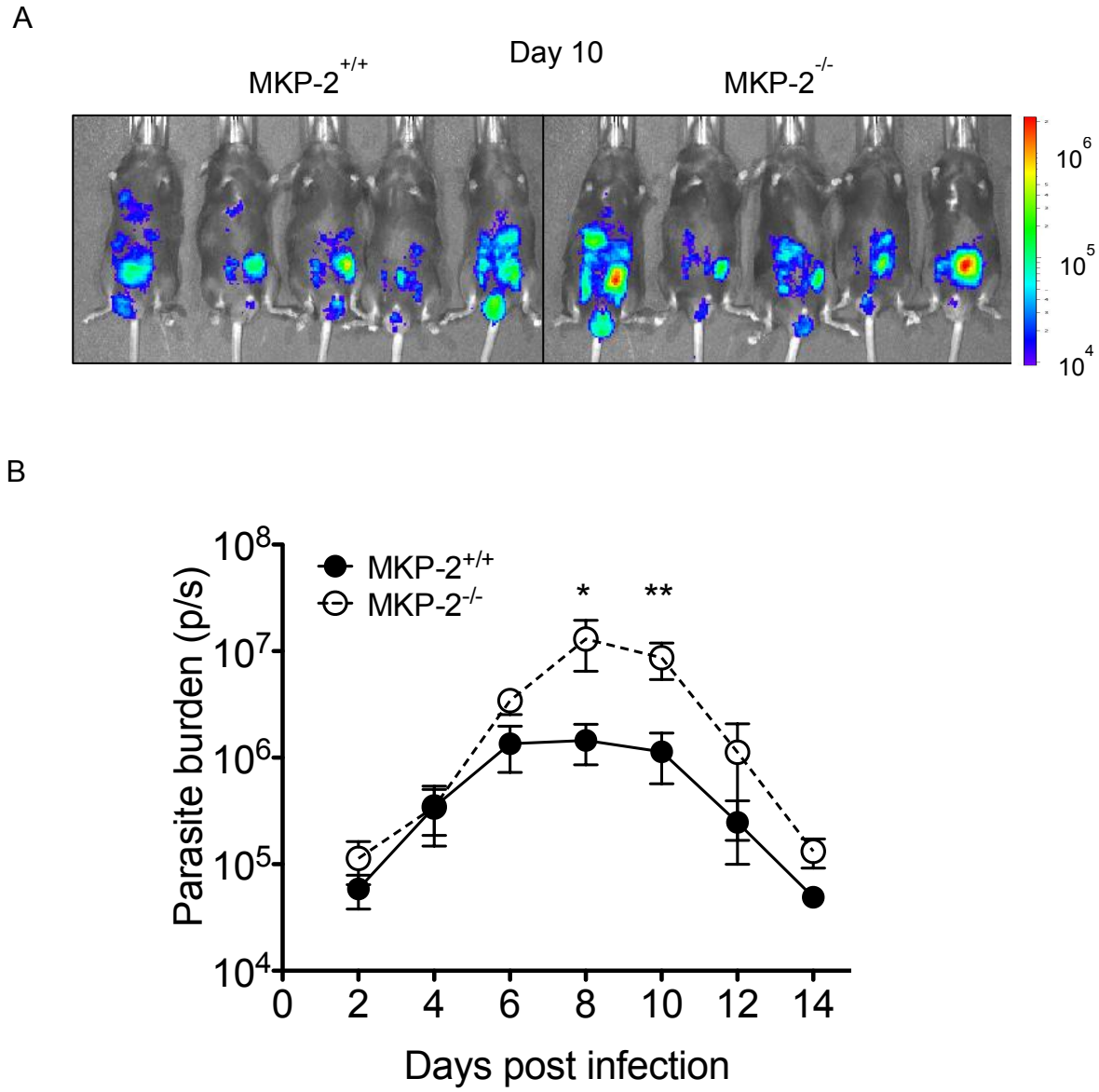
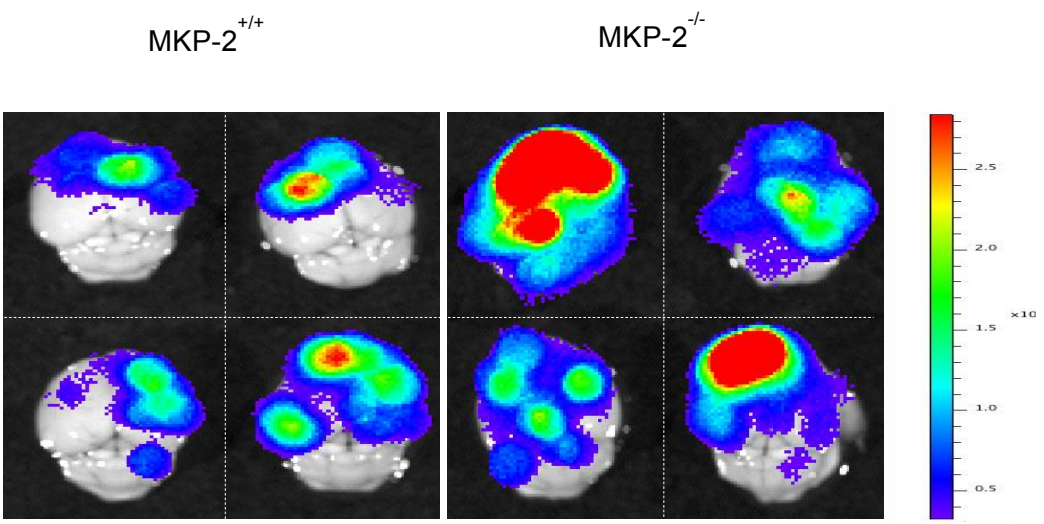


Figure 3.4.5. Increased parasite burden in MKP-2$^{-/-}$ mice infected with *T. gondii*.

Mice were infected with 20,000 type II Prugniaud tachyzoites, expressing firefly luciferase. Mice were imaged as outlined in the methods (A). Parasite burdens were determined by measuring whole body Total Flux (Photons/second) using LivingImage4.0 (B). Each value represents the mean of 5 animals per experimental group ± SEM. * $P < 0.05$, ** $P < 0.005$. All experiments were carried out on at least 2 occasions.

A



B

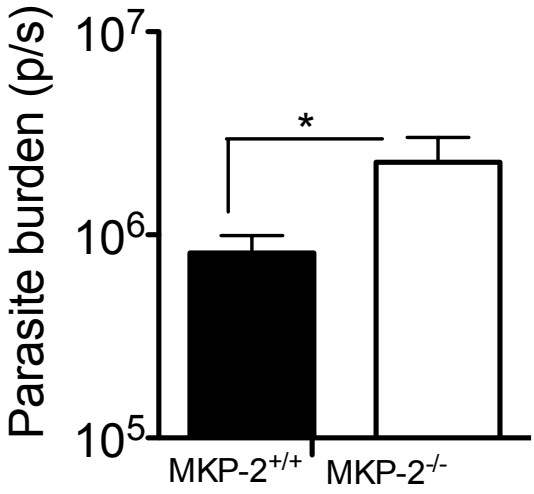


Figure 3.4.6. MKP-2 deficient mice have an increased parasite burden during chronic infection.

On day 30 post-infection, brains were imaged *ex vivo* as outlined in the methods (A). Chronic parasite burden was determined by measuring Total Flux (photons/second) from each brain (B). Each value represents the mean of 4 animals per experimental group ± SEM. * $P < 0.05$. All experiments were carried out on at least 2 occasions.

3.4.3 Serum nitrite levels are reduced in MKP-2$^{-/-}$ mice while tissue arignase-1 expression is up regulated during acute *T. gondii* infection.

The enhanced susceptibility of MKP-2$^{-/-}$ mice following infection with the intracellular parasite *L. mexicana* has been attributed to enhanced macrophage arginase expression concomitant with decreased iNOS expression compared to wild-type mice. (AL-Mutairi *et al.,* 2010). Consequently we measured serum nitrite levels at day 10 post-infection with *T. gondii* when differences in *T. gondii* parasite burdens between MKP-2$^{-/-}$ mice and MKP-2$^{+/+}$ mice were most pronounced. At this stage of infection nitrite levels were significantly greater ($p<0.005$) in wild-type mice compared with their MKP-2 deficient counterparts (Figure 3.4.7). At the same time spleen arginase-1 expression was not only enhanced in infected mice compared to uninfected mice irrespective of MKP-2 deficiency but MKP-2$^{-/-}$ mice expressed higher levels of arginase-1 than wild-type mice whether uninfected or infected with *T. gondii* (Figure 3.4.8).

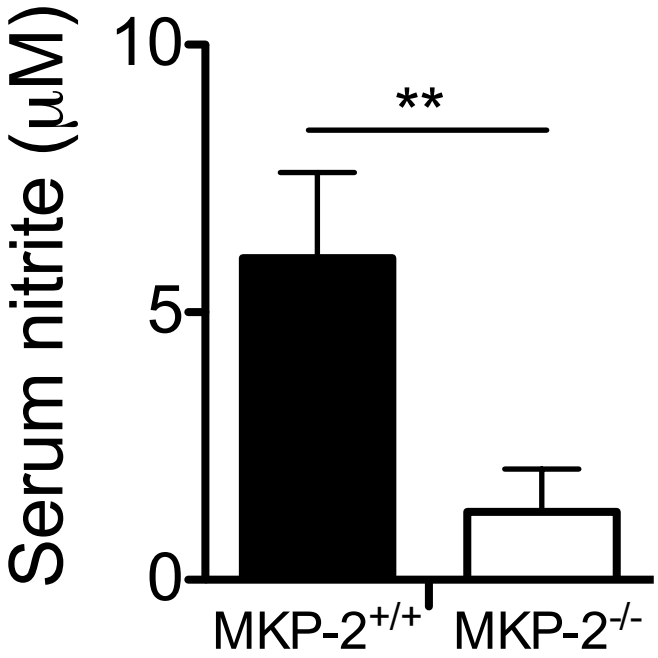


Figure 3.4.7. Systemic serum nitrite levels are reduced in MKP-2$^{-/-}$ mice infected with *T. gondii.*

Serum from infected animals was assessed for its nitrite content by Griess assay. Each value represents the mean from 5 animals per experimental group ± SEM. ** P < 0.005. All experiments were carried out on at least 2 occasions

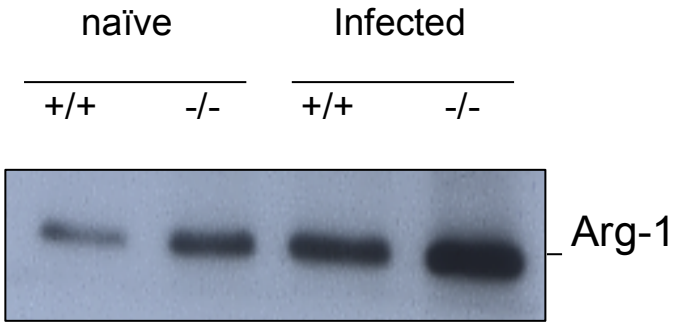


Figure 3.4.8 MKP-2$^{-/-}$ mice display increased tissue arginase-1 expression.

