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Immunity to *Toxoplasma gondii*

by

Harshita Satija Grover

A dissertation submitted in partial satisfaction

of the requirements for the degree of

Doctor of Philosophy

In

Molecular and Cellular Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Nilabh Shastri, Co-chair
Professor Ellen Robey, Co-chair
Professor Russell Vance
Professor Suzanne Fleiszig

Spring 2012

Immunity to *Toxoplasma gondii*

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by

Harshita Satija Grover

Abstract

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Harshita Satija Grover

Doctor of Philosophy in Molecular and Cellular Biology

University of California, Berkeley

Professor Nilabh Shastri, Co-chair
Professor Ellen Robey, Co-chair

Toxoplasma gondii (*T. gondii*) is an obligate intracellular protozoan parasite, capable of infecting all warm-blooded animals, and can cause a severe disease in immunocompromised individuals. Protection to *T. gondii* is largely mediated by CD8 T cells, although CD4 T cells have also been shown to be important in immunity to *T. gondii*. However, the natural antigens recognized by these CD8 T cells, and how they mediate immune protection *in vivo* is poorly understood. Recently, it has been discovered that in mice with H-2^d MHC molecules, there is an immunodominant CD8 T cell response to GRA6, a dense granule *T. gondii* protein. In the absence of the T cell response to GRA6, such as in ERAAP-/- animals, mice succumb to infection. On the contrary, C57BL/6 (H-2^b) mice do not mount a response to GRA6. Studying antigen presentation dependent on MHC haplotype is important because the response to *T. gondii* in mice is controlled by genes in the H-2 region, wherein mice with the H-2^b haplotype are more susceptible to infection than mice with H-2^d MHC molecules. In addition, C57BL/6 (B6) mice are good models because a large number of genetic mutations in the innate and adaptive immune system have been established in this strain.

To better understand T cell responses in the susceptible strain, we immunized B6 mice with *T. gondii*, and found that it leads to potent CD4 T cell but weak CD8 T cell response. To identify the CD4 T cell stimulating antigens, we generated a *T. gondii*-specific, lacZ inducible, CD4 T cell hybridoma and used it as a sensitive probe to screen a *T. gondii*-cDNA library. We isolated a cDNA encoding a putative secreted protein of unknown function that we named CD4Ag28m and identified the minimal peptide, AS15 that was presented by MHC class II molecules to the CD4 T cells. Immunization of mice with the AS15 peptide provided protection to subsequent parasite challenge, resulting in a lower parasite burden and cyst load.

Furthermore, in order to characterize the CD8 T cell response to *T. gondii* in B6 mice, we restimulated T cells from mice immunized with *T. gondii*, with MHC Class II-/- bone marrow dendritic cells (BMDCs) to allow for the proliferation of CD8 T cells. This allowed us to generate a CD8 T cell hybridoma that was *T. gondii* specific and MHC Class I H-2D^b restricted. This hybridoma was used as a probe to screen the *T. gondii* cDNA library, which revealed that it recognizes ROP5, a rhoptry protein from *T. gondii*. We identified that YAL9 is the minimal antigenic peptide recognized by CD8 T cells. In chronically infected mice, response towards YAL9 in the brain and spleen is minor but detectable, and unlike the protective GRA6 response in H-2^d mice, immunization of B6 mice with YAL9 does not provide protection in B6 mice challenged with lethal dose of *T. gondii*. Most importantly, we found that altering the expression and trafficking of ROP5 from rhoptries to dense granules in parasites enhances the immunogenicity of ROP5 in B6 mice and now protects the mice from lethal challenge with transgenic parasites after peptide immunization.

Identification of the antigens in the H-2^b mice will enable us to study antigenic specific T cell responses, including effector functions such as clonal expansion and memory response at different stages of infection. We can now generate T cell receptor transgenic mice and use the antigen specific T cells from these mice to visualize and measure the dynamics of T cell interaction with antigen presenting cells during infection. Most significantly, understanding the nature of how these antigens are presented to T cells will allow us to design new and improved vaccine candidates against *T. gondii* and other apicomplexan parasites.

Dedication

I would like to dedicate my dissertation to my

Grandfathers,

Mr. Laxmi Chand Satija, who instilled in me the love of learning and fondness for education.

Dr. Hem Raj Seth, for living his life as an example for others and I.

&

Grandmothers,

Mrs. Bhagwanti Satija and Mrs. Ved Seth, who made sure that no matter what we do in life, their children and grandchildren never forget the basic human values.

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My parents, Alka and Surendra Satija, for supporting & encouraging me in all my decisions, and always being there for every step of the way!

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CRL Flow facility and the NIH tetramer facility.

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Abbreviations

APC = Antigen presenting cell
ICCS = Intracellular cytokine staining
TCR = T cell receptor
BMDC = Bone-marrow derived dendritic cell
MHC = Major histocompatibility complex
pMHC = Peptide-MHC complex
PV = Parasitophorous vacoule
ERAAP = Endoplasmic reticulum aminopeptidase associated with antigen presentation
TAP = Transporter associated with antigen processing
GRA = Refers to dense granule proteins in *T. gondii*
ROP = Refers to rhoptry proteins in *T. gondii*

Chapter 1: Introduction

***Toxoplasma gondii* biology**

Toxoplasma gondii (T. gondii), an obligate intracellular protozoan parasite, is a significant human and veterinary pathogen. It is classified in the phylum Apicomplexa, which includes parasites such as *Plasmodium*. It is known to cause an opportunistic ą⌐˄&ą̨} in immunocompromised individuals, and poses a severe risk for congenitally infected infants (Carruthers, 2002; Montoya and Liesenfeld, 2004). *T. gondii* enters the host via the gastrointestinal tract (Buzoni-Gatel and Werts, 2006) and is the third most common cause of food borne deaths in the United States (Carruthers, 2002). There are three &ommon strains of *T. gondii* in North America and Europe. Type I strains, ex: RH, are the most virulent, while type II strains, ex: Me49 or Prugniaud are moderately virulent and type III strains, ex: CTG or VEG are avirulent (Black and Boothroyd, 2000).

Although *T. gondii* has a wide host range, which includes all warm-blooded animals, the only known definitive hosts for *T. gondii* are those of feline species where the sexual stages of the *T. gondii* life cycle occur. *T. gondii* exists in the fast replicating form as tachyzoites that cause the acute phase of infection, which then converts into slow dividing bradyzoites stage that form tissue cysts in brain and muscles. Parasite spread to the next host by ingestion of oocysts released in feces of these animals or by ingestion of tissue cysts through consumption of undercooked meat or unwashed fruits and vegetables (Black and Boothroyd, 2000; Carruthers, 2002). In the intermediate host, during the acute phase of infection, tachyzoites that invade a cell reside and replicate in a specialized non-fusogenic compartment, known as parasitophorous vacuole (PV) (Fig.1). Eventually the host cell membrane ruptures to release the replicated parasites, which can now infect any nucleated cell that they encounter. However, in the presence of an adequate immune response, the tachyzoite stage is cleared but bradyzoites still persist within cysts, which effectively escape the host immune system leading to life-long persistent infection. These cysts can reemerge to cause an acute infection when the host's immune system is compromised, leading to lethal neural damage and Toxoplasmic encephalitis (TE) (Black and Boothroyd, 2000; Carruthers, 2002).

T. gondii invasion is an active process, distinct from endocytosis, requiring parasite motility. As *T. gondii* infects a nucleated cell it generates a parasitophorous vacuole (PV), which is where it resides and replicates. This vacuole, unlike endocytic and exocytic vesicles, it is thought to be non-fusogenic to any cellular organelles and is necessary for the intracellular survival of the parasites (Jones et al., 1972). Once generated, the PV does not acidify (Sibley et al., 1985) and dense granule proteins are secreted to generate the tubulovesicular network that extends from the PV membrane into the PV lumen (Mercier et al., 2005). There have been few models that have been proposed for

the generation of the PV around the parasites. One theory suggests that suggests that during invasion, discharged proteins from rhoptries form the PV de novo (Boothroyd and Dubremetz, 2008; Dubremetz, 2007), while the other theory suggests that PV forms by direct invagination of the host plasma membrane (Carruthers, 2002). Interestingly, it has also been shown that upon cytochalasin D treatment of parasites, which inhibits invasion, rhoptry proteins are still discharged into the host cytosol leading to the generation of the PV without the parasite, which is termed as 'evacoule'. These evacuoles do not contain any host membrane proteins but have been shown to co-localize with host cell mitochondria and endoplasmic reticulum (ER) (Hakansson et al., 2001). Even in actively infected cells, it has been shown that the PV membrane (PVM) associates with the host ER and mitochondria in a process termed as PVM-organelle association (Sinai et al., 1997), which could be dependent on proteins such as ROP2 (Sinai and Joiner, 2001). More recently, using immunofluorescence and electron microscopy studies, it was shown that the PV in actively infected cells interacts and fuses with the host ER, and allows for the cross presentation of *T. gondii* antigens for presentation to CD8 T cells in a retrograde manner involving Sec61. This was determined using transgenic parasites that express OVA, which can stimulate CD8 and CD4 T cells that recognize OVA (OT-I and OT-II cells, respectively). Furthermore, this was shown to happen only in actively infected cells but not in cells that have phagocytosed live parasites or parasitic debris (Goldszmid et al., 2009). Additionally, *T. gondii* was shown to take advantage of the host endo-lysosomal pathway to sequester nutrients with the help of the protein GRA7. Although PVM allows for the diffusion of small molecules (up to 1300 Daltons), in order to access larger nutrients, the microtubular invaginations of the PVM, steal away the intact endo-lysosomal vesicles into the vacuolar space (Coppens et al., 2006).

On the contrary, from the host defense point of view, it has been demonstrated that in actively infected cells, PV are disrupted by the p47-GTPases in an IFN-γ inducible manner (IGTPases). Upon induction, GTPases are known to interact with intracellular membranes, and in the case of infection with *T. gondii*, they accumulate around the PVM. Accumulation of IGTPase allows for the maturation of PV reflected by disrupted morphology. A loss of GRA7 signal is seen and finally PVM disintegration leads to the clearance of the parasites (Martens et al., 2005). Nonetheless, knowing the mechanisms in which the PV creates a barrier between the parasite and host cytosol can allow us to better understand how the parasite proteins enter the endogenous antigen presentation pathway to be presented on MHC I class molecules for detection by CD8 T cells.

Secretory proteins of *Toxoplasma gondii* and their role in infection

T. gondii, like the other apicomplexans, carries out all the essential functions of its life cycle by help of its secretory proteins. These secretory proteins help it infect, egress and invade the neighboring cells. Three distinct secretory

organelles, rhoptries, micronemes, and dense granules contain and discharge these proteins during the appropriate part of the life cycle. During budding, each daughter cell gets the secretory organelles that are formed de novo, anterior to the nucleus (Striepen et al., 2007). While dense granules are distributed throughout the parasite, rhoptries and micronemes are located at the apical surface of the parasite forming the apical complex (Carruthers, 1999). It was defined that the default pathway of all secretory proteins with signal sequence are the dense granules (Carruthers, 1999; Mercier et al., 2005) and cell surface, rhoptry and micronemal proteins have positive sorting signals that target them to their respective compartments. After the parasite glides on the host plasma membrane, secretion of micronemes allow for the motility of the parasite and attachment to the host cell membrane (Besteiro et al., 2011). This is followed by the sequential secretion of one set of rhoptry proteins called, rhoptry neck proteins (RONs). RONs in combination with microneme proteins form a central structure called moving junction complex (MJ) that propels the parasite through the host cell membrane. Following the secretion of RONs, rhoptry proteins (ROPs) that are part of the bulb portion of the organelle are secreted into the host cytosol. Here they either function in the biogenesis of the PV, migrate to the lumen of the PV, or enter the host nucleus (Boothroyd and Dubremetz, 2008). Lastly, dense granule proteins are secreted to be part of the tubular network of the PV and allow for acquisition of nutrients from the host cell (Carruthers, 2002).

Among the secretory proteins, of most interest to us are rhoptry proteins, specifically, ROP5, as we have identified it to be an antigen recognized by CD8 T cells in C57BL/6 mice. Rhoptry proteins, part of the club shaped secretory organelle, rhoptries, are secreted during the invasion process immediately after the secretion of microneme proteins, and they are involved in the invasion of the parasite and the generation of the PV. So far, there have been 30 rhoptry bulb proteins identified (ROPs) and 5 rhoptry neck proteins (RONs) identified (Dubremetz, 2007). ROP proteins are homologues of higher eukaryotic protein kinases and proteases. Furthermore, most of ROP proteins belong to the ROP2 family of protein kinases, of which only one, ROP18, has shown to have catalytic activity (El Hajj et al., 2007). The rest of them all are currently known to contain an inactive catalytic pseudokinase domain. Although most ROP2 family proteins are known to be associated with the PV membrane facing the host cytosol, there are two of them, ROP16 and PP2C- that localize in the host cell nucleus (Boothroyd and Dubremetz, 2008).

To understand the mechanism of localization of other rhoptry proteins, most recently, it was shown that the ROP2 family of rhoptry proteins, contain arginine-rich amphipathic helix domains (RAH domains) that allow them to interact and bind with the PV membrane upon secretion during invasion (Reese and Boothroyd, 2009). Additionally, rhoptry proteins have now been known to play a role in virulence. ROP16Ás known to enter the host nucleus, and interfere with the host STAT pathways involving IL-12 regulation that are central
to the host defense against *T. gondii* (Boothroyd, 2009). Although not fully

understood, ROP18, the catalytically active kinase can alter the parasite replication as shown by its over-expression (El Hajj et al., 2007). Most recently, despite being catalytically inactive, ROP5, has been shown to play role in virulence, wherein deletion of the protein from the RH, the type I virulent strain of parasites, renders mice resistant to infection (Reese et al., 2011). Furthermore, ROP5 is encoded by a cluster of tandem genes that express three different paralogs of the protein. These paralogs are highly divergent among the three different strains of *T. gondii* and are in different copy numbers among the strains. In fact, the copy numbers of the different isoforms of ROP5 was also shown to influence the virulence of the parasites and most of the polymorphisms were to found in the pseudokinase domain (Reese et al., 2011). Rhoptry proteins are one of the key molecules that are necessary not only for successful invasion, generation of PV and virulence of the parasite, but can also play a role in immune evasion by subverting function of host proteins.

Immune response to *Toxoplasma gondii*

An adequate and persistent immune response is critical to not only prevent the tachyzoites from killing the host, but also to prevent their reemergence from the dormant cysts. Much of what is known about the immune response to *T. gondii* comes from studies in the mouse model of infection. Studies have shown that strong and persistent T-cell mediated immunity is important for resistance to *T. gondii*, and athymic nude mice, which lack functional T cells, are extremely susceptible to both virulent and avirulent parasite strains (Denkers and Gazzinelli, 1998; Lindberg and Frenkel, 1977). It has been shown in the murine model of infection that CD8 T cells are the major effectors of immunity during *T. gondii* infection (Brown and McLeod, 1990; Gazzinelli et al., 1991) and in-vivo imaging studies in the brains of infected animals have shown the recruitment and persistent presence of antigen specific CD8 T cells (Schaeffer et al., 2009).

Although *T. gondii* is a strong inducer of Ag-specific CD8 T lymphocytes, very little is known about what the actual parasite antigens are and how they are efficiently loaded onto the MHC molecules. An important approach to understand the T cell response to infection is to define the peptide-MHC ligands recognized by the TCR at a molecular level. This is especially important since PV, where the parasites reside within the cell, is a non-fusogenic vacuole, limiting the access of the parasitic proteins to the cytosol for antigen processing (Fig.1). A proposed model of how peptide loading can occur on MHC is by the transportation of antigen from within the parasitophorous vacuole to the cytosolic presentation pathway (Blanchard et al., 2008; Denkers and Gazzinelli, 1998; Goldszmid et al., 2009) (Fig.1). One of the earliest potential antigen that had been identified is the major surface glycoprotein, SAG-1 (Khan et al., 1994; Kim and Boothroyd, 2005), but this identification is based on prediction and immunization of mice with recombinant SAG-1 to generate antigen specific T cells (Khan et al., 1988). Since then additional antigens were also identified using an epitope prediction
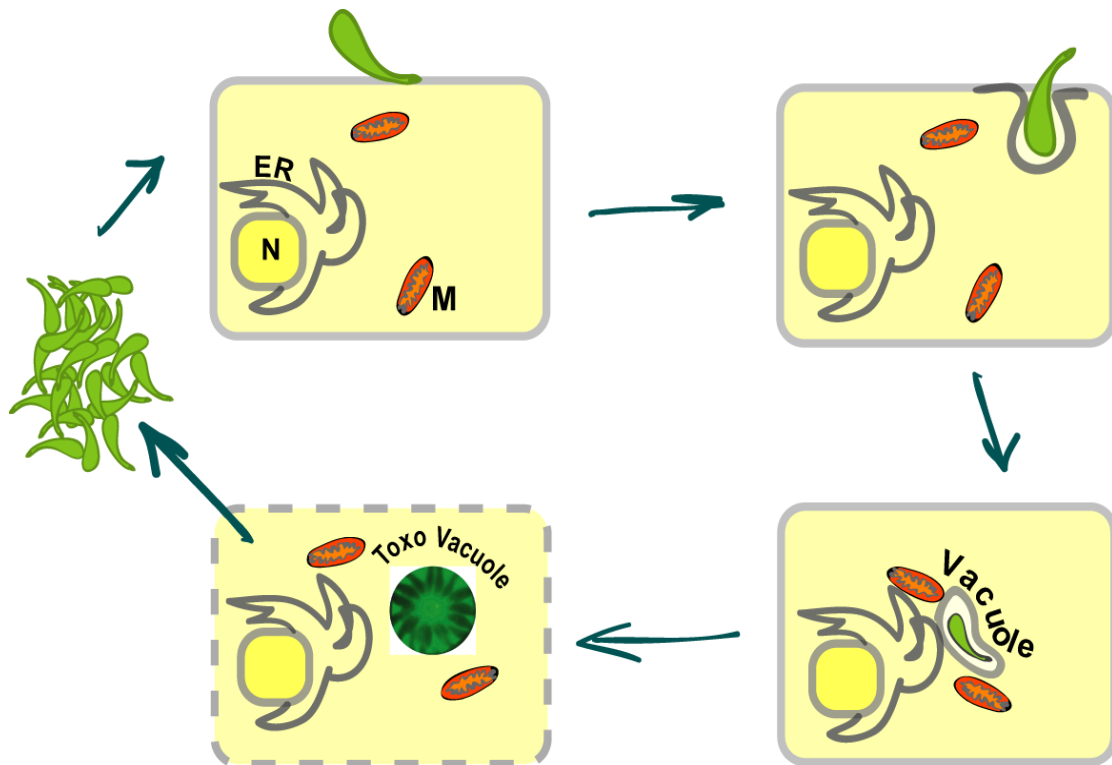
Created by Dr. Nilabh Shastri.
Adapted from:
Black and Boothroyd, *Microbiol Mol Biol Rev*, 2000

Figure 1: Life cycle of *Toxoplasma gondii* (tachyzoite stage).
T. gondii can infect any nucleated cell by first gliding along the cell surface and then actively penetrating the host plasma membrane with the help of its secretory proteins. As it infects, it generates a non-fusogenic parasitophorous vacuole. In this vacuole, it resides and replicates until it lyses the host cell, egresses and infects other neighboring cells.

Table 1: *Toxoplasma gondii* antigens recognized by T cells in mice

MHC molecule	Antigen (Epitope)	Reference
D^b	ROP5 (YAL9)	Grover, HS., et. al, unpub.
K^b	Tgd057 (SVL8)	Wilson, DC., et. al, *PLoS Pathog*, 2010
D^b	SAG1 (TL9)	Mendes, EA., et. al., *Vaccine*, 2011
L^d	GRA6 (HF10)	Blanchard, N., et. al., *Nat. Immunol*, 2008
L^d	GRA4 (SM9)	Frickel, EM., et. al., *J Infect Dis*, 2008
L^d	ROP7 (IF9)	Frickel, EM., et. al., *J Infect Dis*, 2008
I-A^b	CD4Ag28m (AS15)	Grover, HS et. al, unpub.
I-A^b	Profilin (WN15)	Yarovinsky, F., et. al, *Immunity*, 2006

approach, recognized by CD8 T cells in H-2^d mice, included ROP7 and GRA4 (Frickel et al., 2008) and Tgd057 (Wilson et al., 2010) in H-2^b mice (Table 1). However, for the first time, a natural immunodominant CD8 T cell antigen, the dense granule protein, GRA6, had been identified *in-vivo* from *T. gondii* challenged mice with H-2^d MHC haplotype (Blanchard et al., 2008).

While CD8 T cells play an important role in resistance to *T. gondii*, CD4 T cells also provide protection, especially when CD8 T cells are absent (Araujo, 1991; Gazzinelli et al., 1991; Lutjen et al., 2006; Suzuki and Remington, 1988). Moreover, CD4 T cells play an important immunoregulatory role during infection by producing the immunosuppressive cytokine IL-10, and can also contribute to immune-mediated pathology (Jankovic et al., 2007b; Liesenfeld et al., 1996; Oldenhove et al., 2009; Stumhofer et al., 2006). To date, our understanding of CD4 T cell responses during *T. gondii* infection have been based on analysis of polyclonal CD4 T cell populations of poorly defined specificity. Some CD4 Th1 clones reactive to *T. gondii,* and the parasite proteins that they respond to, have been described, although the identity of peptide epitopes and the relative abundance of responding T cells is not known (Reichmann et al., 1997a, b; Reichmann et al., 2001; Reichmann et al., 1997c). In one recent study, CD4 T cells were raised against a lysed parasite extract, and were found to respond predominantly to the parasite protein profilin. However, the contribution of this response during infection was not characterized and the antigenic peptide was not defined (Yarovinsky et al., 2006). Most importantly, whether the parasite specific CD4 T cells identified so far can protect animals from toxoplasmosis remains unresolved. In most of these studies, protection was not examined (Reichmann et al., 1997a, 1997b, 1997c; Yarovinsky et al., 2006) and in one case the relevant Th1 cells failed to protect (Reichmann et al., 2001). Thus, the question of how CD4 T cells detect and respond to *Toxoplasma gondii* remains largely unknown.

Toxoplasma gondii infection and MHC molecules

Studying antigen responses in a different MHC haplotype is important because it has been shown that genetic resistance to *T. gondii* is linked to the H-2 chromosomal region. C57BL/6 (B6) mice with H-2^b MHC haplotype have a higher susceptibility to infection than BALB/c mice (H-2^d) (Araujo et al., 1976). Studies in congenic strains of mice confirmed that C57BL/10 mice (H-2^b) also have a much higher susceptibility than B10.D2 mice (H-2^d) (Deckert-Schluter et al., 1994; Williams et al., 1978). Further linkage analysis showed that MHC class I genes among the H-2 region and in specific L^d gene confers resistance in the H-2^d mice (Brown et al., 1995; Brown and McLeod, 1990; Suzuki et al., 1994; Suzuki et al., 1991). Therefore, studying the mechanism of CD8 T cell immune response to *T. gondii* in the absence of the gene that causes resistance to *T. gondii* infection (i.e. in H-2^b mice) will allow us to study how alteration of immune response in the susceptible mice can lead to resistance to *T. gondii*.

Toxoplasma gondii and protective cytokines

The production of cytokines such as IFN-γ and TNF-α plays an important role in controlling tachyzoite replication during the acute or the chronic phase of infection (Gazzinelli et al., 1993; Schluter et al., 2003; Suzuki et al., 1989; Suzuki et al., 1988). Ablation of IFN-γ, using monoclonal anti-bodies against rendered mice susceptible to *T. gondii* infection (Suzuki et al., 1988). Furthermore, depending upon on the strain of *T. gondii*, treatment with antibodies against IFN-γ caused a range of augmentation of inflammatory changes in the brains of chronically infected mice (Suzuki and Joh, 1994). Both cells of the innate and adaptive immune system, i.e., NK cells and CD4 and CD8 T cells, respectively, generate IFN-γ in response to *T. gondii* infection (Gaddi and Yap, 2007).

Furthermore, differentiation of T helper cells into Th1 cells to generate IFN-γ is dependent upon the production of IL-12 by innate cells (Gaddi and Yap, 2007). However, in the absence of IFN-γ, such as in IFN-γ knockout mice, there was a comparable production of IL-12 and ablation of IL-12 in these mice did not render them more susceptible (Scharton-Kersten et al., 1996). Additionally, both dendritic cells and macrophages have been shown *in-vivo* to produce the IFN-γ polarizing cytokine IL-12 (Liu et al., 2006; Robben et al., 2004). In the absence of dendritic cells that make IL-12, mice succumb to infection (Liu et al., 2006).

On the other hand, to counter balance the immunpathological effects of the proinflammatory cytokines, it was shown that IL-10 is generated by IFN-γ producing CD4 T cells that are FoxP3-Tbet+ (Jankovic et al., 2007a). IL-10 deficient mice displayed hypersensitivity and with increased levels of IFN-γ and TNF-α. In a separate study, IL-10 independent STAT3 activation in infected macrophages was shown to induce the suppression of IL-12 and TNF-α responses (Butcher et al., 2005). The fine balance between pro-inflammatory cytokine production and regulation allows for protective immune responses against *T. gondii*.

Antigen presentation to T cells

In response to foreign pathogens and tumors, CD4 and CD8 T cell responses comprise an important arm of the adaptive immune system. CD4 T cells recognize antigenic peptides of variable lengths presented to them on MHC class II molecules, which unlike MHC Class I molecules, are only present on professional antigen presenting cells and upregulated upon maturation of these cells. CD8 T cells recognize antigenic peptides for fixed length presented to them on MHC class I molecules, which are present on most cells and upregulated upon inflammatory signals (Trombetta and Mellman, 2005). Table 2 lists the currently identified mouse MHC class I and class II restricted antigens from different pathogens. It is important to keep the antigen presentation pathways

8
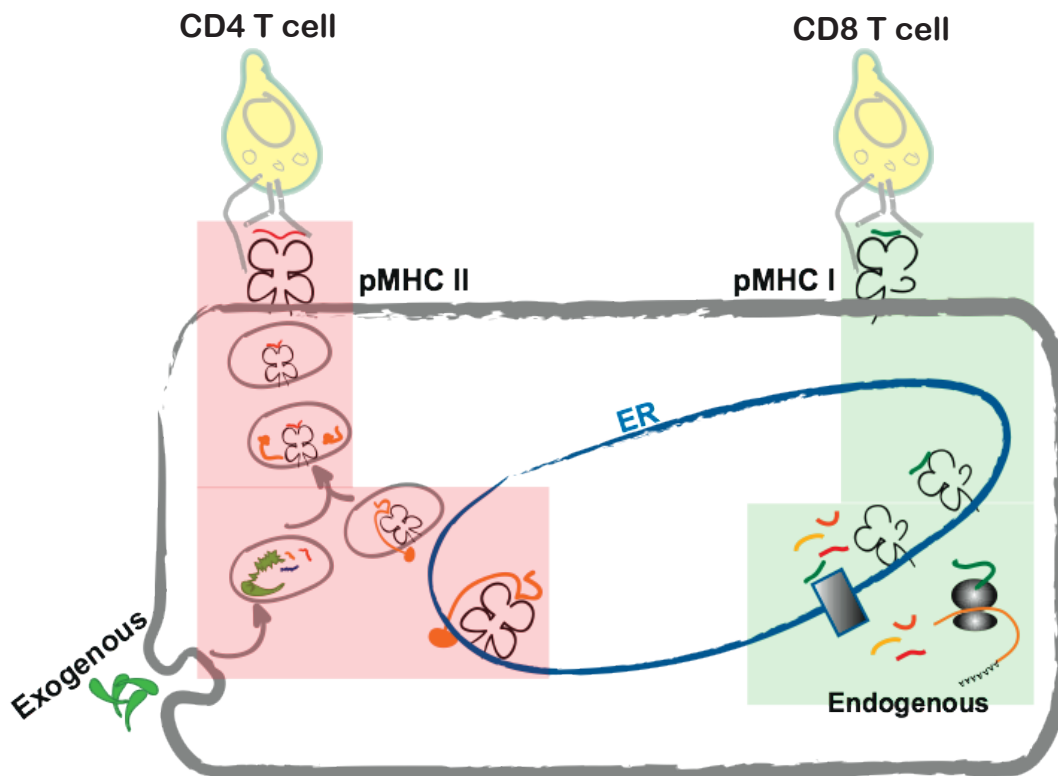
Created by Dr. Nilabh Shastri.

Figure 2: Antigen sources for pMHCI and pMHCII complexes.
Peptides presented on MHC class I molecules come from an endogenous source, where they are first broken down by the proteasome in the cytosol, then transported into the ER via TAP to finally trimmed by ERAAP and loaded onto MHC I molecules with the help of the peptide loading complex. The pMHCI molecules then traffic to cell surface to present the antigenic peptide to CD8 T cells. On the other hand, peptides presented on MHC class II molecules come from an exogenous source. They have to be taken up by the antigen presenting cell via phagocytosis or other processes such as autophagy. Once inside the endosomes, antigenic peptides replace CLiP and are loaded onto MHC class II molecules to be presented on the cell surface for recognition by CD4 T cells.

Table 2: Currently known mouse MHC class I and II antigens to intracellular pathogens

Pathogen	Mouse MHC molecules	Antigen	Reference
Plasmodium berghei	L^d	Circumsporozoite protein	Romero et al., 1989
Plasmodium falciparum	L^d	Circumsporozoite protein	Blum-Tirouvanziam et al., 1994
Plasmodium yoelli	Ld	Circumsporozoite protein	Weiss et al., 1990
Plasmodium yoelli	I-Ab	Circumsporozoite protein	Takita-Sonada et al., 1996; Grillot et al., 1990.
Trypansome cruzi	Kk, Kb, H-2a	ASP-2	Dominguez et al., 2011
Mouse cytomegalovirus	Db	M45	Munks et al., 2006
Mouse cytomegalovirus	Kb	m141	Munks et al., 2006
Mouse cytomegalovirus	Ld	M84	Holtappels et al., 2000
Mouse cytomegalovirus	Ld	IE1	Holtappels et al., 2002
Mouse cytomegalovirus	Dd	m164	Holtappels et al., 2002
Mouse cytomegalovirus	Kb	m38a	Munks et al., 2006
Mouse cytomegalovirus	Dd	gp34	Holtappels et al., 2000
Mouse cytomegalovirus	Kb	m139	Munks et al., 2006
Mouse cytomegalovirus	Kb	m57	Munks et al., 2006
Mouse cytomegalovirus	Kb	m102	Munks et al., 2006
Mouse cytomegalovirus	Db	m86	Munks et al., 2006
Mouse cytomegalovirus	Kb	m97	Munks et al., 2006
Mouse cytomegalovirus	Ld	pp89	Reddehase et al., 1989
Lymphocytic choriomengitis virus	Ld	NP	Whiton et al., 1989
Lymphocytic choriomengitis virus	Ld	GP	van der Most et al., 1996
Influenza (PR8)	Ld	NP	Rotzschke et al., 1991
Influenza A	Ld	HA	Kuwano et al., 1988

Table 2 continued

Pathogen	Mouse MHC molecules	Antigen	Reference
Influenza A	Ld	HA	Cao et al., 1995
Influenza A (JAP)	Ld	HA	Braciale et al., 1987
Influenza (PR8)	Ld	NP	Deng et al., 1997
Dengue	Ld	NS3	Spaulding et al., 1999
HIV	Ld	gp160	Bergman et al., 1994
HIV	Ld	gp160	Tobery et al., 1997
HIV	Ld	gag p24	Doe et al., 1996
HIV	Ld	gp120	Egan et al., 1999
Measles virus	Ld	HA	Beauverger et al., 1994
Measles virus	Ld	NP	Beauverger et al., 1993
HBV	Ld	NP	Bergmann et al., 1996
HBV	Ld	Core protein	Kuhober et al., 1997
HBV	Ld	Surface antigen	Sette et al., 2001
HSV	Ld	ICP27	Banks et al., 1993
Mouse mammary tumor virus	Ld	Env	Gill et al., 1994
Mouse mammary tumor virus	Ld	Gag	Gill et al., 1994
Polio virus	Ld	VP1	Kutubuddin et al., 1992
Ross river virus	Ld	T48	May la Linn et al., 1998
Respiratory syncytial virus	Ld	F64-72	Chang et al., 2001
Respiratory syncytial virus	Ld	F228-236	Johnstone et al., 2004
Respiratory syncytial virus	Ld	Matrix protein	Kulkarni et al., 1993
Simian rotavirus	Ld	VP	Buesa et al., 1999
Francisella tularenis	I-Ab	IgIB	Valentino et al., 2011
Francisella tularenis	I-Ab	GroEL	Valentino et al., 2011
Francisella tularenis	I-Ab	DNAK	Valentino et al., 2011
Francisella tularenis	I-Ab	Tul4 (LpnA)	Valentino et al., 2011

Table 2 continued

Pathogen	Mouse MHC molecules	Antigen	Reference
Chlamydophila pneumonia	Ld	OMP2	Saren et al., 2002
Chlamydophila pneumoniae	Ld	HSP60	Saren et al., 2002
Listernia monocytogenes	Ld	Mettaloproteinase	Busch et al., 1997
Listeria monocytogenes	Ld	LLO	Pamer et al., 1991
Listeria monocytogenes	Ld	p60	Pamer et al., 1994
Listeria monocytogenes	Ld	p60	Sijts et al., 1996
Listeria monocytogenes	I-Ad	p60	Geginat et al., 1998
Influenza A (PR8)	I-Ad	HA	Shih et al., 1997
HIV	I-Ed	gag p17	Sette et al., 1993
Influenza A (PR8)	I-Ed	HA	Fahrer et al., 1995
Influenza A (PR8)	I-Ed	HA	Spouge et al., 1987
Influenza A (PR)	Db	NP	Falk et al., 1991
Chlamydophila pneumonia	Db/Dd	Outer membrane protein	Saren et al., 2002
Lymphocytic choriomengitis virus	Db	GP	Hudrisier et al., 1999
Mouse cytomegalovirus	Db	Major capsid protein	Munks et al., 2006
Vaccinia	Db	A47	Mathew et al., 2005
Vaccinia	Db	Profilin	Tscharke et al., 2005
Vaccinia	Db	K3	Tscharke et al., 2005
Mouse mammary tumor virus	Kb	gp70	Malarkannan et al., 1996
Lymphocytic choriomengitis virus	Kb	GPI	Hudrisier et al., 1997
Mycobacterium tuberculosis	Kb	38kD protein antigen B	Vordermeier et al., 1997
Polyoma virus	Kb	Large T antigen	Kemball et al., 2005
Sendai virus	Kb	NP	Kast et al., 1991, Schumacher et al., 1988
Vaccinia	Kb	DNA directed RNA polymerase	Mathew et al., 2005

Table 2 continued

Pathogen	Mouse MHC molecules	Antigen	Reference
Vaccinia	Kb	A19	Tscharke et al., 2005
Vaccinia	Kb	A47	Tscharke et al., 2005
Vaccinia	Kb	B8	Tscharke et al., 2005
Leishmania major	I-Ab	36 kD LACK antigen	Mougneau et al., 1995
Leishmania major	I-Ad / I-Ed	Soluble leishmanial antigen	Scott et al., 1988
Leishmania major	I-Ad / I-Ed	gp63	Jardim et al., 1990
Salmonella typhimurium	I-Ad / I-Ed	Salmonella invasion protein (SipC)	Musson et al., 2002
Salmonella enterica	I-Ak	Flagellar filament protein (FliC)	Cookson et al., 1996
Trypanosoma cruzi	I-Ab	Surface glycoproteins	Kahn et al., 1997
Mycobacterium tuberculosis	I-Ab	Antigen 85B	Andersen et al., 1995; Yanagisawa et al., 1996
Mycobacterium tuberculosis	I-Ab	ESAT-6	Andersen et al., 1995
Mycobacterium tuberculosis	I-Ab	PT19	Harris et al., 1995
Mycobacterium leprae	I-Ab	PL28	Harris et al., 1995
Plasmodium yoelli	I-Ab	Meroziote surface protein-1	Hirunpetcharat et al., 1997
Listeria monocytogenes	I-Ak	12A4.G7	Campbell et al., 1998
Listeria monocytogenes	I-Ak	p3A1	Sanderson et al., 1995
Listeria monocytogenes	I-Ad	p60	Geginat et al., 1997
Listeria monocytogenes	Kd	LLO	Vijh et al., 1996
Listeria monocytogenes	Kd	p60	Vijh et al., 1996

Data in this table was partially obtained from SYFPETHI database: Rammenesee et al., 1999

(Fig. 2) in mind when designing techniques such as an expression-cloning screen to identify natural antigens that are recognized by T cells. Identification of MHC class I and II restricted antigens presented to CD4 and CD8 T cells is important because not only does it give us insight into the mechanism of what is recognized and how the adaptive immune responses are generated to clear the pathogen, but it also allows us to generate new and improved vaccines for protection against lethal infections.

