UNIVERSITY OF CALIFORNIA, MERCED

Host and Parasite Requirements for CD8 T cell IFNγ Responses to *Toxoplasma gondii* Infections

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Quantitative and Systems Biology

by

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List of Abbreviations

41BBL	41BB ligand
ACK	Ammonium chloride-potassium
ANOVA	Analysis of variance
ASC	Apoptosis-associated speck like protein
ASP5	*Toxoplasma gondii* aspartyl protease 5
ATF6β	Activated transcription factor 6 beta
ATP	Adenosine triphosphate
B6	C57BL/6
BMDM	Bone marrow-derived macrophages
CASP	Caspase
CD	Cluster of differentiation used in CD8, CD40, etc.
CRISPR	Clustered regularly interspaced short palindromic repeats
DAMP	Damage-associated molecular pattern
DHFR	Dihydrofolate reductase
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERGIC	ER-derived Golgi Intermediate Compartments
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
GAS	Gamma-activated sequence
GBP	Guanylate-Binding Proteins
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GOI	Gene of interest
GRA	*Toxoplasma gondii* dense granule used in GRA15, GRA43, etc.
GREAT	Interferon-gamma reporter with endogenous polyA transcript
GSDMD	Gasdermin D
GTP	Guanosine triphosphate
HA	Hemagglutinin
HCE1	Host cyclin E, also “TEEGR”
HFF	Human foreskin fibroblasts
HG	Haplogroup
HRP	Horse radish peroxidase
HXGPRT	Hypoxanthine-xanthine-guanine phosphoribosyltransferase
ICOSL	Inducible co-stimulator ligand
IFNGR	Interferon gamma receptor
IFNγ	Interferon gamma
IL	Interleukin

	used in IL-1β, IL-12p40, IL-18, etc.
IRE1	Inositol-requiring enzyme 1
IRES	Internal ribosome entry site
IRF4	Interferon regulatory factor 4
IRG	Immunity-Related GTPases
	used in Irga6, Irgb6, IRGM
IVN	Intravacuolar network
Jak	Janus kinase
KLRG1	Killer cell lectin-like receptor subfamily G member 1
LCAT	Lecithin–cholesterol acyltransferase
LOD	Logarithm of odds
LOPIT	Location of organelle proteins by isotype tagging
LUC	Firefly luciferase
MHC	Major histocompatibility complex
ml	Milliliter
MOI	Multiplicity of infection
MPA	Mycophenolic acid
mtROS	Mitochondrial reactive oxygen species
MYR1	Myc regulation 1
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptor
NLRP	Nucleotide binding and leucine-rich repeat-containing protein
	used in NLRP1, NLRP3
NOD	Nucleotide-binding oligomerization domain
	used in NOD2
NTPase	Nucleoside triphosphate hydrolase
OT1	OVA antigen-specific CD8 T cells
OVA	Ovalbumin
OX40L	OX40 ligand
P2X7R	Purigenic P2X7 receptor
p62	ubiquitin-binding protein “p62”, also SQSTM1
PBS	Phosphate buffered saline
PD-L1	Programmed death-ligand 1
PEXEL	*Plasmodium* export element
pg	Picogram
PI	Propidium iodine
pMHC	Peptide-loaded major histocompatibility complex
PRR	Pattern recognition receptors
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuole membrane
QTL	Quantitative trait loci

RFX	Regulatory factor X
RIPK2	Receptor-interacting serine/threonine-protein kinase 2
ROP	*Toxoplasma gondii* rhoptry proteins used in ROP5, ROP16, etc.
ROS	Reactive oxygen species
SD	Standard deviation
SNARE	Soluble N-ethylmaleimide–sensitive fusion protein attachment protein receptor
SPF	Specific pathogen free
SQSTM1	Sequestosome 1, also “p62”
STAT	Signal transducer and activation of transcription used in STAT1, STAT6, etc.
T57	*T. gondii* antigen “TGD057”
TCR	T cell receptor
TEEGR	*Toxoplasma* E2F4-associated EZH2-inducing gene regulator, also “HCE1”
TEXEL	*Toxoplasma* export element
TgIST	*T. gondii* inhibitor of STAT1 transcriptional activity, also “IST”
T-GREAT	IFNγ reporter mouse line, from T57 mice and GREAT mice cross
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF receptor-associated factor 2 used in TRAF2, TRAF6
UC	University of California
UPR	Unfolded protein response
WNG2	With-No-Gly-loop 2, *T. gondii* secreted kinase, also “ROP34”
WT	wildtype
YFP	Yellow fluorescent protein