Splenocyte lysates were prepared and assayed for Arginase-1 by western blot. The blot represents a pool of 5 animals per experimental groups, standardised for protein concentration. ** $P < 0.005$. All experiments were carried out on at least 2 occasions

3.4.4. MKP-2$^{-/-}$ mice do not display an altered Th-1 phenotype during infection with *T. gondii*

In order to determine whether the increased susceptibility of MKP-2$^{-/-}$ mice infected with *T. gondii* was the result of an impaired adaptive immune response spleens were removed and splenocytes stimulated with TLA antigen. Flow cytometry analysis of splenocytes day 10 post-infection either under resting conditions, following antigen stimulation or following treatment with ionomycin and PMA revealed no differences in either the overall numbers of CD4^{+} or CD8^{+} T cells between infected MKP-2$^{-/-}$ and MKP-2$^{+/+}$ mice nor in the frequencies of CD4^{+} (Figure 3.4.9.A) or CD8^{+} (Figure 3.4.9.B) T cells producing either IFN-γ, TNF-α or both IFN-γ and TNF-α. At days 10, 20 and 30 post-infection and IFN-γ, IL-4, IL-5, and IL-10 production were measured in the supernatants. There were generally no differences between the ability of splenocytes from MKP-2$^{-/-}$ or MKP-2$^{+/+}$ mice infected with *T. gondii* to produce cytokines when stimulated with parasite lysate antigen (Figure 3.4.9.C). Consequently, there was little evidence that MKP-2$^{-/-}$ mice were limited in their ability to mount a type-1 response. No differences in Th2 cytokine production were noted (results not shown).

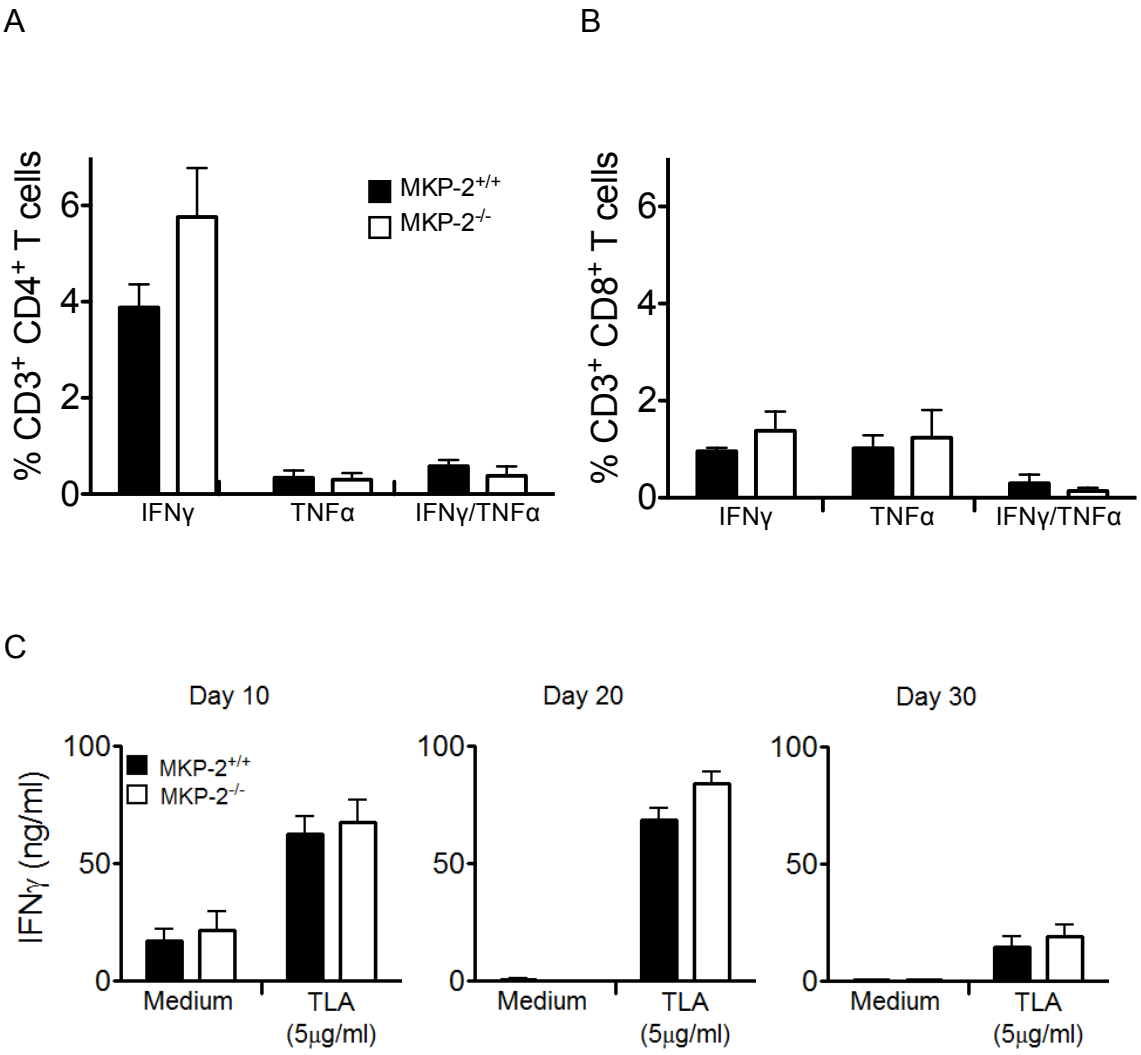


Figure 3.4.9. MKP-2$^{-/-}$ mice do not display an impaired T cell response during infection with *T. gondii.* T cell responses were determined by flow cytometry. Cells were stained for CD3, CD8, CD4 and intracellular stains for IFN-γ and TNF-α. Live cells were gated on forward verses side scatter. T-cells were sub-gated for either CD4 or CD8 and then their antigen specific cytokine production. Specific staining was determined by subtracting the isotype controls (A). Populations of CD3$^+$ CD4$^+$ T cells (B) and CD3$^+$ CD8$^+$ T cells (C) sing or double positive for IFN-γ and TNF-α were determined using FlowJo software. Splenocytes from *T. gondii* infected mice were stimulated with TLA and IFN-γ in the supernatant determined by ELISA (C). Each value represents the mean of 4 animals per experimental group ± SEM. All experiments were carried out on at least 2 occasions.

3.4.5. Inhibition of NO production by L-NAME enhances the susceptibility of MKP-$2^{+/+}$ but not MKP-$2^{-/-}$ mice to *T. gondii* infection.

In order to determine whether the apparently enhanced NO production by *T. gondii* infected MKP-$2^{+/+}$ mice over their MKP-2 deficient counterparts was responsible for their increased resistance to parasite growth and survival *in vivo* NO production was inhibited in infected mice by intraperitoneal injection with the iNOS inhibitor L-NAME. While L-NAME treatment of infected MKP-$2^{+/+}$ mice resulted in 100% mortality by day 10 post-infection the majority of infected MKP-$2^{-/-}$ mice survived until day 12 post-infection (Figure 3.4.10). All non-treated infected MKP-$2^{-/-}$ and MKP-$2^{+/+}$ mice survived until day 12 (Figure 3.4.10). Measurement of bioluminescence (Figure 3.4.11A) allowed quantification of parasite burdens and demonstrated significantly greater ($p<0.05$) parasite growth days 6 and 8 post-infection in L-NAME treated as apposed to non-treated MKP-$2^{+/+}$ mice (Figure 3.4.11.B). By comparison treatment of infected MKP-$2^{-/-}$ mice with L-NAME did not significantly alter parasite growth (Figure 3.3.11.B). At day 8 post-infection there were significantly more parasites ($p<0.05$) in L-NAME treated MKP-$2^{+/+}$ mice than either L-NAME treated or non-treated MKP-$2^{-/-}$ mice.

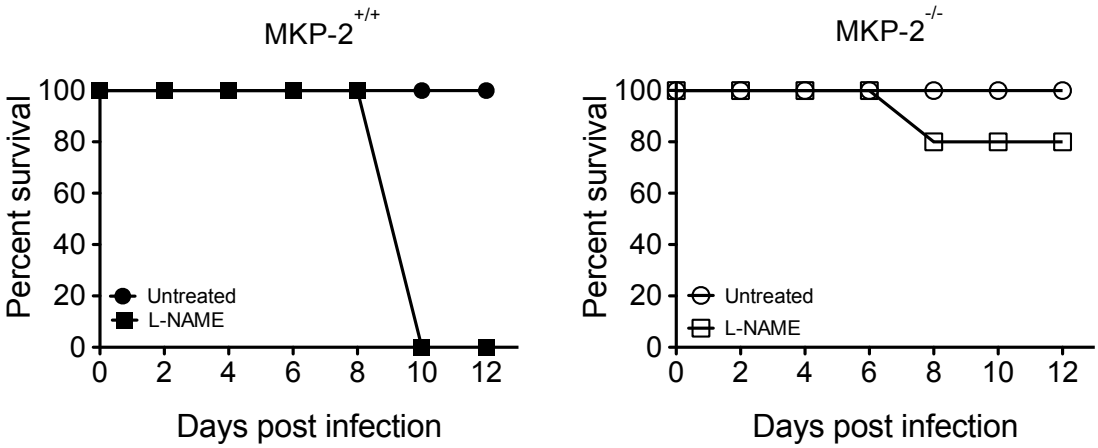


Figure 3.4.10. NO inhibition by L-NAME enhances susceptibility of MKP-2$^{+/+}$ but not MKP-2$^{-/-}$ mice to *T. gondii* infection.

Mice were pre-treated with L-NAME and subsequently treated with L-NAME (200mg/kg) daily following infection with 20,000 Pru tachyzoites expressing firefly luciferase Mortality was measured over 12 days. Experiments were repeated on at least 2 occasions with 5 animals per group.