In brief, MHC class II presentation pathway involves the processing of exogenous antigens that can either be phagocytosed from the extracellular environment via phagocytosis, micropinocytosis or receptor-mediated uptake (Lennon-Dumenil et al., 2002; Trombetta and Mellman, 2005). These self and foreign proteins are taken up into compartments known as phagosomes, which mature and eventually fuse to the lysosomes to generate phagolysosomes within the antigen presenting cells. It is in the phagolysosomes, the antigens are unfolded and then acidification and enzymatic activity of proteases such as cathepsins (ex: cathepsin B, S and L) (Bryant and Ploegh, 2004), allow for the degradation of the antigenic proteins. Within the lysosomes, MHC class II molecules are also present after being trafficked from the ER and Golgi. MHC class II molecules within the lysosomes are bound to an invariant chain. Upon maturation, the invariant chain is cleaved leaving only the CLIP peptide bound to the MHC class II molecules. CLIP peptide then competes with foreign peptides present in the lysosome that bind to MHC class II molecules with higher affinity than CLIP and this is facilitated by the help of MHC class II like molecule HLA-DM in humans or H-2M in mice (Bryant and Ploegh, 2004; Trombetta and Mellman, 2005; Wolf and Ploegh, 1995). Eventually the antigenic peptide loaded MHC class II molecules is transported to the cell surface via endolysosomal tubules (Vyas et al., 2008).

Additionally, more recently, it has been shown that pathways such as autophagy also contribute to MHC class II antigen presentation pathway, especially in the case of the presentation of endogenous antigens. This is an important immune defense mechanism in infections with pathogens such as *Toxoplasma gondii*, *Mycobacterium spp*. that interfere with the canonical MHC class II antigen presentation pathway by either blocking the acidification of the lysosomes and residing in an non-fusogenic vacuole or by blocking phagosome maturation or intercepting vesicular traffic from the ER to form an ER-like compartment that avoids fusion with lysosomes, respectively (Vyas et al., 2008; Wong and Pamer, 2003). Although not fully characterized, there are three types of autophagy, i.e., macroautophagy, microautophagy and chaperone-mediated autophagy that allow for the maintenance of cell homeostasis. During autophagy, an autophagosomal membrane forms, expands and engulfs cytoplasmic contents, eventually leading to a formation of an autophagosome. Autophagosomes then fuse with lysosomes where they serve as an additional source of peptide to be presented on MHC class II molecules (Munz, 2006; Vyas et al., 2008).

On the contrary, antigenic peptides presented on MHC class I molecules to CD8 T cells coventionally come from an endogenous source (Fig. 2). The sources of endogenous peptide can include, self or foreign pathogenic proteins present in the cytosol, cryptic translation products, defective ribosomal products (DRiPs), proteins retrotranslocated from the ER to the cytosol, internalized proteins transferred to the cytosol and aberrant proteins due to premature termination or misincorporation (Trombetta and Mellman, 2005; Vyas et al., 2008).

The cytosolic proteins are further subject to degradation into polypeptide subunits by ubiquitnation and then proteasomal degradation, or just proteasomal degradation, where IFN-γ inducible LMP2 and LMP7 subunits belonging to the β-subunit of family of proteasome subunits play an important role. The antigenic peptide precursors are then delivered to the ER via the transporter associated with antigen processing (TAP) (Holtappels et al., 2008; York and Rock, 1996). Within the ER, peptides can be further trimmed by endoplasmic reticulum aminopeptidase associated with antigen processing (ERAAP) before being loaded onto the MHC class I molecules (Serwold et al., 2002). TAP is known to be part of the peptide-loading complex and before associating with β_2-microglobulin (β_2-m), MHC class I heavy chain binds to the chaperone protein, calnexin. After dissociation with calnexin, MHC class I molecules dimerize with β_2-m and are part of the peptide-loading complex which consists of TAP, tapasin, an ER chaperone – calreticulin, and a soluble thiol oxidoreductase, ERp57. Peptides are loaded onto the MHC molecules with the help of the peptide-loading complex, which upon binding of the peptide disassociates from the peptide-MHC (pMHC) molecule (Cresswell, 2005). After assembly, pMHC class I molecules traffic through the Golgi and transported to the cell surface via the canonical exocytic pathway (York and Rock, 1996).

Lastly, it has been shown that presentation of antigenic peptides can also happen via cross-presentation. It has been proposed that cross-presentation of exogenous antigens can happen via the loading of MHC class I molecules in the endo-lysosomes when they are being recycled back from the cell surface. Alternately, cross-presentation is also possible as suggested by the ER-phagosome fusion model, which is supported by proteomic analysis of the phagosomes that revealed the presence of many ER-derived proteins (Cresswell, 2005; Vyas et al., 2008). Nonetheless, cross-presentation allows for the presentation of exogenous proteins, thereby providing an additional source of antigenic peptides that can be presented on MHC class I molecules to CD8 T cells.

Thesis research summary: Immunity to *Toxoplasma gondii*

As part of my thesis research, in the next few chapters, you will see results that define mechanisms of immunity to *T. gondii* in the susceptible strain of mice, C57BL/6. In chapter 2, we identify an endogenous *T. gondii* antigen, CD4Ag28m recognized by CD4 T cells and characterize the responses against this antigen. We further show that immunization of mice with the antigenic peptide, AS15, from CD4Ag28m, generates protection against lethal infection in B6 mice. In chapter 3, we identify an endogenous *T. gondii* antigen, ROP5, which is recognized by CD8 T cells. We determine the responses towards the antigenic peptide, YAL9 from ROP5 throughout the course of infection. Lastly, we show that by altering the expression of ROP5, i.e. from a rhoptry protein to a dense granular protein, we can enhance the immunogenicity of ROP5 in B6 mice. Chapter 4 discusses the potential hypotheses for why the immunogenicity of ROP5 is enhanced when it is secreted like a dense granular protein. We also discuss potential mechanisms in which CD4Ag28m can be presented and the possible hypotheses for the mechanisms of protection.

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Chapter 2: CD4 T cell responses to *Toxoplasma gondii* in C57BL/6 mice

Summary

Toxoplasma gondii can cause severe disease in immunocompromised individuals. Previous studies in mice have shown that genetic resistance to disease is linked to the MHC locus and protection is largely mediated by T cells. CD8 T cells are known to be the primary mediators of resistance to *T. gondii*. In contrast, the role of CD4 T cells in immunity to *T. gondii* remains poorly characterized. In this chapter, it is shown that *T. gondii* immunized B6 mice elicit primarily a CD4 rather than a CD8 T cell response. To identify the CD4 T cell stimulating antigens, *T. gondii*-specific, lacZ inducible, CD4 T cell hybridoma was generated and used as a sensitive probe to screen a *T. gondii*-cDNA library expressed in *E. coli* fed to bone marrow derived dendritic cells. A cDNA was isolated encoding a putative secreted protein of unknown function that we referred to as CD4Ag28m. The 15-mer, antigenic peptide in CD4Ag28m, AS15, presented by MHC class II molecules to the CD4 T cells was identified. Immunization of mice with the AS15 peptide provided partial protection to subsequent parasite challenge, resulting in a lower parasite burden and enhanced survival. These findings identify the first CD4 T cell stimulating peptide that can confer protection against toxoplasmosis and show that the knowledge of CD4 T cell stimulating antigens can help in development of more effective vaccines.

Toxoplasma gondii immunization of C57BL/6 mice elicits a potent CD4 T cell response

In order to characterize the T cell response in the C57BL/6 (B6, H-2^b) mice, the animals were immunized with irradiated tachyzoites from the Type II *T. gondii* strain, *Prugniaud* (Pru). Irradiated parasites can invade host cells, but they do not replicate, and intraperitoneal (i.p) injection of irradiated parasites can induce robust T cell mediated immunity that provides protection against lethal challenge with live parasites (Chtanova et al., 2009; Gazzinelli et al., 1991; Suzuki and Remington, 1988). One week post-immunization, splenocytes harvested from immunized B6 mice were examined for *T. gondii*-specific CD4 and CD8 T cell responses *ex-vivo* by measuring intracellular cytokine staining (ICCS) for IFN-γ. In contrast to the H-2^d strain of mice, in which the CD8 T cell response dominates over the CD4 T cell response (Blanchard et al., 2008), B6 mice generated a robust CD4 T cell response but a far weaker CD8 T cell response towards *T. gondii* (Fig. 3A-C). This strong CD4 T cell response was observed *ex-vivo*, as well as in cultures after *in-vitro* restimulations with infected syngeneic APCs (Fig. 3A). Furthermore, during the *in-vitro* restimulations, IFN-γ producing CD4 T cells proliferated better than their CD8 counterparts and thus accumulated to larger numbers (Fig. 3B versus Fig. 3C).

Generation of *Toxoplasma gondii* specific CD4 T cell hybridomas

To further characterize the CD4 T cell response at a clonal level, we generated lacZ inducible T cell hybridomas, as described previously (Karttunen et al., 1992; Sanderson and Shastri, 1994). Briefly, after the second *in-vitro* restimulation, the expanded population of *T. gondii* specific CD4 T cells was fused to the BWZ.36/CD8α fusion partner that lacks the alpha and beta TCR chains and contains a β-galactocidase (lacZ) inducible reporter gene (Karttunen and Shastri, 1991). Upon appropriate TCR stimulation, the hybridomas express lacZ, which can be measured at an absorbance of 595nm by conversion of the substrate to chlorophenol red (Sanderson and Shastri, 1994). After fusion, we successfully generated 14 *T. gondii* specific CD4 T cell hybridomas and confirmed their specificity for *T. gondii* using uninfected versus infected B6 APCs. Additionally, the MHC-restriction was also confirmed with infected APCs lacking MHC Class II I-A^b molecules (Fig. 4A). Hybridoma BTg01Z.A was sub-cloned and chosen for further experiments. Sub-cloned hybridoma BTg01Z.A produced lacZ specifically in response to *T. gondii* infected wild-type cells, but not in response to those lacking I-A^b (Fig. 4B and C). The T cell hybridoma did not recognize *T. gondii* derived recombinant profilin, which was earlier shown to be recognized by some CD4 T cells in B6 mice (Yarovinsky et al., 2006).
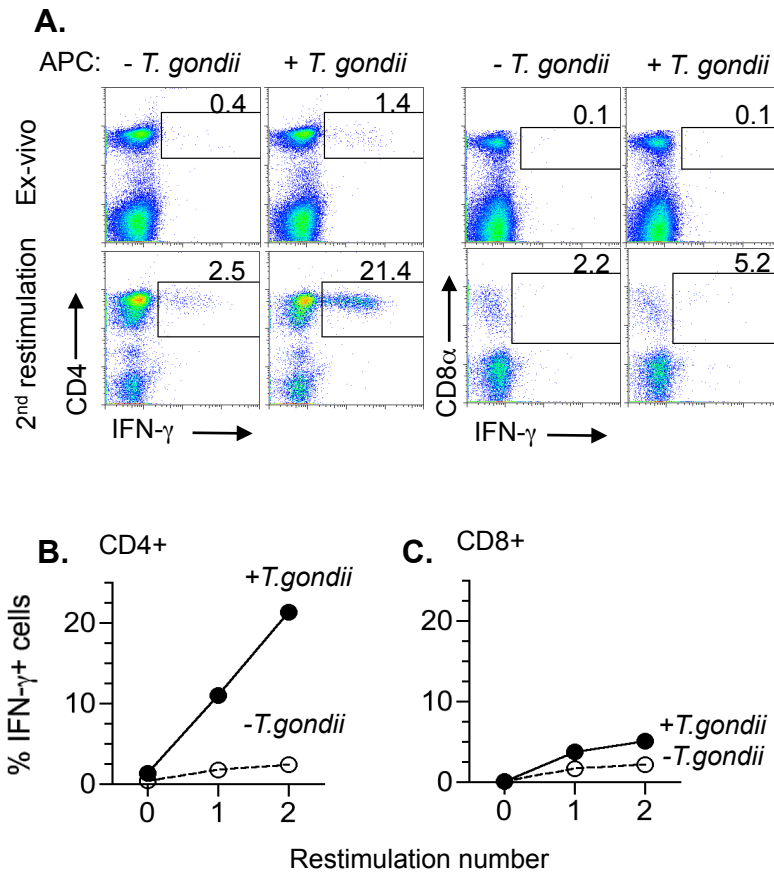
Figure 3: *T. gondii* **immunization of C57BL/6 mice elicits a potent CD4 but weak CD8 T cell response.**
C57BL/6 mice were immunized with irradiated *T. gondii* tachyzoites. Splenocytes were harvested from mice 1 wk post immunization and *T. gondii* specific CD4 and CD8 T cell responses were measured by intracellular cytokine staining for IFN-γ. Antigen presenting cells +/- *T. gondii* were used as stimulators for *ex-vivo* and *in-vitro* restimulations. *Ex-vivo* and *in-vitro* restimulation results are shown as **A)** Representative flow cytometry plots **B and C)** Plots depicting the expansion of **B)** CD4 T cells and **C)** CD8 T cells over the course of two *in-vitro* restimulations. Data are representative of three experiments.
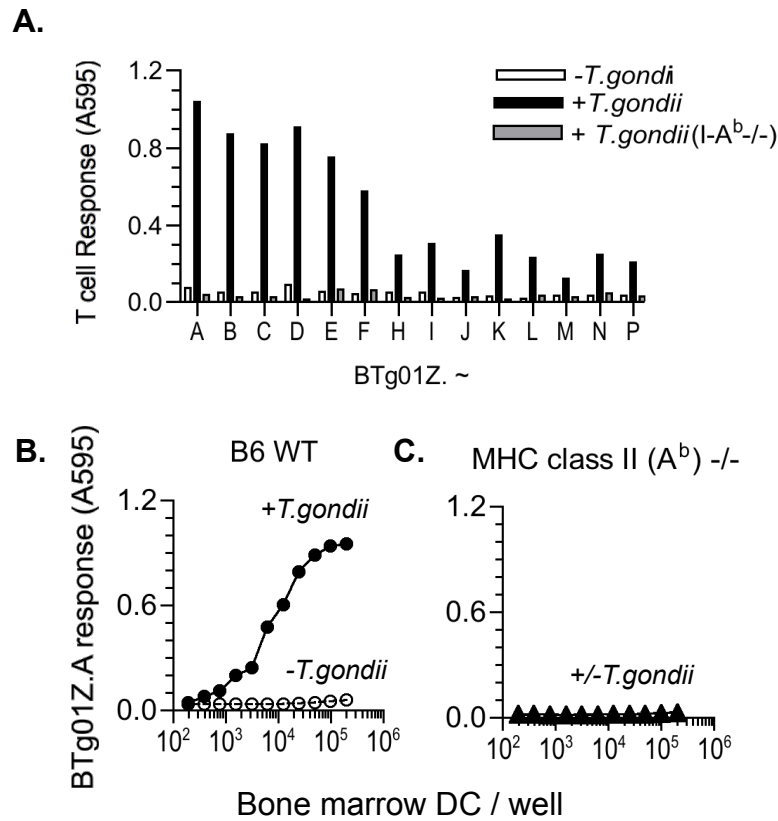
Figure 4: Generation of *T. gondii* specific CD4 T cell hybridomas that are antigen specific and MHC-restricted.

A) C57BL/6 mice were immunized with irradiated *T. gondii* tachyzoites. Splenocytes were harvested from mice 1 wk post immunization. After two *in-vitro* restimulations, proliferating CD4 T cells were fused to lacZ containing fusion partner (Kartunnen et al., 1991) to generate *T. gondii* specific CD4 T cell hybrid. LacZ response of CD4 hybrids after an overnight culture with wildtype APCs +/- *T. gondii* or APCs lacking I-A^b MHC molecules + *T. gondii*. **B, C)** Hybridoma BTg01Z.A was sub-cloned and lacZ response of sub-cloned CD4 hybridoma (BTg01Z.A) after an overnight culture with **B)** wild-type APCs +/- *T. gondii* or **C)** APCs lacking I-A^b MHC molecules + *T. gondii*. Data is representative of at least two independent experiments.

Screening for the cognate antigen recognized by BTg01Z.A CD4 T cell hybridoma

The Shastri laboratory has previously shown that recombinant *E. coli* expressing a variety of proteins can serve as an exogenous antigen source for MHC Class II presentation pathway in bone marrow derived DCs (BMDC) (Campbell and Shastri, 1998; Sahara and Shastri, 2003; Sanderson et al., 1995). The pMHC II complex on BMDCs fed recombinant *E. coli* is then detected by activation of the appropriate T cell hybridoma. We therefore used the strategy outlined in Figure 5 to screen a *T. gondii* cDNA library prepared in an IPTG inducible vector to drive expression of recombinant proteins in *E. coli* cells. Small pools of recombinant *E. coli*, grown in 96 well plates, were then fed to BMDCs derived from B6 mice and used as APCs for stimulating BTg01Z.A hybrid. One well (Pool 778) from twelve 96-well plates screened, stimulated lacZ response in the T cell hybrid (Fig. 6A and 6B).

Identification of the cognate antigen recognized by BTg01Z.A CD4 T cell hybridoma

To further narrow down the *T. gondii* cDNA expressed in *E.coli* that yielded a positive hit in the screen, bacterial pool 778 was further fractionated into individual colonies, and rescreened for its ability to stimulate BTg01Z.A hybrid. Six of ten colonies tested scored positive (Fig. 7A). The T cell response to the recombinant *E.coli* was I-A^b restricted because BMDC lacking I-A^b were unable to stimulate the BTg01Z.A hybrid (Fig. 7B). We therefore selected *E.coli* colony pTg778.76 for further characterization (Fig. 7C). Notably as a control in the screening process, the recombinant *E.coli* expressing OVA did stimulate lacZ response in OVA-specific CD4 T cell hybridoma (OTIIZ) (Fig. 7D). Thus, pTg778.76 encoded the BTg01Z.A stimulating antigen presented by MHC class II molecule (Fig. 7C).

The nucleotide sequence of pTg778.76 was blasted against *T. gondii* database (www.toxodb.org) and yielded a match with the TGMe49-012300 (28.m00307), a gene on chromosome X encoding a hypothetical protein of 683 amino acids (Fig 8). We named this protein CD4Ag28m to indicate its function as an antigen for CD4 T cells presented on I-A^b MHC class II molecules. This full-length protein sequence contains a putative signal peptide at its N-terminus and is therefore likely to be secreted by *T. gondii*.

Identification of the minimal antigenic peptide

Compared to the CD4Ag28m sequence in the *T. gondii* database (toxodb.org), plasmid pTg778.76 was missing the sequences corresponding to the 5'UTR and the first 96 amino acids from the N-terminus of the putative protein (Fig. 8). To
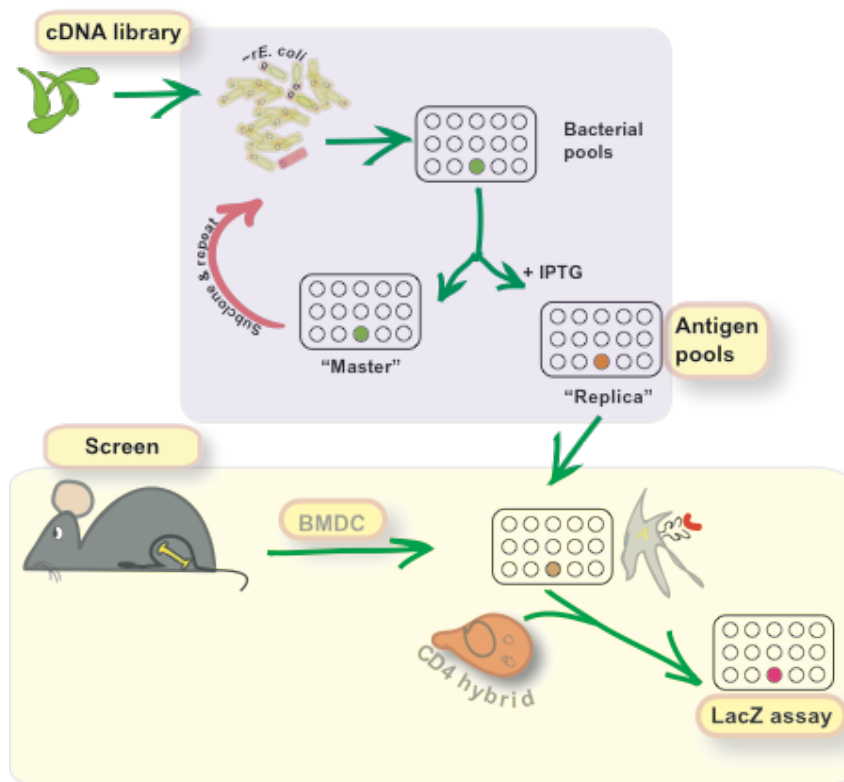
Created by Dr. Nilabh Shastri.

Figure 5: A schematic depicting the strategy used for identifying the antigen recognized by BTg01Z.A as described previously (Sanderson et al., 1995; Campbell et al., 1998).
In brief, the strategy involved the expression of *T. gondii* cDNA expression library into inducible prokaryotic expression vector. Recombinant *E. coli* expressing the different *T. gondii* cDNAs were fed to BMDCs that were incubated with the *T. gondii* specific CD4 hybrid used to probe the cDNA library. A positive pool was identified in the colormetric assay, where upon the CD4 hybrid stimulation, β-galactosidase was expressed converting the substrate from yellow to pink.
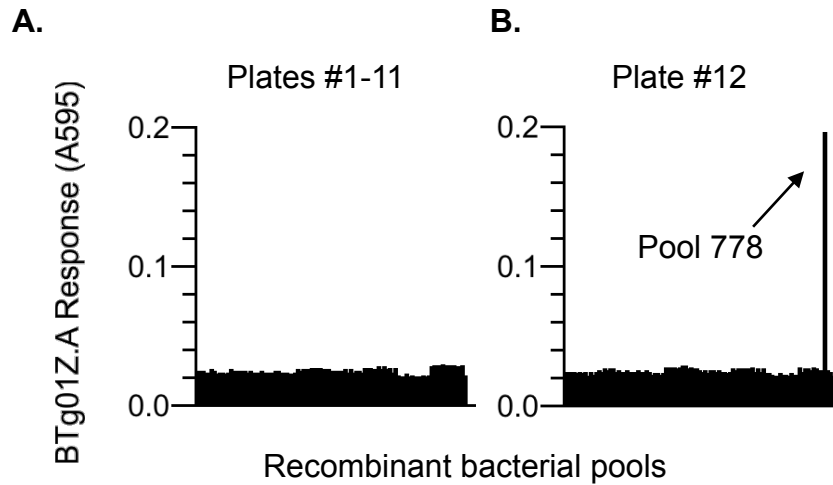
Figure 6: Identification of a recombinant bacterial pool capable of stimulating *T. gondii* specific BTg01Z.A, CD4 hybridoma.
The *T. gondii* cDNA library was prepared into an IPTG inducible prokaryotic expression vector. Small pools of recombinant *E. coli* expressing the cDNAs were then fed to bone-marrow derived dendritic cells (BMDCs) to stimulate the lacZ response of the BTg01Z.A, CD4 T cell hybridoma. **A, B)** One pool of recombinant bacteria (#778) among twelve 96-well plates screened stimulated lacZ response of the hybrid.
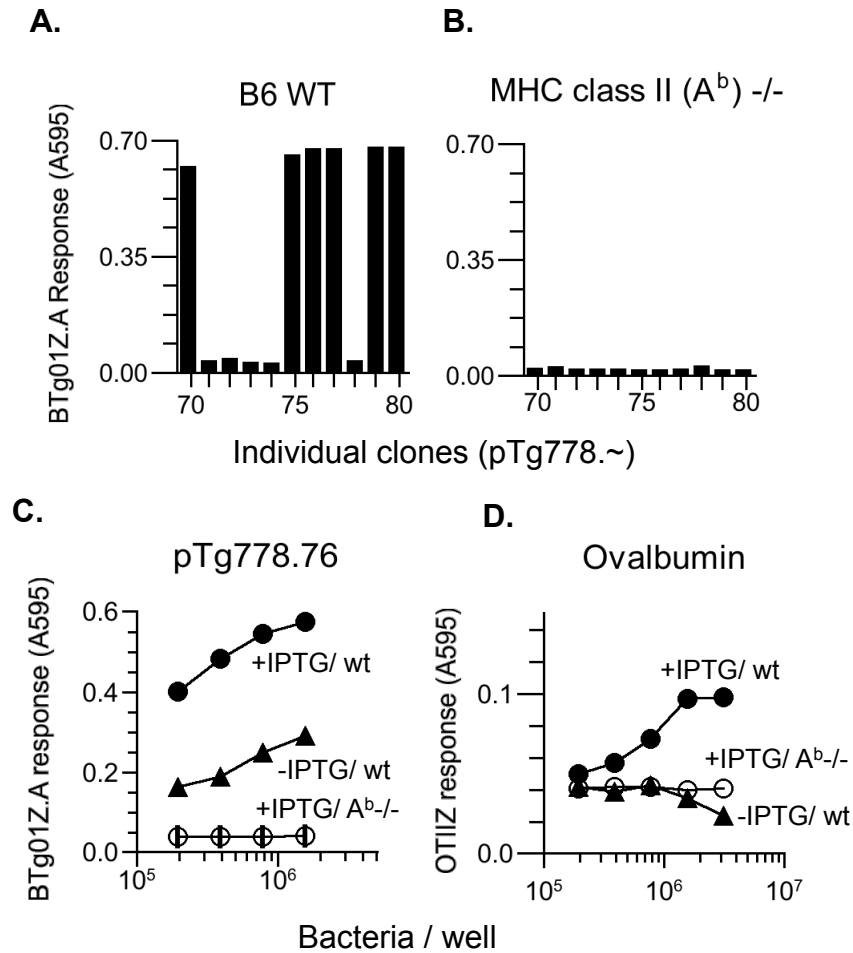
Figure 7: BTg01Z.A response to the positive hit in the screen and its fractionated single clone is antigen specific and MHC restricted.
A) The stimulatory pool found in Fig. 6 was further fractionated until a single stimulatory clone was identified and sequenced. BTg01Z.A response to individual bacterial colonies isolated from pool 778 (Representative 10 clones are shown). Clone 778.76 was selected for further analysis. **B, C)** BTg01Z.A response was antigen specific and MHC restricted. **D)** OTIIZ, I-A^b restricted CD4 hybridoma recognizing OVA, was used as a positive control for the entire screening process.

TGMe49_012300 (28.m00307) = CD4Ag28m

T.gondii Me49, Chr X
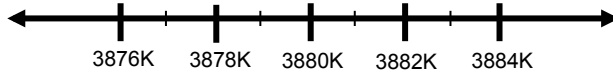

```
                    10        20        30        40        50        60        70        80        90       100
TGMe49_012300  MSAQGQQGVRSTVAAGKNRFWQPAPITVLLVLSSSFLFAFAANTPQNSSTTGQSTPSDDHGVSTEHIHDAVTVDAAEHAGEDEMTACSPEAARVIEARVA
pTg778.76      ----------------------------------------------------------------------------------------------....
                   110       120       130       140       150       160       170       180       190       200
TGMe49_012300  TLKAETEAAVAEVRRTEAMQRELFGRLNAKVRAMNDRLDELSDAQKSLAKRLETTGQLLIADREEQAKRFTTDVETYIIESGLKKAVFQQREIAAKLQTL
pTg778.76      ....................................................................................................
                   210       220       230       240       250       260       270       280       290       300
TGMe49_012300  AESNQKSKQALDALLQHVDDEKEQTKQWISTVVRDLEKYVEARTQTTLESLQRDAQEHANFMQQQLNDMKLDHLLLLQAVSSAETQLNERGIAGVDISQL
pTg778.76      ....................................................................................................
                   310       320       330       340       350       360       370       380       390       400
TGMe49_012300  DESASFERMMTTFEKVLKEQLKGKFVLPIFVVTQCPDSMERVAFMNTYLVPSKRNFRAVFQALGHTPQASAVVIRIESVMVEAWMEVNGKVTGMQYLTPP
pTg778.76      ....................................................................................................
                   410       420       430       440       450       460       470       480       490       500
TGMe49_012300  AHEALTVTTKDRFGFEFLRRVRELETGDFKADPHGRSVVIAFVGNRKGRIGGLPLTDVTEFNKFQALRTLATMHSSPIKETNNLWQIMKTEVDIVATFAA
pTg778.76      ....................................................................................................
                   510       520       530       540       550       560       570       580       590       600
TGMe49_012300  MPRVEVGGSSLRIYQGIAPEPRLPASPFCRNAFTLIRIEVAKSPRGKQEYVYPKASLLKKVFDILVSTNTSQDSGGDGSFAVIRANVSNAGWLQSKLYAT
pTg778.76      ....................................................................................................
                   610       620       630       640       650       660       670       680
TGMe49_012300  ADRMAVEIHRPVPGTAPPSFSSDDVAGEHRANSMAAVVDQIRKQMTDVVLPNIDPEDSTSRRQVLVHICEIGVTCNSDRHPEA
pTg778.76      .................................................................................
```

Figure 8: The cognate antigen recognized by BTg01Z.A hybridoma is a previously unknown protein in the *T. gondii* database, as identified by expression cloning.

T. gondii database (www.toxodb.org) BLAST search results revealed that the antigenic cDNA encoded by clone pTg778.76 corresponded to a truncated version of the *T. gondii* protein, TGMe49-012300 (28.m00307). Here we call the protein CD4Ag28m for "CD4 antigen 28m". pTg778.76 is missing the 5'UTR and first 96 amino acids of ORF. Alignment of the amino acid sequence of the truncated cDNA clone as compared to the full-length clone is depicted here. (-) represent missing amino acids in the sequence and (.) represent alignment between the two sequences. Final antigenic epitope in pTg778.76 is boxed in red.

define the antigenic peptide that stimulates BTg01Z.A in the p778.76 sequence, we first searched for candidate peptides using the appropriate algorithm in the immune epitope database (www.immuneepitope.org) that could bind the I-A^b molecule and thus serve as potential TCR ligands. This analysis suggested 4 candidate peptides that could bind I-A^b MHC Class II molecule and serve as potential TCR ligands. Using PCR mutagenesis with appropriate primers (Table 3), we generated various N-terminal and C-terminal deletion mutant constructs of pTg778.76 (Fig. 9A). The deletion constructs were cloned into the pTRCHis B vector and recombinant *E. coli* expressing the truncated versions were fed to B6 BMDCs, and the cells were used to stimulate BTg01Z.A (Fig. 9B-D). All of the N-terminal deletion constructs, ΔN-B expressing amino acid (aa) 379-683, and ΔN-D expressing aa 577-683, retained the putative antigenic region and stimulated BTg01Z.A (Fig. 9B). On the other hand, C-terminal deletion constructs, ΔC-E expressing aa 97 to 321, and ΔC-G expressing aa 97 to 491, failed to stimulate BTg01Z.A (Fig. 9C). Based upon the positive antigenic activity of ΔN-D expressing aa 577-683 and the lack of antigenic activity by ΔC-G expressing aa 97-491, we concluded that the antigenic activity was located in the C-terminal portion of the protein between aa 577-683. To further narrow this down, we generated deletion constructs missing parts of both the N-terminal and C'terminal region. Construct ΔNΔC-J expressing aa 577-631 as well as deletion construct ΔNΔC-K, stimulated BTg01Z.A and mapped the antigenic activity to a peptide of 24 aa, 602-625 (Fig. 9D).

To independently confirm the assignment of antigenic activity, we tested overlapping synthetic peptides within the 24-mer region 602-625 (Fig. 10A). BMDCs pulsed with peptides IA9 and IA12 failed to stimulate the BTg01Z.A hybridoma (Fig. 10B), while BMDCs pulsed with AA12 stimulated BTg01Z.A response. However, this response was substantially enhanced when BMDCs were pulsed with the 15-mer peptide, AS15 (Fig. 10B). We therefore concluded that AS15 is the optimal peptide contained within CD4Ag28m that is presented by I-A^b and recognized by the BTg01Z.A T cell.

Detection of AS15 specific CD4 T cells in mice immunized with *Toxoplasma gondii*

The identification of AS15 peptide allowed us to detect this defined specificity of CD4 T cell response that makes up part of the total response towards *T. gondii*. To determine the fraction that AS15 specific response makes, we immunized C57BL/6 with irradiated Type II *T. gondii*. Two wks post-immunization an *ex-vivo* analysis of splenocytes from immunized mice was assayed through intracellular cytokine staining (ICCS) for IFN-γ or MHC Class II tetramer staining. *Ex-vivo* restimulation in the presence of *T. gondii* infected or AS15 peptide pulsed APCs revealed that AS15 specific response by ICCS averaged 0.2% of the total CD4 T cells, which calculates to 4% out of the total *T. gondii*-specific CD4 T cell response (Fig. 11A and 11B). These responses were corrected for background
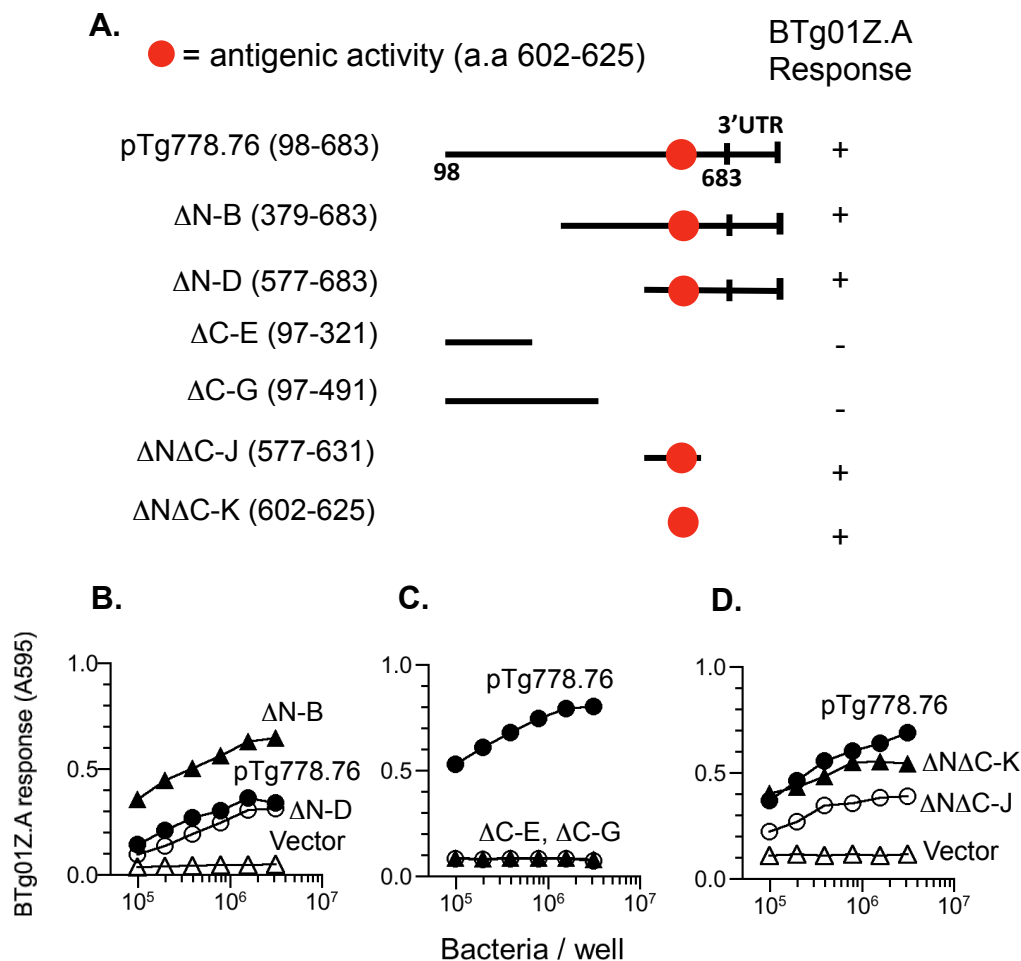
Figure 9: Identification of the antigenic peptide within CD4Ag28m that stimulates BTg01Z.A hybridoma.
A) Schematic representation of the N-terminal and C-terminal deletion constructs generated to identify the antigenic epitope within clone pTg778.76. The clone itself was truncated and missing the 5'UTR and the first 96 amino acids. The specific amino acids tested in each construct are shown in parentheses and the region with the antigenic activity is indicated by the red circle. **B, C, D)** BTg01Z.A lacZ response against different deletion constructs expressed in *E.coli* that were incubated with wild-type BMDCs for presentation after an overnight stimulation.

A.

```
                610          620
        DRMAVEIHRPVPGTAPPSFSSEDV
```

```
    IA9           IHRPVPGTA

    IA12          IHRPVPGTAPPS

    AA12        AVEIHRPVPGTA

    AS15        AVEIHRPVPGTAPPS
```

B.
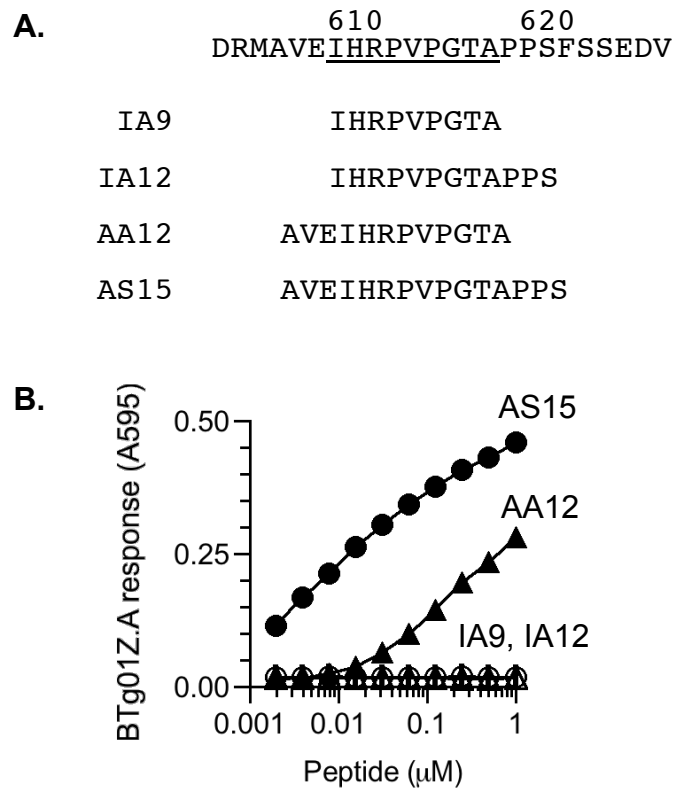
Figure 10: BTg01Z.A recognizes the 15-mer epitope, AS15, from the parasite protein CD4Ag28m.
A) Sequences of overlapping peptides within the 24-mer antigenic region were used for fine mapping of the BTg01Z.A epitope. The core sequence that binds I-A^b MHC class II molecule is underlined. **B)** BTg01Z.A lacZ response to wild-type BMDCs that were pulsed with titrating concentrations of indicated peptides.
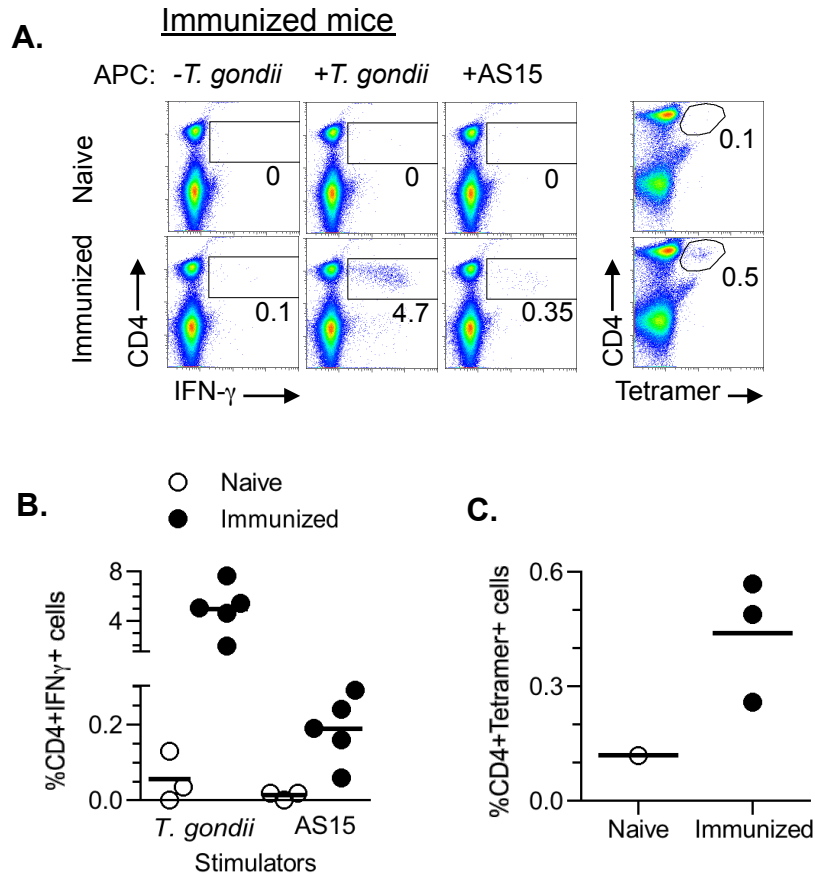
Figure 11: A fraction of the *T. gondii* specific CD4 T cell response is AS15 specific in *T. gondii* immunized mice.

A-C) C57BL/6 mice were immunized with irradiated $5x10^6$ *T.gondii* tachyzoites. Splenocytes were harvested from mice 2 wks post immunization and *T. gondii* specific CD4 T cell responses were measured by intracellular cytokine staining for IFN-γ or by staining with tetramers. **A)** Representative flow cytometry plots from naïve and immunized mice. Left panels show intracellular IFN-γ staining after *in vitro* restimulation with antigen presenting cells either, +/- *T. gondii*, or 10μM AS15 peptide. Right panels show MHC class II I-A^b –AS15 tetramer staining. **B)** Compiled data showing splenic *T. gondii* specific and AS15 specific responses from immunized mice. Data are corrected for background based on responses by CD4 T cells towards uninfected APCs or APCs with irrelevant peptide. Each dot represents an individual mouse. **C)** Compiled data showing MHC class II I-A^b –AS15 tetramer staining on splenocytes from immunized mice. Cells were also co-stained with CD4 antibody. Data are representative of at least three independent experiments.

responses to uninfected or irrelevant peptide pulsed APCs. No significant response was seen in naïve mice. Similarly, peptide MHC-Class II I-A^b AS15 tetramer staining showed that AS15 specific responses were on average 0.4% of the splenic CD4 T cell response (Fig. 11A and 11C). Overall, we concluded that AS15 specific CD4 T cells represent a readily detectable population of the total CD4 T cell response to *T. gondii* in C57BL/6 mice.

Similar fraction of AS15 specific CD4 T cells were detected in *Toxoplasma gondii* infected mice as measured in immunized mice

To determine the fraction of the total *T. gondii* specific CD4 T cell response directed towards the AS15 peptide in chronically infected animals, we fed B6 mice orally 25-40 Type II cysts. Splenocytes or brain leukocytes were harvested 3-4 weeks post infection. *Ex-vivo* restimulation of CD4 T cells from infected mice, in the presence of APCs pulsed with AS15 peptide, followed by intracellular cytokine staining (ICCS) for IFN-γ revealed that on average 0.6% (spleen) and 1.5% (brain) of the total CD4 T cells responded to this peptide (Fig. 12A-C). No significant response was observed in naive mice or in *T. gondii* infected mice with APCs pulsed with an irrelevant peptide (OVAp) that also binds to I-A^b MHC molecule, which was corrected for in the depicted response. Similarly, 1.3% (spleen) and 3% (brain) of CD4 T cells from infected mice stained with the AS15 loaded MHC class II tetramers (Fig. 12A and 12D-E). Given that on average 8% of CD4 T cells in the spleen responded to *T. gondii* infected APCs in these experiments, we estimate that 7.5% of *T. gondii*-specific CD4 T cell response is specific for the AS15 peptide. Likewise, in the brain, 17% of CD4 T cells responded to *T. gondii*. Therefore, we estimate that on average, 9% of the *T. gondii*-specific CD4 T cells are also specific for the AS15 peptide in the brains of chronically infected mice. Similar to immunized mice, AS15 specific CD4 T cell response constitutes a readily detectable fraction of the overall *T. gondii* specific response in C57BL/6 mice.

Kinetics of AS15 specific CD4 T cell response over the course of *Toxoplasma gondii* infection

Since the AS15 specific response constitutes a detectable fraction of the total *T. gondii* specific response in chronically infected animals, we also wanted to determine the kinetics of AS15 response throughout the course of infection. To assess the kinetics, we infected B6 mice orally with 25-40 Type II cysts and harvested splenocytes 1-4 wks post-infection. Splenocytes were restimulated *ex-vivo* in the presence of infected or peptide-pulsed APCs, followed by ICCS for IFN-γ or staining with MHC-Class II, AS15 loaded tetramers. AS15 specific CD4 T cell response expanded, as did *T. gondii* specific response during the acute phase of infection (2 wks) (Fig. 13A). However, during the chronic phase of infection, both AS15 and *T. gondii* specific responses declined. Despite the

decline, the responses observed during chronic phase were still higher than those observed at 1 wk. post-infection (Fig. 13A). No significant response was seen in naïve mice throughout. Similarly, staining with MHC Class II I-A^b AS15 tetramers also showed same results where maximum percentage of AS15 cells were detected 2 wks post infection followed by a contraction and decline during the chronic stages of infection (Fig. 13B).

Polymorphic versions of CD4Ag28m from different strains of *Toxoplasma gondii* express the antigenic peptide

Currently, the only function known for CD4Ag28m is its ability to serve as a CD4 T cell antigen in C57BL/6 mice. According to the *T. gondii* database, CD4Ag28m contains a putative signal sequence indicating that it is most likely a secretory protein. Interestingly, although the full-length protein is polymorphic between type I, II and III strains of *T. gondii*, the AS15 antigenic peptide is conserved in all the different alleles of these strains. Therefore, to assess if the type I (Yarovinsky et al.) and III (*CTG*) strains were equally antigenic to type II (Prugniuad) strain, in terms of AS15 specific CD4 T cell response, we infected BMDCs from B6 mice *in-vitro* with irradiated tachyzoites from type I, II and III strains of *T. gondii*. BMDCs infected with all three strains, stimulated a lacZ response from BTg01Z.A (Fig. 14A and 14B). Uninfected BMDCs failed to stimulate BTg01Z.A. Additionally, immunization of B6 mice with irradiated type II or III strains, showed that AS15 specific response, as measured by ICCS for IFN-γ, in the presence of peptide pulsed APCs was comparable between the two strains (Fig. 14C). Splenocytes were harvested two weeks post-immunization to assess this response. MHC-Class II I-A^b AS15 tetramer staining also revealed that a comparable percentage of AS15 cells was present in mice immunized with either of the strains (Fig. 14D). Therefore, type I (Yarovinsky et al.), II (*Pru*) and III (*CTG*) strains of *T. gondii* express the antigenic peptide, AS15.

BTg01Z.A can recognize AS15 from heat-killed parasites

Presentation to CD4 T cells requires exogenous antigens to be phagocytosed by antigen presenting cells to be presented on MHC Class II molecules (Fig. 2). During *T. gondii* infection, it is known that CD4 antigens can be presented by cells that are directly infected with *T. gondii* or bystander APCs that have phagocytosed parasitic debris. To determine whether presentation of AS15 to BTg01Z.A requires direct secretion of CD4Ag28m via infection of the APC or if heat-killed parasites, which can only be phagocytosed, can also present AS15, we infected B6 BMDCs *in-vitro* with either irradiated or heat-killed GFP expressing tachyzoites of Type II strain (*Prugniaud*-GFP). LacZ response of BTg01Z.A was much enhanced when stimulated with BMDCs that had phagocytosed heat-killed parasites as compared to irradiated parasites at a MOI of as low as 5:1 (Fig. 15A-C), even when the infection index of heat-killed
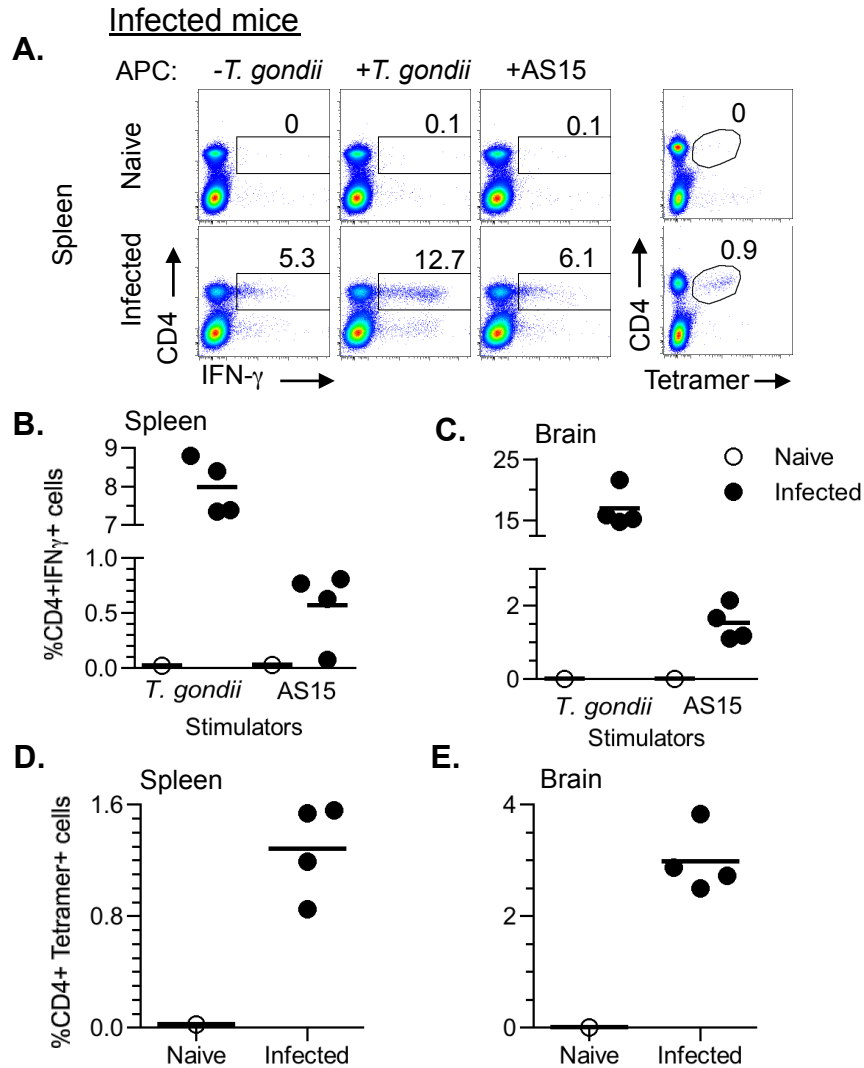
Figure 12: A fraction of the *T. gondii* specific CD4 T cell response is AS15 specific in mice infected chronically with *T. gondii*.
A-E) C57BL/6 mice were orally infected with 25-40 *T. gondii* cysts. Splenocytes and brain leukocytes were harvested from mice 3-4 wks post infection, *T. gondii* specific CD4 T cell responses were measured by intracellular cytokine staining for IFN-γ or by staining with peptide-MHC tetramers. **A)** Representative flow cytometry plots of splenocytes from naïve and infected mice. Left panels show intracellular IFN-γ staining after *in vitro* restimulation with antigen presenting cells either, +/- *T. gondii*, or 10μM AS15 peptide. Right panels show MHC class II I-A^b –AS15 tetramer staining. **B, C)** Compiled data showing *T. gondii* specific and AS15 specific responses from **B)** spleen and **C)** brain of infected mice. Data are corrected for background based on responses by CD4 T cells towards uninfected APCs or APCs with irrelevant peptide. Each dot represents an individual mouse. **D, E)** Compiled data showing flow cytometry analysis of MHC class II I-A^b –AS15 tetramer staining on splenocytes from **D)** spleen and **E)** brain of mice. Cells were also co-stained with CD4 antibody. Data are representative of at least three independent experiments.

39

A.

Spleen

%CD4+IFNγ+ vs Wks post infection

—○— Naive ——●—— T. gondii —▲— AS15

B.

Spleen

%CD4+Tetramer+ vs Wks post infection

—○— Naive ——●—— Infected
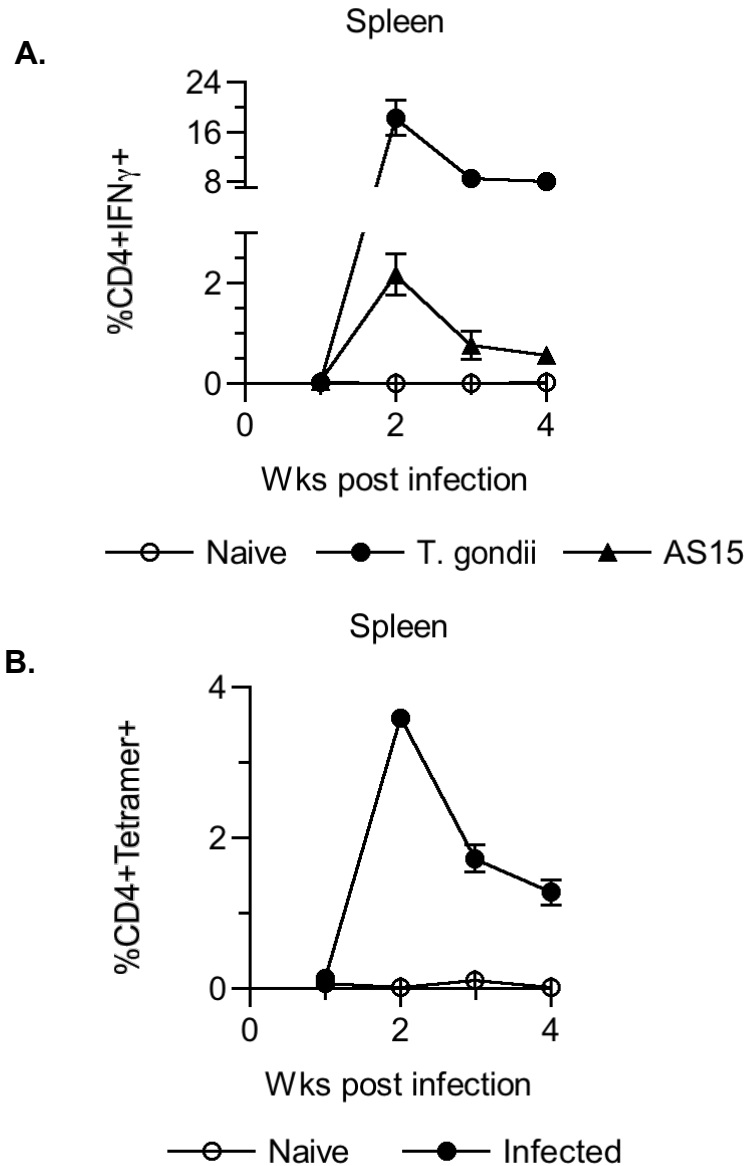
Figure 13: Expansion of AS15 CD4 T cell response occurs during the acute phase of infection.
C57BL/6 mice were orally infected with 25-40 *T. gondii* cysts. Splenocytes were harvested from mice 1-4 wks post infection. *T. gondii* specific CD4 T cell responses were measured by intracellular cytokine staining for IFN-γ or by staining with peptide-MHC tetramers. **A)** Compiled data showing *T. gondii* and AS15 specific responses from spleens of infected or naïve mice over the course of infection. Each dot represents average of four mice. Data are corrected for background based on CD4 T cell responses towards uninfected or irrelevant peptide pulsed APCs. **B)** Compiled data showing MHC class II I-A^b-AS15 tetramer staining on splenocytes from infected or naïve animals over the course of infection. Each dot represents an average from four mice. Cells were also co-stained with CD4 antibody. Data are representative of two independent experiments.
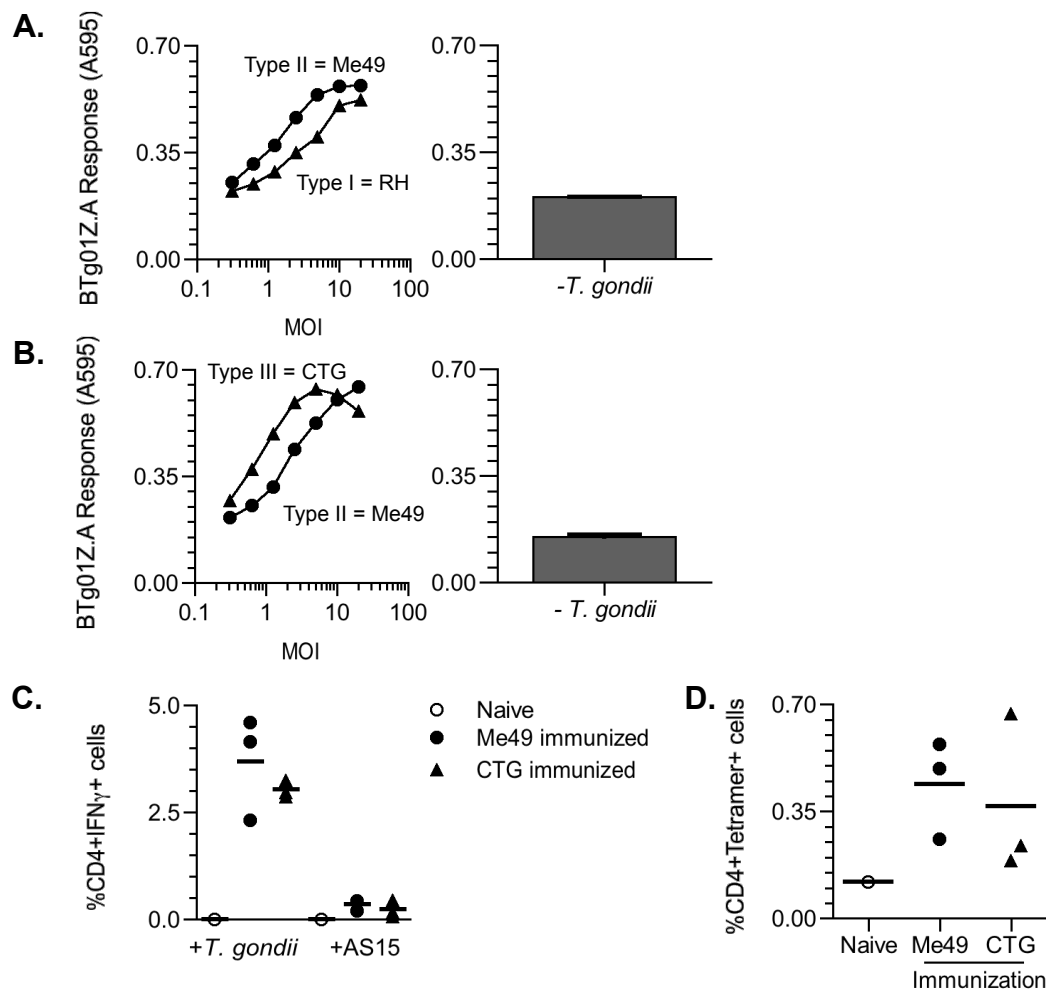
Figure 14: BTg01Z.A recognizes the 15-mer epitope, AS15, from different strains of *T. gondii*.

A, B) BMDCs from C57BL/6 mice were infected *in-vitro* with irradiated Type I (RH), II (Me49) or III (CTG) strain of *T. gondii*. BTg01Z.A lacZ response after an overnight stimulation with **A)** Type I and II or **B)** Type II and III infected BMDCs. Right panel depicts background lacZ response against uninfected BMDCs. **C, D)** C57BL/6 mice were immunized with irradiated 5x10^6 *T.gondii* tachyzoites from Type II (Me49) and III (CTG) strain. Splenocytes were harvested from mice 2 wks post immunization and *T. gondii* or AS15 specific CD4 T cell responses were measured by intracellular cytokine staining for IFN-γ or by staining with tetramers. **C)** Compiled data showing CD4 T cell responses from immunized or naïve mice towards *T. gondii* infected or AS15 peptide pulsed APCs. Each dot represents an individual mouse. Data are corrected for background based on CD4 T cell responses towards uninfected or irrelevant peptide pulsed APCs. **D)** Compiled data showing MHC class II I-A^b-AS15 tetramer staining on splenocytes from immunized or naïve animals. Each dot represents an individual mouse. Cells were also co-stained with CD4 antibody. Data are representative of three independent experiments.

parasites was much lower than that of irradiated parasites (Fig. 15E). At a MOI of 1:1 there was no difference in the lacZ response of the hybrid to the two different treatments of the parasites (Fig. 15D). This was not surprising because at MOI 1:1, the infection index of heat-killed parasites was barely detectable (Fig. 15E).

Additionally, the T cell response to AS15 is more efficient when APCs are not directly infected with the parasites. This was evident when we infected BMDCs *in-vitro* with irradiated Type II strain of parasites, expressing a derivative of the red fluorescent protein (RFP) (*Prugniuad*-tomato), and 16h post infection, sorted the RFP+ and RFP- cells using fluorescence activated cell-sorting (FACS). RFP+ BMDC cells were actively infected, while RFP- cells could have come in contact with the parasites or parasitic debris, but did not contain any RFP. Both sets of cells were used to as APCs for stimulation of BTg01Z.A and interestingly, BTg01Z.A lacZ response to RFP- cells was much enhanced in comparison to RFP+ cells (Fig. 16A). CTgEZ hybridoma that recognizes *T. gondii* antigen GRA6 was used as a control (Fig. 16B). It has been shown that the presentation of GRA6 requires direct infection of APCs (Blanchard et al., 2008). Presentation by heat-killed parasites, and by APCs not containing parasites shows that phagocytosis of parasites or parasitic debris can be a component of presentation of AS15 to CD4 T cells. In contrast, it is possible that the presence of an active infection allows the parasite to undergo an immune evasion mechanism, which can involve manipulation of the components of the presentation pathway leading to the reduction of presentation of antigenic peptides such as AS15.

BTg01Z.A response to AS15 is partially dependent on optimal phagocytosis

With the findings that both phagocytosed parasites or parasitic debris and direct invasion by parasites can allow for the presentation of AS15 to CD4 T cells, we wanted to determine if impairment of phagocytosis will alter presentation of AS15. We addressed this by using BMDCs from either wildtype B6 or UNC93b mutation (3d) mice. UNC93b is an ER resident protein important for TLR signaling. In a forward genetic screen using germline mutagen N-ethyl-N-nitrosourea, a single nucleotide substitution was identified, which abrogates signaling via TLR3, 7, and 9, prevents cross presentation, and diminishes MHC Class II presentation of exogenous antigen (Tabeta et al., 2006). More recently, it has been shown that UNC93b mutant mice are more susceptible to *T. gondii* because they abolish TLR11 dependent IL-12 secretion by DCs leading to attenuated Th1 responses against *T. gondii* (Pifer et al., 2011). In another study, it was shown that UNC93b deficient mice are susceptible to *T. gondii* infection due to uncontrolled parasite replication as UNC93b protein was shown to be recruited to the PV during infection and mediated resistance by controlling parasite replication in the PV (Melo et al., 2010).
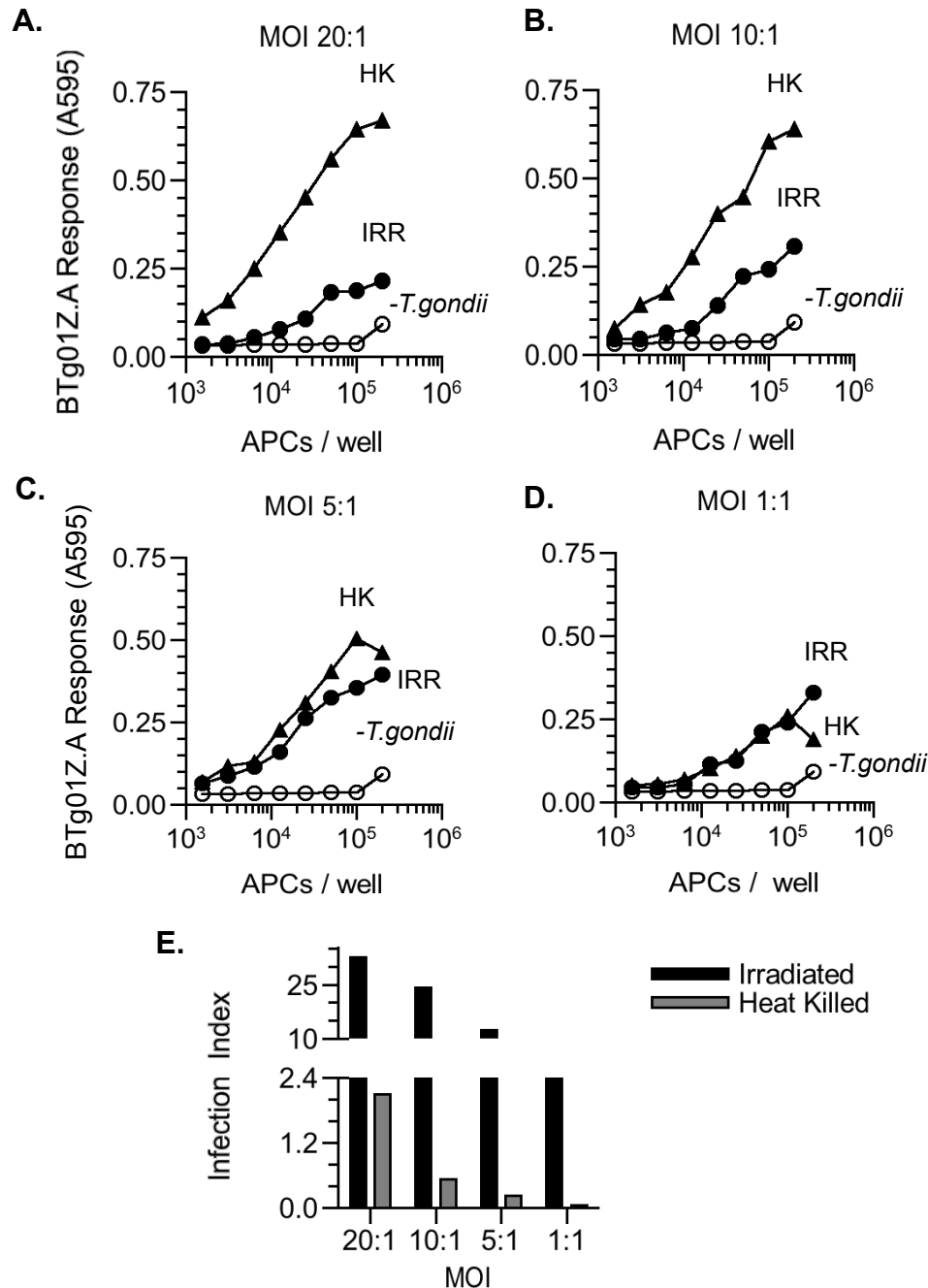
Figure 15: BTg01Z.A stimulation is enhanced in the presence of heat-killed parasites.
A-D) BMDCs from C57BL/6 mice were infected *in-vitro* with irradiated or heat-killed GFP expressing Pruginaud (Pru) strain*T. gondii*. Heat-killed parasites have to be phagocytosed in order to be presented by BMDCs while irradiated parasites can also directly invade. BTg01Z.A lacZ response was measured against BMDCs infected at MOI **A)** 20:1 **B)** 10:1 **C)** 5:1 **D)** 1:1 after an overnight stimulation. **E)** Infection index of both irradiated and heat-killed parasites was measured using flow cytometry by assessing the mean fluorescence of infected cells. Infection index was normalized for GFP loss due to heat.

A.

CD4Ag28m

RFP-

RFP+

-*T. gondii*

BTg01Z.A Response (A595)

APCs/well

B.

GRA6

RFP+

RFP-

-*T. gondii,*

CTgEZ Response (A595)

APCs/well
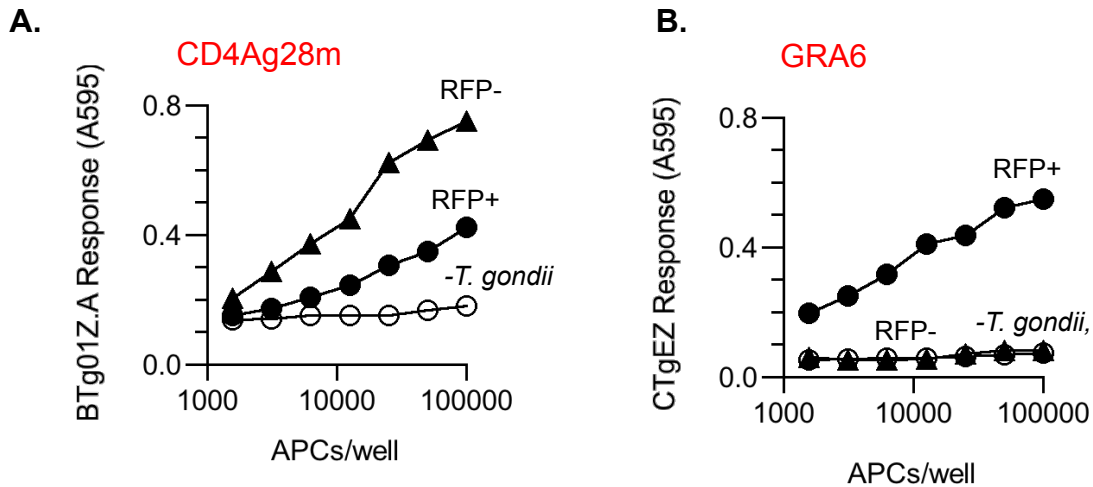
Figure 16: BTg01Z.A can also be stimulated by APCs that are not directly infected but cultured with *T. gondii* infected APCs.
A, B) BMDCs from C57BL/6 mice were infected *in-vitro* with irradiated RFP expressing Pruginuad (Pru) strain of *T. gondii*. After an overnight infection, RFP expressing BMDCs were sorted from the pool of infected BMDCs. **A)** BTg01Z.A lacZ response was measured against both RFP expressing (directly infected) BMDCs and the RFP negative population (co-cultured but not directly infected) BMDCs. **B)** CTgEZ hybridoma recognizing the GRA6 antigen only from directly infected BMDCs (Blanchard et al., 2008) was used as a control.
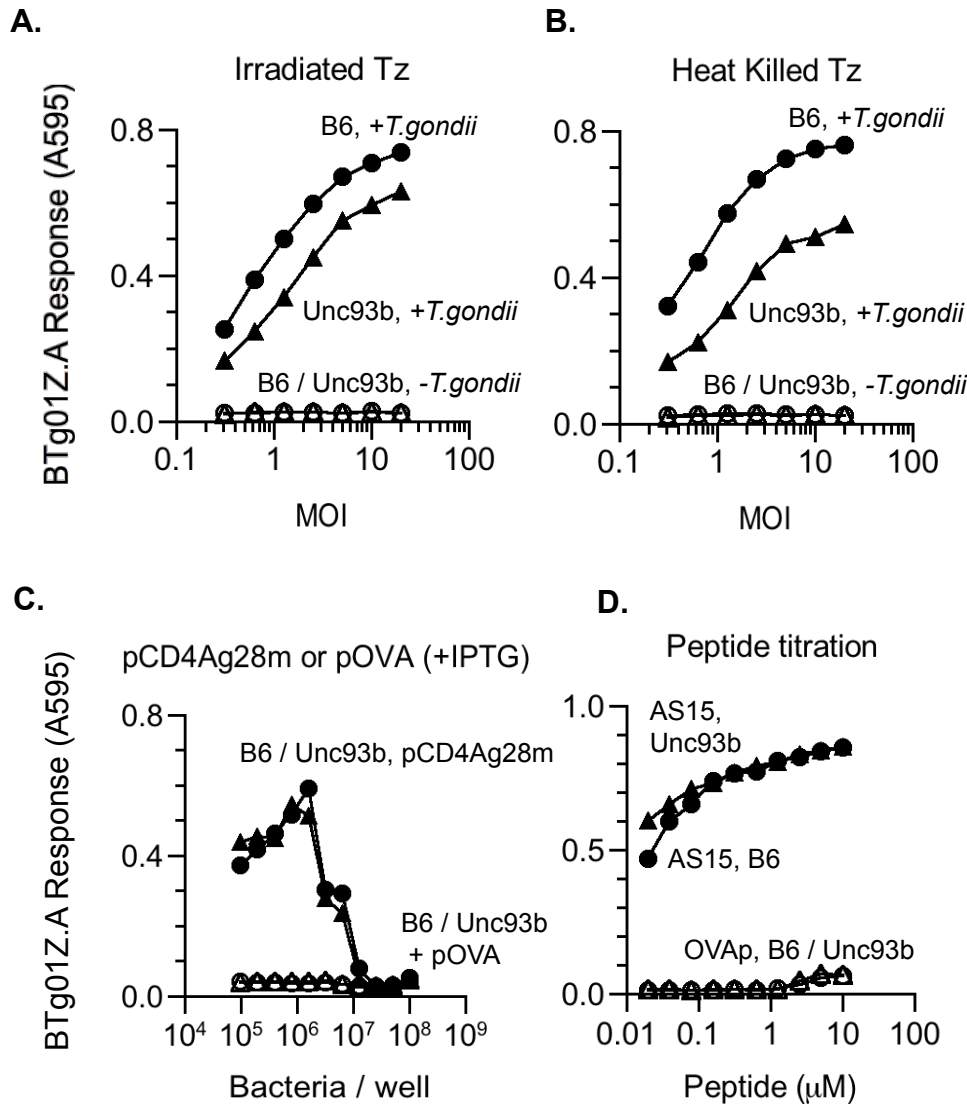
Figure 17: Impaired phagocytosis reduces BTg01Z.A response towards heat-killed parasites.
BMDCs from C57BL/6 and Unc93b mice were infected *in-vitro* with irradiated or heat-killed *T. gondii,* type II, Me49 strain. BTg01Z.A lacZ response against BMDCs infected with **A)** irradiated tachyzoites or **B)** heat-killed tachyzoites measured after an overnight stimulation with infected BMDCs. **C)** BTg01Z.A lacZ response against BMDCs that were fed induced recombinant *E.coli* expressing CD4Ag28m. **D)** BTg01Z.A lac Z response against BMDCs that were pulsed with different concentrations of the AS15 peptide or the irrelevant OVA peptide.