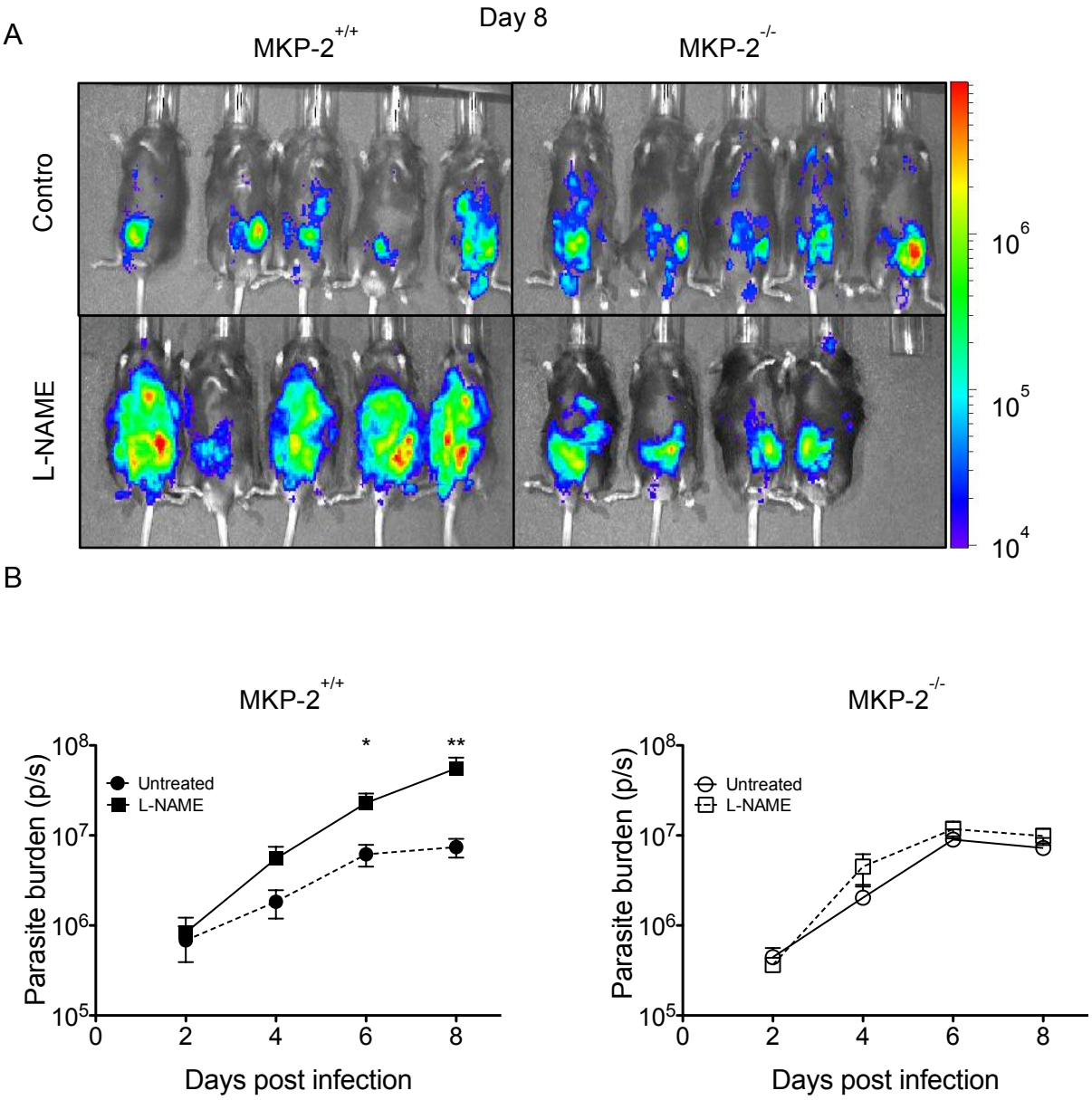


Figure 3.4.11. MKP-2$^{+/+}$ mice display increased parasite burden following treatment with L-NAME.

Mice infected with *T. gondii* were imaged every second day. (A) Represents day 8 post-infection. The parasite burden was determined by measuring the total flux (photons/second) for each group (B). Each value represents the mean of 5 mice per group ± SEM * P < 0.05. All experiments were carried out on at least 2 occasions.

3.4.6. Treatment with nor-NOHA during *T. gondii* infection resulted in increased parasite burden in MKP-2$^{-/-}$ mice

Unlike there wild-type counterparts, inhibition of NO production does not increase parasite growth or the early mortality of MKP-2$^{-/-}$ mice infected with *T. gondii.* This would imply an alternative mechanism of controlling early infection in the absence of MKP-2. As *T. gondii* is an L-arginine auxotroph (Fox *et al*., 2004) we suspected that over expression of arginase-1 in MKP-2$^{-/-}$ mice could be playing a protective role by depleting the parasite of this metabolite. This was tested by the intraperitoneal inoculation of infected MKP-2$^{-/-}$ and MKP-2$^{+/+}$ mice with the arginase-1 inhibitor nor-NOHA. Nor-NOHA did not significantly increase mortality in either MKP-2$^{-/-}$ nor MKP-2$^{+/+}$ mice infected with *T. gondii* compared with non-drug treated controls (Figure 3.4.12). Measurement of bioluminescence (Figure 3.4.13.A) allowed quantification of parasite burdens and demonstrated significantly greater ($p<0.05$) parasite growth day 8 post-infection in nor-NOHA treated as apposed to non-treated MKP-2$^{-/-}$ mice (Figure 3.4.13.C). By comparison nor-NOHA treatment had little effect on the course of early *T. gondii* infection in MKP-2$^{+/+}$ mice (Figure 3.4.13.C).

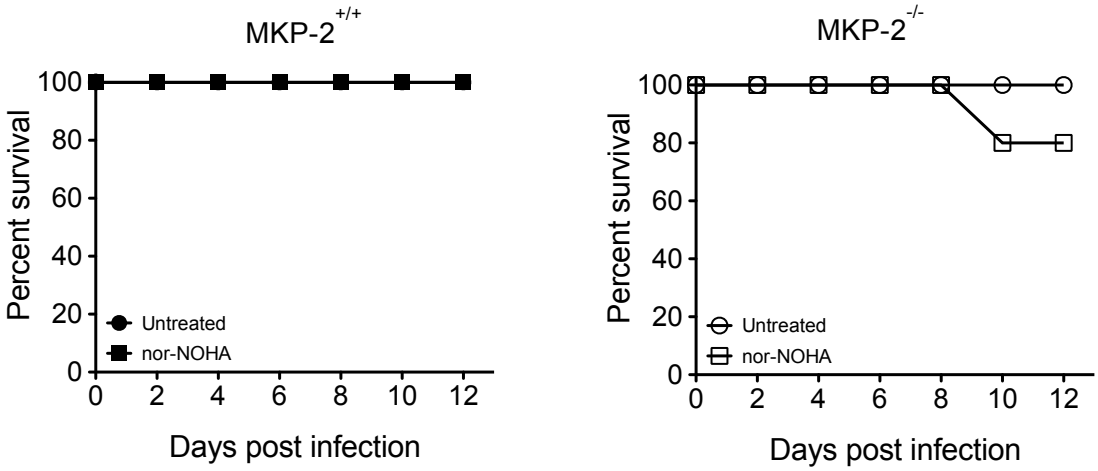


Figure 3.4.12. Nor-NOHA treatment of MKP-2 deficient mice does not increase mortality.

Mice were pre-treated with nor-NOHA (100μg/mouse) and treated daily following infection with 20,000 Pru tachyzoites expressing firefly luciferase and monitored for mortality. All experiments were carried out on at least 2 occasions with 5 animals per group.

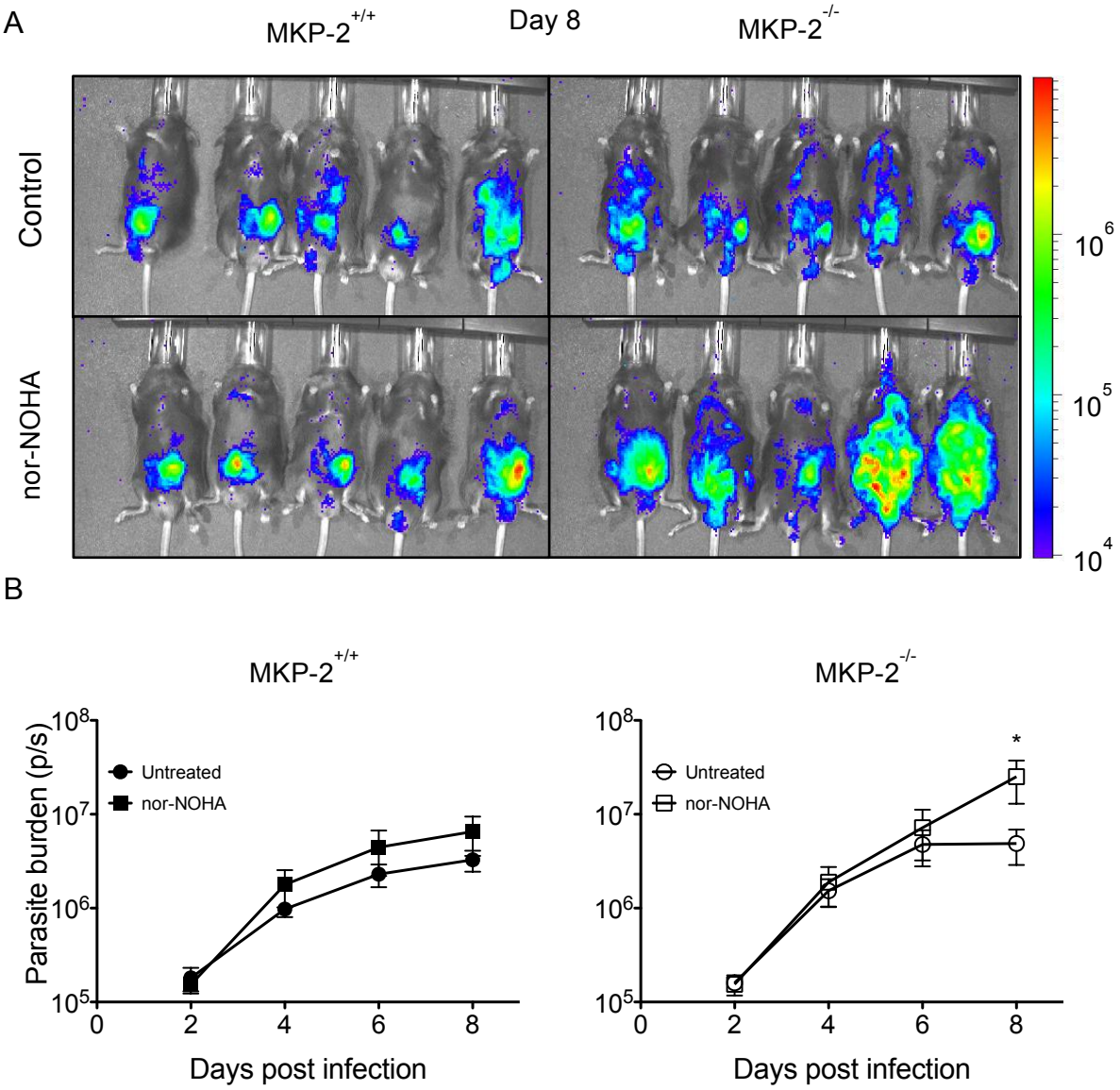


Figure 3.4.13. MKP-2$^{-/-}$ mice have an increased parasite burden following nor-NOHA treatment.

Mice were pre-treated with nor-NOHA (100μg/mouse) and treated daily following infection with 20,000 Pru tachyzoites expressing firefly luciferase. Mice were imaged every second day post infection. (B) Represents day 8 post-infection. Total flux (photons/second) was determined for each animal to determine parasite burden (C). Each value represents the mean of 5 mice per group ± SEM * P < 0.05. All experiments were carried out on at least 2 occasions

3.4.7. MKP-2 deficiency does not make macrophages more susceptible to infection with *T. gondii*.

We next determined whether the increased susceptibility of MKP-2$^{-/-}$ mice to infection with *T. gondii* was a result of their macrophages being more permissive to parasite growth as previously demonstrated for *L. mexicana* (AL-Mutairi *et al*., 2010). MKP-2$^{-/-}$ and MKP-2$^{+/+}$ bone marrow derived macrophages were infected with YFP expressing Pru *T. gondii* tachyzoites at a 1:1 ratio. In addition the macrophages were either treated with LPS and IFN-γ with or without L-NAME or nor-NOHA. Parasite growth was then determined by assessing YFP fluorescence. At 24, through to 72 hours post-infection parasite growth was similar in non-stimulated MKP-2$^{-/-}$ and MKP-2$^{+/+}$ macrophages (Figure 3.4.14). In addition, following LPS/IFN-γ stimulation parasite growth was significantly, and equally, reduced over the non-treated macrophages and this was irrespective of whether the macrophages were derived from MKP$^{+/+}$ or MKP$^{-/-}$ mice (Figure 3.4.14). Treatment of LPS/IFN-γ activated MKP-2$^{-/-}$ and MKP-2$^{+/+}$ macrophages with L-NAME partially ablated their ability to control parasite growth at 24 hours and totally ablated their ability to control parasite growth through 48 hours and 72 hours post-infection (Figure 3.4.14). Conversely, treatment of MKP-2$^{-/-}$ and MKP-2$^{+/+}$ macrophages stimulated with LPS/IFN-γ, with nor-NOHA partially ablated their ability to control parasite growth at 24 hours but not at 48 or 72 hours post-infection (Figure 3.4.14). While LPS/IFN-γ activation stimulated NO production as measured by nitrite production 72 hours post-infection in the supernatants of *T. gondii* infected MKP-2$^{-/-}$ and MKP-2$^{+/+}$ macrophages, this was significantly higher in the infected MKP-2$^{+/+}$ culture supernatants (Figure 3.4.15 A). Treatment of activated macrophages with L-NAME but not nor-NOHA largely ablated NO production by both MKP-2$^{-/-}$ and MKP-2$^{+/+}$ macrophages (Figure 3.4.15.B). As previously demonstrated (Butcher *et al*., 2011; Marshall *et al*., 2011) infection with *T. gondii* tachyzoites was found to enhance macrophage arginase-1 expression (Figure 3.4.15.B) although this was consistently higher in MKP-2$^{-/-}$

than in MKP-$2^{+/+}$ macrophages up to 24 hours post-infection. Consequently both iNOS and arginase-1 activities contribute to MKP-$2^{-/-}$ and MKP-$2^{+/+}$ macrophage control of *T. gondii* infection and despite differences in comparative expression macrophages from either MKP-$2^{-/-}$ or MKP-$2^{+/+}$ mice are equally able to control parasite growth.

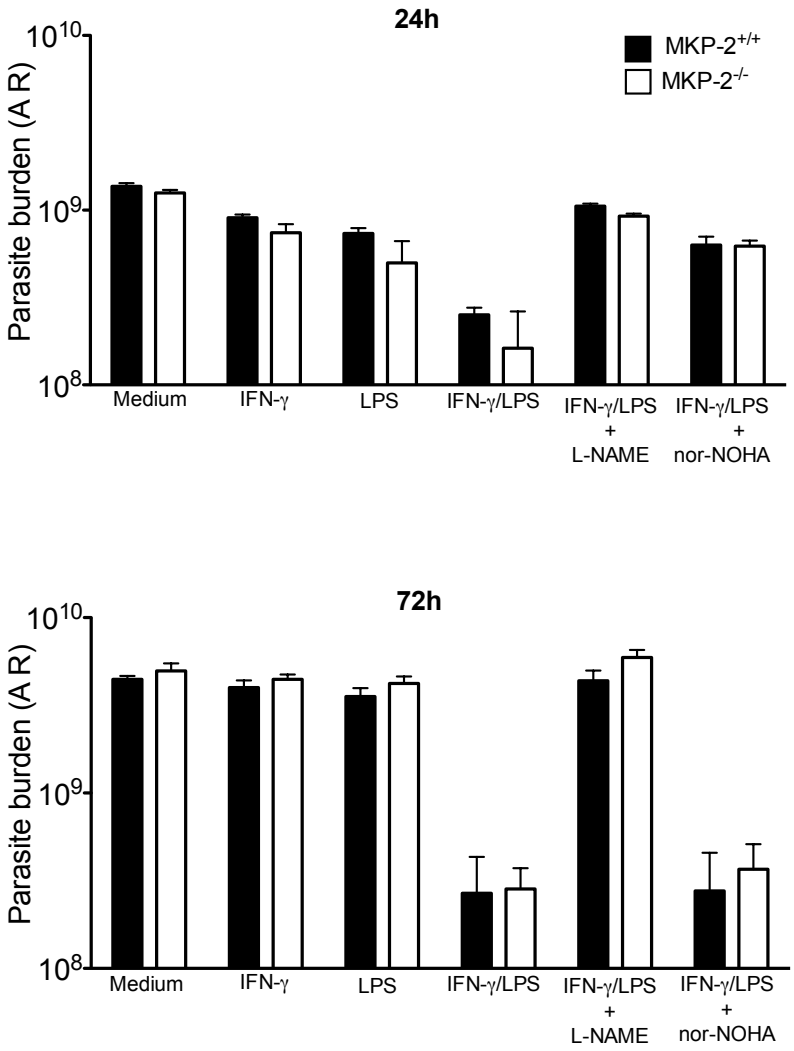
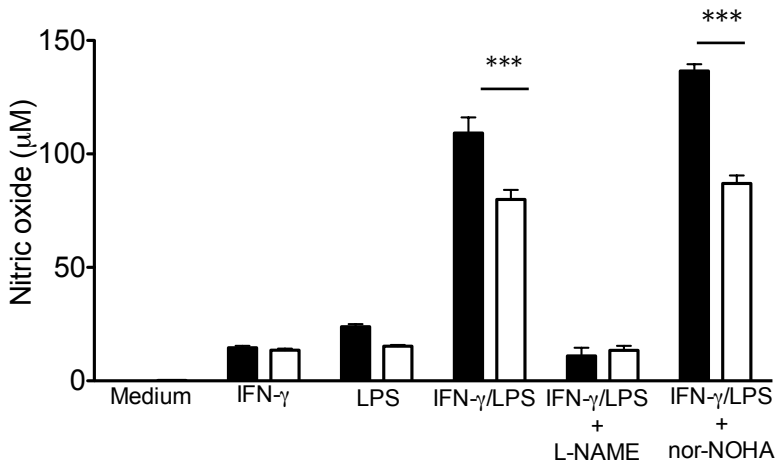


Figure 3.4.14. MKP-2 deficient macrophages are no more permissive to infection with *T. gondii* than wild-type macrophages.

BMD macrophages were treated as indicated with L-NAME, nor-NOHA, LPS and IFN-γ and subsequently infected with Pru tachyzoites expressing YFP. At 24 and 72 hours parasite burdens was determined by measuring YFP fluorescence in average radiance (AR). Each value represents 4 replicates ± SEM. All experiments were carried out on at least 2 occasions.

A.



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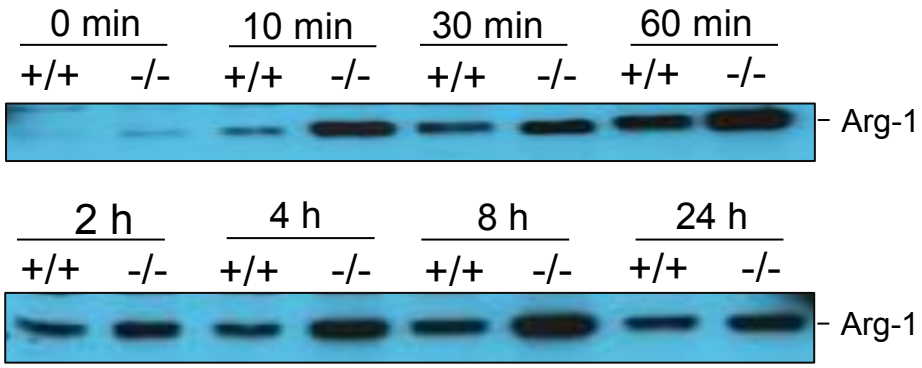

Figure 3.4.15. Infected MKP-2 deficient macrophages produce less NO but have increased arginase-1 than MKP-2$^{+/+}$ macrophages.

BMD macrophages were treated as indicated with L-NAME, nor-NOHA, LPS and IFN-γ and subsequently infected with Pru tachyzoites expressing YFP. Supernatants from cultures were assayed for nitrite content by Griess assay (A). BMD macrophage cell lysates were examined for arginase-1 expression following infection with type II *T. gondii*. Cells were not stimulated by other means. (B). Each value represents 3 replicates ± SEM. * P <0.05. All experiments were carried out on at least 2 occasions

3.5 Discussion

This study demonstrates an important role for MKP-2 in controlling infection with *T. gondii*. Infected MKP-$2^{-/-}$ C57BL/6 mice were found to be less able to control parasite growth during the both acute and chronic infection as well as increased mortality compared with their wild-type counterparts. The enhanced susceptibility of MKP-$2^{-/-}$ mice was associated with increased tissue arginase-1 expression, generally associated with Th2 responses, and at the same time serum nitrite levels, generally associated with type-1 responses, were down regulated. Nevertheless increased susceptibility was not associated with any significant modifications of the adaptive immune response between MKP-2 deficient and wild-type mice and the type-1 responses generated in infected MKP-$2^{-/-}$ mice were at least as strong as in their MKP-$2^{+/+}$ counterparts. The recently identified unique feature of MKP-2 as a negative regulator of macrophage arginase-1 expression and a positive regulator of macrophage iNOS expression (Al-Mutairi *et al*., 2010) would appear to underlie the important role of this member of the dual specific phosphatase family in controlling infection with *T. gondii.* While highlighting the importance of iNOS and NO production in controlling *T. gondii* infection the present study also uncovered a protective role for arginase-1 in controlling parasite multiplication that can compensate for NO deficiency during early infection.

Protective immunity against *T. gondii* is associated with a type-1 response where IFN-γ synergizes with TNF-α to activate macrophages and induce the expression of inducible nitric oxide synthase (iNOS) that catalyzes the formation of nitric oxide (NO) from L-Arginine. While NO can directly kill the parasites (Gazinelli *et. al*., 1993; Jun *et al*., 1993) some studies have also shown that NO promotes tachyzoite conversion to the much slower dividing bradyzoite form of the parasite through inhibition of mitochondrial and nuclear enzymes essential for parasite respiration (Bohne *et al*., 1993). Although iNOS seems to be the predominant pathway used by classically activated macrophages to control *T. gondii* proliferation in tissue culture, the role of NO during *in vivo* infection is less clear. Studies using iNOS-deficient mice have shown that mice

lacking iNOS are able to survive and control tachyzoite growth during the acute stage of infection via an IFN-γ dependent mechanism and only succumb during chronic toxoplasmosis (Scharton-Kersten *et al*., 1997). Death was associated with uncontrolled proliferation of tachyzoites in the brain, suggesting that the protective anti-*Toxoplasmic* effect in the brain is iNOS-dependent (Scharton-Kersten *et al*., 1997, Khan et al., 1997). Nevertheless, the observation that iNOS deficient mice were able to survive acute infection in an IFN-γ mediated manner suggested that there were alternative pathways other than NO production mediating anti-*Toxoplasma* resistance *in vivo* (Khan *et al*., 1998).