We decided to use UNC93b mutant (3d) mice to determine if mutation of UNC93b will impair presentation of AS15 as a result of diminished MHC Class II presentation. BTg01Z.A lacZ response was slightly diminished when stimulated with BMDCs from 3d versus wild-type mice that were infected *in-vitro* with irradiated parasites (Fig. 17A). On the other hand, BTg01Z.A lacZ response was significantly reduced when stimulated with BMDCS from 3d mice that were incubated with heat-killed parasites (Fig. 17B). Interestingly, BTg01Z.A lacZ response against wild type or 3d BMDCs fed with recombinant *E. coli* bacteria expressing CD4Ag28m was comparable (Fig. 17C). Recombinant *E. coli* need to be phagocytosed in order to be presented, but perhaps level of the antigen in *E. coli* expressing pCD4Ag28m was saturating that no difference was seen even with 3d mice. Additionally, 3d are not fully defective in MHC Class II antigen presentation and the reduced presentation may have been enough to present antigen from recombinant *E. coli*. Lastly, 3d mice still possess comparable levels of surface MHC Class II as compared to wildtype animals (Tabeta et al., 2006). Therefore, BTg01Z.A lacZ response to presentation of AS15 from peptide-pulsed BMDCs was comparable from both strains of mice (Fig. 17D).

Immunization with AS15 peptide protects B6 mice against *Toxoplasma gondii*

Given previous indications that CD4 T cells can play a protective role during *T. gondii* infection (Araujo, 1991; Lutjen et al., 2006; Suzuki and Remington, 1988), we asked whether immunization with the AS15 peptide alone could elicit immunity to *T. gondii* infection. We immunized B6 mice in the footpad with LPS activated BMDCs pulsed with AS15 or an irrelevant peptide (OVAp). We then challenged mice 7 d post immunization with a lethal dose of type II strain of *T. gondii* (10,000 Pru tz) and monitored survival over time. We also measured the CD4 T cell responses, cyst number and parasite load in the brains of surviving mice. The lethal dose for infection was optimized in B6 mice infected intraperitoneally with varying doses of type II strain of *T. gondii* (Fig. 33). Overall, we saw enhanced survival in the AS15 immunized mice compared to control mice (Fig. 18A). Both groups of surviving mice produced a robust CD4 T cell response to *T. gondii* infected BMDCs and a detectable response to AS15 pulsed BMDCs (Fig. 18B), as measured by ICCS for IFN-γ, confirming that the mice were infected and that the AS15-specific response constitutes a proportion of the overall CD4 T cell response in the brain. In addition, mice immunized with AS15 made a greater response to AS15 than mice that were immunized with OVAp (Fig. 18B), confirming that the peptide immunization boosted the overall CD4 T cell response to AS15. Staining with MHC class II tetramers confirmed that AS15 specific CD4 T cells were detectable in the brains of both groups of surviving mice with a greater response in AS15 immunized mice (Fig. 18C). In order to assess protection directly, we also examined parasite loads in the brains of the surviving mice. Mice that had been immunized with the AS15 peptide had a significantly lower cyst burden (Fig. 18D) and reduced parasite number (Fig.

18E) compared to mice immunized with the irrelevant OVA peptide. These data indicate that AS15-specific T cell responses can partially protect mice against *T. gondii* infection, and that peptide immunization can enhance this response over that generated during infection.

CD4 T cell responses to other *Toxoplasma gondii* proteins

AS15 specific CD4 T cell response constitutes a readily detectable response out of the total *T. gondii* specific response in both *T. gondii* infected and immunized B6 mice (Figs. 11-13). Nonetheless, no hierarchical dominance can be assigned to this response since the nature of other CD4 T cell responses to *T. gondii* proteins is not known. Recently, it was also shown that the B6 mice generate a CD4 T cell response to the *T. gondii* protein profilin (Yarovinsky et al., 2006). However, we do not know the identity of the final antigenic peptide that is recognized, to be able to compare peptide specific CD4 T cell responses from infected animals. Therefore to better characterize the repertoire of CD4 T cell response in B6 mice, we assessed the antigen specificity of other known *T. gondii* specific CD4 T cell hybridomas (Fig. 4A). We chose 7 hybridomas (BTg01Z.B to H) and measured their lacZ response against *T. gondii* infected or uninfected BMDCs (Fig. 19A). Surprisingly, only two hybrids (BTg01Z.B and BTg01Z.A) showed specificity towards *T. gondii*. It is possible that after the initial screen as shown in figure 4, these hybrids lost their sensitivity as result of being frozen without subcloning for many years. None of the other hybrids were specific for recombinant *E.coli* expressing CD4Ag28m or OVA (Fig. 19B and 19C). Similarly, no lacZ response of other hybrids against AS15 peptide-pulsed BMDCs was measured except hybrid BTg01Z.C (Fig. 19D). Therefore, it is likely that hybridomas BTg01Z.B and BTg01Z.E recognize antigens that are different from CD4Ag28m. Further identification of these antigens will allow us to assess the repertoire of *T. gondii* specific CD4 T cell responses in B6 mice.
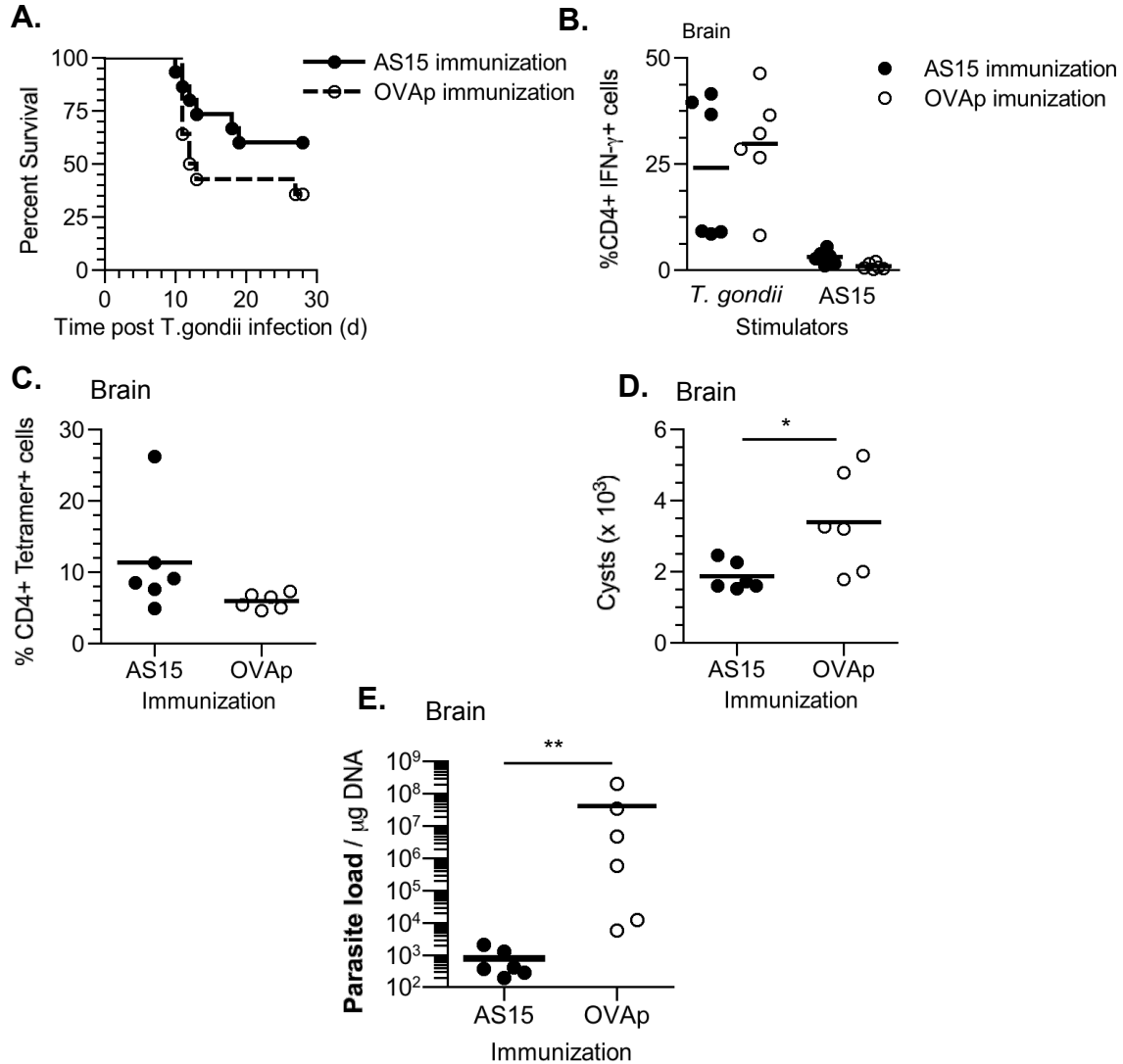
Figure 18: Immunization with AS15 peptide lowers the cyst burden and parasite load in the brains of infected mice.

C57BL/6 mice were immunized with LPS activated BMDCs pulsed with AS15 or control peptide (OVAp). 7 days post immunization, these mice were infected with 1×10^4 live *T. gondii* tachyzoites, intraperitoneally. **A)** Kaplan-Meier survival curves between the two groups. **B-E)** Analysis of CD4 responses and parasite loads from surviving mice. **B)** IFN-γ response by brain CD4 T cells as measured by ICCS for IFN-γ using flow cytometry after *ex-vivo* restimulation with *T. gondii*-infected APCs or AS15-pulsed APC. Data are background corrected based on the values from uninfected APCs or APC pulsed with irrelevant peptide. **C)** MHC class II I-A^b –AS15 tetramer staining on brain leukocytes. Cells were also co-stained with CD4 antibody. **D)** Number of cysts in the brain as measured by staining a portion of the brain with fluorescent lectin to detect the cysts. **E)** The parasite load in the brain measured using semi-quantitative PCR on genomic DNA extracted from the tissue. Data is representative of at least three experiments with at least 4 mice per condition in each experiment. (*=p<0.05, **=p<0.01).
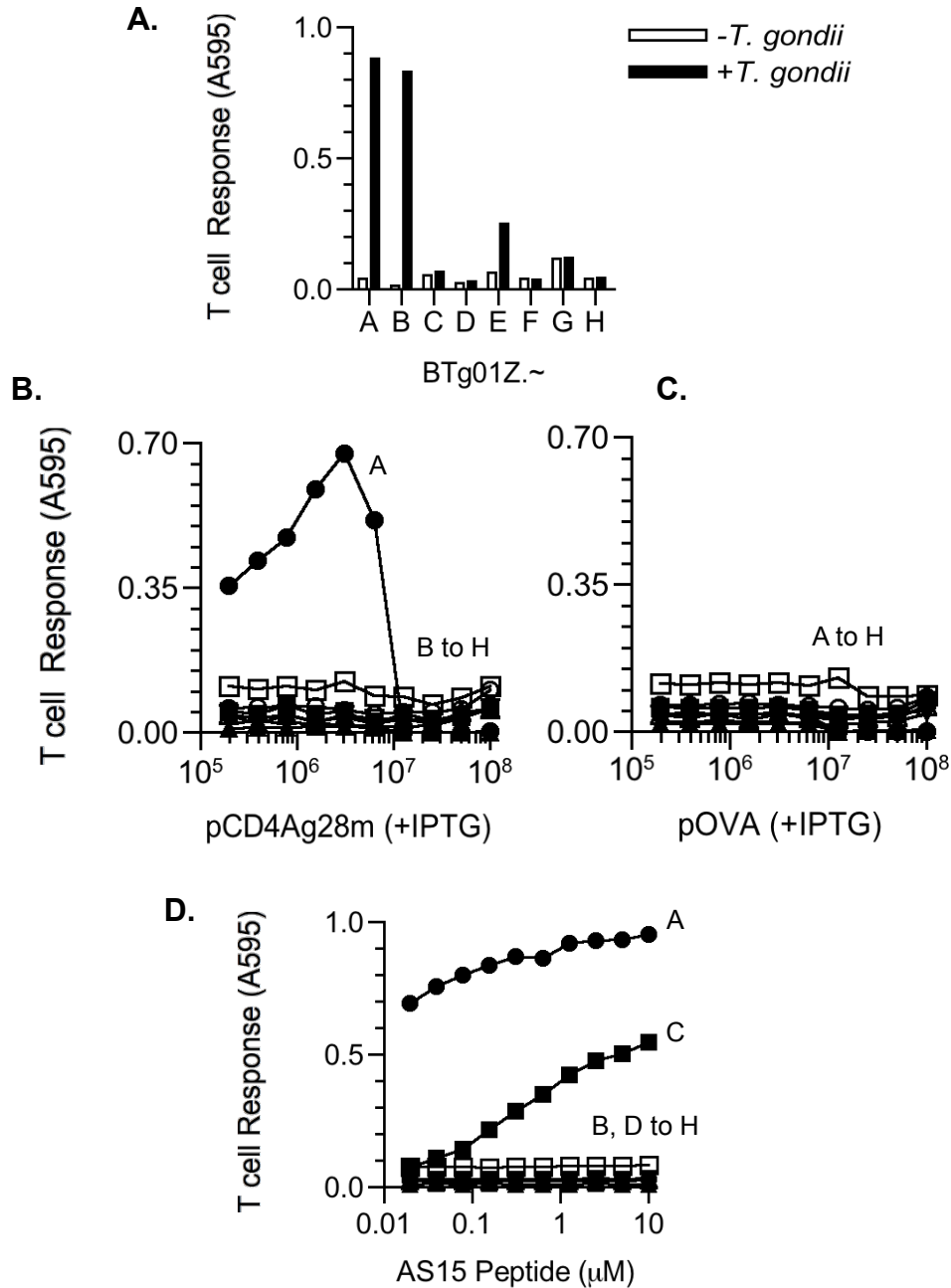
Figure 19: CD4 T cell hybridomas that are specific for other *T. gondii* proteins.

C57BL/6 mice were immunized with irradiated *T.gondii* tachyzoites. Splenocytes were harvested from mice 1 wk post immunization. After two weeks of *in-vitro* restimulations, proliferating CD4 T cells were fused to lacZ containing fusion partner (Kartunnen et al., 1991) to generate *T. gondii* specific CD4 T cell hybrid (Fig. 4). LacZ response of CD4 hybrids after an overnight culture with **A)** wild-type APCs +/- *T. gondii*; **B, C)** against BMDCs that were fed induced recombinant *E.coli* containing **B)** CD4Ag28m or **C)** OVA; **D)** against BMDCs that were pulsed with different concentrations of the AS15 peptide.

Conclusions

CD4 T cells are an important component of the immune response to *Toxoplasma gondii*, but the nature of the parasite antigens recognized by CD4 T cells is largely unknown. Here we examined the CD4 T cell response in *T. gondii* infected C57BL/6 (B6) mice, a strain that is widely used by immunologists, and is relatively susceptible to infection. We observe that CD4 T cell responses dominate over CD8 T cell response in B6 mice and we define a parasite antigen, CD4Ag28m (Fig. 8), which accounts for a fraction of the CD4 T cell response in immunized or infected mice (Fig. 11 and 12). Identification of other CD4 *T. gondii* antigens will allow us to determine the hierarchy of T cell responses towards various *T. gondii* antigens (Fig. 19). It is possible that all *T. gondii* specific CD4 antigens are a small fraction constituting the overall CD4 response. Even though profilin was published to be an immunodominant CD4 T cell response towards *T. gondii* (Plattner et al., 2008; Yarovinsky et al., 2006), knowing the final antigenic peptide from profilin will allow us to make a direct comparison between the antigenic peptides from the known CD4 antigens.

Furthermore, we show that a 15-mer AS15 peptide derived from the CD4Ag28m protein is presented by I-A^b MHC molecule (Fig. 10). Currently, based on the database CD4Ag28m is known to be a hypothetical protein in *T. gondii*, with no known function. It would be interesting to determine what is the function and location of this protein within *T. gondii*. Knowing the function and location can further help us in understanding how this protein can enter the antigen presentation pathway to be presented to CD4 T cells.

Although CD4Ag28m is polymorphic between the type I, II, and III strains of *T. gondii*, AS15 is conserved among then and is antigenic (Fig. 14). Can this protein play an antigenic role in other species that are infected with *T. gondii*? Additionally, both infected and bystander APCs can present the antigenic peptide, and AS15 response from heat-killed parasites is much enhanced compared to response from irradiated parasites indicating that phagocytosis is an important step for the presentation of AS15 on MHC Class II molecules (Fig. 15-17). Determining the exact process of phagocytosis or utilization of pathways such as autophagy will give us a better understanding of how *T. gondii* antigens are accessed by the immune system to be presented to CD4 T cells. Finally, we have demonstrated that immunization of mice with the AS15 peptide protects the animals from subsequent infection, resulting in enhanced survival and lower parasite burden (Fig. 18).

Importantly, identification of the antigen, CD4Ag28m, allowed us to better understand the role of *T. gondii*-specific CD4 T cell response in B6 mice. Since, the peptide-MHC class II ligands recognized by CD4 T cells are generated in specialized APCs using unique antigen processing pathways, identifying pathogen antigens that elicit CD4 T cell responses is a challenging undertaking. Even when the genome sequences are known, candidate peptides are difficult to

predict due to poor definition of MHC class II binding consensus motifs. Direct purification of the processed peptides from infected cells is also difficult because the peptides are not only present in small amounts but also represent a heterogeneous mixture with ragged N- and C-termini. Thus, unlike MHC class I binding homogenous peptides that elute in a single peak on High Performance Liquid Chromatography, the active MHC II binding peptides elute in multiple peaks that effectively reduce recovery. Genetic approaches are also difficult, because MHC class II molecules generally present peptides obtained from exogenous sources; endogenous expression of transfected cDNAs in APCs does not yield appropriate pMHC II ligand. We overcame these limitations by developing a unique expressing cloning strategy for identifying CD4 T cell stimulating antigens as described previously (Campbell and Shastri, 1998; Mougneau et al., 1995; Sahara and Shastri, 2003; Sanderson et al., 1995). Currently, CD4 epitopes for pathogens such as *Salmonella spp* (Cookson and Bevan, 1997; Musson et al., 2002), *Mycobacterium spp* (Andersen et al., 1995; Harris et al., 1995; Yanagisawa et al., 1997), *Tyrpsanoma cruzi* (Kahn and Wleklinski, 1997), *Leishmania spp* (Jardim et al., 1990; Scott et al., 1988; Yang et al., 1991), and *Plasmodium spp* (Grillot et al., 1990; Hirunpetcharat et al., 1997; Reece et al., 2004; Takita-Sonoda et al., 1996), have all been identified against previously known conserved surface, abundant or secreted proteins. In contrast, the cDNA expression library provides an unbiased and immunologically relevant approach that can be applied to identify any CD4 T cell-stimulating antigen.

Moreover, the identification of an immunogenic peptide, which is capable of generating protection in mice may aid in the development of more effective vaccines against Toxoplasmosis. In this regard, it is encouraging that, although CD4Ag28m is polymorphic, the AS15 epitope is conserved between the three North American and European strains of *T. gondii* (Fig. 14). It will be important to determine if this protein or peptide is also immunogenic and protective in other species, including humans. Inclusion of CD4 epitopes such as AS15 can potentially be used boost responses to CD8 T cell epitopes (Cong et al., 2011) and thus improve the efficacy of vaccination.

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Chapter 3: CD8 T cell responses to *Toxoplasma gondii* in C57BL/6 mice

Summary

The parasite, *Toxoplasma gondii* (*T. gondii*) can cause severe disease in immunocompromised individuals. Susceptibility to *T. gondii* is also linked to the MHC haplotype: B10.D2 (H-2^d) mice are relatively resistant to *T. gondii* infection than C57BL/6 mice (B6, H-2^b). CD8 T cells, activated by MHC molecules, are known to be that primary mediators of protection from *T. gondii*, and it is possible that differences in their responses might determine susceptibility to infection. Indeed, in H-2^d mice, Shastri lab recently identified the key immunodominant peptide, HF10, contained in the GRA6 protein that elicited protective CD8 T cell responses. In contrast, in this chapter, it is shown that the CD8 T cell response to *T. gondii* in susceptible B6 mice is weaker and specific for a distinct 9-mer peptide, YAL9, derived from the ROP5 protein. Furthermore, unlike the HF10 peptide that generated protective CD8 T cells in H-2^d mice, immunization with the YAL9 peptide failed to diminish the parasite burden in chronically infected H-2^b mice. Additionally, unlike HF10, YAL9 specific responses were a minor but detectable fraction of the *T. gondii* specific CD8 T cell response in chronically infected mice. To further understand the lack of protection and poor CD8 T cell response, we generated transgenic parasites that express ROP5 as part of the dense granules. Immunization or infection with these parasites allowed for an enhancement of YAL9 specific T cell response. Further, immunization with YAL9 protected the mice against challenge with lethal dose of the transgenic parasites during the acute phase on infection. Thus, susceptibility to *T. gondii* correlates with MHC–linked processing of specific parasite proteins that based on their expression and secretion within *T. gondii* can elicit differential CD8 T cell responses in mice.

***Toxoplasma gondii* immunization of C57BL/6 mice elicits a potent CD4 and weak CD8 T cell response: expansion of CD8 T cells**

In order to characterize the T cell response in the C57BL/6 (B6, H-2^b) mice, we immunized B6 mice with irradiated tachyzoites from the type II *T. gondii* strain, *Prugniaud* (Pru). One week post-immunization, splenocytes harvested from immunized B6 mice were examined for *T. gondii*-specific CD4 and CD8 T cell responses *ex-vivo* by measuring intracellular cytokine staining (ICCS) for IFN-γ. In contrast to the H-2^d strain of mice, in which the CD8 T cell response dominates over the CD4 T cell response (Blanchard et al., 2008), B6 mice generated a robust CD4 T cell response but a far weaker CD8 T cell response towards *T. gondii* (Fig. 3A-C and Fig. 20A, top panels). The nature of the CD4 T cell response is discussed in detail in Chapter 2. However, to compare the CD8 T cell responses in B6 mice to that observed in H-2^d mice, especially since CD8 T cells are the primary mediators of resistance to *T. gondii* infection, we decided to expand *T. gondii* specific CD8 T cells isolated from *T. gondii* immunized mice, *in-vitro* using MHC Class II A$^{b-/-}$ BMDCs. This allowed for the expansion of *T. gondii* specific CD8 T cells over the course on *in-vitro* restimulations as measured by ICCS for IFN-γ (Fig. 20A and 20B). At the end of ten *in-vitro* restimulations, only the IFN-γ producing CD8 T cells rigorously expanded while CD4 T cells failed to proliferate and were barely present in the culture (Fig. 20A and 20B).

Generation of *Toxoplasma gondii* specific CD8 T cell hybridoma

To further understand *T. gondii* specific CD8 T cell response at a clonal level and for overcoming the challenges of working with primary T cell lines we generated *T. gondii* specific CD8 T cell hybridomas. After ten *in-vitro* restimulations, proliferating *T. gondii* specific IFN-γ producing CD8 T cells were used to generate lacZ inducible T cell hybridomas, as described previously (Karttunen et al., 1992; Sanderson and Shastri, 1994). In brief, an expanded population of *T. gondii* specific CD8 T cells was fused to the BWZ.36/CD8α fusion partner that lacks the alpha and beta TCR chains and contains a β-galactocidase (lacZ) inducible reporter gene (Karttunen and Shastri, 1991). Upon TCR stimulation, the hybridomas express lacZ, which can be measured at an absorbance of 595nm by conversion of the substrate to chlorophenol red (Sanderson and Shastri, 1994). After fusion, we successfully generated two *T. gondii* specific CD8 T cell hybridomas and confirmed their specificity for *T. gondii* using uninfected versus infected B6 BMDCs. We chose one of the hybridomas, referred as BTg45Z, sub-cloned it and used it for all further experiments. Additionally, the MHC-restriction of BTg45Z was confirmed using infected BMDCs that were blocked with antibodies for MHC Class I molecules, H-2K^b and H-2D^b. Blocking H-2D^b abrogated the lacZ response of BTg45Z, while blocking H-2K^b did not alter the response (Fig. 20D). BTg45Z lacZ response against infected and uninfected BMDCs was also measured for comparison. Thus, we were able to conclude that BTg45Z is *T. gondii* specific and H-2D^b restricted.
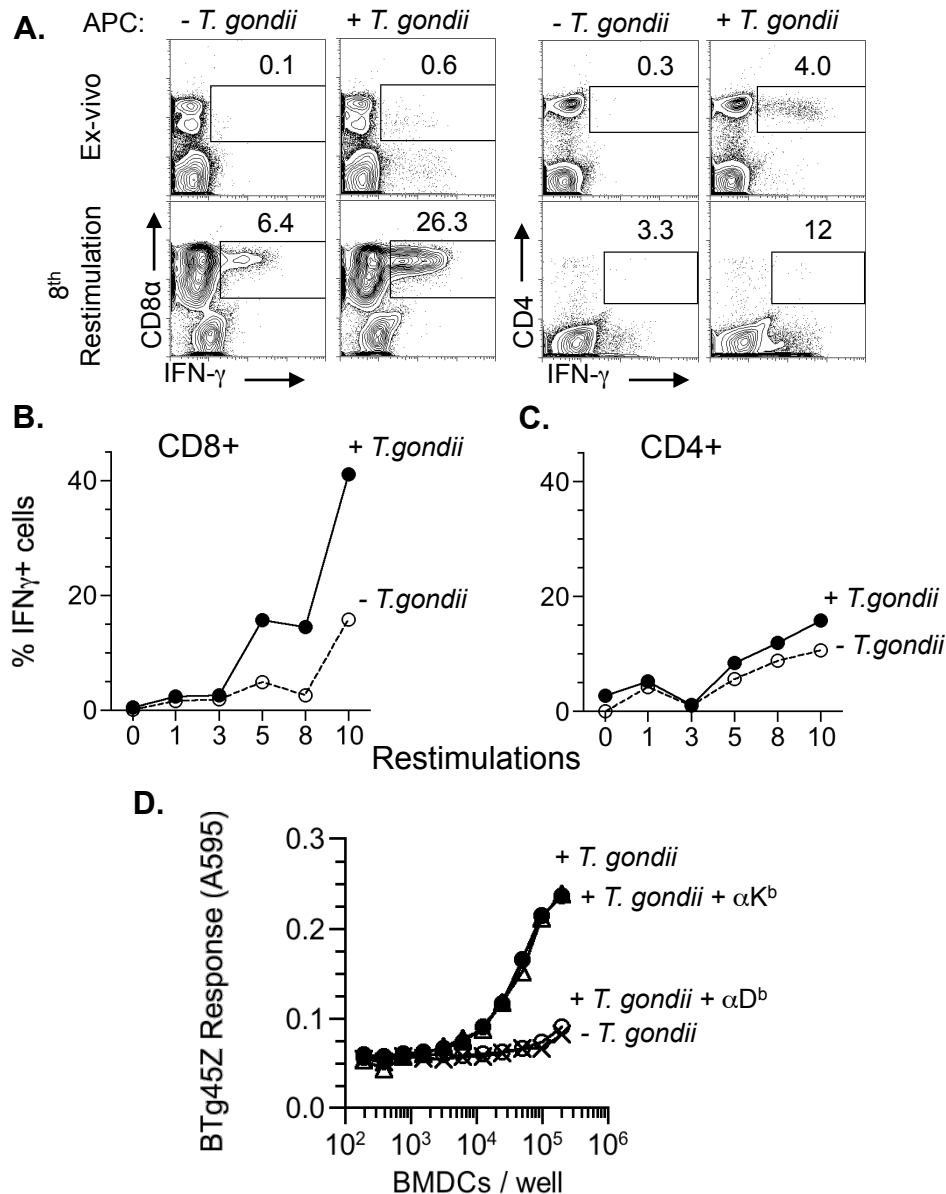
Figure 20: Expansion of CD8 T cells from *T. gondii* immunized C57BL/6 mice, using MHC Class II-/- infected BMDCs.

C57BL/6 mice were immunized with irradiated *T. gondii* tachyzoites. Splenocytes were harvested from mice 2 wks post immunization and *T. gondii* specific CD8 and CD4 T cell responses were measured by intracellular cytokine staining for IFN-γ. Antigen presenting cells +/- *T. gondii* were used as stimulators for *ex-vivo* and *in vitro* restimulations. *Ex-vivo* and *in-vitro* restimulation results are shown as **A)** Representative flow cytometry plots **B and C)** Plots depicting the expansion of CD8 T cells **B)** and CD4 T cells **C)** over the course of ten *in-vitro* restimulations. Data are representative of three experiments. **D)** LacZ response of sub-cloned CD8 hybridoma (BTg45Z) after an overnight culture with wild-type APCs +/- *T. gondii* or APCs blocked with antibodies against H-2K^b or H-2D^b MHC I molecules + *T. gondii*. Data is representative of at least two independent experiments.

Screening for the cognate antigen recognized by BTg45Z CD8 T cell hybridoma

The Shastri laboratory has previously shown that *T. gondii* and other antigens can be identified by generating a cDNA expression library and using the lacZ inducible T cell hybridoma as a probe to screen the library (Blanchard et al., 2008). We therefore used the strategy outlined in Figure 21 to screen a *T. gondii* cDNA library expressed in a eukaryotic expression vector, pcDNA. In brief, *T. gondii* cDNA library was transfected into APCs that were expressing H-2D^b MHC Class I molecules. 48h post-transfection, antigen presenting cells expressing the different pMHC I complexes were incubated with the T cell hybridoma, BTg45Z, and the lacZ response was measured to determine hybridoma activation in stimulating wells.

Identification of the cognate antigen recognized by BTg45Z CD8 T cell hybridoma

The results of expression library screen yielded two wells (Pool pTg8E6 and pTg13B7) that stimulated lacZ response in the T cell hybrid, BTg45Z (Fig. 22A). To further narrow down the *T. gondii* cDNA among the pool of cDNA in the stimulatory well that yielded a positive hit in the screen, pool pTg8E6 was further fractionated into individual colonies, and rescreened for its ability to stimulate BTg45Z hybrid. Two of twelve colonies tested scored positive (Fig. 22C). The T cell response to the individual pools was H-2D^b restricted because APCs lacking H-2D^b were unable to stimulate the BTg45Z hybrid (Fig. 22C). We therefore selected *E.coli* colony pTg8E6.1 for further characterization (Fig. 22C). Notably as a control the screening process, *T. gondii* infected APCs stimulated BTg45Z, while uninfected APCs failed to stimulate the hybrid (Fig. 22B). Thus, pTg8E6.1 encoded the BTg45Z stimulating antigen presented by MHC class I molecule (Fig. 22C).

The nucleotide sequence of pTg8E6.1 was blasted against *T. gondii* database (www.toxodb.org) and yielded a match with the rhoptry protein 5 (ROP5), a gene on chromosome XII encoding a protein of 549 amino acids (Fig. 23). Further alignment to different polymorphic versions of ROP5 in type II strain of *T. gondii* (Reese et al., 2011) revealed that pTg8E6.1 matched to ROP5 version IIC (Fig. 23). Incidentally, fractionation of pool pTg13B7, further sequencing and database blast search also resulted in a match with ROP5IIC.

Identification of the minimal antigenic peptide

Compared to the ROP5 sequence in the *T. gondii* database (www.toxodb.org), plasmid pTg8E6.1 was missing the sequences corresponding to the 5'UTR and the first 222 amino acids from the N-terminus of the putative protein (Fig. 23). To

T. gondii mRNA

↓

cDNA library pools

↓ Transfection into
Ag-, MHC-I-D^b(+) cells

↓ 48h

+ BTg45Z (LacZ inducible
Ag specific T cell hybrid)

↓ 16-20h

BTg45Z LacZ response (A595)
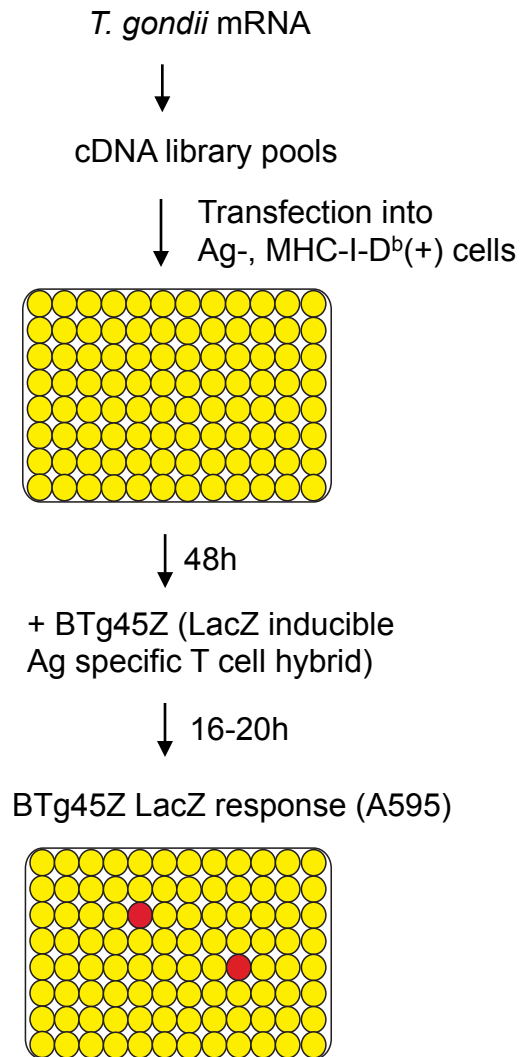
Figure 21: A schematic depicting the strategy used for identifying the antigen recognized by BTg45Z as described previously (Mendoza et al., 2001).
In brief, the strategy involved the expression of *T. gondii* cDNA expression library into eukaryotic expression vector. DNA was extracted from recombinant *E.coli* expressing the different *T. gondii* cDNAs, which was transfected into APCs expressing H-2D^b MHC I molecule. 48h post-transfection, *T. gondii* specific CD8 hybrid, BTg45Z, used to probe the cDNA library, was incubated with the APCs. Positive pools were identified in the colormetric assay, where upon the CD8 hybrid stimulation, β-galactosidase was expressed converting the substrate from yellow to pink.
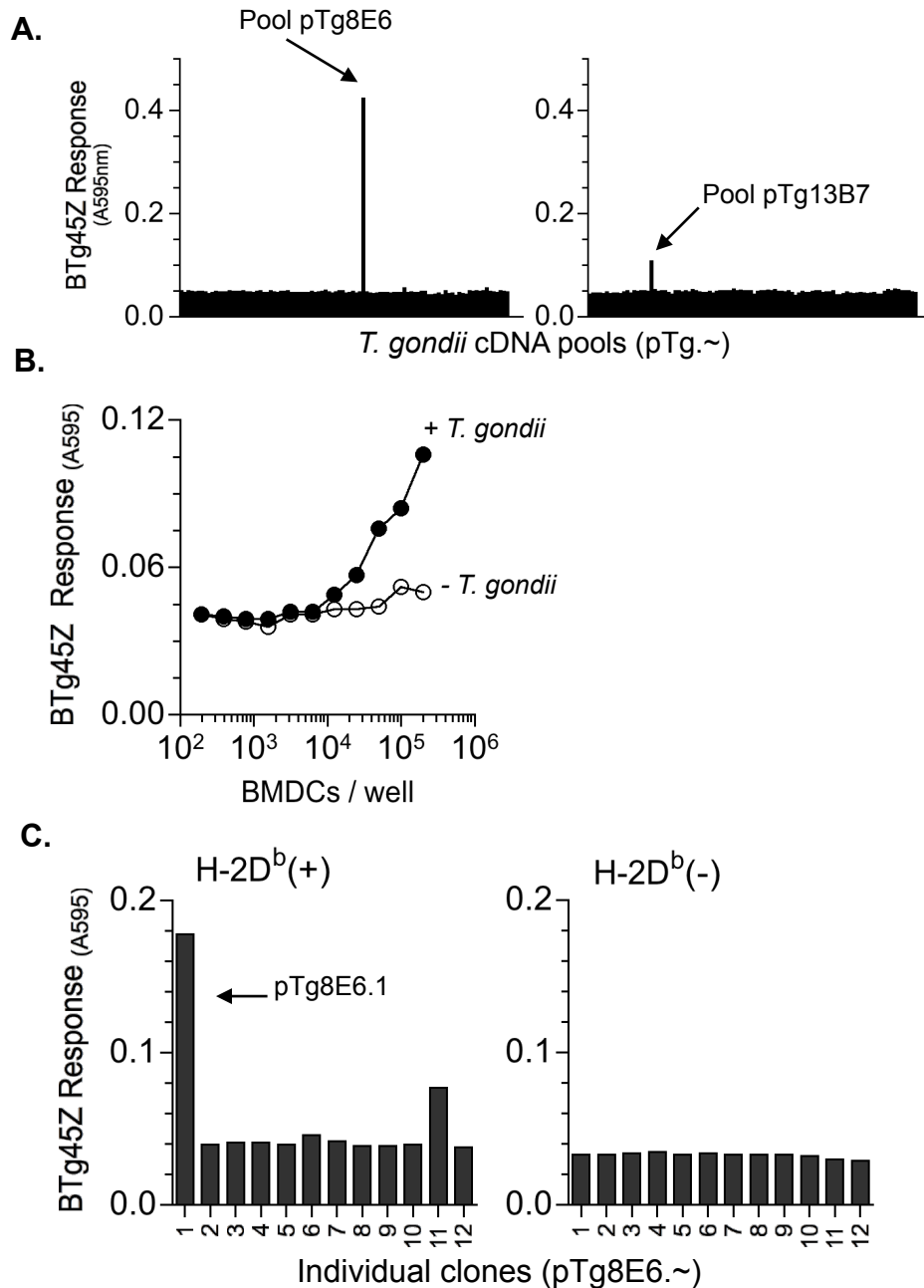
Figure 22: BTg45Z response to the positive hits in the screen and the fractionated single clone is antigen specific and MHC restricted.
A) T. gondii cDNA library was screened to identify the antigen recognized by BTg45Z. Two cDNA pools (pTg8E6 and pTg13B7) stimulated the lacZ response of the hybrid. **B)** As a positive control for the screening process, lacZ response of the hybrid against BMDCs +/- T. gondii was measured. **C)** pTg8E6 was further fractionated until a single stimulatory clone was identified and sequenced. BTg45Z response to individual cDNA clones isolated from pool pTg8E6. Clone pTg8E6.1 was selected for further analysis. BTg45Z response was antigen specific and MHC restricted.