In the present study we found that L-NAME treatment of wild-type mice infected with 20,000 type-II Prugniaud-FLUC tachyzoites, but not MKP-2$^{-/-}$ mice, resulted in enhanced parasite growth and mortality early in infection. This indicated that an NO independent mechanism was playing a protective role in MKP-2$^{-/-}$ mice and controlling parasite growth under conditions where NO generation was being inhibited. Previously induction of the IFN-γ inducible gene Indoleamine 2,3 dioxygenase (IDO) has been implicated in mediating some of the IFN-γ dependent NO independent anti-*toxoplasma* activity (Pfefferkorn, & Guyre, 1984). IDO catalyzes the degradation of the essential amino acid L-tryptophan through the kynurenine pathway, thereby compromising metabolic processes of the parasite (Fujigaki *et al.*, 2002). Interestingly the relative contributions of iNOS and IDO to parasite control appear to be tissue specific (Fujigaki *et al.*, 2002). More recently immunity-related GTPases (IRGs) have emerged as potent effectors of *T gondii* killing in mice (Taylor *et al*., 2000; Collazo *et al*., 2001). Thus, murine astrocytes have been shown to have the ability to kill intracellular *T. gondii* independently of iNOS and IDO via IFN-γ inducible IRGs (Halonen et al 1998 and 2001; Melzer *et al*., 2008). Different IRGs have been shown to play roles in controlling acute and chronic infection (Martens *et al*., 2005, Hunn *et al.*, 2008) although the mechanisms through which p47 GTPases confer resistance to *T. gondii* infection have not been determined (Butcher *et* al., 2005; Howard *et al*., 2011). However, as no

difference in IFN-γ production was noted between infected MKP-$2^{+/+}$ and MKP-$2^{-/-}$ mice infected with *T. gondii* this would suggest that neither differential IDO or GTPase production were associated with the NO independent resistance demonstrated by MKP-$2^{-/-}$ mice and that an IFN-γ independent mechanism was operating to protect these mice in the absence of NO production.

Butcher and colleagues (2011) have recently demonstrated that the type-1 strain *T. gondii* parasites deficient in ROP16 have enhanced growth in macrophages, and *in vivo* infection results in increased parasite multiplication and dissemination in the host. This has been associated with the inability of ROP16 deficient parasites to induce STAT-6 mediated arginase-1 expression which is needed to deplete the host cell L-arginine which is required by the parasite. It has previously been demonstrated that *T. gondii* is an L-arginine auxotroph (Fox *et al.*, 2004) and parasite multiplication in the host cell is L-arginine dependent. Type-2 parasites lack ROP-16 expressed by type-1 strains (Butcher *et al.*, 2011; Jensen *et al.*, 2011) but in the course of the present study these parasites were shown to increase arginase-1 expression in both MKP-$2^{+/+}$ and MKP-$2^{-/-}$ macrophages. While often thought of as a Th2 associated product of alternative macrophage activation, innate macrophage activation via TLR-4 ligation (Menzies *et al.*, 2011; Shweash *et al.*, 2011) has also been shown to induce arginase-1 expression. As *T gondii* has been demonstrated to have a number of TLR-4 ligands such as GPI anchors (Yap *et al.*, 2006) and HSP70 (Debierre-Grockiego *et al.*, 2007; Mun *et al.*, 2005) this is the likely source of the STAT-6 independent induction of arginase-1 by type-2 strain parasites as demonstrated in the present study. *In vivo* treatment with nor-NOHA to inhibit arginase-1 activity during the course of the present study resulted in enhanced parasite multiplication in MKP-$2^{-/-}$ mice. This indicated that the enhanced expression of arginase-1 expression in these mice could in some part compensate for iNOS deficiency compared with MKP-$2^{+/+}$ animals.

Many studies suggest that macrophage killing of parasites via iNOS catalyzed NO production is the main mechanism by which *T. gondii* parasite multiplication

is controlled (El Kasmi *et al*., 2008; Li *et al*., 2012) and that L-arginine depletion by arginase-1 counter-regulates the effectiveness of iNOS and facilitates parasite growth. Our *in vitro* studies utilizing classically activated BMDM clearly demonstrate that iNOS catalyzed NO production plays the major role in controlling parasite growth and this could be reversed by treatment of cultures with L-NAME. This was irrespective of whether MKP-$2^{-/-}$ or MKP-$2^{+/+}$ macrophages were examined. Interestingly despite producing less NO than activated MKP-$2^{+/+}$ macrophages activated MKP-$2^{-/-}$ macrophages were equally able to control parasite growth. Indeed inhibition of arginase-1 activity by nor-NOHA treatment did not facilitate parasite killing in activated MKP-$2^{-/-}$ or MKP-$2^{+/+}$ macrophages but in fact reversed inhibition of parasite growth early under conditions of activation. This clearly indicates a role for arginase-1 in protection against *T. gondii*. Surprisingly no differences in intracellular parasite growth in MKP-$2^{-/-}$ versus MKP-$2^{+/+}$ macrophages *in vitro* were noted suggesting the differential weighting of the alternative control mechanisms were compensating for each other over the course of the experiment. Nevertheless the *in vivo* consequences of MKP-2 deficiency are significant in terms of parasite growth and long-term survival that would be in keeping with NO playing a role post-acute infection (Scharton-Kersten *et al*., 1997). It is also likely that cells other than macrophages are contributing to the dysregulated iNOS/arginase-1 expression bias *in vivo* in infected MKP-$2^{-/-}$ mice. Of particular note is that neutrophils, that have been shown to play a significant role in early *T. gondii* infections (Bliss *et al.,* 2001), are major sources of arginase-1 activity *in vivo* (Munder *et al*., 2009).

Our studies on the consequences of MKP-2 deficiency have revealed some fascinating insights into the control of *T. gondii* infection. Firstly NO induction is ultimately of paramount significance in controlling parasite multiplication and host survival. However, in the absence of NO production enhanced arginase-1 is able to in part compensate for this deficiency presumably by starving the parasite of L-arginine as previously demonstrated (Butcher *et al.*, 2011). Previously it has been suggested that arginase-1 stimulates parasite growth by

converting L-arginine to the polyamines needed by the parasite (Cook *et al.,* 2007) or inhibiting iNOS activity by L-arginine depletion (El-Kasmi *et al*., 2008). We could find no evidence for this. Rather we would propose that arginase-1 and iNOS work together to control parasite multiplication by a combination of L-arginine starvation (arginase-1 and iNOS) and NO killing (iNOS).

Overall our results demonstrate that MKP-2 through its ability to reciprocally modulate arginase-1 and iNOS expression is a key regulator in L-arginine metabolism and consequently this has clear consequences for the control of intracellular parasites. Furthermore as arginase-1 has also been shown to have potent T cell modulatory effects (Modolell *et al*., 2009) MKP-2 influences are likely to have significant consequences for inflammatory disease and cancers where arginase-1 and iNOS have already been identified as key players (Cao *et al*., 2009; Thanan *et* al 2012; Waha *et al.*, 2010). These observations identify manipulation of MKP-2 expression or activity as a significant target for future therapeutic strategies.

3.6 Critical discussion

This chapter focused on examining the outcome of infection of MKP-2 deficient mice with *Toxoplasma gondii* in order to determine any possible role that MKP-2 may have in the immune response. Immunity to infection and control of the growth of the parasite has been shown to be largely but not wholly dependent on the production of Nitric Oxide (NO) as a result of the enzymatic activity of inducible nitric oxide synthase (iNOS) on the substrate L-arginine. Previous studies have suggested that arginase-1 can enhance parasite growth by depleting the L-arginine store needed by iNOS and MKP-2 was recently demonstrated to play a role in regulating iNOS and arginase so this became a major focus of the work carried out.

The initial phenotype following infection with *T. gondii* was established using Beverely strain *T. gondii.* However, this model is limited due to excess mortality following infection. The decision was made to use a less virulent Prugniaud *T. gondii* transfected with firefly luciferase. This allowed the infection and parasite burden to be tracked using an *in vivo* bioluminescent imaging system. This allowed a more accurate determination of how the knockout was affecting parasite numbers. This determination is possible using the wild type strains although this can only be established after death and requires a large number of mice in each group in order that the group sizes at each time point can provide reliable statistical analysis. *In vivo* bioluminescent imaging offers a non-invasive means of tracking infection over time in the same group of animals, so reducing the number of animals needed for an experiment. However it must be noted that *in vivo* imaging can be influenced by a number of factors such as the location of infection, which may limit the light being emmitted. The luciferase activity is ATP and oxygen dependent so as mice get sicker the availability of oxygen may become limited so reducing the signal.

The work presented in this chapter demonstrates that the increased susceptability of MKP-$2^{-/-}$ mice to infection was not as a result of a down-regulated type 1 phenotype as determined by IFN-γ production. As stated an important mediator in protection against *T. gondii* in mice, is IRG proteins, induced by IFN-γ (Howard *et al*., 2011). As no difference in IFN-γ was detected between MKP-$2^{-/-}$ and MKP-$2^{+/+}$ mice it would seem unlikely that differential IRG expression would be affecting parasite growth. However, during the course of the study IRG expression was not determined to definitively say this mechanism of protection against *T. gondii* was not altered by the MKP-2 deletion. If this work were to be taken forward it would be prudent to determine any differences in IRG expression and this could be achieved by real-time PCR or western blot analysis of cell extracts. It should also be taken into account that there may be localised differences in immune phenotype. IFN-γ production was established in splenocytes. However IFN-γ from cells located at

other infection sites, such as cytotoxic $CD8^+$ T cells and NK T cells may have a different profile and this should be investigated further.

A focus of this study was the effect of the enzymes iNOS and Arginase-1 that compete for the same substrate, L-arginine, in controlling *T. gondii* infection. To achieve this, the competitive inhibitors L-name and nor-NOHA to inhibit iNOS and arginase-1 respectively were used during infections with *T. gondii*. Although established in the literature that these enzyme inhibitors are specific it is well established that the enzymes can counteract each other as they compete for their common substrate L-arginine (Chicoine *et al.,* 2004; Stanley *et al*., 2006). The extent of these possible effects *in vivo* was not established and further analysis would be required to check for unexpected effects. In an *in vivo* setting establishing this is complicated by the fact that both enzyme inhibitors are reversible and it is unknown as to what exactly the concentration of the inhibitors are in the tissues. So removing the tissues for analysis of enzyme activity may result in loss of the inhibitor. Consequently determination of any off target effect is best established by looking at the enzymatic by products. In nor-NOHA treated groups this would be achieved by measuring nitrite by Griess assay of the serum from treated animals. The effect of L-name on arginase activity could be determined by using mass spectrometery analysis of the serum for L-ornithine, one of the by products of arginase-1 activity. *In vitro,* where the concentrations of the inhibitors are known throughout the experiment, it is easier to determine the effects of the inhibitors using methods such as the arginase activity assay where the inhibitor concentration can be maintained throught the course of the assay.