TGMe49_108080 (ROP5IIC, Rhoptry Kinase
family protein 5, IIC)

T. gondii Me49, Chr XII

```
        539K    540K   541K    542K    543K
```

Full length:

Truncated cDNA (pTg8E6.1):

```
ROP5TypeIIC    MATKLARLATWLVLVGCLLWRAGAVQLSPPNSRTNDLASGTPHVARGDTEAQSGTGDDSD 60
pTg8E6.1       ------------------------------------------------------------
ROP5TypeIIA    MATKLARLATWLVLVGCLLWRAGAVQLSPPNSRTNDLASGTPHVARGDTEAQSGTGDDSD 60


ROP5TypeIIC    FPQGVVEEVADMSGGRVPRVPASSTTTSASEGIFRRLVRRLRRGRGTADGAGVADETHQG 120
pTg8E6.1       ------------------------------------------------------------
ROP5TypeIIA    FPQGVVEEVADMSGGRVPRVPASSTTTSASEGIFRRLVRRLRRGRGTADGAGVADETHQG 120


ROP5TypeIIC    PRPPLRKRLAQHFRRLRGFFGRLTPRWLSGLGRRAQRWWRGRQRPLLDPSFHGLEAGDSF 180
pTg8E6.1       ------------------------------------------------------------
ROP5TypeIIA    PRPPLRKRLAQHFRRLRGFFGRLTPRWLSGLGRRAQRWWRGRQRPLLDPSFHGLEAGDSF 180


ROP5TypeIIC    MRDLLKREEELIGYCREEALKEPAAMVEAVMATVWPQNAETTVDSLLSQGERKLKLVEPL 240
pTg8E6.1       -------------------------------------------VDSLLSQGERKLKLVEPL 18
ROP5TypeIIA    MRDLLKHEKELIGYCREEALKEPAAMVEAVMATVWPQNAETTVDSLLSQGERKLKLVEPL 240
                                                          ******************

ROP5TypeIIC    RVGDRSVVFLVRDVERLEDFALKVFTMGAENSRSELERLHEATFAAARLLGESPEEARDR 300
pTg8E6.1       RVGDRSVVFLVRDVERLEDFALKVFTMGAENSRSELERLHEATFAAARLLGESPEEARDR 78
ROP5TypeIIA    RVGDRSVVFLVRDVERLEYFALKVFTMGAENSRSELERLHEATFAAARLLGESPEEARDR 300
               ******************* ****************************************

ROP5TypeIIC    RRLLLPSDAVAVQSQPPFAQLSPGQSDYAVANYFFLMPAASVDLELLFRTLDFVYVFRGE 360
pTg8E6.1       RRLLLPSDAVAVQSQPPFAQLSPGQSDYAVANYFFLMPAASVDLELLFRTLDFVYVFRGE 138
ROP5TypeIIA    RRLLLPSDAVAVQSQPPFAQLSPGQSDYAVANYFLMPAASVDLELLFRTLDFVYVFRGE 360
               **********************************:***********************

ROP5TypeIIC    EGILARHLLTAQLIRLAANLQSKGLVHGRFTPENLFIMPDGRLMMGDVSTLRKVGTRGPA 420
pTg8E6.1       EGILARHLLTAQLIRLAANLQSKGLVHGRFTPENLFIMPDGRLMMGDVSTLRKVGTRGPA 198
ROP5TypeIIA    EGILARHLLTAQLIRLAANLQSKGLVHGRFTPDNLFLMGYGPVMLGDASALWKVGTRGPA 420
               ********************************:***:*   * :*:**.*:* *******

ROP5TypeIIC    SSVPVTYAPREFLNANTATFTHALNAWQLGLSIYRVWCLVLPFGLVTPGIKRTWKRPSLR 480
pTg8E6.1       SSVPVTYAPREFLNANTATFTHALNAWQLGLSIYRVWCLVLPFGLVTPGIKRTWKRPSLR 258
ROP5TypeIIA    SSVPVTYAPREFLNANTATFTHALNAWQLGLSIYRVWCLVLPFGLVTPGIKRTWKRPSLR 480
               ************************************************************

ROP5TypeIIC    VPGTDSLLFDSCIPVPDFVQTLTRRFLNFDRRRRLLPLEAMETPEFLQLQNEISSSLSTG 540
pTg8E6.1       VPGTDSLLFDSCIPVPDFVQTLTRRFLNFDRRRRLLPLEAMETPEFLQLQNEISSSLSTG 318
ROP5TypeIIA    VPGTDSLLFDSCIPVPDFVQTLTRRFLNFDRRRRLLPLEAMETPEFLQLQNEISSSLSTG 540
               ************************************************************

ROP5TypeIIC    QPTAAPSVA 549
pTg8E6.1       QPTAAPSVA 327
ROP5TypeIIA    QPTAAPSVA 549
               *********
```

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Figure 23: The cognate antigen recognized by BTg45Z hybridoma is a Rhoptry protein, ROP5, in the *T. gondii* database as identified by expression cloning.

T. gondii database (www.toxodb.org) BLAST search results revealed that the antigenic cDNA encoded by clone pTg8E6.1 corresponded to a truncated version of the *T. gondii* protein, TGMe49_018080 (Rhoptry kinase family protein 5). More specifically after aligning with the different polymorphic versions of ROP5 (Reese et al., 2011), pTg8E6.1 corresponded to the ROP5 type IIC version. pTg8E6.1 is missing the 5'UTR and first 222 amino acids of ORF. Alignment of the amino acid sequence of the truncated cDNA clone as compared to the full-length polymorphic clones (ROP5 IIA and ROP5IIC) is depicted here. (-) represent missing amino acids in the sequence, (*) represent alignment and (: or gap) represents alignment between ROP5IIC and pTg8E6.1.

define the antigenic peptide that stimulates BTg45Z in the pTg8E6.1 sequence, we first searched for candidate peptides using the appropriate algorithm in the immune epitope database (www.immuneepitope.org) that could bind the H-2D^b MHC Class I molecule and thus serve as potential TCR ligands. This analysis suggested multiple candidate peptides within ROP5 that could bind H-2D^b MHC Class I molecule and serve as potential TCR ligands. We chose the top four peptides that bind H-2D^b with the greatest affinity from the list of potential peptides. Using PCR mutagenesis with appropriate primers (Table 4), we generated various N-terminal and C-terminal deletion mutant constructs of pTg8E6.1 (Fig. 24A). The deletion constructs were cloned into the pcDNA vector, the truncated versions were transfected into H-2D^b expressing APCs, and the cells were then used to stimulate BTg45Z (Fig. 24B and 24C). The N-terminal deletion construct, ΔN-C7 expressing aa 414-550 containing two of the predicted epitopes failed to stimulate BTg45Z (Fig. 24B). Additionally, N and C-terminal deletion constructs ΔNΔC-3 and ΔNΔC-5 also failed to stimulate BTg45Z (Fig. 24B). On the other hand, N and C-terminal deletion constructs ΔNΔC-8 and ΔNΔC-9 containing predicted epitopes stimulated BTg45Z hybridoma (Fig. 24B). Based on the negative antigenic activity of construct ΔN-7 and the positive antigenic activity of ΔNΔC-9, we concluded that the antigenic activity could be present in the predicted epitope aa 328-336, YAVANYFFL (YAL9).

To independently confirm the assignment of antigenic activity to YAL9 peptide based on prediction and deletion constructs, we tested 9-mer YAL9 synthetic peptide and its N'terminal extended versions DL10, SL11 and QL12 (Fig. 24C). BMDCs pulsed with peptides DL10, SL11. QL12 stimulated BTg45Z to far lesser extent than the 9-mer, YAL9 (Fig. 24C). The antigenic activity of YAL9 was further corroborated with high-performance liquid chromatography (HPLC) fractionation of extracts from APCs transfected with pTg8E6.1 or spiked with synthetic peptide. BTg45Z cells were stimulated by the same fractions, 31-32, in both conditions, while no stimulation was observed with extracts from APCs transfected with pcDNA vector alone (Fig. 25A-C). We therefore concluded that YAL9 is the optimal peptide contained within ROP5IIC that is presented by H-2D^b and recognized by the BTg45Z T cell hybridoma.

Presentation of YAL9 to BTg45Z requires the proteasome, TAP but not necessarily ERAAP

Conventionally, antigens that are presented on MHC Class I molecules to CD8 T cells are endogenous wherein they require processing in the cytosol. Foreign or endogenous proteins are broken down into peptide precursors in the cytosol by the proteasome, and are transported to the ER via TAP. Finally in the ER, they can be further processed by ERAAP and loaded on to MHC Class I molecules (Serwold et al., 2001) (Figure 2). Since *T. gondii* is an intracellular parasite, we wanted to assess if the presentation happens in this canonical manner where *T. gondii* proteins are accessible to the antigen processing machinery. To address
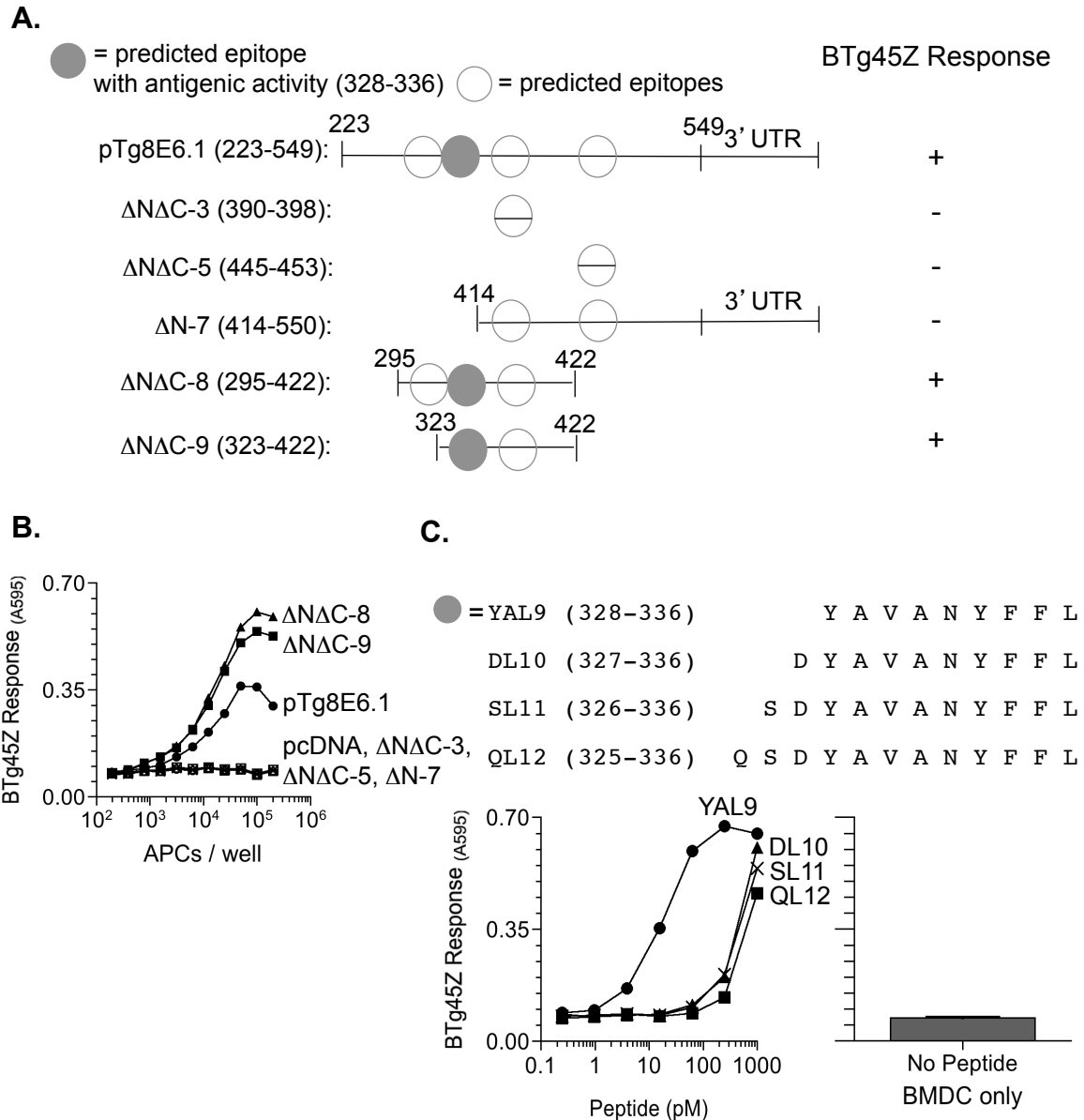
Figure 24: Identification of the antigenic epitope within ROP5IIC that stimulates BTg45Z hybridoma.

A) Schematic representation of the N-terminal and C-terminal deletion constructs generated to identify the antigenic epitope within clone pTg8E6.1. The clone itself was truncated and missing the 5'UTR and the first 222 amino acids. The specific amino acids tested in each construct are shown in parentheses and the region with the antigenic activity is indicated by the filled gray circle. Open circles represent other putative H-2D^b binding epitopes as predicted by the immune epitope database (www.immuneepitope.org) **B)** BTg45Z lacZ response against different deletion constructs that were transfected in H-2D^b expressing APCs for presentation after an overnight stimulation. **C)** Sequences of antigenic peptide and its N'terminal extended versions. BTg45Z lacZ response to wild-type BMDCs alone or BMDCs that were pulsed with titrating concentrations of indicated peptides.

65
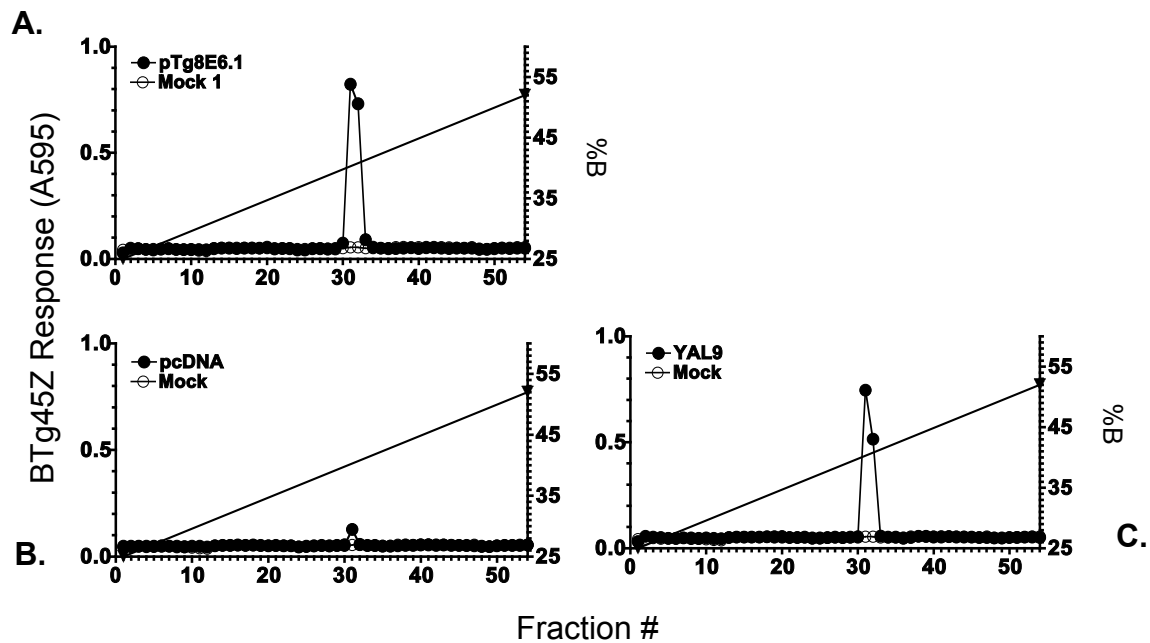
Figure 25: BTg45Z recognizes the 9-mer antigenic peptide, YAL9, from HPLC fractionated pTg8E6.1 transfected cells.
A, B) Cellular extracts from COS cells transfected with **A)** cDNA clone pTg8E6.1 or **B)** pcDNA vector alone were generated and fractionated through HPLC. **C)** As a control, synthetic 9-mer peptide, YAL9, alone was also fractionated. Lyophilized peptides fractions were pulsed onto H-2D^b expressing APCs and incubated with BTg45Z hybrid. LacZ response of BTg45Z to **A)** pTg8E6.1 or **B)** pcDNA fractionated peptides, or **C)** Synthetic peptide YAL9. Mock represents response to HPLC buffer alone.

this, we infected BMDCs from wild-type B6, TAP-/- or ERAAP-/- mice and cultured them with BTg45Z hybridoma. BTg45Z generated a lacZ response against infected ERAAP-/- BMDCs, albeit it was slightly lower than seen with infected wildtype BMDCs (Fig. 26A). However, BTg45Z failed to generate a lacZ response against infected TAP deficient BMDCs (Fig. 26A). No lacZ response was detected against uninfected wildtype, TAP-/- or ERAAP-/- BMDCs (Fig. 26A). There was no difference measured in the lacZ response seen with either type of BMDCs when they were pulsed with YAL9 peptide. They all stimulated the hybridoma equally (Fig. 26B). To test for requirement of proteasome, wildtype BMDCs were treated with lactacystin prior to infection. BTg45Z lacZ response against infected and treated BMDCs reduced to levels of uninfected BMDCs with increasing dose of lactacystin (Fig. 26C). Treatment with lactacystin did not impact surface levels of MHC Class I molecules, since BMDCs treated with lactacystin and then pulsed with YAL9 stimulated the hybridoma at levels comparable to no treatment (Fig. 26C). Overall, it seems that processing of ROP5 leading to the final antigenic peptide YAL9 can happen via the cytosolic pathway requiring the proteasome and TAP but not necessarily ERAAP.

Detection of YAL9 specific CD8 T cells in mice immunized with _Toxoplasma gondii_

The identification of YAL9 antigenic peptide allowed us to detect this defined specificity of CD8 T cell response that makes up part of the total response towards _T. gondii_. To determine the fraction that makes up YAL9 specific response, we immunized C57BL/6 with irradiated type II _T. gondii_. Two wks post-immunization an _ex-vivo_ analysis of splenocytes from immunized mice was assayed through intracellular cytokine staining (ICCS) for IFN-γ or MHC Class I tetramer staining. _Ex-vivo_ restimulation in the presence of _T. gondii_ infected or YAL9 peptide pulsed APCs revealed that YAL9 specific response by ICCS averaged 0.3% of the total CD8 T cells, which calculates to 60% out of the total _T. gondii_-specific CD8 T cell response (Fig. 27A and 27B). These responses were corrected for background responses to uninfected or irrelevant peptide (WI9) pulsed APCs. No significant response was seen in naïve mice. Similarly, peptide MHC-Class I H-2D^b YAL9 tetramer staining showed that YAL9 specific responses were on average 0.5% of the splenic CD8 T cell response (Fig. 27A and 27C). Although the CD8 T cell response is weak in C57BL/6 immunized mice, we were able to conclude that YAL9 specific CD8 T cells represent a measurable population of the total CD8 T cell response to _T. gondii_.
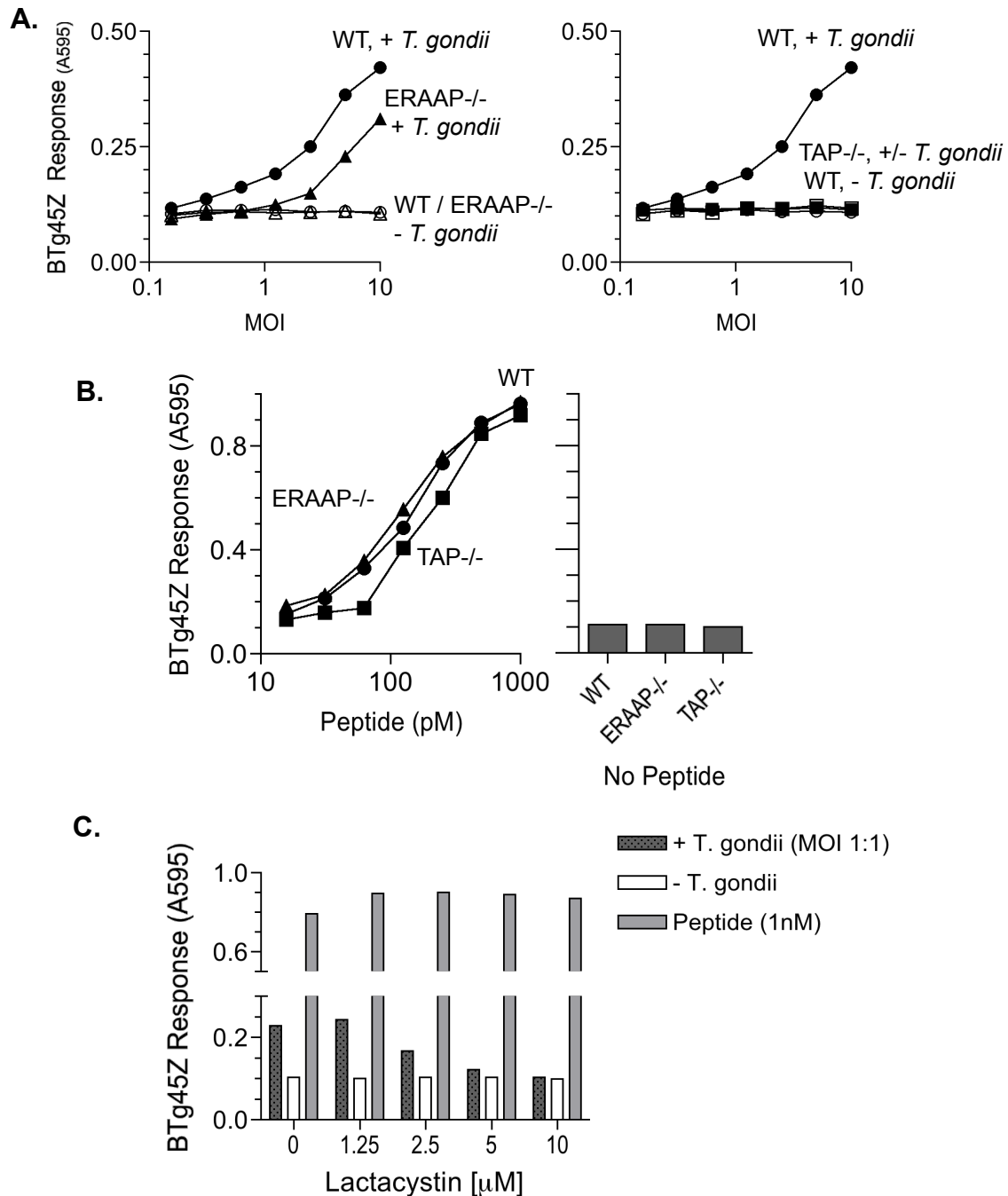
Figure 26: Presentation of YAL9 to BTg45Z is TAP and proteasome dependent but ERAAP independent.
BMDCs from C57BL/6, ERAAP-/- or TAP-/- were **A,C)** infected *in-vitro* with irradiated *T. gondii*, type II, Me49 strain or **B,C)** pulsed with YAL9 antigenic peptide. BTg45Z lacZ response after an overnight stimulation against wild-type, ERAAP-/- or TAP-/- ,**A)** +/- T. gondii or **B)** BMDCs that were pulsed with titrating concentrations of YAL9 peptide. **C)** BTg45Z lacZ response against wild-type BMDCs that were treated with proteasome inhibitor, lactacystin, +/- *T. gondii* or pulsed with the antigenic peptide, YAL9.

Figure 27: A fraction of the *T. gondii* specific CD8 T cell response is YAL9 specific in *T. gondii* immunized mice.
C57BL/6 mice were immunized with irradiated 5×10^6 *T.gondii*, type II, Me49 tachyzoites. Splenocytes were harvested from mice 2 wks post immunization and *T. gondii* specific CD8 T cell responses were measured by intracellular cytokine staining for IFN-γ or by staining with tetramers. **A)** Representative flow cytometry plots from naïve and immunized mice. Left panels show intracellular IFN-γ staining after *in vitro* restimulation with antigen presenting cells either, +/- *T. gondii*, or 1μM YAL9 peptide. Right panels show MHC class I H-2D[b] –YAL9 tetramer staining. **B)** Compiled data showing splenic *T. gondii* specific and YAL9 specific responses from immunized mice. Data are corrected for background based on responses by CD8 T cells towards uninfected APCs or APCs with irrelevant peptide. Each dot represents an individual mouse. **C)** Compiled data showing MHC class I H-2D[b] –YAL9 tetramer staining on splenocytes from immunized mice. Cells were also co-stained with CD8 antibody. Data are representative of at least three independent experiments.

YAL9 specific CD8 T cells constitute a similar percentage of the overall CD8 T cell response, but a lower fraction of the overall *Toxoplasma gondii* specific response in chronically infected mice

Encouraged with the dominance of YAL9 seen in *T. gondii* immunized animals, we wanted to determine if similar fraction of the total *T. gondii* specific CD8 T cell response directed towards the YAL9 peptide was also observed in chronically infected animals. To assess this, we fed B6 mice orally 25-40 type II cysts and harvested splenocytes or brain leukocytes 3-4 weeks post infection. *Ex-vivo* restimulation of CD8 T cells from infected mice, in the presence of APCs pulsed with YAL9 peptide, followed by intracellular cytokine staining (ICCS) for IFN-γ revealed that on average 0.25% (spleen) and 0.7% (brain) of the total CD8 T cells responded to this peptide (Fig. 28A-C). No significant response was observed in naive mice or in *T. gondii* infected mice with APCs pulsed with an irrelevant peptide (WI9) that also binds to H-2D^b MHC class I molecule, which was corrected for in the depicted response. Similarly, 0.6% (spleen) and 0.8% (brain) of CD8 T cells from infected mice stained with the YAL9 loaded MHC class I tetramers (Fig. 28A and 28D-E).

Given that on average 3.3% of CD8 T cells in the spleen responded to *T. gondii* infected APCs in these experiments, we estimate that 7.5% of *T. gondii*-specific CD8 T cell response is specific for the YAL9 peptide. Likewise, in the brain, 8% of CD8 T cells responded to *T. gondii*. Therefore, we estimate that on average, 9% of the *T. gondii*-specific CD8 T cells are specific for the YAL9 peptide in the brains of chronically infected mice. Similar to immunized mice, YAL9 specific CD8 T cell response constitutes a similar percentage of the overall CD8 T cell response, however, out of the total *T. gondii* specific response, YAL9 specific response represents a measurable fraction that is <10%. Also, unlike immunized mice, the percentage of *T. gondii* specific T cell responses by both CD8 and CD4 T cells as measured by ICCS for IFN-γ are greater in infected animals, and as observed before, CD4 responses are still more robust than CD8 T cell responses (Fig.12A-C vs. Fig. 27A-C).

Kinetics of YAL9 specific CD8 T cell response over the course of *Toxoplasma gondii* infection

Since YAL9 specific response constitutes a small but detectable fraction of total *T. gondii* specific response in chronically infected animals, we further wanted to understand the kinetics of YAL9 response throughout the course of infection to determine if it undergoes an expansion during the acute phase and then contraction during the chronic phase of infection. To assess the kinetics, we infected B6 mice orally with 25-40 type II cysts and harvested splenocytes 7-28 days post-infection. Splenocytes were restimulated *ex-vivo* in the presence of infected or peptide-pulsed APCs, followed by ICCS for IFN-γ. YAL9 specific CD8 T cell response expanded, as did *T. gondii* specific response during the acute
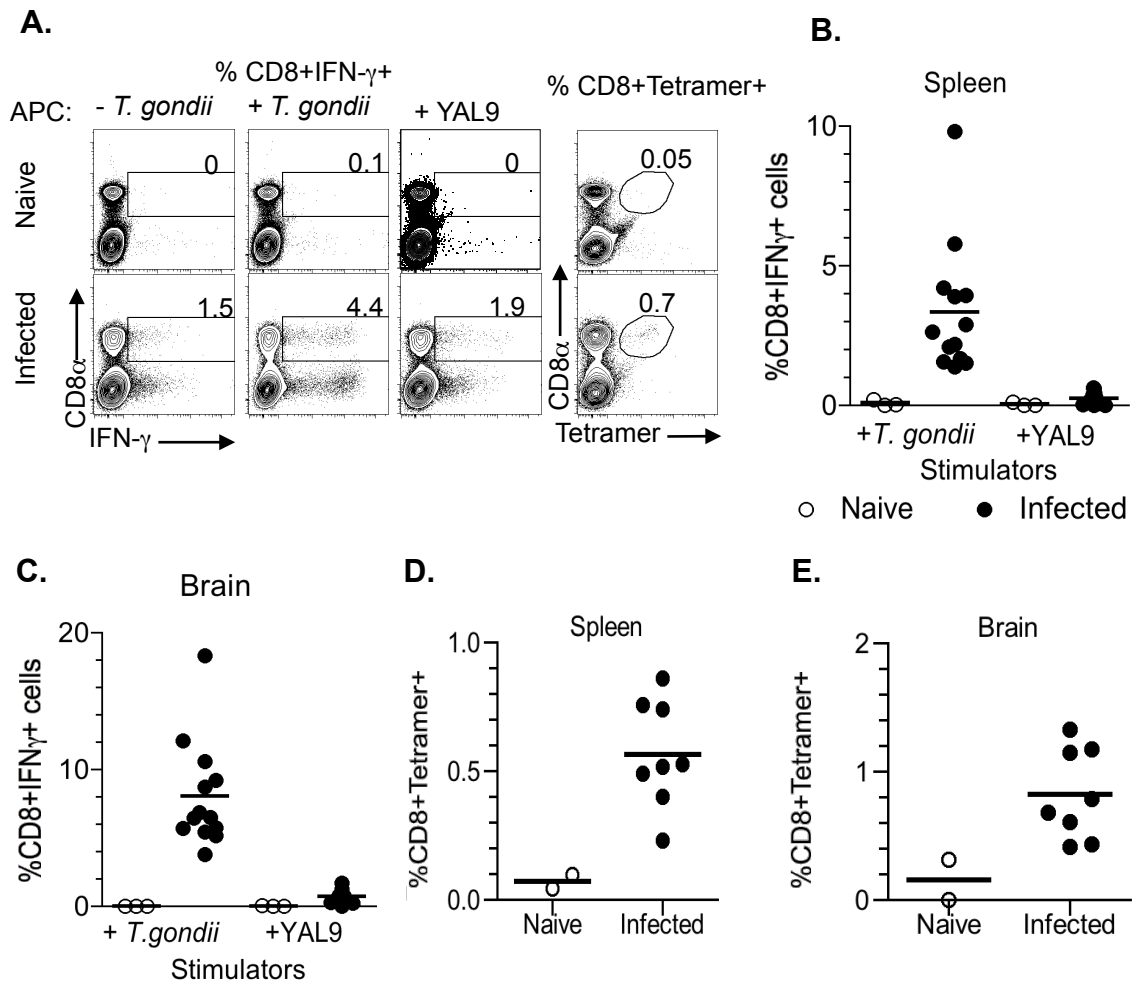
Figure 28: YAL9 specific CD8 T cell response constitutes a minor but detectable fraction of the *T. gondii* specific response in mice chronically infected with *T. gondii*.

A-E) C57BL/6 mice were orally infected with 25-40 *T. gondii* cysts. Splenocytes and brain leukocytes were harvested from mice 3-4 wks post infection, *T. gondii* specific CD8 T cell responses were measured by intracellular cytokine staining for IFN-γ or by staining with peptide-MHC tetramers. **A)** Representative flow cytometry plots of splenocytes from naïve and infected mice. Left panels show intracellular IFN-γ staining after *ex-vivo* restimulation with antigen presenting cells either, +/- *T. gondii*, or 1μM YAL9 peptide. Right panels show MHC class I H-2D[b] –YAL9 tetramer staining. **B, C)** Compiled data showing *T. gondii* specific and YAL9 specific responses from **B)** spleen and **C)** brain of infected mice. Data are corrected for background based on responses by CD8 T cells towards uninfected APCs or APCs with irrelevant peptide. Each dot represents an individual mouse. **D, E)** Compiled data showing flow cytometry analysis of MHC class I H-2D[b] – YAL9 tetramer staining on splenocytes from **D)** spleen and **E)** brain of mice. Cells were also co-stained with CD8 antibody. Data are representative of at least three independent experiments.
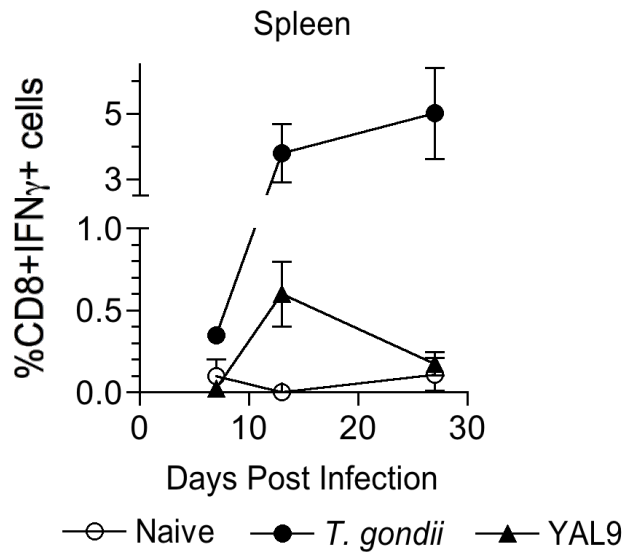
Figure 29: Expansion of YAL9 CD8 T cell response occurs during the acute phase and recedes during the chronic phase of infection.
C57BL/6 mice were orally infected with 25-40 *T. gondii* cysts. Splenocytes were harvested from mice 1-4 wks post infection. *T. gondii* specific CD8 T cell responses were measured by intracellular cytokine staining for IFN-γ. Compiled data showing *T. gondii* and YAL9 specific responses from spleens of infected or naïve mice over the course of infection. Each dot represents average of four mice. Data are corrected for background based on CD8 T cell responses towards uninfected or irrelevant peptide pulsed APCs. Data are representative of two independent experiments.

phase on infection (14 days) (Fig. 29). However, during the chronic phase of infection, YAL9 and *T. gondii* specific responses both crashed and declined, to the level that was observed previously in chronically infected mice (Fig. 29A and 28B). No significant response was seen in naïve mice throughout. Overall, during the acute phase of infection, YAL9 specific response was on average 0.6% of the overall CD8 T cell response. Given that *T. gondii* specific response was approximately 4% of the CD8 T cell response, we can calculate that YAL9 specific response was 15% of the total *T. gondii* specific response. Therefore, YAL9 specific cells expand during the acute phase of infection and then decline by the chronic stages. This response is unlike the response seen in H-2^d mice, where GRA6 (HF10) response immunodominates and makes up >90% of the T. *gondii* specific T cell response (Blanchard et al., 2008).

GRA6 (HF10) dominates in comparison to ROP5 (YAL9) specific CD8 T cell response in F1 (C57BL/6 x B10.D2) mice immunized with *Toxoplasma gondii*

H-2^d mice generate an immunodominant CD8 T cell response to HF10 from the *T. gondii* protein GRA6 presented on L^d MHC molecule (Blanchard et al., 2008). To determine if YAL9 specific CD8 T cell responses are enhanced or masked in the presence of a robust CD8 T cell response, such as in H-2^d mice to HF10, we immunized F1 (C57BL/6 x B10.D2) mice with irradiated tachyzoites from type II strain of *T. gondii*. 2 wks post immunization we harvested splenocytes from immunized mice and restimulated *ex-vivo* in the presence APCs +/- *T. gondii* or respective peptide pulsed APCs to measure using ICCS the IFN-γ response by CD8 T cells. Response to YAL9 was approximately 0.5% of the overall CD8 T cell response and was masked by the immunodominant CD8 T cell response to HF10, which was approximately 8.5% of the overall CD8 T cell response (Fig. 30A). No significant response was observed in naïve mice or in splenocytes from immunized mice restimulated with uninfected and irrelevant peptide pulsed APCs. MHC Class I H-2D^b YAL9 tetramer staining of splenocytes from immunized mice also revealed that on average 0.6% of the CD8 T cells were specific for YAL9 (Fig. 30B). On the other hand, HF10 loaded H-2L^d DimerX staining showed that 7% of the CD8 T cells were specific for HF10 (Fig. 30C).

Lastly, we also measured CD4 T cell response to *T. gondii* infected APCs or to APCs pulsed with the I-A^b restricted antigen AS15. Unlike B6 mice, in FI mice, CD4 responses to *T. gondii* were comparable to CD8 T cell response (Fig. 30A and 30D). In F1 mice, AS15 specific CD4 responses averaged to approximately 0.1%, which is similar to that observed in B6 mice (Fig. 30D). Therefore, even in the presence of robust CD8 T cell response to *T. gondii*, YAL9 responses remain a minor but detectable fraction of the overall *T. gondii* specific response especially in the presence of the dominating antigenic peptide, HF10. Antigen specific CD4 T cell response to AS15 in F1 immunized mice is similar to that seen in B6 mice.

HF10 specific response dominates over all the CD8 T cell responses against known *Toxoplasma gondii* antigens, including YAL9

Knowing the hierarchy of CD8 T cell responses to GRA6 vs. ROP5 in F1 (C57BL/6 x B10.D2) mice immunized with *T. gondii*, we wanted to determine, if the similar trends are also seen in infected F1 animals over the course of infection. Furthermore, in addition to HF10, we also wanted to assess the hierarchy of CD8 T cells that respond to other known *T. gondii* antigens, Tgd057, GRA4, ROP7 and OVA (Table 1). To determine the kinetics of CD8 T cell response, we orally infected F1 (C57BL/6 x B10.D2) mice with 50 cysts of *Prugniuad*-OVA, type II strain of *T. gondii*. 1-4 weeks post-infection, splenocytes were harvested and an *ex-vivo* restimulation was performed in the presence of infected APCs or various antigenic peptide pulsed APCs followed by ICCS for IFN-γ. Expansion of CD8 T cells in response to *T. gondii* or any of the antigenic peptides was seen during the acute phase of infection (Fig. 31A and 31B), and only the expansion of SM9 specific CD8 T cells seems to have declined during the chronic stages of infection. YAL9 specific CD8 T cell percentage did not change during the course of infection. This is consistent with kinetics of GRA6 specific response in H-2^d mice, where the contraction of specific CD8 T cells only happens around 6 weeks post infection (Blanchard et al., 2008). Expectedly, HF10 specific CD8 T cell responses were immunodominant throughout the course of infection ranging from approximately 2% during the acute phase and 10% during the chronic stages of infection (Fig. 31B). Other H-2^d antigenic response from GRA4 and ROP7 antigens expressing SM9 and IF9 antigenic peptide, respectively (Frickel et al., 2008), were also a minor fraction of the overall CD8 T cell response. SM9 responses ranged from 0.3% (acute phase) to 0.1% (chronic phase) of CD8 T cell responses (Fig. 31B). IF9 responses were on average 0.3% of total CD8 T cell responses throughout the course of infection (Fig. 31B). Interestingly, results from H-2^b specific responses showed that OVA (SL8) specific responses were also robust during the course of infection, even though OVA is not an endogenous antigen. They ranged from 1% (acute phase) to 5% (chronic phase) of the total CD8 T cell response (Fig. 31A). Tgd057 specific responses ranged from 1% (acute phase) to 1.25% (chronic phase) of the total CD8 T cell responses (Fig. 31A). YAL9 specific responses were the weakest and were 0.1% or less of the total CD8 specific CD8 T cell responses throughout the course of infection (Fig. 31A). We also measured overall CD4 T cell responses against *T. gondii*, against MHC Class II restricted *T. gondii* endogenous antigenic peptide, AS15, and against the antigenic peptide from OVA. Overall CD4 T cell responses to *T. gondii* were comparable to CD8 T cell response in F1 mice (Fig. 31A-C). The fraction of CD4 T cells responding to AS15 was similar to that seen in B6 mice wherein they expanded during the acute phase of infection (~1.5%) and declined during the chronic phases (~1%) (Fig. 13A-B and 31C). Meanwhile, OVA specific response displayed a similar trend but was much lower than AS15 specific response. They ranged from 0.75% (acute phase) to 0.5% (chronic phase) of total CD4 T cells (Fig. 31C). No response was seen by naïve mice (Fig. 31A-C) or by infected mice towards
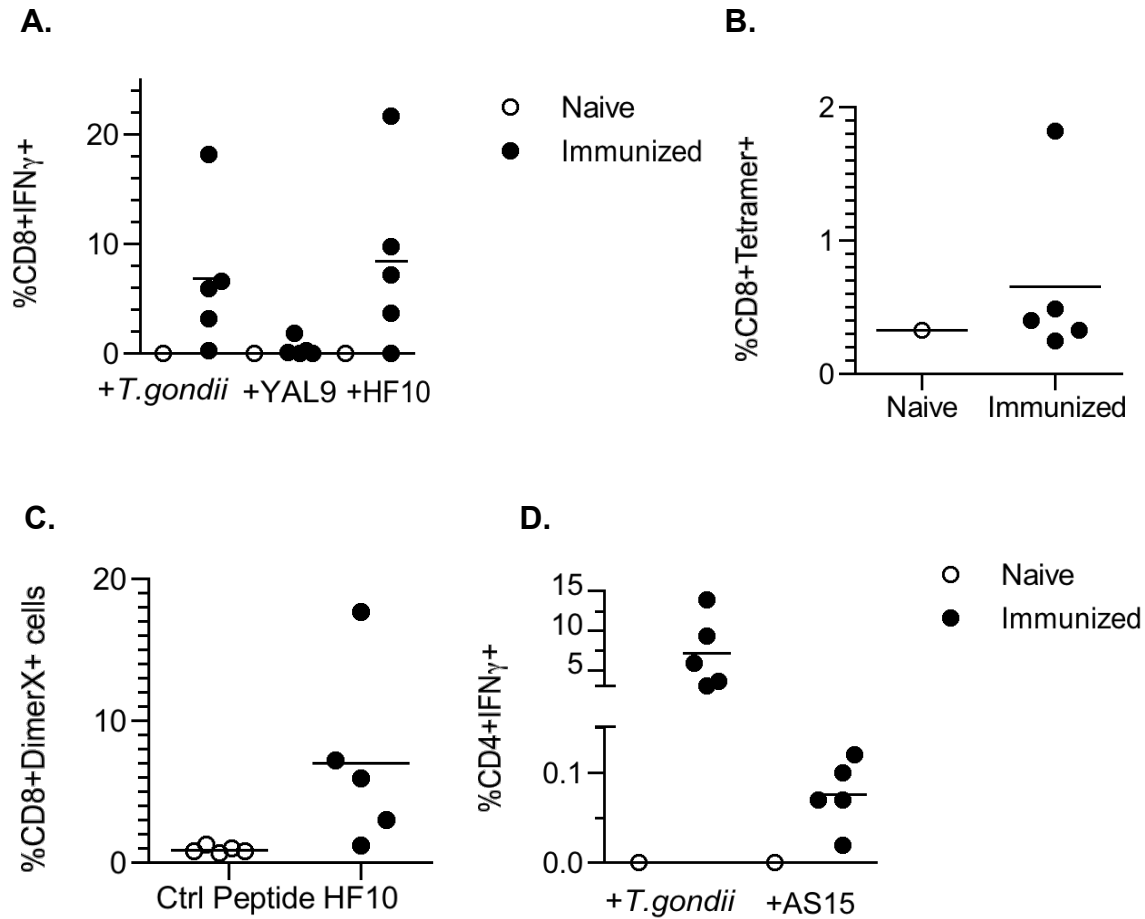
Figure 30: HF10 specific response immunodominates in comparison to YAL9 specific CD8 T cell response in *T. gondii* immunized F1 mice containing both H-2^b and H-2^d MHC Class I molecules.
A-D) F1 mice (C57BL/6 x C67BL/10.D2 mice) were immunized with irradiated 5x10^6 *T.gondii,* Me49 tachyzoites. Splenocytes were harvested from mice 2 wks post immunization and *T. gondii* specific T cell responses were measured by intracellular cytokine staining for IFN-γ or by staining with either tetramers or L^d-dimerX. **A)** Compiled data showing splenic *T. gondii*, YAL9 and HF10 specific responses from immunized mice. Compiled data showing **B)** MHC class I H-2D^b –YAL9 tetramer staining or **C)** L^d-HF10 DimerX staining on splenocytes from immunized mice. Cells were also co-stained with CD8 antibody and an irrelevant L^d binding peptide was used as control for DimerX staining. **D)** *T. gondii* and AS15 (I-A^b) specific CD4 T cell responses from immunized mice. Data are corrected for background based on responses by CD8 or CD4 T cells towards uninfected APCs or APCs with irrelevant peptide. Each dot represents an individual mouse. Data are representative of at least three independent experiments.
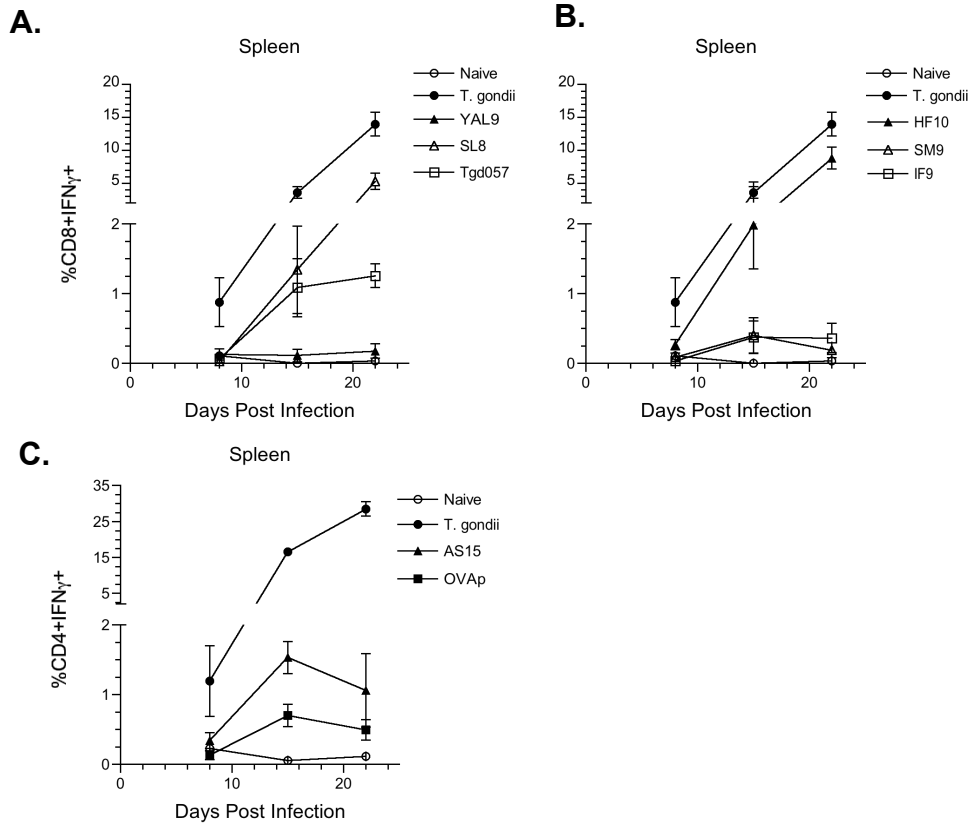
Figure 31: HF10 specific response dominates over all the CD8 T cell responses against known *Toxoplasma gondii* antigens, including YAL9, in infected F1 mice containing both H-2^b and H-2^d MHC Class I molecules.
A-C) F1 mice (C57BL/6 x C67BL/10.D2 mice) mice were orally infected with 50 *T. gondii*, Pruginuaud-OVA cysts. Splenocytes were harvested from mice 1-4 wks post infection, *T. gondii* specific T cell responses were measured by intracellular cytokine staining for IFN-γ. Compiled data showing *T. gondii* specific and different antigen specific responses from **A)** H-2^b specific antigens YAL9 (ROP5), SL8 (OVA), SVL8 (Tgd057); **B)** H-2^d specific antigens HF10 (GRA6), SM9 (ROP7), IF9 (GRA4), and **C)** CD4 T cell responses for MHC Class II-IA^b restricted antigen AS15 (CD4Ag28m). Data are corrected for background based on responses by T cells towards uninfected APCs or APCs with irrelevant peptide. Each dot represents an individual mouse. Cells were also co-stained with CD8 or CD4 antibody.

uninfected or irrelevant peptide pulsed APCs, which has been corrected from depicted responses. Measuring the kinetics of infection in F1 mice against different *T. gondii* antigens shows that the hierarchy of CD8 T cell responses favors GRA6 (HF10) as the immunodominant antigen, while others constitute minor but detectable fraction of the overall *T. gondii* specific response.

Immunization with YAL9 fails to generate protection in C57BL/6 mice challenged with a lethal dose of infection

It has been shown previously that immunization of H-2^d mice with BMDCs pulsed with HF10 peptide generates protection in the mice following a lethal dose of infection (Blanchard et al., 2008). To determine if YAL9 has similar protective potential in B6 mice, we immunized the animals in the footpad with LPS activated BMDCs pulsed with YAL9 or an irrelevant peptide, WI9. We then challenged mice 7-21 days post immunization with a lethal dose of type II strain of *T. gondii* (10,000 Pru tz) and monitored survival over time. The lethal dose for infection was optimized in B6 mice infected intraperitoneally with varying doses of type II strain of *T. gondii* (Fig. 33). From the surviving mice, we measured the CD8 T cell responses, cyst number and parasite load in the brain. Overall, we did not see a significant difference in survival between the mice immunized with the relevant peptide or the irrelevant peptide (Fig. 32A). To ensure, that the surviving mice were indeed immunized and challenged, we measured CD8 T cell responses to infected or peptide pulsed APCs in an *ex-vivo* restimulation, which was followed by ICCS for IFN-γ. CD8 T cell responses to *T. gondii* infected APCs revealed that indeed surviving mice were infected and generated a response in both spleen (Fig. 32A) and brain (Fig. 32B). Additionally, YAL9 immunized and infected mice generated a greater response to YAL9 pulsed APCs than WI9 immunized mice in both the spleen and brain, confirming that peptide immunization boosted a response to YAL9 (Fig. 31B and 32C). Both sets of infected mice generated a robust CD4 T cell response towards infected APCs as measured by ICCS for IFN-γ in the brain and spleen, also indicating that they were infected (Fig. 32D and 32E). No response was seen towards uninfected or irrelevant peptide pulsed APCs, which has been corrected from depicted responses. YAL9 loaded MHC Class I tetramer staining of brain leukocytes and splenocytes also revealed a greater percentage of YAL9 specific CD8 T cells in YAL9 immunized mice as compared to WI9 mice, confirming a boost in YAL9 specific CD8 T cells due to immunization (Fig. 32F and 32G). Furthermore, a measurement of the number of cysts and the parasite load from surviving mice showed that there was no difference between relevant or irrelevant peptide immunization (Fig. 32H and 32I). Therefore, unlike HF10, immunization with BMDCs pulsed with YAL9 peptide does not generate protection in B6 mice as there was no difference in survival, cyst number and parasite load between YAL9 and irrelevant peptide (WI9) immunized mice.
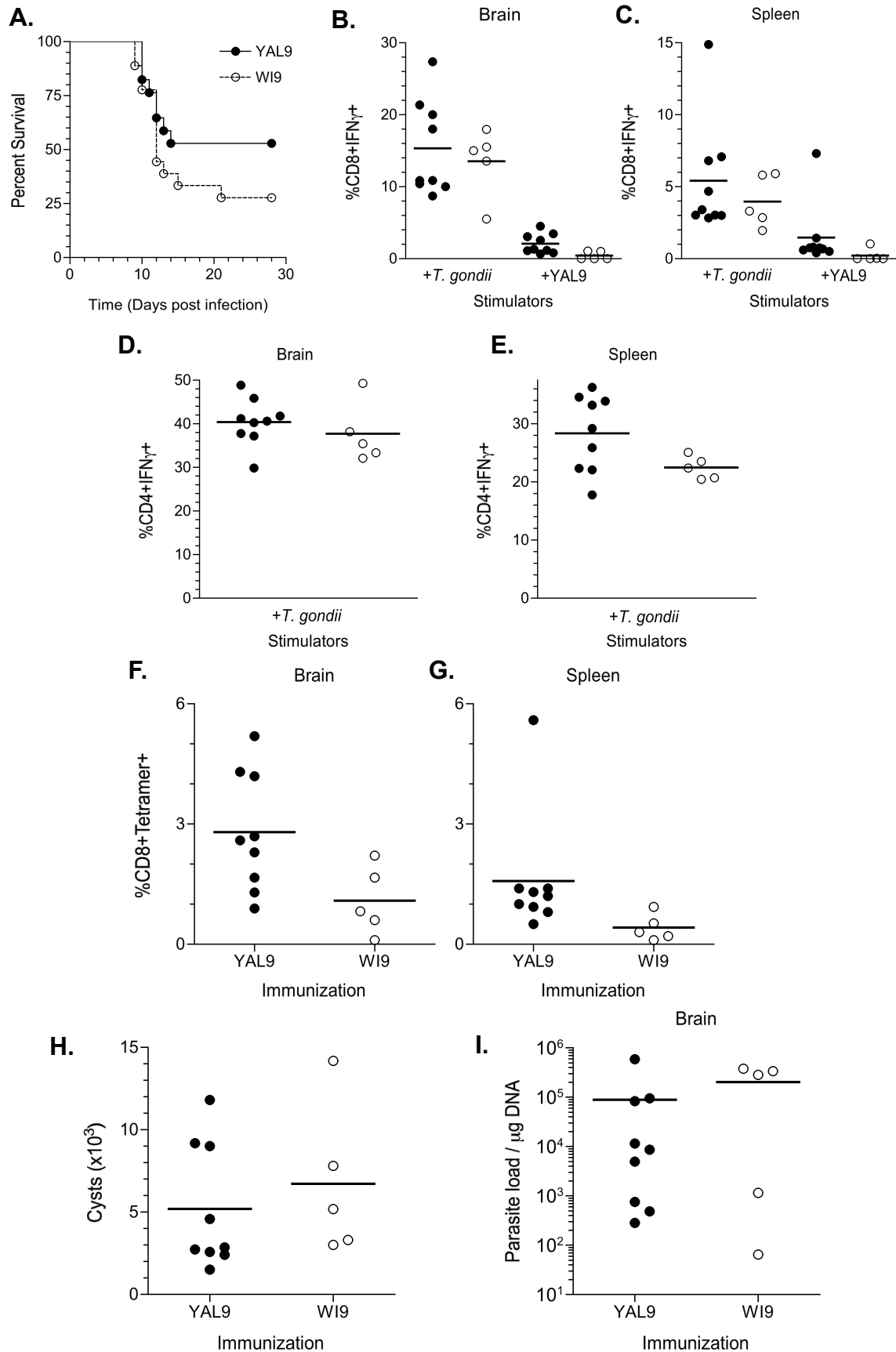
Figure 32: Immunization with YAL9 does not protect C57BL/6 mice from lethal challenge with *T. gondii*.
C57BL/6 mice were immunized with LPS activated BMDCs pulsed with YAL9 or control peptide, WI9. 7-21 days post immunization, these mice were infected with 1×10^4 live *T. gondii* tachyzoites, intraperitoneally. **A)** Kaplan-Meier survival curves between the two groups. **B-I)** Analysis of the T cell responses and parasite loads from surviving mice. IFN-γ response by **B, C)** CD8 T cells, **D, E)** CD4 T cells, as measured by ICCS for IFN-γ using flow cytometry after *ex-vivo* restimulation with *T. gondii*-infected APCs or peptide-pulsed APCs in **B, D)** brain **C, E)** spleen. Data are background corrected based on the values from uninfected APCs or APC pulsed with irrelevant peptide. **F, G)** MHC class I H-2D^b –YAL9 tetramer staining on **F)** brain leukocytes **G)** splenocytes. Cells were also co-stained with CD8 antibody. **H)** Number of cysts in the brain as measured by staining a portion of the brain with fluorescent lectin to detect the cysts. **I)** The parasite load in the brain measured using semi-quantitative PCR on genomic DNA extracted from the tissue. Data is representative of at least three experiments with at least 4 mice per condition in each experiment.
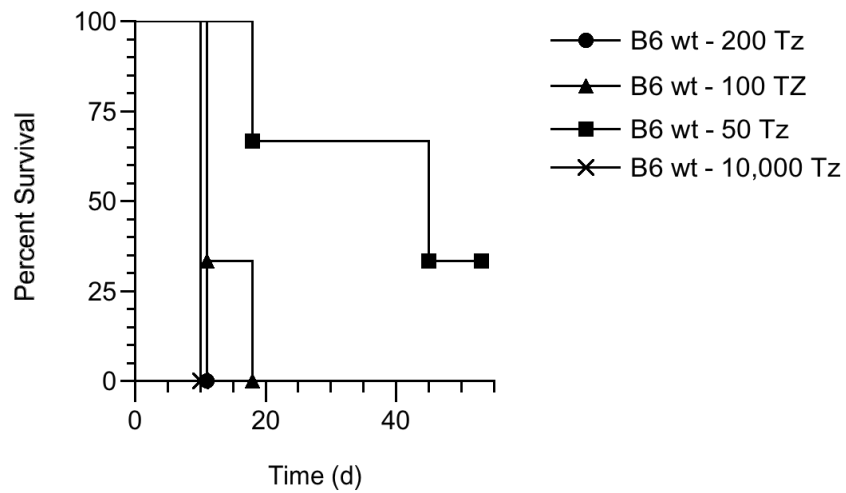
Figure 33: Optimization of the lethal dosage for infection of wild-type or peptide immunized C57BL/6 mice.
C57BL/6 mice were infected intraperitoneally with varying doses of live *T. gondii* tachyzoites, type II, Prugniaud strain. Kaplan-Meier survival curves between the different groups of infected mice (d = days post infection). Data is representative of at least two experiments with 3 mice per condition in each experiment.

Lack of antigenic activity among the polymorphic versions of ROP5 from different paralogs within the strains of *Toxoplasma gondii*

Our expression cloning screen to identify the antigen recognized by *T. gondii* specific CD8 hybridoma BTg45Z, yielded in the hit that corresponded to ROP5 in the *T. gondii* database from the type II strain of *T. gondii* (Fig. 23). Additional sequence information from recently published paper (Reese et al., 2011) revealed that it was the type II, ROP5 version C paralog that we had sequenced in our screen. To determine, if other versions of ROP5, including the type II paralog A or paralogs A, B or C from type I or III strain are antigenic, we infected B6 BMDCs with irradiated type I or III strains of *T. gondii* or pulsed them with peptides from different versions of ROP5, followed by an incubation with hybridoma, BTg45Z. CTG, the type III strain and RH, the type I strain of *T. gondii* were not able to stimulate the BTg45Z hybridoma as compared to the Me49, the type II strain of *T. gondii* (Fig. 34A and 34B). Baseline response was seen with uninfected APCs (Fig. 34A and 34B). Additionally, peptide, YAVANYLLL, from paralogs A, B, and C from both type I and type III strains of *T. gondii*, was not able to stimulate the hybridoma, while type II version A paralog peptide, YAVANYFLL stimulated the hybridoma to levels seen with the type II version C peptide, YAVANYFFL (Fig. 34C). No response was seen with irrelevant WI9 peptide (Fig. 34C).

Similar results were seen *in-vivo* when B6 mice were immunized with irradiated tachyzoites from Me49, the type II or CTG, the type III strain of *T. gondii*. *Ex-vivo* restimulation with infected or peptide pulsed APCs, followed by ICCS for IFN-γ, showed that both groups of mice generated a robust response to *T. gondii* infected APCs (Fig. 34D). However, only ME49 immunized mice were able to generate a response above background (naïve mice) towards YAL9 (type IIC version) (Fig. 34D). CTG immunized mice generated a response to YAL9 from the type III A-C version, but not to the type II version C (Fig. 34D). Staining with YAL9 (IIC) peptide loaded MHC Class I tetramers also showed that YAL9 (IIC) specific CD8 T cells were only present in animals immunized with Me49 and not CTG (Fig. 34E). Overall, we were able to conclude that even though the identified antigen was from the ROP5 version IIC paralog in type II strain, version IIA is also antigenic, since it stimulates BTg45Z. On the other hand, type I or III strain parasites do not stimulate the hybridoma and *in-vivo,* mice immunized with type III strain do not generate a cross reactive response towards the YAL9 type IIC peptide.

Generation of transgenic parasites that allow for the secretion of ROP5 as a dense granular protein

To understand why a dense granular protein such as GRA6 generates an immunodominant and protective response while ROP5 generates a much weaker response that fails to protect, we asked the question whether the type of
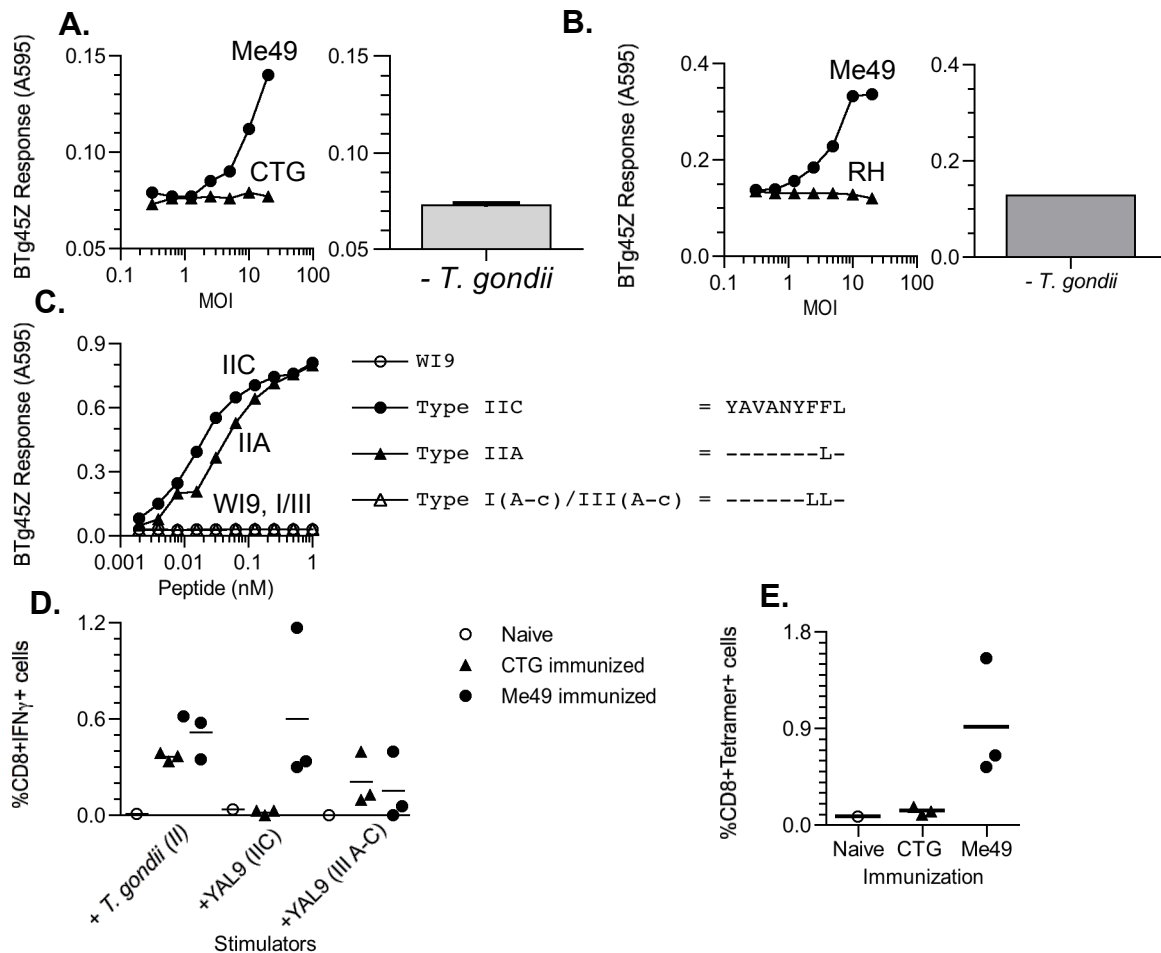
Figure 34: BTg45Z hybridoma does not recognize the polymorphic versions of the 9-mer epitope, YAL9, from different strains of *T. gondii*.
A, B) BMDCs from C57BL/6 mice were infected *in-vitro* with irradiated type I (RH), type II (Me49) or type III (CTG) strain of *T. gondii*. BTg45Z lacZ response after an overnight stimulation with **A)** type II and III or **B)** type I and II infected BMDCs. Right panel depicts background lacZ response against uninfected BMDCs. **C)** BTg45Z lacZ response after an overnight stimulation against BMDCs pulsed with titrating concentrations of polymorphic YAL9 peptides and irrelevant WI9 peptide. **D, E)** C57BL/6 mice were immunized with irradiated 5×10^6 *T. gondii* tachyzoites from type II (Me49) and type III (CTG) strain. Splenocytes were harvested from mice 2 wks post immunization and *T. gondii* or YAL9 specific CD8 T cell responses were measured by intracellular cytokine staining for IFN-γ or by staining with tetramers. **D)** Compiled data showing CD8 T cell responses from immunized or naïve mice towards *T. gondii* infected or YAL9 (type IIC or type IIIA-C) peptide pulsed APCs. Each dot represents an individual mouse. Data are corrected for background based on CD8 T cell responses towards uninfected or irrelevant peptide pulsed APCs. **E)** Compiled data showing MHC class I H-2D[b]-YAL9 tetramer staining on splenocytes from immunized or naïve animals. Each dot represents an individual mouse. Cells were also co-stained with CD8 antibody. Data are representative of three independent experiments.

secretory proteins within the parasite caused a difference in the amount of antigen availability or accessibility leading to a differential T cell response. For example, the rhoptry proteins are only secreted during the initial phase of infection, while dense granules proteins are secreted throughout the life cycle of the parasite within the infected cell. In order to address the question whether altering the protein trafficking of ROP5 from rhoptries to dense granules can enhance YAL9 specific responses in B6 mice, we decided to generate transgenic parasites that allow the secretion of ROP5IIC to be like that of a dense granular protein. All the transgenic strains were made by Michael Reese in the Boothroyd lab at Stanford University. All transgenic parasites were generated using the CTG, the type III strain of *T. gondii*. Because type III paralog versions of YAL9 were not antigenic and did not cross-react with YAL9IIC (Fig. 34), type III parasites served as a null strain in which we can express ROP5 type IIC version either in the rhoptries or dense granules and monitor response specifically against YAL9IIC. We generated three different versions of the transgenic parasites that are depicted in the schematic in Figure 35. Incidentally, the parental type III CTG strain used also expresses luciferase and green-fluorescent protein (Boothroyd laboratory) that could be used for detection of infection and fluorescent studies. CTG/ROP5IIC parasites allow for the expression of ROP5IIC, in its native context as a rhoptry protein (Fig. 35A). CTG/DG-ROP5IIC parasites allow for the expression of the pseudo-kinase domain from ROP5IIC into the dense granules under the control of the GRA6 promoter and signal sequence eventually allowing for the secretion of ROP5IIC in a dense granular context. YAL9 is located in the pseudo-kinase domain (Reese and Boothroyd, 2009). The transgene in these parasites is under the GRA6 targeting promoter to allow for the appropriate timing and expression level during *T. gondii* cell cycle (Fig. 35A). The third transgenic strain that we generated was CTG/GRA6II-YAL9, in which GRA6 from type II is transgenically expressed in the type III strain as part of the dense granules with its antigenic epitope, HF10 that is recognized in H-2^d mice is replaced by YAL9IIC (Fig. 35A). Notably, all the transgenes were expressing an FLAG or HA tag, i.e., ROP5IIC or DG-ROP5IIC or GRA6II-YAL9 were fused with FLAG or HA tag. Furthermore, immunofluorescence staining was performed using antibodies against HA or FLAG (Fig. 35B) to ensure the correct expression and localization of the respective transgenic proteins among the different strains.

Enhanced stimulation of BTg45Z hybridoma by CTG/DG-ROP5IIC transgenic parasites expressing ROP5IIC as a dense granule protein

To determine, if transgenic parasites CTG/ROP5IIC (parasites that express ROPIIC in the rhoptry context), CTG/DG-ROP5IIC (parasites that express ROP5IIC in dense granular context) and CTG/GRA6II-YAL9 (parasites that express the antigenic peptide, YAL9, at the end of GRA6 type II dense granule protein) can stimulate BTg45Z hybridoma, we infected BMDCs with irradiated tachyzoites from these strains. As a control, we also infected BMDCs with

A.

CTG (Null) WT Parental Type III strain

GRA6 (III)

ROP5 (III)

PV lumen

Host Cytosol

Rhoptries

PV Membrane

Dense Granules

CTG/ROP5IIC

ROP5 (IIC)

YAL9

CTG/DG-ROP5IIC

GRA6p

ROP5 (IIC) Pseudokinase domain

CTG/GRA6II-YAL9

GRA6 (II) – YAL9

B.

CTG/ROP5IIc

CTG/DGROP5IIC

αFLAG

αHA

CTG/GRA6II-YAL9

αHA

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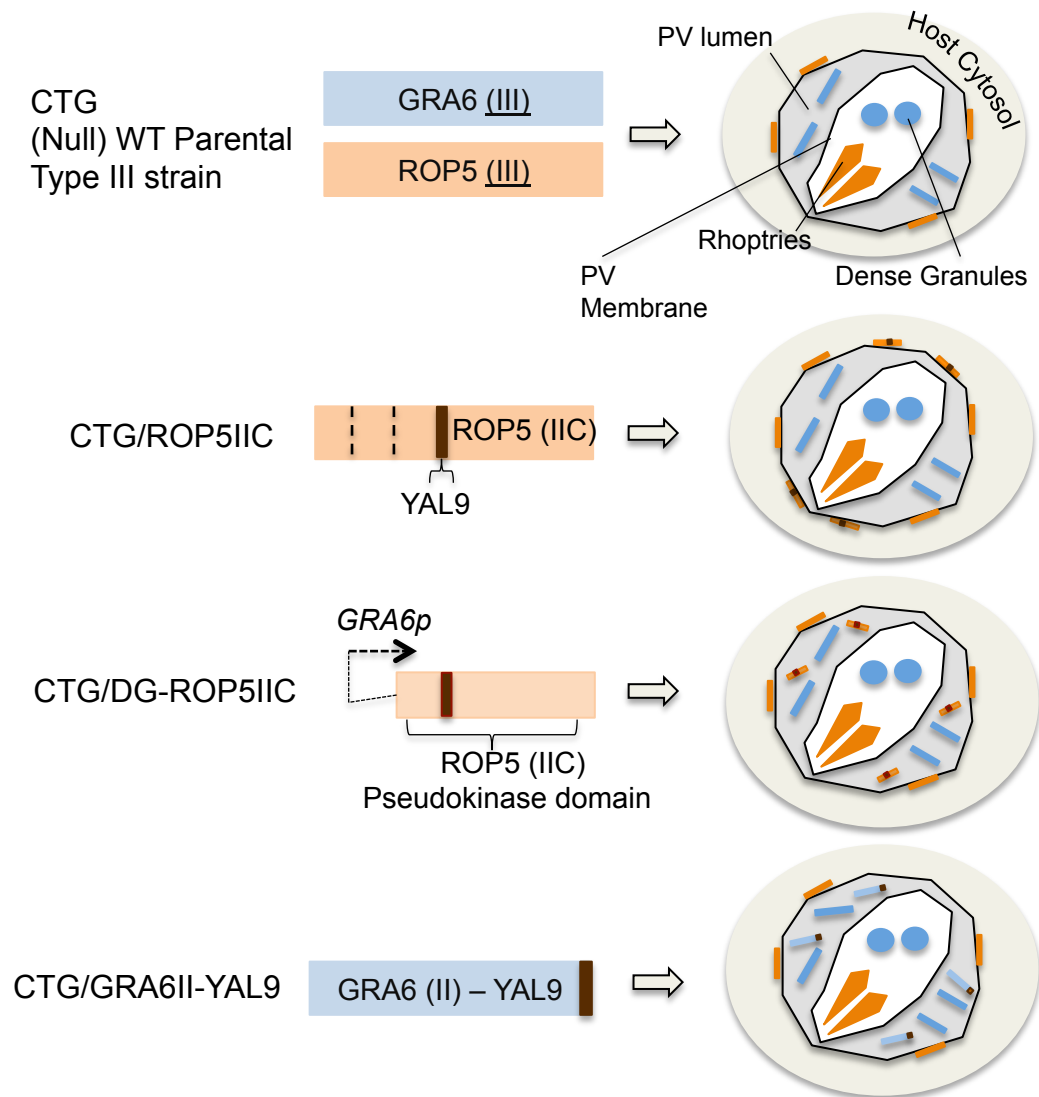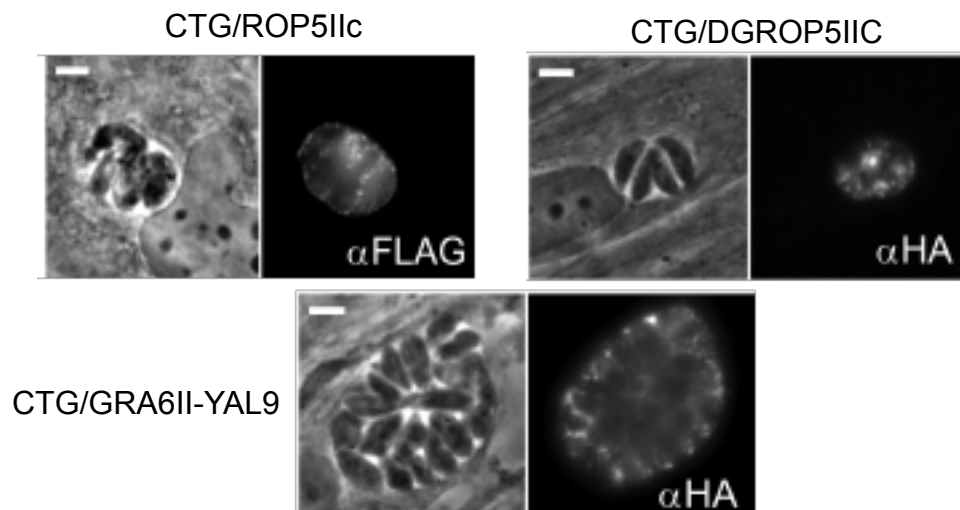
Figure 35: Transgenic parasites that allow the expression of ROP5IIC or YAL9 from type IIC to be part of the dense granules.
A) Schematic depicting the different versions of transgenic parasites that allow the expression of ROP5IIC or YAL9 from type IIC as part of the different secretory organelles in *T. gondii*. Type III strain, CTG, was the parental "null" strain used to generate the transgenic parasites. CTG/ROP5IIC parasites allow the expression of ROP5IIC in the rhoptries. CTG/DG-ROP5IIC parasites allow the expression of ROP5IIC in the dense granules, under the control of the GRA6 promoter. CTG/GRA6II-YAL9 parasites, allow the expression of GRA6 from type II in the dense granules, with the exception that the antigenic epitope in GRA6 (HF10) has been replaced by YAL9IIC. **B)** Immunofluorescence images depicting the presence of either ROP5 or GRA6 in the respective location depending upon the transgenic parasite strain. All transgenic parasites express an HA or FLAG tag linked either with ROP5IIC or GRA6.

irradiated tachyzoites from the type II, Me49 strain of *T. gondii*. Upon incubation of BTg45Z with infected or uninfected BMDCs, it was evident that BTg45Z response was much enhanced with CTG/DG-ROPIIC parasites that allow the expression of ROP5IIC into the dense granules than compared to either the transgenic parasites CTG/ROP5IIC that express ROP5IIC into the rhoptries or even with the type II Me49 strain (Fig. 36). Interestingly, BTg45Z response with CTG/GRA6II-YAL9 transgenic parasites was slightly enhanced as compared to the CTG/ROP5IIC or type II Me49 strain (Fig. 36). No BTg45Z response was seen with uninfected BMDCs (Fig. 36).