Summary Discussion

Toxoplasma gondii is a globally ubiquitous pathogen capable of causing a persistent infection in the host. Currently there is no vaccine available for use in humans. In this thesis we examine the potential of supertype HLA restricted peptide and a live attenuated vaccine to protect against oocyst initiated infections and we investigate the role of MKP-2 during *T. gondii* infection. Recently MKP-2 has been demonstrated to regulate Arginase-1 and iNOS expression (Al-Mutairi *et al.*, 2010) both of which play significant roles in determining the outcome of infection with intracellular parasites (El-Kasmi *et al.*, 2008). Therefore, in parallel as part of studies to determine the mechanisms underlying the induction of protective immune responses against *T. gondii* we used a recently generated MKP-2 deficient mouse strain on a C57BL/6 background to study the effect of MKP-2 on *T. gondii* infection.

Our studies determined that vaccines composed of HLA-A*0201, HLA-A*1101 or HLA-B*0702 restricted peptides are not protective during oocyst challenge in HLA-A*0201, HLA-A*1101 or HLA-B*0702 transgenic mice. This is in contrast to previous studies that demonstrated that the same peptide vaccinations are protective during tachyzoite challenge (Cong *et al.,* 2010, 2011, 2012). This lack of vaccine efficacy may be due to a number of reasons. As discussed earlier the peptide pools for each HLA supertype group contain peptides from a limited repertoire of *T. gondii* proteins and contained epitopes absent from or that have reduced expression in the sporozoites which are contained within the oocyst and are released after rupture. Consequently, the immune system is not primed against the first stage of the *T. gondii* life cycle that it will encounter. However, this study demonstrates the potential of the ΔRPS13 live attenuated strain of *T. gondii* as a vaccine candidate as it enhanced survival during oocyst infection and proved to be a more potent inducer of SLEC $CD8^+$ T cells than the peptide vaccines.

The natural route of infection for *T. gondii* is via the mucosal surfaces of the body, typically by ingestion. Thus development of a protective mucosal immune response is therefore an important goal of vaccination. This is something that is

difficult to achieve with systemic vaccination strategies and best achieved with oral or nasal delivery (Holmgren & Svennerholm 2012). Oral vaccination with recombinant *T. gondii* products has been a successful strategy in a number of studies (Dimier-Poisson *et al*., 2006, Qu *et al.,* 2009). The efficacy of the current class 1 HLA restricted vaccine strategy could be improved by inducing mucosal immunity. Alternative vaccine delivery methods would be needed to ensure the epitopes survive the oral route.

One possible adjuvant that could be used are bilosomes. Bilosomes are vesicles comprised of non-ionic surfactants ands and bile salts and as such they are able to survive oral administration (Conacher *et al*., 2001). Previously bilosomes have been demonstrated to be capable of inducing strong systemic and mucosal immunity in models using measles, influenza and tetanus epitopes (Conacher *et al*., 2001; Mann *et al*., 2006). However, the incompatibility of the peptides utilised in this study with NISV entrapment, and therefore most likely bilosomes, rules out the use of bilosomes for oral delivery. Currently studies are under way utilising DNA immunisition using poly epitope constructs of the HLA restricted epitopes using a number of vectors such adenovirus and entrapment of the plasmid constructs and proteins within bilosomes. Preliminary data, not included as part of the current thesis, are promising and suggests that such a strategy might promote protective immunity.

In this study as in the studies undertaken by Cong *et al.,* (2010, 2011, 2012) antigen specific IFN-γ production was analysed as a read out for the protective immune response induced by the HLA restricted epitopes given with the PADRE and GLA-SE adjuvants. While this gives an indication of immunological activity, it does not provide information of the quality of the immune response or the potential for memory. It is now generally accepted that a high quality response is one, in which not only IFN-γ^+ T cells are induced but also IFN-γ/TNF-α double positive T cells. The strongest response has been shown to be associated with IFN-γ, TNF-α, and IL-2 triple positive T cells (Makedonas & Betts 2006, Darrah *et al.*, 2007), with the additional IL-2 being correlated with an effective memory

response. Durrah *et al*., (2007) demonstrated that double or triple positive T cells were in fact better producers of TNF-α and IFN-γ than single positive cells in a vaccine model against *L. major.* Further studies demonstrated that polyfunctional $CD8^+$ T-cells are important for controlling infection, and inducing them during vaccination can lead to enhanced survival upon challenge (Hersperger *et al*., 2012; Rodrı *et al*., 2012). A recent study by Bhadra *et al*., (2011, 2012) have highlighted the importance of polyfunctional $CD8^+$ T cells during *T. gondii* infection, with their depletion leading to an increased susceptibility to infection. Using the peptide pools selected for their class I HLA restriction and re-screening with these conditions for inducing double or triple positive cells may improve the selection of peptides.

Oocyst challenge is considered the gold standard for successful vaccination in *T. gondii* infection. Oocysts are widely accepted to be the most infectious stage of the parasite life cycle. Dubey *et al*., (2011) used the same HLA transgenic mice and their BALB/c and C57BL/6 parental strains in oocyst challenge assays with a large array of *T. gondii* strains. They actually found that the HLA transgenic mice displayed a greater susceptibility to infection than their parental strains (Dubey *et al*., 2011). The underlying mechanism for this still remains unknown. One hypothesis is that the HLA transgene may not be properly interacting with the murine T cell receptor. An experiment that would determine if this is the case would be to infect the HLA-A*0201, HLA-A*1101 and HLA-B*0702 transgenic mice and their parental strains with ΔRPS13. Dendritic cells derived from naïve mice, from each of the mouse strains would then be pulsed with lysate antigen and incubated with splenocytes derived from the infected mice. For example, HLA-B*0702 T cells with HLA-B*0702 DCs, BALB/c T cells with HLA-B*0702 DC's, and BALB/c T cells with BALB/c DCs as a baseline. A difference in functional response, determined by $CD8^+$ T cell populations and their effector response could indicate a problem with the HLA-TCR interaction. If this is the case then it could pose a problem in using these transgenic mice as a model for developing fully protective vaccines against the *T. gondii* oocyst.

The vaccine efficacy may also be improved by screening for immunodominant epitopes that are HLA class II CD4 restricted. Although their use would be limited in the current class I HLA transgenic mouse model, if the ultimate goal is a vaccine for use in humans, then *T. gondii* specific CD4 T cell epitopes may induce greater CD4 help than the non-specific pan CD4 PADRE epitope.

Currently seven class II HLA supertypes have been identified (Greenbaum *et al.*, 2011). These supertypes are now available on the immune epitope database so a similar approach of screening *T. gondii* proteins against these alleles has the potential to enhance the vaccines overall efficacy. A combined vaccine including HLA class I and class II restricted peptides, which are present in the tachyzoite, bradyzoite and sporozoite stages of infection would likely prove more efficacious against oocyst challenge.

As previously discussed the HLA transgenic mice being used in this study may not be a totally reliable model of the efficacy of the HLA restricted peptide vaccines, owing to species differences in antigen processing and presentation and the interaction of human HLA moleucles with murine T cell receptors. A potential means of overcoming this in order to test the viability of a vaccine designed with an immunogenetic approach, with the ultimate goal of use in humans, would be the use of NOD-SCID/γ-chain$^{-/-}$ mice transfected with an HLA transgene (Koo *et al*., 2009; Strowig *et al*., 2009). These mice lack both T cells and B cells and possessing the common gamma chain knockout are profoundly deficient in an innate immune response. If these mice were reconstituted with, HLA matched, human hematopoietic stem cells (Koo *et al*., 2009; Strowig *et al*., 2009; Lepus *et al* 2009) and treated with human GM-CSF and IL-4 (Chen *et al*., 2013) then they would have a functioning human immune system. Peptides selected by bioinformatics analysis and screened for their ability to elicit an IFN-γ response from HLA matched PBMCs derived from chronically infected humans could then be accurately analysed for their vaccine efficacy in this *in vivo* model. The resulted yielded would be more applicable to the ultimate goal of the vaccine, which is use in humans.

It should be noted that such an approach to vaccine design, while giving a more accurate readout of the vaccine efficacy in a human system has some considerable ethical considerations owing to the need to use human stem cells derived from foetal liver. Stem cells derived from cord blood could also be used. However, the number of cells derived from this source may be a limiting factor. It should also be considered that there might be practical difficulties in getting enough mice, which have been successfully reconstituted so as to give group sizes that will yield statistical significance. Nevertheless this *in vivo* model presents an interesting and innovative way of furthering the search for HLA restricted vaccines against *Toxoplasma gondii* that theoretically would be directly translatable to humans.

In this thesis we also demonstrated the dual specific phosphatase MKP-2 results in an increased susceptibility to infection with *T. gondii*. The infected mice displayed increased parasite burden and mortality rate. We also highlighted that early resistance to *T. gondii* in MKP-$2^{+/+}$, but not MKP-$2^{-/-}$, mice was NO dependent as infected MKP-$2^{+/+}$, but not MKP-$2^{-/-}$ mice succumbed within 10 days post–infection with increased parasite burdens following treatment with the iNOS inhibitor L-NAME. Conversely, treatment of infected MKP-$2^{-/-}$ but not MKP-$2^{+/+}$ mice with nor-NOHA increased parasite burdens indicating a protective role for arginase-1 in MKP-$2^{-/-}$ mice. *In vitro* studies using tachyzoite-infected bone marrow derived macrophages and selective inhibition of arginase-1 and iNOS activities confirmed that both iNOS and arginase-1 could mediate anti-parasite growth. However, the effects of arginase-1 were transient and ultimately the role of iNOS was important in facilitating long-term inhibition of parasite multiplication within macrophages.

To take this work forward it would be interesting to examine the intraperitoneal wash of infected MKP-$2^{+/+}$ and MKP-$2^{-/-}$ mice by flow cytometery to sequentially examine what cells have infiltrated into the infection site following intaperitoneal infection. Neutrophils would be of particular interest and have been previously shown to be present early in *T. gondii*

infections (Abi-Abdallah *et al.,* 2011 and 2012). In humans neutrophils have been shown to be major arginase-1 producers (Munder *et al*., 2009). Are murine neutrophils major producers of arginase-1 and do MKP-$2^{-/-}$ neutrophils have enhanced arginase-1 and reduced nitric oxide profiles compared with MKP-$2^{+/+}$ neutrophils? This remains to be examined but could be extremely influential in determined the outcome of *T. gondii* infection.