Enhanced immunogenicity of YAL9 is observed in mice immunized with CTG/DG-ROP5IIC transgenic parasites expressing ROP5IIC as a dense granule protein

Since YAL9 specific CD8 T cell responses were a smaller fraction of the total *T. gondii* specific T cell responses in comparison to HF10 specific responses, we next wanted to assess, if the alteration of ROP5IIC to a dense granular protein changes this fraction in mice that have been immunized with different transgenic strains. We immunized B6 mice with 5×10^6 irradiated tachyzoites from transgenic strains: CTG/ROP5IIC (express ROP5IIC in the rhoptry context), CTG/DG-ROP5IIC (express ROP5IIC as a dense granular protein), and CTG/GRA6II-YAL9 (express just the antigenic peptide YAL9 in the dense granules in place of HF10 in GRA6II). 2 wks post-immunization splenocytes were harvested and *ex-vivo* restimulation was performed in the presence of APCs +/- *T. gondii* or peptide pulsed APCs followed by ICCS for IFN-γ. Indeed, immunization with CTG/DG-ROP5IIC parasites enhanced CD8 T cell responses against YAL9 approximately 20x as much as compared to immunization with CTG/ROP5IIC (~2% vs. ~0.1% of total CD8 T cell response, respectively) (Fig. 37A and 37B). Meanwhile, YAL9 specific CD8 T cell responses in mice immunized CTG/GRA6II-YAL9 strain were enhanced approximately 6x as compared to immunization with CTG/ROP5IIC (~0.6% vs. ~0.1% of total CD8 T cell response) (Fig. 37A and 37B). Overall, *T. gondii* specific CD8 T cell responses were comparable in all three strains of immunized animals (Fig. 37A and 37B).

Interestingly, as the YAL9 specific CD8 T cell responses increased based upon the immunization, *T. gondii* specific CD4 T cell responses decreased proportionally (Fig. 37C) indicating a change in the balance of responses where having a potent CD8 T cell response reduces the overall CD4 T cell response unlike seen with B6 mice immunized with wildtype type II, Me49 strain (Fig. 3). No significant response was observed naïve mice or in immunized mice restimulated with uninfected or irrelevant peptide pulsed APCs. Similar results were seen with staining for MHC Class I YAL9 H-2D^b tetramers, where immunization with CTG/ROP5IIC resulted in the presence of 0.25% of YAL9 specific CD8 cells, while immunization with CTG/DG-ROP5IIC resulted in
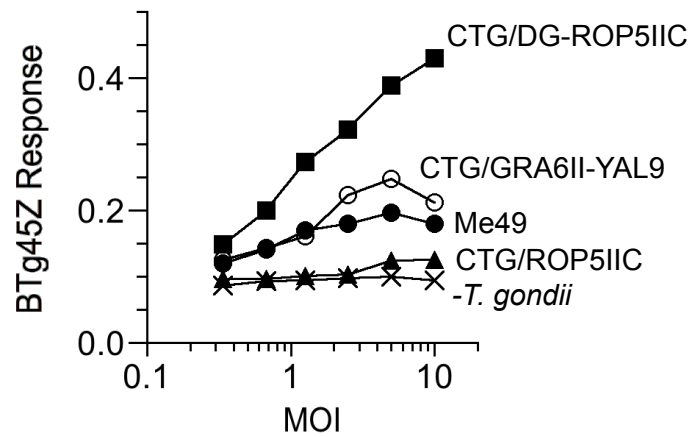
Figure 36: BTg45Z hybridoma response to YAL9 is enhanced against transgenic *T. gondii* that express ROP5IIC as a dense granular protein. BMDCs from C57BL/6 mice were infected *in-vitro* with irradiated transgenic *T. gondii* tachyzoites at different MOI. BTg45Z lacZ response was measured after an overnight stimulation with infected and uninfected BMDCs. Data are representative of three independent experiments.
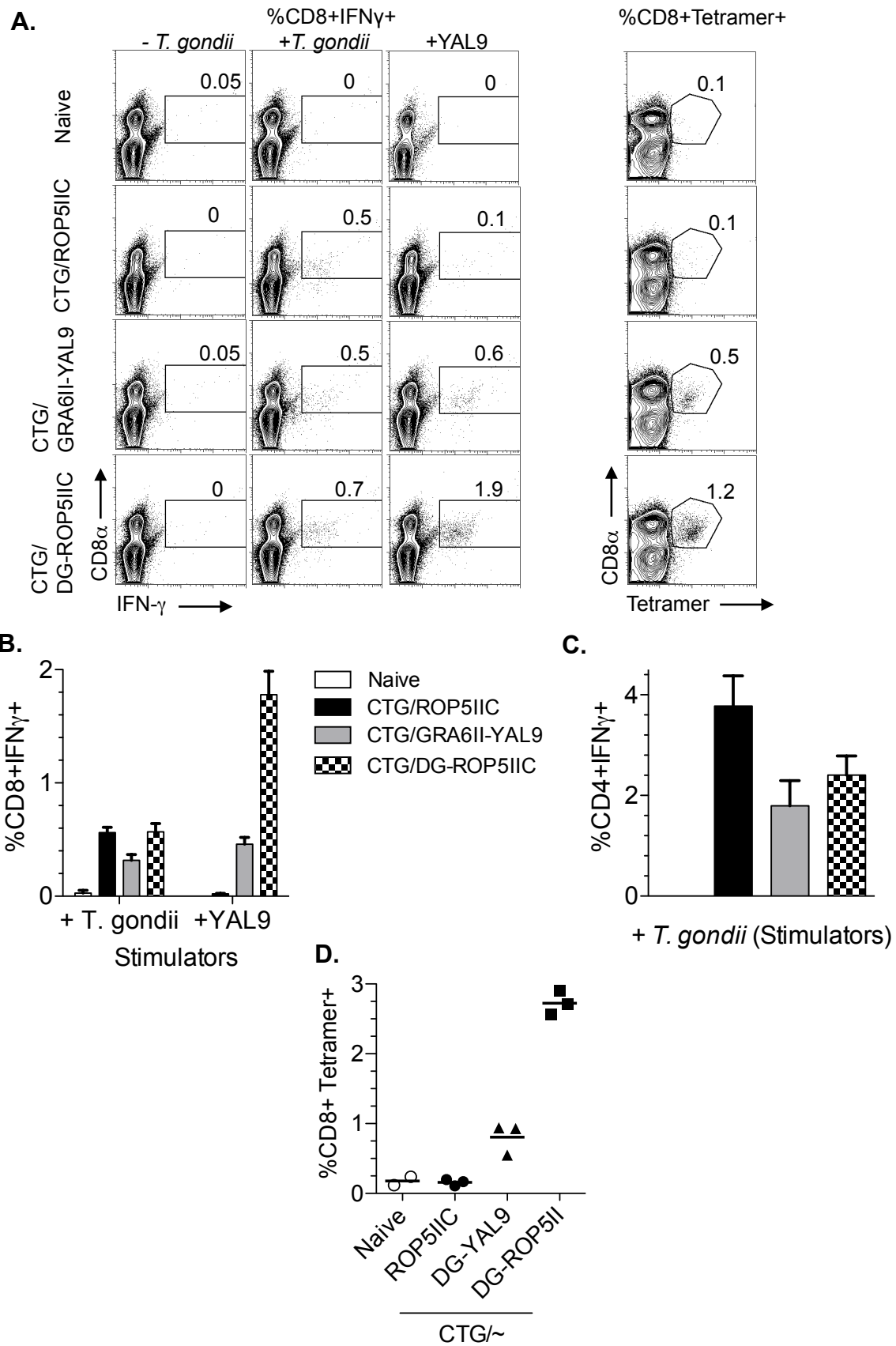
Figure 37: YAL9 specific response is enhanced in mice immunized with transgenic *T. gondii* that express ROP5IIC as a dense granular protein.
A-D) C57BL/6 mice were immunized with irradiated 5x10^6 transgenic *T.gondii* tachyzoites. Splenocytes were harvested from mice 2 wks post immunization and *T. gondii* specific T cell responses were measured by intracellular cytokine staining for IFN-γ or by staining with tetramers. **A)** Representative flow cytometry plots from naïve and immunized mice. Left panels show intracellular IFN-γ staining after *in vitro* restimulation with antigen presenting cells either, +/- *T. gondii*, or 1μM YAL9 peptide. Right panels show MHC class I H-2D^b –YAL9 tetramer staining. **B, C)** Compiled data showing splenic **B)** *T. gondii* specific and YAL9 specific CD8 T cell responses **C)** *T. gondii* specific CD4 T cell responses from immunized mice. Data are corrected for background based on T cell responses by towards uninfected APCs or APCs with irrelevant peptide. Each dot represents an individual mouse. **D)** Compiled data showing MHC class I H-2D^b – YAL9 tetramer staining on splenocytes from immunized mice. Cells were also co-stained with CD8 antibody. Data are representative of at least two independent experiments.

presence of 2.75% of YAL9 specific CD8 T cells, an enhancement of approximately 11x (Fig. 37D). CTG/GRA6II-YAL9 immunization resulted in an intermediate increase of 3x (~0.8% YAL9 specific CD8 T cells) (Fig. 37D). Overall, expression of ROP5 as a dense granular protein enhances the immunogenicity of YAL9 as compared to its expression in its native context as a rhoptry protein. Additionally, just having the antigenic peptide in the dense granules increases the immunogenicity intermediately in comparison to having the entire ROP5IIC pseudo-kinase domain as a dense granule protein.

Enhanced immunogenicity of YAL9 and reduced cyst numbers are observed in mice infected with CTG/DG-ROP5IIC transgenic parasites

In line with data observed from immunized mice, we also wanted to assess if the fraction of YAL9 specific CD8 T cells is altered in mice that are chronically infected with transgenic parasites that express ROP5IIC as a dense granule protein. This was especially demanding, since YAL9 specific T cells were a small but measurable fraction of the *T. gondii* specific response as seen in figure 28. We infected B6 mice with 5×10^3 tachyzoites, intraperitoneally, from transgenic strains, CTG/ROP5IIC (express ROP5IIC in the rhoptries), CTG/DG-ROP5IIC (express ROP5IIC pseudokinase domain as a dense granular protein), and CTG/GRA6II-YAL9 (express just the antigenic peptide YAL9 in the dense granules in place of HF10 in GRA6II). 3-4 wks post-infection splenocytes and brain leukocytes were harvested and *ex-vivo* restimulation was performed in the presence of APCs +/- *T. gondii* or peptide pulsed APCs followed by ICCS for IFN-γ. Infection with CTG/DG-ROP5IIC parasites enhanced CD8 T cell responses against YAL9 in the spleen approximately 15x as much as compared to infection with CTG/ROP5IIC (~4.5% vs. ~0.3% of total CD8 T cell response, respectively, Fig. 38A and 38B). In the brain, this enhancement was about 220x, (~4.5% vs. 0.02% of total CD8 T cell response, respectively, Fig. 38C).

Meanwhile, YAL9 specific CD8 T cell response in the spleen of mice infected CTG/GRA6II-YAL9 strain was enhanced approximately 6x as compared to infection with CTG/ROP5IIC (~1.8% vs. ~0.3% of total CD8 T cell response, Fig. 38A and 38B). In the brain, this enhancement was 50x (~0.95% vs. 0.02%, of total CD8 T cell response, respectively, Fig. 38C). No significant response was observed naïve mice or in infected mice restimulated with uninfected or irrelevant peptide pulsed APCs.

Overall, *T. gondii* specific CD8 T cell responses increased in both the spleen and the brain as did responses towards YAL9 among the animals that were infected with the either CTG/DG-ROP5IIC or CTG/GRA6II-YAL9 (Fig. 38A-C). Furthermore, as the YAL9 specific CD8 T cell responses increased with respective infections, *T. gondii* specific CD4 T cell responses decreased proportionally in the spleen (Fig. 38D) but not in the brain (Fig. 38E) indicating a change in the balance of responses where having a potent CD8 T cell response

reduces the overall CD4 T cell response. However, there is no change in the CD4 T cell response in the brain even when there are potent CD8 T cell responses, suggesting that CD4 T cells in the brain perhaps expand in the periphery before accumulating in the brain.

Similar results were seen with staining for MHC Class I YAL9 H-2D^b tetramers, where infection with CTG/ROP5IIC resulted in the presence of 0.3% of YAL9 specific CD8 cells in the spleen, while infection with CTG/DG-ROP5IIC resulted in presence of 6.5% of YAL9 specific CD8 T cells, an enhancement of approximately 20x (Fig. 38F). In the brain ~0.2% of CD8 T cells were YAL9 specific with CTG/ROP5IIC infection and ~11.5% with CTG/DG-ROP5IIC infection, which calculates to an enhancement of 60x (Fig. 38G). CTG/GRA6II-YAL9 infection resulted in an intermediate increase of ~10x (~2.7% YAL9 specific CD8 T cells) in the spleen (Fig. 38F) and an increase of 20x (~4.4% of YAL9 specific CD8 T cells) in the brain (Fig. 38G).

Lastly, an increase in the number of CD8 T cells specific for YAL9 in the brain and spleen resulted in a 50% decrease of number of cysts observed in the brains of mice infected with CTG/DG-ROP5IIC and CTG/GRA6II-YAL9 (~5x10^3 cysts in CTG/ROP5IIC vs. ~2.5x10^3 cysts in CTG/DG-ROP5IIC or CTG/GRA6II-YAL9 infected mice). Interestingly, an intermediate increase of enhancement of YAL9 specific T cell responses by CTG/GRA6II-YAL9 strain was sufficient enough to decrease the cyst numbers by 50%, the same decrease seen by CTG/DG-ROP5IIC strain, even though the enhancement of YAL9 specific T cells is much greater with this strain.

YAL9 peptide immunization of C57BL/6 mice protects mice during the acute phase of infection against a lethal dose of CTG/DG-ROP5IIC transgenic parasites expressing ROP5IIC as a dense granule protein

Earlier in this chapter, we had shown that peptide immunization with YAL9 does not protect mice against a challenge with a lethal dose of infection with type II strain of *T. gondii*. However, with the transgenic strain CTG/DG-ROP5IIC we were able to enhance the immunogenicity of YAL9 to about 15x in the spleen and 220x in the brains of chronically infected mice (Fig. 38B and 38C). Therefore, we wanted to determine if immunization with YAL9 followed by a lethal challenge with the transgenic parasites can generate protection in B6 mice. To assess this, we immunized B6 mice in the footpad with LPS activated BMDCs pulsed with YAL9 or an irrelevant peptide, WI9. We boosted the mice with the same dose of peptide immunization two weeks after the first immunization. We then challenged mice intraperitoneally 21 days post immunization with a lethal dose of CTG/ROP5IIC (express ROP5IIC in the rhoptry context) or CTG/DG-ROP5IIC (express ROP5IIC in the dense granular context) transgenic strains and monitored survival over time (Fig. 39A). We only looked at protection with CTG/DG-ROP5IIC parasites where the maximum enhanced responses were

A.

%CD8+IFNγ+

%CD8+Tetramer+
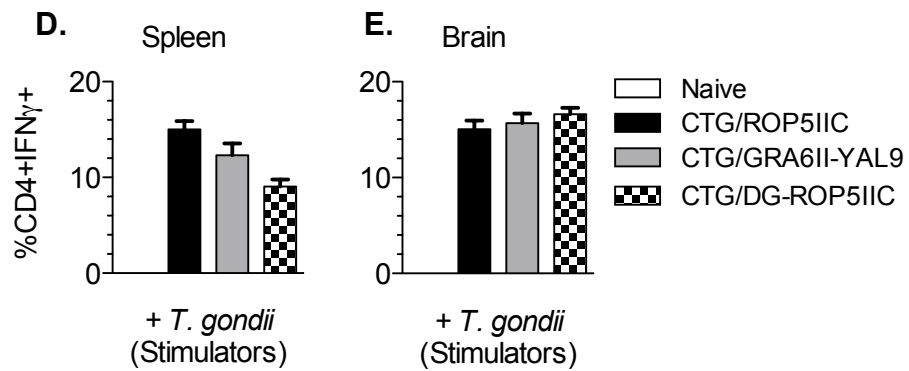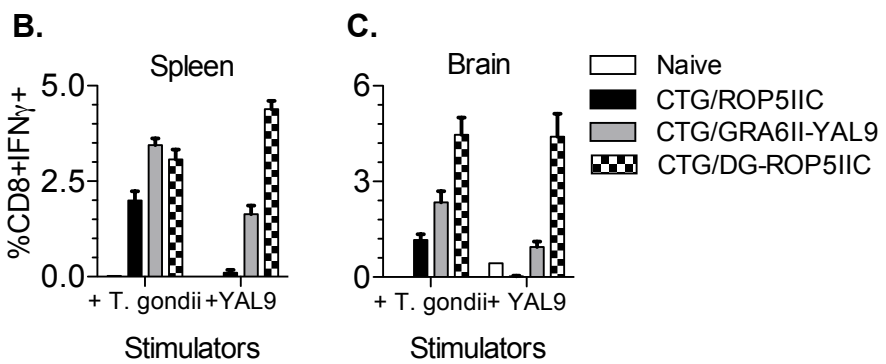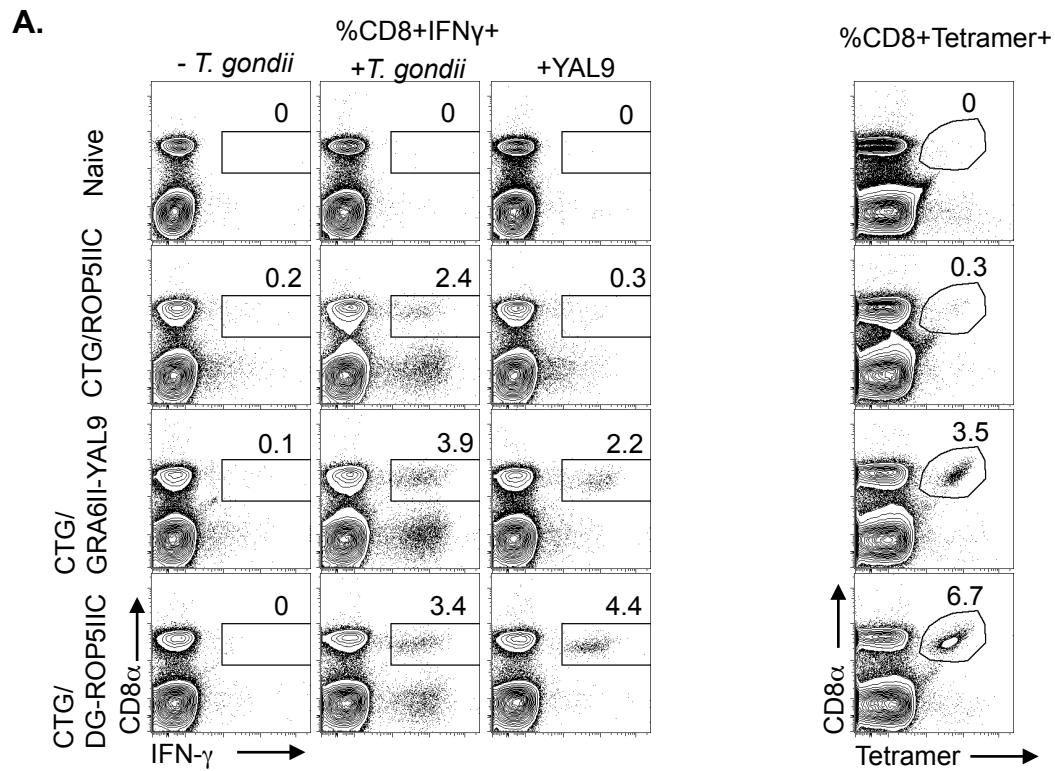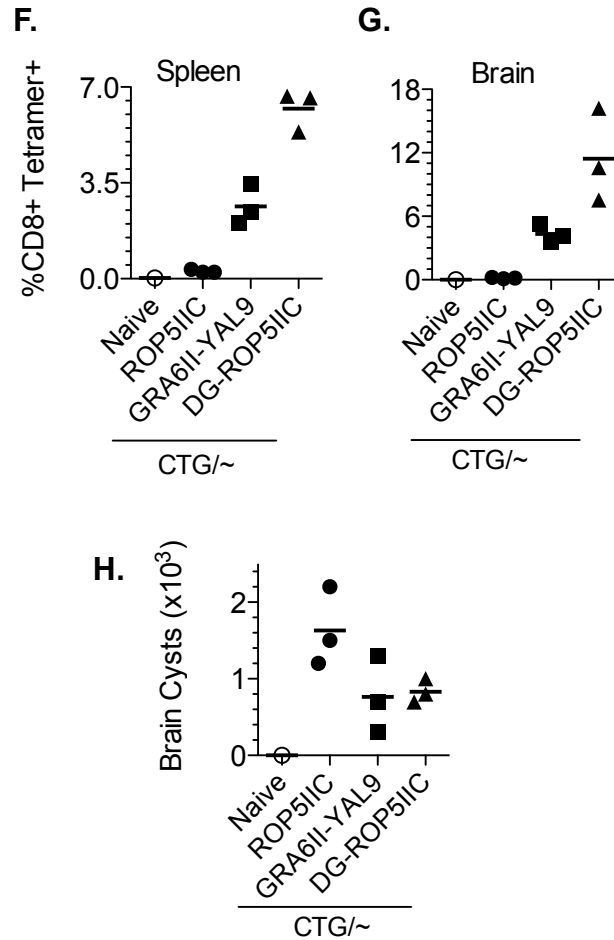
Figure 38: YAL9 specific response in enhanced in mice chronically infected with transgenic *T. gondii* that express ROP5IIC as a dense granular protein. C57BL/6 mice were infected with 5×10^3 transgenic *T.gondii* tachyzoites. Splenocytes or brain leukocytes were harvested from mice 3-4 wks post infection and *T. gondii* specific T cell responses were measured by intracellular cytokine staining for IFN-γ or by staining with tetramers. **A)** Representative flow cytometry plots from naïve and infected mice. Left panels show intracellular IFN-γ staining after *in vitro* restimulation with antigen presenting cells either, +/- *T. gondii,* or 1μM YAL9 peptide. Right panels show MHC class I H-2D^b –YAL9 tetramer staining. **B-E)** Compiled data showing **B)** splenic and **C)** brain *T. gondii* specific and YAL9 specific CD8 T cell responses; and **D)** splenic **E)** brain *T. gondii* specific CD4 T cell responses from infected mice. Data are corrected for background based on responses by T cells towards uninfected APCs or APCs pulsed with irrelevant peptide. Each dot represents an individual mouse. **F, G)** Compiled data showing MHC class I H-2D^b –YAL9 tetramer staining on **F)** splenocytes and **G)** brain leukocytes from infected mice. Cells were also co-stained with CD8 antibody. **H)** Number of cysts in the brain as measured by staining a portion of the brain with fluorescent lectin to detect the cysts. Data are representative of at least two independent experiments.

observed in comparison with CTG/ROP5IIC parasites. Overall, we saw a significant difference in survival between mice that were immunized with YAL9 and infected with CTG/ROP5IIC or with CTG/DG-ROP5IIC (p<0.01 Fig. 39B). Also, there was a significant difference in survival between mice that were immunized with WI9 or YAL9 and then infected with CTG/DG-ROP5IIC parasites (p<0.01 Fig. 39B). Only 3 out of the 10 mice immunized with WI9 and then infected with CTG/DG-ROP5IIC survived, while all 10 out of 10 animals immunized with YAL9 and then infected with CTG/DG-ROP5IIC survived. However, as expected, there was no difference in survival between mice that were immunized with WI9 or YAL9 and then infected with CTG/ROP5IIC parasites (Fig. 39B).

From the surviving mice, we measured the T cell responses in the spleen and brain by tetramer staining and *ex-vivo* restimulation followed by ICCS for IFN-γ. We also counted cyst numbers in the brains of the surviving mice. *Ex-vivo* restimulation results revealed:
1) There was an overall greater percentage of CD8 T cells that responded to *T. gondii* infected or YAL9 pulsed APCs in the brain vs. the spleen of mice immunized with YAL9 and WI9 and infected with either of the parasite strains (Fig. 39C-D, left panels).
2) The percentage of CD8 T cells that responded to *T. gondii* in the brain was greater in mice infected with CTG/DG-ROP5IIC in comparison to the percentage of CD8 T cell response observed with CTG/ROP5IIC, irrespective of the peptide immunization (Fig. 39C, left panel).
3) There was an enhanced YAL9 specific response in the brains of CTG/DG-ROP5IIC infected animals irrespective of the peptide immunization in comparison to mice infected with CTG/ROP5IIC, both immunizations (Fig. 39C, right panel).
4) There was an enhanced YAL9 specific response in the spleens of YAL9 immunized animals that were infected with either CTG/ROP5IIC and CTG/DG-ROP5IIC animals indicating that prior immunization with the peptide boosted the percentage of YAL9 specific T cells (Fig. 39D, right panels). Furthermore, this enhancement was the maximal in mice that were infected with CTG/DG-ROP5IIC (Fig. 39D, right panels).
5) Unlike the spleen, in the brain, YAL9 specific responses were not greater in YAL9 immunized animals possibly indicating that expansion and boost of T cells is happening in the periphery before they get to the brain.
6) Restimulation of T cells followed by ICCS for IFN-γ also revealed that CD4 T cells generated a comparable response to *T. gondii* infected APCs in the brains of all the 4 sets of mice, irrespective of immunization and infection (Fig. 39E).
7) In the spleen, however, there seems to be a decrease in the CD4 T cell response to *T. gondii* infected APCs in YAL9 or WI9 mice infected with CTG/DG-ROP5IIC (Fig. 39F). This could be due to a proportional increase in CD8 T cell response in mice infected with this strain.
8) Furthermore, this also shows since there is a comparable number of CD4 T cells in the brain but not the spleen, the expansion of T cells is happening in periphery before reaching the brain.

Staining with YAL9 loaded MHC Class I tetramers showed similar results:
1) There is a much greater percentage of YAL9 specific CD8 T cell population in the brains of YAL9 and WI9 immunized mice infected with CTG/DG-ROP5IIC as compared to immunized mice that were infected with CTG/ROP5IIC (Fig. 39G).
2) In the spleen, there is a greater percentage of YAL9 specific CD8 T cells in mice immunized with either YAL9 or WI9 and then infected with CTG/DG-ROP5IIC (Fig. 39H).
3) YAL9 immunized mice have a greater percentage of YAL9 specific T cells than WI9 immunized mice, irrespective of the infection, confirming that peptide immunization boosted this population (Fig. 39G-H). (A greater percentage of YAL9 specific T cells were also seen YAL9 vs. WI9 immunized mice that were infected with CTG/ROP5IIC, albeit, it was overall much lower than observed with CTG/DG-ROP5IIC parasites).

Lastly, we also counted the number of cysts in the brains of surviving mice. We observed a significant reduction in cysts (p<0.001) between mice that were immunized with YAL9 and then infected with CTG/DG-ROP5IIC vs. CTG/ROP5IIC strains (Fig. 39I). Interestingly, we also saw a significant difference (p<0.01) between WI9 immunized mice that were infected with CTG/DG-ROP5IIC vs. CTG/ROP5IIC (Fig. 39I). However, we did not see a difference between YAL9 or WI9 immunized mice that were infected with CTG/DG-ROP5IIC strain (Fig. 39I), keeping in mind that with this infection only 3 out of 10 WI9 immunized mice survived vs. all 10 out 10 YAL9 immunized mice survived the infection. Therefore, it seemed apparent that if CTG/DG-ROPIIC infected mice survived past the acute phase of infection, then regardless of immunization, enhanced T cells responses were sufficient to reduce the number of cysts in the brain. As a control, to ensure that the virulence of with CTG/ROP5IIC and CTG/DG-ROP5IIC strains was comparable, we performed plaque assay on fibroblasts, which revealed a comparable number of plaques between the two strains (Fig. 40A). Additionally, infection of TAP deficient animals, known to be having a greatly reduced number of CD8 T cells, also showed a comparable survival after infection with the two transgenic strains (Fig. 40B).

Based on these results, we can conclude that immunization with YAL9 prior to lethal infection with transgenic parasite strain CTG/DG-ROP5IIC protects the mice during the acute phase of infection. Once the mice make it to the chronic stages, they are protected irrespective of immunization possibly due to a robust expansion of YAL9 specific CD8 T cells.

A.

-35   -21   0   49

Immunization with LPS activated BMDCs + YAL9 or WI9

Booster Immunization

Transgenic *T. gondii* Infection

Assay Surviving Mice

B.

	Immunization	Infection
	--△-- WI9	CTG/ROP5IIC
	--▲-- YAL9	
	--○-- WI9	CTG/DG-ROP5IIC
	--●-- YAL9	

Percent Survival

Time post infection (days)

**

C.

Brain

%CD8+IFNγ+ cells

Immunization	Naive	YAL9	WI9	YAL9	WI9	Naive	YAL9	WI9	YAL9	WI9
Infection	-	CTG/ROP5IIc		CTG/DG-ROP5IIC		-	CTG/ROP5IIC		CTG/DG-ROP5IIC	
Stimulators	+ *T.gondii* (II)					+ YAL9				

D.

Spleen

Immunization	Naive	YAL9	WI9	YAL9	WI9	Naive	YAL9	WI9	YAL9	WI9
Infection	-	CTG/ROP5IIC		CTG/DG-ROP5IIC		-	CTG/ROP5IIC		CTG/DG-ROP5IIC	
Stimulators	+ *T.gondii* (II)					+ YAL9				

E.

Brain

Immunization	Naive	YAL9	WI9	YAL9	WI9
Infection	-	CTG/ROP5IIC		CTG/DG-ROP5IIC	
Stimulators	+ *T.gondii* (II)				

F.

Spleen

Immunization	Naive	YAL9	WI9	YAL9	WI9
Infection	-	CTG/ROP5IIC		CTG/DG-ROP5IIC	
Stimulators	+ *T.gondii* (II)				

Brain

G.

Immunization	Naive	YAL9	WI9	YAL9	WI9
Infection	-	CTG/ROP5IIC		CTG/DG-ROP5IIC	

Spleen

H.

Immunization	Naive	YAL9	WI9	YAL9	WI9
Infection	-	CTG/ROP5IIC		CTG/DG-ROP5IIC	

I.

Immunization	Naive	YAL9	WI9	YAL9	WI9
Infection	-	CTG/ROP5IIC		CTG/DG-ROP5IIC	

Figure 39: Immunization with YAL9 protects C57BL/6 mice from lethal challenge with transgenic *T. gondii* that express ROP5IIC as a dense granular protein during acute phase of infection.
A) C57BL/6 mice were immunized with LPS activated BMDCs pulsed with either YAL9 or control peptide, WI9, followed by a booster dose 2 weeks post primary immunization. 3 weeks post boost immunization, mice were infected with 5×10^5 live transgenic *T. gondii* tachyzoites (either CTG/ROP5IIC or CTG/DG-ROP5IIC), intraperitoneally. **B)** Kaplan-Meier survival curves between the four groups. **C-I)** Analysis of the T cell responses and cyst numbers from surviving mice. IFN-γ response by CD8 T cells in the **C)** brain **D)** spleen, and by CD4 T cells in **E)** brain **F)** spleen, as measured by ICCS for IFN-γ using flow cytometry after *ex-vivo* restimulation with *T. gondii*-infected APCs or peptide-pulsed APCs. Data are background corrected based on the values from uninfected APCs or APC pulsed with irrelevant peptide. **G, H)** MHC class I H-2D^b –YAL9 tetramer staining on **G)** brain leukocytes **H)** splenocytes. Cells were also co-stained with CD8 antibody. **I)** Number of cysts in the brain as measured by staining a portion of the brain with fluorescent lectin to detect the cysts. Data is pooled from two representative experiments with at least 5 mice per condition in each experiment. ** = p<0.01, *** = p<0.001.
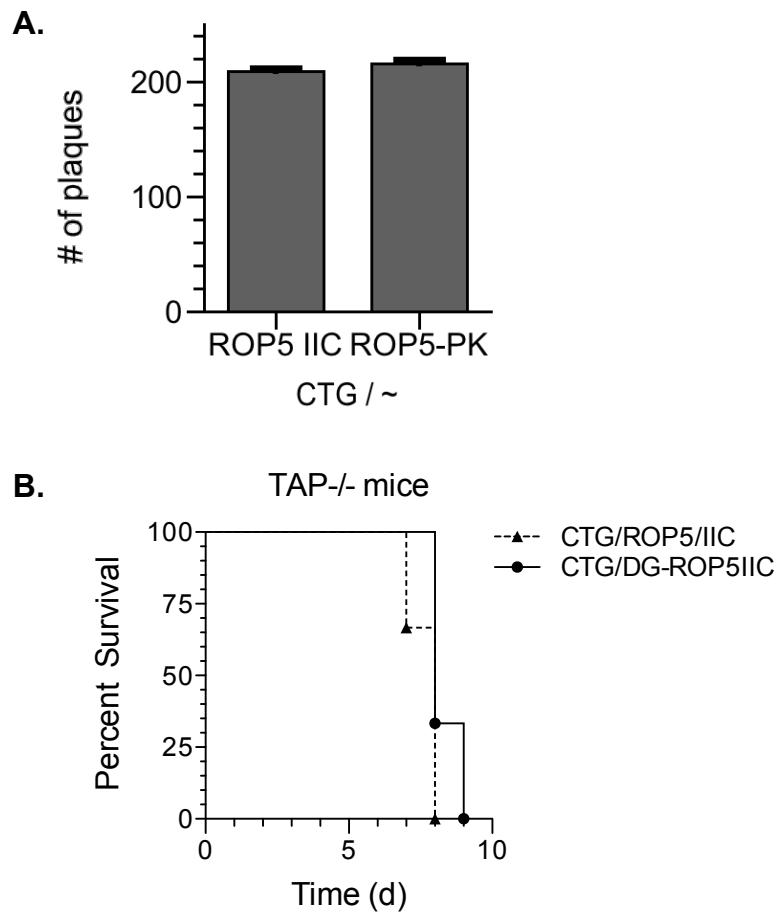
Figure 40: CTG/ROP5IIC and CTG/DG-ROP5IIC transgenic *T. gondii* parasites have comparable relative virulence.
A) *In-vitro* plaque assay on human foreskin fibroblasts with 1000 tachyzoites from CTG/ROP5IIC and CTG/DG-ROP5IIC transgenic *T. gondii*. **B)** Kaplan-Meier survival curves on TAP-/- mice that were infected intraperitoneally with 5×10^{5} tachyzoites from CTG/ROP5IIC and CTG/DG-ROP5IIC transgenic *T. gondii*.

Conclusions

Previous studies have shown that there is a difference in susceptibility to *T. gondii* infection in mice depending upon strain of mice. Specifically, immunity to *T. gondii* in mice has been shown to be linked to MHC class I molecules, wherein H-2^d mice are more resistant to infection while H-2^b mice are susceptible to infection. MHC Class I molecules are known to present antigenic peptides to CD8 T cells, which are known to be the primary mediators of resistance to *T. gondii*, along with the help of CD4 T cells (Gazzinelli et al., 1991; Suzuki and Remington, 1988). Earlier work from the Shastri lab had shown that, H-2^d mice are resistant to infection because they generate an immunodominant CD8 T cell response to HF10 from the *T. gondii* protein, GRA6, presented on H-2L^d MHC Class I molecule. However, B6 mice fail to generate a response to GRA6 and we asked the question what is the mechanism in the absence of a robust L^d restricted response that makes B6 mice susceptible?

In this chapter, we have shown that B6 mice generate a CD8 T cell response to the *T. gondii* protein ROP5 that contains the antigenic peptide YAL9 presented on the H-2D^b MHC Class I molecule (Fig. 20 and 23-24). Presentation of YAL9 was proteasome and TAP dependent and primarily ERAAP independent (Fig. 26). YAL9 specific response constitutes a minor but detectable fraction of the overall CD8 T cell response in immunized and infected animals (Fig. 27 and 28). The overall CD8 T cell response in immunized animals is much lower in comparison to infected animals. Additionally, kinetics of infection in B6 mice showed that YAL9 specific CD8 T cells expand during the acute phase of infection and crash by the chronic phases of infection (Fig. 29). In comparison to other known *T. gondii* CD8 T cell epitopes identified from both H-2^b and H-2^d strains of mice, HF10 was the immunodominant epitope and even in the presence of a robust CD8 T cell response to HF10, YAL9 specific T cell response remained to be a minor but measureable fraction in F1 (C57BL/6 x B10.D2) mice (Fig. 30-31). Unlike immunization with HF10 (Blanchard et al., 2008), immunization with YAL9 followed by a challenge with a lethal dose of infection failed to protect B6 mice (Fig. 32). Analyzing the precursor frequencies from the T cell point of view comparing the different *T. gondii* antigens can establish that whether a differential response with YAL9 specific T cells is due to a lack of the presence of T cells to begin with or, insufficient expansion due to reasons such as lack of available antigen for presentation.

To understand the differential T cell response towards the dense granule protein, GRA6 leading to protection vs. the rhoptry protein, ROP5, that fails to protect, we asked the question whether being the type of a secretory protein, i.e., rhoptry vs. dense granule, can make a difference in the antigen availability, accessibility and presentation to T cells. To address this, we generated transgenic parasites that allowed the expression and trafficking of ROP5IIC as a dense granular protein (Fig. 35), using the parental CTG (antigen null), type III *T. gondii* strain. In comparison to the parasites that allow the expression and trafficking of ROP5IIC

to rhoptries (CTG/ROP5IIC), dense granular ROP5IIC expressing parasites (CTG/DG-ROP5IIC) had an enhanced YAL9 immunogenicity both *in-vitro* and *in-vivo* (Fig. 36-38). Moreover, an intermediate level of enhancement was also seen when just the antigenic peptide, YAL9 was expressed at the C' terminus of GRA6II replacing HF10 (CTG/GRA6II-YAL9). It is not clear to us why enhancement with CTG/GRA6II-YAL9 parasites was not as high as that seen with CTG/DG-ROP5IIC parasites. One possibility is that YAL9 peptide needs it native location within ROP5 for efficient processing by the antigen presentation machinery. Also, it is known based on immunofluorescence and microscopy studies that most dense granule proteins are detected in close association with the membranous tubulo-vesicular network of the parasitophorous vacuole (Labruyere et al., 1999; Lecordier et al., 1995; Mercier et al., 2005). Therefore, it could be possible that the GRA6II-YAL9 protein is tethering itself within the tubulo-vesicular network, while *gra6* promoter driven ROP5IIC pseudokinase domain protein, is primarily in the lumen of PV and thereby more accessible. Perhaps generation of transgenic parasites that express ROP5IIC pseudokinase domain downstream of GRA6 (ex: CTG/GRA6II-ROP5IIC) can dissect this possibility.

Lastly, we showed that, YAL9 immunization of B6 mice was protective, during the acute phase of infection, against challenge to lethal dose of infection with transgenic parasites that expressed ROP5IIC as a dense granular protein (Fig. 39). Interestingly, in mice infected with CTG/DG-ROP5IIC strain of parasites, there was a proportionate decrease in CD4 T cells as there was an increase in YAL9 specific CD8 T cells, indicating that a robust CD8 T cell response to an antigenic *T. gondii* protein can change the balance of responses between CD4 and CD8 T cells. Additionally, this difference was only seen in the spleens and not the brains of infected animals, raising the possibility that expansion of *T. gondii* specific T cells is happening in the periphery and are then they recruited to the brains even though *T. gondii* is mostly present in the brain during chronic stages of infection. Overall, these findings implicate that expression and trafficking of an antigen as a dense granular protein in *T. gondii* can make it a more potent antigen by enhancing antigen-specific T cell responses that can lead to an efficient immune response against the pathogen.

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Chapter 4: Future Directions

It is known that CD4 and CD8 T cells play an important role in protection against *T. gondii* infection. In comparison to what is previously known about T cell responses in the H-2^d, the resistant strain of infection, we wanted to define the mechanisms of T cell responses in the C57BL/6 mice, the susceptible strain of infection with *T. gondii*. In chapter 2, we have shown you that *T. gondii* immunization of C57BL/6 mice elicits a potent CD4 T cell response and a weak CD8 T cell response. Upon generating a CD4 T cell hybridoma using CD4 T cell lines from *T. gondii* immunized animals, we were able to identify that the CD4 T cells in B6 mice recognize the antigenic peptide, AS15, from the *T. gondii* protein CD4Ag28m. AS15 specific response was readily detectable in mice immunized or infected with *T. gondii*. It is possible that presentation of AS15 is dependent upon phagocytosis as heat-killed parasites were better presented than irradiated parasites. Furthermore, the all the three North American and European strains of *T. gondii* (type I, II and III) were able to present this antigen to the hybridoma. Lastly, we have shown that immunization with AS15 can partially protect B6 mice against lethal dose of infection by lowering the parasite load and cysts numbers in brains of infected animals. Knowing this data allows us to ask the following questions:

What is the location of CD4Ag28m?

Currently, the only function known for CD4Ag28m is its ability to serve as a CD4 T cell antigen in C57BL/6 mice. According to the *T. gondii* database, it is known to contain a putative signal sequence indicating that it most a likely a secretory protein. It is generally known that proteins with signal sequences by default end up in the dense granules, and are secreted like the dense granule proteins (Mercier et al., 2005). Therefore, it is possible that CD4Ag28m is a dense granular protein. In order to formerly test that, we can generate antibodies against this protein and study its location within *T. gondii* via immunofluorescence studies. Alternately, we can generate transgenic parasites that perhaps either express or overexpress a FLAG or HA tagged version of this protein. Again using antibodies to the tags, we can find out the location using immunofluorescence studies. To better understand a functional role of this protein, we can generate knockout-transgenic parasites that do not express CD4Ag28m. By doing virulence studies in mice or *in-vitro* on HFF layers we can determine if this protein is necessary for virulence. If not virulence, by knocking out this protein we can study if it disrupts any function or architecture with the parasite or the parasitophorous vacuole.

How is CD4Ag28m presented to CD4 T cells?

Currently, CD4Ag28m contains a predicted signal sequence suggesting that is a secretory protein. Secretion of parasite antigens into the host cell is known to be important for presentation via the MHC class I pathway, which samples the host cytosol and stimulates CD8 T cell responses to intracellular pathogens. This is in line with evidence that secretion into the host cell promotes recognition by CD8 T cells (Kwok et al., 2003) and the fact that all of the *T. gondii* CD8 epitopes identified to date are derived from secreted parasite proteins (Blanchard et al., 2008; Frickel et al., 2008; Mendes et al., 2011; Wilson et al., 2010). On the other hand, the impact of secretion on MHC class II presentation of potential parasite antigens is less clear, since both secreted and non-secreted parasite antigens should have ready access to the class II MHC pathway via phagocytosis of intact parasites and debris. Indeed, we observe robust AS15 specific T cell response when APCs were provided heat-killed parasites (Fig. 15 and 16). However, while both secreted and non-secreted parasite proteins may be presented by bystander (non-invaded) APCs, secreted proteins may be preferentially presented by invaded APCs and this pathway may be particularly important *in vivo* where antigen concentration is often limiting.   Indeed, enhanced recognition by CD4 T cells of a secreted version of the model antigen OVA has been reported (Pepper et al., 2004), and CD4 responses to *Salmonella* are enhanced by secretion into host cells (Hess et al., 1996).

Furthermore, our data suggested that the hybridoma response towards non-invaded APCs was better than that observed towards infected APCs (Fig. 16). Perhaps this fits in with data from other groups that have shown that manipulation of MHC class II antigen presentation by *T. gondii* (Lang et al., 2007; Luder et al., 2003; Luder et al., 1998; McKee et al., 2004) may represent a way for the parasite to evade the most effective CD4 responses directed toward secreted antigens on invaded host cells. Therefore, it is possible that other mechanisms such as autophagy may be involved in presentation of secreted antigens or presentation from directly invaded cells. Autophagy has been shown to be involved in the clearance of parasites from infected macrophages in an IGTP dependent manner (Ling et al., 2006) or with induction by CD40 (Andrade et al., 2006). Using autophagy inhibitors such as wortmannin. 3-methyladenine, and other PI3K inhibitors we can test if the presentation of AS15 to the hybridoma is altered. Overall, we think that there is no one possible mechanism but a combination of many, depending on the type and route of infection, which allows for the presentation of AS15.

What is the mechanism of protection after AS15 immunization?

A CD4 T cell response directed toward a single parasite peptide, AS15, is sufficient to mediate immune partial protection as indicated by the enhanced survival and decreased parasite load in vaccinated mice. This raises the question

of how AS15-specific CD4 T cells contribute to the control of infection. One possibility is that these CD4 cells directly contribute to protection via expression of the protective cytokine IFN-γ (Suzuki et al., 1989; Suzuki et al., 1988).

Alternatively, AS15-specific CD4 cells may aid in promoting CD8 responses, as suggested by the impairment of intracerebral CD8 T cells in CD4 T cell depleted mice (Lutjen et al., 2006). An achievable way to test for this phenomenon would be look at the cytokine profile, using reagents such as cytokine bead array in mice that are immunized with AS15 and then infected with *T. gondii*.

A third possibility is that immunization with the peptide may alter the balance between different types of T helper cells, such as between Tregs and Th1 cells, as suggested by the plasticity of Tregs and Th1 function during lethal *T. gondii* infection (Oldenhove et al., 2009). Furthermore the plasticity of effector Th1 cells was shown by (Jankovic et al., 2007), where they reported that conventional IFN-γ producing Tbet+FoxP3- Th1 cells were the major source of IL-10 in mice infected with *T. gondii*. It is possible that the balance of IL-10 and IFN-γ production is altered upon immunization with AS15. Initially, we would need to see if there are Tregs that are also AS15 specific. This can be tested using FoxP3+GFP mice, or by staining for FoxP3 intracellularly after restimulation of splenocytes ex-vivo in presence of AS15 pulsed APCs. Intracellular staining of splenocytes from infected mice with other Th cytokines such as IL-10, IL-17 and IL-4 can help us gain insight into the possible mechanism of protection that involves the plasticity of Th cells during infection. Lastly, the ability to track AS15 specific T cells in vivo by generating TCR transgenic mice can also allow us to dissect the mechanism by which vaccination generates protective T cell responses, and how these responses provide protection.

What are other CD4 T cell antigens?

Identification of other CD4 *T. gondii* antigens will allow us to determine the hierarchy of CD4 T cell responses towards *T. gondii* antigens (Fig. 19). It is possible that all *T. gondii* specific CD4 antigens are a small fraction constituting the overall CD4 response. Even though profilin was published to be an immunodominant CD4 T cell response towards *T. gondii* (Plattner et al., 2008; Yarovinsky et al., 2006), knowing the final antigenic peptide from profilin will allow us to make a direct comparison between the antigenic peptides from the known CD4 antigens. Additionally, we were able to generate panel of *T. gondii* specific CD4 T cell hybridomas (Fig. 4 and 19). Screening using an expression-cloning library, as we did for the identification of CD4Ag28m will allow us to identify the other antigens. Prior to screening, it would be appropriate to test the hybridomas for their specificity against profilin or CD4Ag28m. Knowing different antigenic specificities will further provide us insight into immune responses to *T. gondii*, the biology of *T. gondii*, and a design of possibly new or improved vaccine candidates.

In chapter 3, we have shown that upon an expression-cloning screen using a *T. gondii* specific CD8 T cell hybridoma, generated from T cell lines obtained from immunized animals, we found that CD8 T cells in B6 mice recognize the antigen, ROP5 that contains the antigenic peptide YAL9 presented on H2-D^b MHC Class I molecule. Presentation of YAL9 was shown to be proteasome and TAP dependent, while ERAAP independent. Expansion of YAL9 specific cells happens during the acute phase of infection and during the chronic phase of infection, YAL9 specific CD8 T cells represent minor but detectable fraction of the overall *T. gondii* specific CD8 T cell response. As a result, immunization with YAL9 does not protect B6 mice against challenge with a lethal dose of infection. However, altering the expression and trafficking of ROP5 as a rhoptry protein to dense granular protein enhances the immunogenicity of YAL9, due to robust T cell expansion. Furthermore, immunization with YAL9 is now able to protect B6 mice against a challenge with lethal dose of transgenic *T. gondii* that express and traffic ROP5 as a dense granular protein. Enhanced immunogenicity by altering the expression and trafficking of *T. gondii* proteins now drives us ask the following questions:

Can mice immunized with CTG/DG-ROP5IIC transgenic parasites be protected by lethal infection?

Various studies have shown that immunization of mice with heat-killed or irradiated parasites can protect susceptible C57BL/6 mice from lethal challenge. However, it would be interesting to show if protection in B6 mice can be generated with an enhancement of responses against just particular antigen specificity. To test for this we can immunize B6 mice with irradiated CTG/DG-ROP5IIC or the CTG/ROP5IIC strain of parasites and then challenge them with a lethal dose of type II strain of *T. gondii*. If protection is achieved then it can further shed light into the physiological relevance of what impacts an antigen from a particular expression and trafficking location within *T. gondii* can have on immune responses.

Are levels of YAL9 higher in CTG/DG-ROP5IIC transgenic parasites expressing ROP5 as a dense granule protein?

Perhaps, from the antigen perspective, it is possible that the enhancement of T cell responses when mice are infected with CTG/DG-ROP5IIC are due to the presence of higher levels or amount of antigenic peptide present in APCs. A possible way to test for this is by generating extracts from cells infected with CTG/ROP5IIC or CTG/DG-ROP5IIC parasites. These extracts can be fractionated using high performance liquid chromatography (HPLC), in comparison to cellular extracts spiked with synthetic peptide. Additionally, extracts from infected cells can be titrated onto to APCs to be presented to the CD8 T cell hybridoma. If indeed there is a difference between the levels of

antigenic peptide present, we should be able to see that in a differential hybridoma response.

Is the YAL9 antigenic peptide presented in the same manner, i.e., dependent on TAP in CTG/DG-ROP5IIC transgenic parasites?

In addition to a possibility of differential levels of the antigenic peptide among the transgenic parasite strains, it is also probable that the antigen is better accessible to the antigen presentation pathway. We have shown that the presentation of YAL9 from ROP5 to BTg45 hybridoma was proteasome dependent, TAP dependent but mostly ERAAP independent. Perhaps, having ROP5IIC, now secreted, expressed and trafficked as a dense granular protein present in the PV lumen, as in CTG/DG-ROP5IIC parasites, makes the antigen more easily degradable to be presented on MHC Class I molecules. In addition to presented in the canonical manner, the antigenic peptide may now be directly accessible from the PV to the ER bypassing use of TAP, especially since studies have shown the close interaction between the PV membrane and the host ER (Goldszmid et al., 2009). To test this hypothesis, we can test the presentation of YAL9 from CTG/DG-ROP5IIC or CTG/ROP5IIC infected ERAAP or TAP deficient APCs to the YAL9 specific CD8 T cell hybridoma. In the same manner TAP independent presentation may happen in a proteasome independent manner, wherein ROP5 degradation can be happening within the PV or within host ER. Presentation of YAL9 from the transgenic strains to the hybridoma can also be tested in the presence of proteasome inhibitors. Answers to these questions will provide us with clarity from the antigen presentation perspective as to why the T cell responses are enhanced with CTG/DG-ROP5IIC parasites.

Development of TCR transgenic mice.

To understand the dynamics of ROP5 specific T cell responses, it is equally important to approach this from the T cell point of view, and one way to address that is by generating TCR transgenic mice. As shown previously with OVA model (Chtanova et al., 2009), now with endogenous antigens, *in-vivo* imaging studies and transfer studies of antigen specific T cells from these mice can be used to study interaction between APCs and T cells *in-vivo* in different organs throughout the course of infection.

***Toxoplasma gondii* as a vector for vaccine?**

Among parasites, *T. gondii* is known to be relatively easy to manipulate, transfect and generate transgenic strains. It would be of great interest to test the possibility, whether avirulent and irradiated transgenic *T. gondii* strains can serve as an effective vector for vaccines to boost antigen specific T cell responses, if

antigens from other pathogens, especially other apicomplexans such as *Plasmodium spp*., are expressed and trafficked as dense granular proteins.

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Chapter 5: Materials and Methods

Mice and parasites

C57BL/6J, CBA, the MHC class II - deficient B6.129S-H2 $^{dlAb1-Ea}$ and the TAP deficient B6.129S2-*Tap1^{tm1Arp}*/J mice were obtained from Jackson Laboratory. MHC class II - deficient B6.129S-H2 $^{dlAb1-Ea}$ and the TAP deficient B6.129S2-*Tap1^{tm1Arp}*/J mice were then bred at UC Berkeley animal facility. For all immunization and infection experiments sex and age matched mice were used. Mice were used with the approval of the Animal Care and Use Committee of the University of California.

The parental Prugniaud or Me49 strain of *T. gondii* (PruΔhpt;hypoxanthine-xanthine-guaninephosphoribosyltransferase deficient) was a gift from J. Boothroyd (Stanford University). Transgenic parasites, CTG/ROP5IIC, CTG/g6-ROP5IIC-PK and CTG/GRA6II-YAL9 were generated by Michael Reese in the Boothroyd lab at Stanford university (as described below). Tachyzoites were maintained by passage in confluent monolayers of human foreskin fibroblasts (HFF) grown in DMEM (Invitrogen) containing 10%FCS (Hyclone), and 1%Pen-strep glutamine (Invitrogen). Tachyzoites were passaged by scraping the infected HFF layer with a cell scraper and homogenizing through a 21 gauge needle and then transferring a small aliquot ranging between (200μl-1mL) into a new confluent flask of HFFs.

In-vivo infection and immunization

Mice were immunized intraperitoneally with 1-5x10^6 tachyzoites that were irradiated (14,000 rads) and resuspended in 100μl PBS. For inducing protection, B6 BMDCs were activated with LPS for 24h (100ng/mL, Sigma), incubated for 90 min with 10μM of synthetic peptide, MHC Class II peptide or 1μM of MHC class I binding synthetic peptide, washed twice with PBS and used for footpad immunization. Mice were immunized with 5x10^6 peptide-loaded BMDCs for 7 days and then infected with a lethal dose of live tachyzoites (1x10^4) intraperitoneally. For generating chronic infections, mice were orally fed 25-50 cysts of the Prugniuad-tomato-OVA strain. Cysts obtained from CBA mice infected for 3-5 weeks intraperitoneally with live 400 Prugniuad-tomato-OVA tachyzoites. Mice were also chronically infected by intraperitoneal injection of (5x10^3 - 5x10^5) live tachyzoites from parental of different transgenic strains.

Ex-vivo analysis

Mice were euthanized 3-7 wks post infection or 1-2 wks post immunization. Spleens and/or brains were collected and immediately processed or stored at -

80°C for DNA extraction and further analysis. Spleens were dissociated into single-cell suspensions in complete RPMI media (Invitrogen) supplemented with 10%FCS (Hyclone). Erythrocytes were removed from the suspension using Ammonium chloride potassium chloride lysis buffer (10μM EDTA, 160mM NH$_4$Cl and 10mM NaHCO$_3$). Brains were homogenized and digested for 1h at 37°C with collagenase type IA (1mg/mL; Sigma) and DNaseI (100μg/mL; Roche) in serum free RPMI media. Brains were further dissociated and filtered through 70μcons cell strainers and centrifuged 20 min at 1000g. Cells were resuspended in 60% (vol/vol) Percoll (GE Healthcare), were over layered on 30% (vol/vol) Percoll followed by a centrifugation at 1000g for 2 min. Infiltrating mononuclear cells were collected from the gradient interface and red blood cells were removed via lysis with ammonium chloride potassium chloride lysis buffer. Cells were washed twice in complete RPMI medium before analysis. The proportion of *T. gondii* specific or antigen specific cells was monitored by intracellular cytokine staining (ICCS) for IFN-γ on CD4$^+$ or CD8α^+ cells. APCs were either infected the day before (as described below) or pulsed with antigenic peptide on the same day and were used for the ex-vivo IFN-γ assay. Antigen specific CD4$^+$ or CD8α^+cells were also detected via staining using MHC Class I and class II peptide loaded tetramers loaded with *T. gondii* antigenic peptide (as described below).

Parasite load analysis

Genomic DNA was extracted from brain and spleen using Wizard genomic DNA purification kit (Promega). Parasite burden in the spleen and brain was assessed by semiquantative PCR as described (Kirisits et al., 2000). The number of cysts in the brain was determined by labeling a portion of the brain with fluorescein-conjugated *Dolichos biflorus* agglutinin (FL-1031, Vector Laboratories). This lectin stains the cyst wall and the cysts were counted using an inverted fluorescence microscope.

Generation of *Toxoplasma gondii*-specific T cell hybridomas

C57BL/6 mice were immunized with 1x10^6 irradiated Pru tachyzoites (14,000 rads) intraperitoneally. Mice were euthanized 7 days post infection and spleens were harvested. Spleens were dissociated into single-cell suspensions in complete RMPI as described above. *T. gondii* specific populations were expanded in-vitro by weekly restimulations with irradiated syngeneic splenocytes or irradiated MHC class II-deficient, B6.129S-H2$^{dlAb1-Ea}$ bone-marrow dendritic cells (BMDCs) (2000 rads) that were infected with irradiated Pru tachyzoites the day before. The proportion of *T. gondii* specific T cells was measured weekly by intracellular cytokine staining (ICCS) for IFN-γ. APCs were infected the day before (as described below).

For the generation of CD4 T cell hybridoma, after two weeks of *in-vitro* restimulation, responding T cells were fused to TCR$\alpha\beta$-negative lacZ inducible BWZ.36.CD8α fusion partner as described before (Malarkannan et al., 2001). The antigen-specificity and MHC-restriction of the hybridomas were assessed by overnight incubation with infected or uninfected splenocytes from wildtype C57BL/6 mice or MHC class II-deficient, B6.129S-H2 $^{dlAb1-Ea}$ mice. For the generation of CD8 T cell hybridoma, after 8 weeks of *in-vitro* restimulation, responding T cells were fused to TCR TCR$\alpha\beta$-negative lacZ inducible BWZ.36.CD8α fusion partner as described before (Malarkannan et al., 2001). The antigen-specificity and MHC-restriction of the hybridomas were assessed by overnight incubation with infected or uninfected BMDCs from wildtype C57BL/6 mice containing MHC class I blocking antibodies. Anti-H-2K^b antibody clone used was 5F1.5 and anti-H-2D^b antibody clone used was B22.249. Prior to infection, the BMDCs were incubated with the blocking antibodies for 30 min at 4°C. The CD4 or CD8 hybridoma response was quantitated by TCR-mediated induction of β-galactosidase upon the addition of chromogenic substrate CPRG (chrolophenol red-β-D-galactopyranoside; Roche). The absorbance of the cleaved purple product was measured at 595nm with a reference at 695nm. The hybridomas were further subcloned to obtain monoclonal population of T cell hybridomas with a single TCR specificity.

Construction of cDNA library and recombinant constructs

Poly(A)+ messenger RNA was isolated from about 10 x 10^8 Pru tachyzoites (that had been passed through a 3μcons filter) using the Oligotex Direct mRNA Midi/Maxi kit (Qiagen). This mRNA was used to generate a cDNA library using Superscript cDNA synthesis kit and oligo(Deckert-Schluter et al.) primers (Invitrogen).

For identification of CD4 T cell antigen:
The cDNA fragments were inserted unidirectionally into the prokaryotic expression vector pTRCHis between SalI and NotI and used to transform TOP10 electrocompetent bacteria (Invitrogen) to yield ~10^9 cfu with the range of the insert size between 0.5-3 kbp. Various C'terminal and N'terminal deletion constructs of the antigenic cDNA (pTg778.76) were generated via PCR using a high fidelity *Pfu* Turbo polymerase system (Stratagene). The 5' forward and 3' reverse PCR primers that were used are listed in Table 3. All forward primers included the SalI site and the reverse primers included NotI restriction sites. The PCR products were purified by agarose gel, digested with restriction enzymes SalI and NotI, subcloned into pTRCHis vector and sequenced directly with gene specific oligos.

Table 3: Oligonucleotide primers for CD4Ag28m deletion constructs

Construct	Forward (5'-3')	Reverse (5'-3')
ΔN–B	ACGGTAGTCGACGACGTTATGGTTGAGG CTTGGATG	AATCTGTATCAGGCTGAAAATC
ΔN–D	ACGGTAGTCGACGACGATGGTAGCTTTG CCGTCATC	AATCTGTATCAGGCTGAAAATC
ΔC–E	GAGGTATATATTAATGTATCG	CTTTTCCTTTTGCGGCCGCGAGCTGTTCCTTC AGGAC
ΔC–G	GAGGTATATATTAATGTATCG	CTTTTCCTTTTGCGGCCGCCTCCGTCTTCATG ATTTG
ΔNΔC–J	ACGGTAGTCGACGACGATGGTAGCTTTG CCGTCATC	CTTTTCCTTTTGCGGCCGCGGCACGGTGTTCC CCCGC
ΔNΔC–K	TCGACGACCGAATGGCAGTCGAAATCCA TCGTCCCGTCCCTGGGACAGCTCCTCCC TCGTTCTCCAGTGAGGATGTTGC	GGCCGCAACATCCTCACTGGAGAACGAGGGAG GAGCTGTCCCAGGGACGGGACGATGGATTTCG ACTGCCATTCGGTCG

For identification of the CD8 T cell antigen:
The cDNA fragments were inserted unidirectionally into the mammalian expression vector pcDNA1 between BstXI and NotI and used to transform MC1061/P3 electrocompetent bacteria (Invitrogen) to yield ~10^9 cfu with the range of the insert size between 0.5-3 kbp. Various C'terminal and N'terminal deletion constructs of the antigenic cDNA (pTg8E6.1) were generated via PCR using a high fidelity Pfu Turbo polymerase system (Stratagene). The 5' forward and 3' reverse PCR primers that were used are listed in Table 4. All forward primers included the BamHI site and the reverse primers included XbaI restriction sites. The PCR products were purified by agarose gel, digested with restriction enzymes BamHI and XbaI, subcloned into pcDNA1 vector and sequenced directly with gene specific oligos.

Table 4: Oligonucleotide primers for ROP5 deletion constructs

Construct	Forward (5'-3')	Reverse (5'-3')
ΔNΔC–3	GATCCATGTTCACACCTGAAAACCTTTT CATTATGTGAT	CTAGATCACATAATGAAAAGGTTTTCAGGTGT GAACATG
ΔNΔC–5	GATCCATGAATGCGTGGCAACTGGGTCT TAGCATATGAT	CTAGATCATATGCTAAGACCCAGTTGCCACGC ATTCATG
ΔN–7	CGCGGATCCGCGATGGTCGGAACCCGAG GACCG	GCTCTAGAGCAGCGACTGAGGGCGCAGC
ΔNΔC–8	CGCGGATCCGCGATGGAGGAGGCACGG	GCTCTAGAGCGCTTGATGCCGGTCCTCG
ΔNΔC–9	CGCGGATCCGCGATGCCAGGACAGAGC	GCTCTAGAGCGCTTGATGCCGGTCCTCG

Expression cloning

For identification of the CD4 T cell antigen:
Bacterial transformants were grown and induced to express the cDNA encoded proteins according to manufacturer's protocol (pTRCHis expression systems,

Invitrogen). Briefly, transformed recombinant bacteria (10 cfus / well) were plated in 96-well U-bottom plates in a total volume of 180μl and allowed to grow overnight at 37°C. The next day, the transformant density was determined by OD_{600} (OD_{600} 1.0 = 5x10^8 cells/mL) and approximately 2-3 x10^7 transformants were plated into new 96-well U-bottom plates in a total volume of 180μl. The bacteria were grown for 2h at 37°C to attain log growth after which they were induced with the addition of 1mM IPTG and allowed further growth for 4h at 37°C. The number of transformants was again estimated by OD_{600} and 1-2 x 10^6 cells/well were transferred into new 96 well plates. The original master plates of bacteria were stored at 4°C. 5x10^4 BMDCs from C57BL/6 mice resuspended in serum and antibiotic free media were added to the plates containing the bacteria and incubated at 37°C for 1h to allow phagocytosis. The plates were centrifuged for 2min at 800g and the supernatant was removed. 1x10^5/well BTg01Z hybridomas were resuspended in complete medium containing 50μg/mL gentamycin (to eliminate residual bacteria) and added to the plates and incubated overnight. T cell activation was measured as the lacZ response described above.

For identification of the CD8 T cell antigen:
Bacterial transformants were grown to express the cDNA encoded proteins in pcDNA (Invitrogen). Briefly, transformed recombinant bacteria (9 cfus / well) were plated in 96-well U-bottom plates in a total volume of 180μl and allowed to grow overnight at 37°C for 48h. DNA was extracted from the bacteria using the alkaline lysis 96-well plasmid prep protocol (Malarkannan et al., 2001). The plasmid DNA was then transfected along with 20ng/mL of H-2D^b and 10ng/mL of B7-2 plasmid DNA into antigen presenting cells. The DNAs were transfected using 10mg/mL DEAE and 10mM chloroquine. The original master plates of plasmid DNA were stored at -20°C. 48h post-transfection, 1x10^5/well BTg45Z hybridoma was resuspended in complete medium were added to the plates and incubated overnight. T cell activation was measured as the lacZ response described above.

BMDC *in-vitro* differentiation and infection

For most experiments, we used BMDCs as the antigen presenting cells (APCs). Bone marrow cells were obtained from mouse femurs and tibias. Bone marrow cells were plated in complete medium containing granulocyte-macrophage colony-stimulating factor (GM-CSF) (10ng/mL; Peprotech) for 6 days to allow for differentiation into DCs. After 6 days, BMDCs were harvested and washed once to remove any GM-CSF. The cells were then infected overnight with irradiated tachyzoites (14,000 rads) or heat-killed parasites (tachyzoites treated for 15 min at 65°C) at a various multiplicities of infection. The next day, cells were washed twice to remove any residual extracellular parasites and were used in the assays described above. For the experiment, assessing the presentation by infected were non-infected but co-cultured BMDCs, cells were infected with Pru-tomato

(derivative of red fluorescence protein) irradiated tachyzoites and then fluorescence-activated cell sorted for RFP+ and RFP- populations.

Proteasome inhibition

Proteasome inhibitor lactacystin was titrated ($10\mu M$ to $0\mu M$) onto 96 well plates containing 5×10^4 BMDCs / well in complete RPMI media. Cells were incubated with the inhibitor for 2hrs at 37^oC. After two hours, irradiated Pru or Me49 tachyzoites (14,000 rads) were added to the treated plate at different multiplicities of infection. 8hrs post-infection, media was removed from treated and infected BMDCs and replaced with fresh complete RPMI media and 1×10^5 T cell hybridoma were added per well. T cell activation was measured as the lacZ response described above.

HPLC fractionation assay

COS-7 cells were transfected with pTg8E6.1 or vector alone (pcDNA) using Fugene tranfection reagent (Promega). 48hrs post-transfection, cellular extracts were generated by resuspending the cell pellets in $500\mu l$ of 10% acetic acid and then boiled for 10 min. The extracts were then spun for 15 min at 4^oC and the supernatant was transferred to 10kDa (Miliopore) cutoff filters and centrifuged for 60 min at 10,000 rpm at 4^oC. Extracts were also generated from untransfected COS-7 cells and 10pmoles of the antigenic peptide, YAL9, was added to the extracts before loading for HPLC fractionation. Extracts were injected onto the C18 column and separated by reverse phase HPLC, with 0.1% TFA in water as the polar buffer and 0.1% TFA in acetonitrile as the non-polar buffer. 5 drop fractions were collected in 96 well plates, dried and analyzed by the addition of 5×10^4 antigen-presenting cells / well and 1×10^5 T cell hybridomas / well. T cell activation to different fractions was measured as the lacZ response described above.

Generation of transgenic parasites

As collaboration, transgenic parasites were generated by the Boothroyd Laboratory, Stanford University.
All of the ROP5IIC transgenic parasites were generated using the parental type III strain deficient in hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPTR) gene (Donald et al., 1996), CTGΔhxgprt. Additionally, this parental strain used was also engineered to express luciferase and green fluorescent protein (GFP) (Boothroyd lab). DNA sequences encoding ROP5 Type IIC, including its native promoter were amplified using Phusion polymerase (NEB) and cloned into plasmid vector pTKO, where they flanked the HXPGRT cassette. This construct was in frame with the FLAG tag. Similarly, DNA sequences

encoding GRA6 type II, including its native promoter were amplified and then mutated so that the last 10 amino acids (HF10) of GRA6 were replaced by the amino acids FAQLSPGQSDYAVANYFFL, where the underlined residues represent the epitope YAL9 from ROP5 type IIC. This construct was in frame with a N'terminal HA tag. Lastly, DNA sequences encoding the pseudo-kinase domain of ROP5 type IIC (Reese and Boothroyd, 2009) were amplified and cloned downstream of native GRA6 type II promoter and signal sequence. This construct was in frame with the HA tag. The transgenic strains were made by electroporation of 15µg of linearized plasmids, described above followed by a selection of HXGPRT+ parasites as described (Donald et al., 1996). Briefly, positive selection for HXGPRT+ parasites were carried out in selection media containing DMEM supplemented with10%FCS (Hyclone), 1%Pen-strep glutamine (Invitrogen), 25µg/mL mycophenolic acid (MPA; Sigma) supplemented with 50µg/mL Xanthine (Sigma). Clonal parasites were grown from populations by limiting dilutions.

For immunofluorescence analysis, cells were fixed, permeabilized, stained with rat anti-HA (3F10, Roche) or mouse anti-FLAG antibodies followed by anti-rat Alexa488 or anti-mouse Alexa594 (Molecular Probes) conjugated-secondary antibodies to be visualized as described previously (Reese and Boothroyd, 2009; Reese et al., 2011).

Plaque Assay

$1x10^3$ tachyzoites from transgenic strains were added to a confluent layer of HFFs in a T-25 flask and incubated at 37°C, without disturbing for 9 days. After 9 days, flasks were washed twice with 1x PBS, fixed with 8% PFA for 2 hours at room temperature and then washed twice again with 1x PBS. This was followed by Giemsa (Sigma) stain for 10 min at room temperature and then washed twice with distilled water. The flasks were then allowed to dry and plaques were counted.

Flow cytometry

Antibodies to mouse CD4 (RM4-5), anti-mouse CD8α (53-6.7) and anti-mouse IFN-γ (XMG1.2) were obtained from BD biosciences. Surface staining with anti-mouse CD4 and CD8α antibodies were performed at 4°C for 30 min in flow buffer containing (3% vol/vol FCS, 1mM EDTA in PBS). Intracellular cytokine staining for IFN-γ was performed using the Cytofix/Cytoperm kit (BD Pharmingen). Fluorescently labeled MHC Class I and II tetramers bound to *T. gondii* antigenic peptide were obtained from the NIH tetramer facility. The concentration and time of staining of the tetramer were optimized and cells were incubated with the tetramer at room temperature for 1hr. The cells were then washed and followed by surface staining at 4°C for 30 min with anti-mouse CD4 or CD8α, and B220

(CD45R) antibody. All flow cytometry data was acquired on an XL Analyzer or FC 500 (Coulter) or BD LSRII and analyzed using FlowJo software (Tree Star).

Statistical analysis

Prism software (GraphPad) was used for statistical analysis. P values were calculated using two-tailed Student's (non-parametric) t-test.

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