Arginase-1 metabolism has been shown to have potent T cell modulatory effects (Modolell *et al*., 2009). L-arginine depletion has been shown to have a detrimental effect on T cell proliferation and IFN-γ production at the infection site but not in the local draining lymph nodes of *Leishmania* infection (Modolell *et al*., 2009. Although the present study did not demonstrate a deficiency in the Th-1 response, cells isolated for this analysis were isolated from the spleens, which have not been shown to be the primary lymph draining site for the peritoneal cavity (Parungo *et al*., 2008), T cells from the peritoneum or other sites where *Toxoplasma* parasites are present could well be displaying a different profile. In addition to increased parasite numbers in the brain in MKP-$2^{-/-}$ mice compared with MKP-$2^{+/+}$ mice there were also increased burdens in the liver and lungs in the present studies.

This work points towards the manipulation of L-arginine metabolism in the host as a potentional method controlling infection with *T. gondii*. It has been established that the bioavilability of L-arginine can affect the outcome of infection; its reduced availability has been demonstrated to hinder T cell activation and as a result impair the T cell response to infection (Rodriguez *et al*., 2008; Bronte *et al*., 2005; Munder *et al.,* 2009). Work carried out by Takele *et al*., (2012) has demonstrated that increased arginase activity, and therefore a reduced L-arginine pool, is associated with poor prognosis in patients infected with visceral leishmaniasis. Depletion of the L-arginine in the microenvironment would result in the eventual impairment of iNOS and arginase activity - the products of which are involved in polyamine biosynthesis. This importance of L-arginine highlights that supplementation of the amino

acid, in parallel with traditional treatments pyrimethamine and sulphadizine, could aid the treatment of *T. gondii* infection. This would help enhance iNOS activity following infection and ensure an adaptive T response to infection. This could be validated *in vivo* through supplementing *T. gondii* infected mice with L-arginine and monitoring the outcome of infection in terms of parasite burden in both the acute and chronic stages of infection, as well as the quality of the immune response generated.

Alternatively, as MKP-2 has been demonstrated to be a negative regulator of arginase 1 expression, the downregulation of arginase 1 through increasing the expression of MKP-2 could potentially be used as a method of aiding the treatment of infection. However, at present there are no drug treatments available to induce the over expression of MKP-2. The only described method of achieving this is with the use of an adenovirus, transfected with MKP-2 (Al-Mutairi *et al*., 2010b). The therapeutic potential of stents coated with adenovirus to induce overexpression of MKP-2 to inhibit apoptosis and restenosisis currently being investigated for use in the treatment of heart disease (personal communication)

To conclude, the work described in this thesis has demonstrated that HLA class I restricted peptide vaccines used in HLA class I transgenic mice were not protective upon oocyst infection. However, we did demonstrate ΔRPS13 to be a potential vaccine candidate. The effectiveness of ΔRPS13 compared to that of the peptide subunit vaccines at inducing a SLEC $CD8^+$ T cell response also highlights the pitfalls in the use of subunit vaccines, which may fail to fully initiate an immune response similar to that of a natural infection. Our results also demonstrate that MKP-2 through its ability to reciprocally modulate arginase-1 and iNOS expression is a key regulator in L-arginine metabolism and consequently this has clear consequences for the control of intracellular parasites. These observations identify manipulation of MKP-2 expression or activity as a significant target for future therapeutic strategies.

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Appendix

Conferences attended

Woods Hole Immunoparasitology Conference, 2010

Woods Hole Immunoparasitology Conference, 2011 – Poster presentation

11th International Congress on Toxoplasmosis, 2011 – Poster presentation

Submitted abstracts for conference

Abstract submitted for Woods Hole Immunoparasitology Conference, 2011 and 11th International Congress on Toxoplasmosis, 2011.

Woods, S., Schroeder, J., Plevin, R., Roberts, C.W. and Alexander, J.

MKP-2 deficiency results in increased susceptibility to *Toxoplasma gondii* infection

A dual specific phosphatase 4 (DUSP-4) deficient murine model backcrossed onto the C57BL/6 background has been used to dissect the role of MAP Kinase phosphatase 2 (MKP-2) during infection with *Toxoplasma gondii.* In vivo infection intra-peritoneally with *T.gondii* (Beverly strain) resulted in greater mortality in MKP-2 deficient mice compared with their wild-type counterparts. Further in vivo studies utilized a type 2 strain *T.gondii,* expressing a firefly luciferase gene to monitor parasite growth longitudinally following intra-peritoneal injection by whole body imaging using a charge-coupled device. This demonstrated a significantly increased parasite burden in MKP-$2^{-/-}$ compared with MKP-$2^{+/+}$ mice throughout the acute stage of infection. At 30 days post infection, during the chronic phase of infection, *ex vivo* imaging of the brain also indicated an increased parasite burden in MKP-$2^{-/-}$ mice compared with their wild-type counterparts. Surprisingly spleen cell stimulation assays on days 7, 9 and 30 post-infection resulted in increased splenocyte IFN-γ production in response to stimulation with *T.gondii* derived antigen in MKP-$2^{-/-}$ compared

with MKP-$2^{+/+}$ mice. MKP-2 has previously been shown to be a negative regulator of arginase 1 expression and it is has been suggested that *T. gondii* promotes infection by upregulating arginase expression and activity and consequently down-regulating NO production. *In vitro* stimulation of bone marrow derived macrophages with LPS or infection with *T. gondii* resulted in significantly increased expression of arginase 1 in MKP-$2^{-/-}$ compared with MKP-$2^{+/+}$ mice. While parasite enumeration using the CCD camera, indicated a trend for an increased parasite number in the MKP-$2^{-/-}$ macrophages compared with wild-type macrophages this was significant when nitric oxide activity was inhibited with L-Name. These results demonstrate that MKP-2 by regulating arginase expression at the level of the host macrophage may have an important role in determining the outcome of infection with *T. gondii.*

MAP kinase phosphatase-2 plays a key role in the control of infection with *Toxoplasma gondii* by modulating iNOS and arginase-1 activities

Stuart Woods, Juliane Schroeder, Robin Plevin, Craig W. Roberts and James Alexander

Strathclyde Institute of Pharmacy and Biological Sciences, University of Strathclyde, Glasgow

INTRODUCTION

Mitogen activated protein (MAP) kinase pathways play a pivotal role in the immune response. MAP kinase phosphatases (MKP) are a family of dual specific phosphatases (DUSP) crucial for the dephosphorylation of threonine and tyrosine and therefore deactivation of MAP kinases. 11 different isoforms are known to exist with a variety of sub-cellular localisations, structures and regulation mechanisms. MKP-2 is a nuclear located class 1 DUSP induced by stress mechanisms such as LPS as well as growth factors and hormones. It exerts its effect through the deactivation of ERK and JNK *in vitro*. The dual specific phosphatase, MAP kinase phosphatase-2 (MKP-2) has recently been demonstrated to negatively regulate macrophage arginase-1 expression, while at the same time to positively regulate iNOS expression. Consequently, MKP-2 was thought to likely play a significant role in the host interplay with intracellular pathogens.

In a recent study, MKP-2 deficient mice displayed an increased susceptibility to *Leishmania mexicana* with increased lesion sizes and parasite burdens (Al-Mutairi *et al.*, 2010). BMD macrophages from MKP-2$^{-/-}$ mice were more permissive to parasite growth than wild-type macrophages and this phenotype could be reversed by the addition of nor-NOHA, a competitive substrate for Arginase 1. During infection with *Toxoplasma gondii* NO plays a key role in parasite control however they are also arginine auxotrophs. Recently an arginase-1 dependent method of parasite control was defined (Butcher *et al.*, 2011). **The aim of the present study was to evaluate the role of MKP-2 during infection with *Toxoplasma gondii*.**

RESULTS

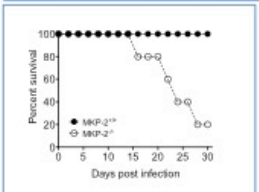

Figure A. MKP-2$^{-/-}$ mice have an increased susceptibility following infection with *T. gondii*. Intraperitoneal infection with 20 cysts of the Beverely (Type II strain) of *T. gondii* resulted in significant mortality 15-25 days post – infection in MKP-2$^{-/-}$ but not it MKP-2$^{+/+}$ mice

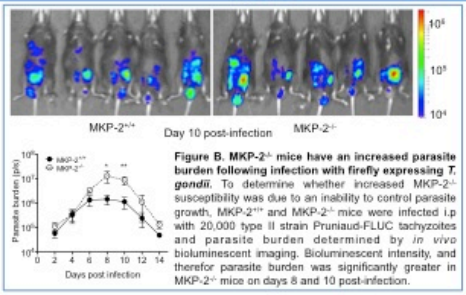

Figure B. MKP-2$^{-/-}$ mice have an increased parasite burden following infection with firefly expressing *T. gondii*. To determine whether increased MKP-2$^{-/-}$ susceptibility was due to an inability to control parasite growth, MKP-2$^{+/+}$ and MKP-2$^{-/-}$ mice were infected i.p with 20,000 type II strain Pruniaud-FLUC tachyzoites and parasite burden determined by *in vivo* bioluminescent imaging. Bioluminescent intensity, and therefor parasite burden was significantly greater in MKP-2$^{-/-}$ mice on days 8 and 10 post-infection.

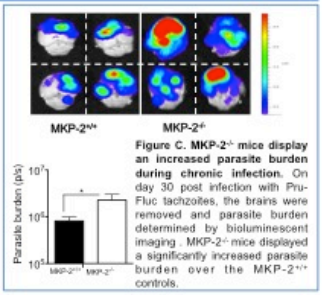

Figure C. MKP-2$^{-/-}$ mice display an increased parasite burden during chronic infection. On day 30 post infection with Pru-Fluc tachzoites, the brains were removed and parasite burden determined by bioluminescent imaging . MKP-2$^{-/-}$ mice displayed a significantly increased parasite burden over the MKP-2$^{+/+}$ controls.

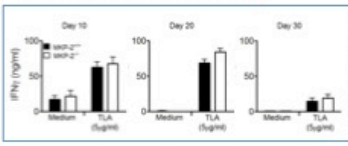

Figure D. MKP-2$^{-/-}$ mice do not have an impaired immune response. To determine if the increased susceptibility to infection was due to an impaired immune response, spleens were taken on 10, 20 and 30 days post-infections and splenocytes stimulated with TLA. IFN-γ, IL-4, IL-5 and IL-10 concentrations in the supernatant were assayed by ELISA. There was no difference between the groups.

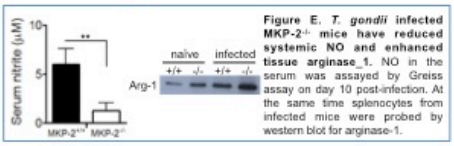

Figure E. *T. gondii* infected MKP-2$^{-/-}$ mice have reduced systemic NO and enhanced tissue arginase_1. NO in the serum was assayed by Greiss assay on day 10 post-infection. At the same time splenocytes from infected mice were probed by western blot for arginase-1.

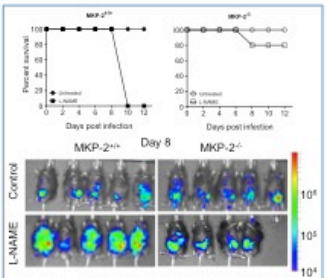

Figure F. Inhibition of NO by L-NAME enhances susceptibility of MKP-2$^{+/+}$ but not MKP-2$^{-/-}$ mice to *T. gondii* infection. To determine if increased NO in the MKP-2$^{+/+}$ mice was responsible for their reduced parasite burden over the MKP-2$^{-/-}$ mice, NO production was inhibited with L-NAME treatment. MKP-2$^{+/+}$ mice rapidly succumbed to infection, with enhanced parasite burden.

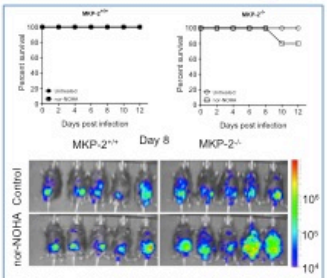

Figure G. Inhibition of Arginase-1 by nor-NOHA enhances susceptibility of MKP-2$^{-/-}$ but not MKP-2$^{+/+}$ mice to *T. gondii* infection. Mice were treated with nor-NOHA to inhibit arginase-1 to determine its role in controling parasite growth. MKP-2$^{-/-}$ mice displayed a small but significant increase in parasite burden over the MKP-2$^{+/+}$ mice.

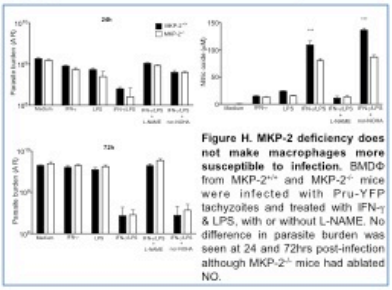

Figure H. MKP-2 deficiency does not make macrophages more susceptible to infection. BMDΦ from MKP-2$^{+/+}$ and MKP-2$^{-/-}$ mice were infected with Pru-YFP tachyzoites and treated with IFN-γ & LPS, with or without L-NAME. No difference in parasite burden was seen at 24 and 72hrs post-infection although MKP-2$^{-/-}$ mice had ablated NO.

SUMMARY

Here we demonstrate that MKP-2$^{-/-}$ mice on the C57BL/6 background have enhanced susceptibility compared with wild-type counterparts following infection with type-2 strains of *Toxoplasma gondii* as measured by increased parasite multiplication during acute infection, increased mortality from day 12 post-infection onwards and increased parasite burdens in the brain, day 30 post-infection. MKP-2$^{-/-}$ mice did not, however, demonstrate defective type-1 responses compared with MKP-2$^{+/+}$ mice following infection although they did display significantly reduced serum nitrite levels and enhanced tissue arginase-1 expression. Early resistance to *T. gondii* in MKP-2$^{+/+}$, but not MKP-2$^{-/-}$, mice was nitric oxide (NO) dependent as infected MKP-2$^{+/+}$, but not MKP-2$^{-/-}$ mice succumbed within 10 days post-infection with increased parasite burdens following treatment with the iNOS inhibitor L-NAME. Conversely, treatment of infected MKP-2$^{-/-}$ but not MKP-2$^{+/+}$ mice with nor-NOHA increased parasite burdens indicating a protective role for arginase-1 in MKP-2$^{-/-}$ mice. In vitro studies using tachyzoite-infected bone marrow derived macrophages and selective inhibition of arginase-1 and iNOS activities confirmed that both iNOS and arginase-1 could mediate anti-parasite growth. However, the effects of arginase-1 were transient and ultimately the role of iNOS was paramount in facilitating long-term inhibition of parasite multiplication within macrophages.

Graphical Summary

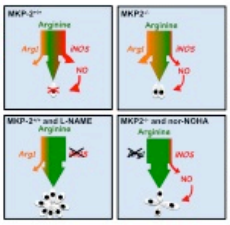

Figure I. Arginine plays a key role. As *T. gondii* is an arginine auxotroph it can be controlled through iNOS and Arg-1 mediated depletion of Arginine as well as the direct cytotoxic effects of NO. MKP-2$^{+/+}$ mice express NO in abundance controlling *T. gondii* through both of these mechanisms. Ablation of NO production by L-NAME in MKP-2$^{+/+}$ mice allows parasite growth. Arg-1 can control *T. gondii* through depletion of arginine, with Arg-1 inhibition by nor-NOHA slightly increasing parasite burden in MKP-2$^{-/-}$ mice.

Publications

Manuscript submitted to PLoS Pathogens and currently under review

MAP kinase phosphatase-2 plays a key role in the control of infection with *Toxoplasma gondii* by modulating iNOS and arginase-1 activities

Stuart Woods, Juliane Schroeder, Helen A. McGachy, Robin Plevin, Craig W Roberts & James Alexander

Abstract

The dual specific phosphatase, MAP kinase phosphatase-2 (MKP-2) has recently been demonstrated to negatively regulate macrophage arginase-1 expression, while at the same time to positively regulate iNOS expression. Consequently, MKP-2 is likely to play a significant role in the host interplay with intracellular pathogens. Here we demonstrate that MKP-$2^{-/-}$ mice on the C57BL/6 background have enhanced susceptibility compared with wild-type counterparts following infection with type-2 strains of *Toxoplasma gondii* as measured by increased parasite multiplication during acute infection, increased mortality from day 12 post-infection onwards and increased parasite burdens in the brain, day 30 post-infection. MKP-$2^{-/-}$ mice did not, however, demonstrate defective type-1 responses compared with MKP-$2^{+/+}$ mice following infection although they did display significantly reduced serum nitrite levels and enhanced tissue arginase-1 expression. Early resistance to *T. gondii* in MKP-$2^{+/+}$, but not MKP-$2^{-/-}$, mice was nitric oxide (NO) dependent as infected MKP-$2^{+/+}$, but not MKP-$2^{-/-}$ mice succumbed within 10 days post-infection with increased parasite burdens following treatment with the iNOS inhibitor L-NAME. Conversely, treatment of infected MKP-$2^{-/-}$ but not MKP-$2^{+/+}$ mice with nor-NOHA increased parasite burdens indicating a protective role for arginase-1 in MKP-$2^{-/-}$ mice. In vitro studies using tachyzoite-infected bone marrow derived macrophages and selective inhibition of arginase-1 and iNOS activities confirmed that both iNOS and arginase-1 could mediate anti-parasite growth. However, the effects of arginase-1 were transient and ultimately the role of iNOS was paramount in facilitating long-term inhibition of parasite multiplication within macrophages.

Manuscripts accepted for publication

T Cell Hypo-Responsiveness against Leishmania major in MAP Kinase Phosphatase (MKP) 2 Deficient C57BL/6 Mice Does Not Alter the Healer Disease Phenotype. ***PLoS Neglected Tropical Diease*** doi: 10.1371/journal.pntd.0002064

Julianse Schroeder, Helen A. McGachy, Stuart Woods, Robin Plevin, & James Alexander

Abstract

We have recently demonstrated that MAP kinase phosphatase 2 (MKP-2) deficient C57BL/6 mice, unlike their wild-type counterparts, are unable to control infection with the protozoan parasite Leishmania 203exicana. Increased susceptibility was associated with elevated Arginase-1 levels and reduced iNOS activity in macrophages as well as a diminished TH1 response. By contrast, in the present study footpad infection of MKP-2−/− mice with L. major resulted in a healing response as measured by lesion size and parasite numbers similar to infected MKP-2+/+ mice. Analysis of immune responses following infection demonstrated a reduced TH1 response in MKP-2−/− mice with lower parasite specific serum IgG2b levels, a lower frequency of IFN-γ and TNF-α producing CD4+ and CD8+ T cells and lower antigen stimulated spleen cell IFN-γ production than their wild-type counterparts. However, infected MKP-2−/− mice also had similarly reduced levels of antigen induced spleen and lymph node cell IL-4 production compared with MKP-2+/+ mice as well as reduced levels of parasite-specific IgG1 in the serum, indicating a general T cell hypo-responsiveness. Consequently the overall TH1/TH2 balance was unaltered in MKP-2−/− compared with wild-type mice. Although non-stimulated MKP-2−/− macrophages were more permissive to L. major growth than macrophages from MKP-2+/+ mice, reflecting their reduced iNOS and increased Arginase-1 expression, LPS/IFN-γ activation was equally effective at controlling parasite growth in MKP-2−/− and MKP-2+/+ macrophages. Consequently, in the absence of any switch in the TH1/TH2 balance in MKP-2−/− mice, no significant change in disease phenotype was observed.

Toxoplasma gondii **HLA-B*0702-restricted GRA7(20-28) peptide with adjuvants and a universal helper T cell epitope elicits CD8(+) T cells producing interferon-γ and reduces parasite burden in HLA-B*0702 mice.** ***Human Immunology*** doi: 10.1016/j.humimm.2011.10.006

Cong H, Mui EJ, Witola WH, Sidney J, Alexander J, Sette A, Maewal A, El Bissati K, Zhou Y, Suzuki Y, Lee D, Woods S, Sommerville C, Henriquez FL, Roberts CW, McLeod R.

Abstract

The ability of CD8(+) T cells to act as cytolytic effectors and produce interferon-γ (IFN-γ) was demonstrated to mediate resistance to Toxoplasma gondii in murine models because of the recognition of peptides restricted by murine major histocompatibility complex (MHC) class I molecules. However, no T gondii-specific HLA-B07-restricted peptides were proven protective against T gondii. Recently, 2 T gondii-specific HLA-B*0702-restricted T cell epitopes, GRA7(20-28) (LPQFATAAT) and GRA3(27-35) (VPFVVFLVA), displayed high-affinity binding to HLA-B*0702 and elicited IFN-γ from peripheral blood mononuclear cells of seropositive HLA-B*07 persons. Herein, these peptides were evaluated to determine whether they could elicit IFN-γ in splenocytes of HLA-B*0702 transgenic mice when administered with adjuvants and protect against subsequent challenge. Peptide-specific IFN-γ-producing T cells were identified by enzyme-linked immunosorbent spot and proliferation assays utilizing splenic T lymphocytes from human lymphocyte antigen (HLA) transgenic mice. When HLA-B*0702 mice were immunized with one of the identified epitopes, GRA7(20-28) in conjunction with a universal CD4(+) T cell epitope (PADRE) and adjuvants (CD4(+) T cell adjuvant, GLA-SE, and TLR2 stimulatory Pam(2)Cys for CD8(+) T cells), this immunization induced CD8(+) T cells to produce IFN-γ and protected mice against high parasite burden when challenged with T gondii. This work demonstrates the feasibility of bioinformatics followed by an empiric approach based on HLA binding to test this biologic activity for identifying protective HLA-B*0702-restricted T gondii peptides and adjuvants that elicit protective immune responses in HLA-B*0702 mice.