List of Symbols

α	alpha; antibody against
β	beta
°	degree
γ	gamma
µ	micro
n	nano
+	positive expression
-	negative expression
-/-	gene deletion

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Chapter 2: Naïve CD8 T cell IFNγ responses to a vacuolar antigen are regulated by an inflammasome-independent NLRP3 pathway and Toxoplasma gondii ROP5

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an inflammasome-independent NLRP3 pathway and *Toxoplasma gondii* ROP5. The 59th Midwinter Conference of Immunologists at Asilomar, January 2020, Pacific Grove, CA.

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Abstract

Host and Parasite Requirements for CD8 T cell IFNγ Responses
to *Toxoplasma gondii* Infections

by

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Host resistance to *Toxoplasma gondii* infections relies on CD8 T cell IFNγ responses. Since the manipulation of CD8 T cells may influence *T. gondii*'s ability to achieve chronic infection, we investigated host and parasite requirements for eliciting this response. Naïve CD8 T cell IFNγ responses to the endogenous *T. gondii* vacuolar resident antigen, TGD057 (T57), were analyzed. T57-specific CD8 T cells, isolated from naïve transnuclear mice, responded to *T. gondii*-infected bone marrow-derived macrophages in an antigen-dependent manner. T57 IFNγ responses to TGD057 were dependent on host immunity pathways downstream of regulatory Immunity-Related GTPases (IRGs), including partial dependence on Guanylate-Binding Proteins (GBPs), but not the parasite's protein export machinery ASP5 and MYR1. Multiple *T. gondii* ROP5 isoforms and allele types, including 'avirulent' ROP5A from clade A and D *T. gondii* strains, were able to suppress CD8 T cell IFNγ responses. However, removal of ROP5 was not enough to elicit maximal CD8 T cell IFNγ production to *T. gondii*. Instead macrophage expression of the pathogen sensor NLRP3 was an absolute requirement while other members of the inflammasome cascade, including ASC, Caspase 1/11 and Gasdermin D, were only partially required. Hence, a novel NLRP3-dependent but inflammasome-independent pathway controls IFNγ differentiation of naïve CD8 T cells to *T. gondii.* Furthermore, a unique phenotypic pattern emerged where CD8 T cells responded vigorously to all *T. gondii* strains except those from clade A, suggesting the presence of a Regulator Of CD8 T cell Response (ROCTR). Genetic mapping implicates multiple ROCTR candidates may be at play, including two encoded on chromosomes X and XII and a weak association with GRA35 as a potential ROCTR candidate. GRA35 has been shown to localize to the parasitophorous vacuole membrane (PVM) with the aid of two dense granules, GRA42 and GRA43. Our studies suggest that GRA35, GRA42, and GRA43 are able to modulate the CD8 T cell response and postulate that ROCTRs may be targeted to the PVM by ASP5 and GRA43. Altogether, our data suggest that *T. gondii* utilize several strategies, perhaps multilayered, to regulate the CD8 T cell response, which is highly sensitive to NLRP3 and PVM integrity.

Chapter 1:

Introduction

1.1 Toxoplasma gondii

Toxoplasma gondii is a globally spread intracellular parasite that can infect nearly all warm-blooded vertebrates, including humans. Toxoplasmosis is often underdiagnosed as it can appear asymptomatic. *T. gondii* can hide from the immune system within its parasitophorous vacuole (PV). The three infectious stages of *T. gondii* include the tachyzoites, bradyzoites (cysts), and oocysts. In addition to vertical transmission which lead to congenital toxoplasmosis, humans acquire *T. gondii* through consumption of either food contaminated with *T. gondii* oocysts shed by the feline definitive hosts, or uncooked meat encysted with *T. gondii* tissue cysts. *T. gondii* can affect both immunocompromised and healthy individuals. When infected, immunocompromised patients are more prone to severe infection, while healthy individuals may exhibit flu-like or no symptoms. If *T. gondii* escapes the immune system's clearance, it can later form cysts in the brain which last a lifetime. Once the cyst structure breaks down, *T. gondii* becomes reactivated; they differentiate into tachyzoites and can further disseminate, leading to more serious complications such as encephalitis (Dubey et al., 1998; Montoya and Liesenfeld, 2004). The severity of toxoplasmosis differs geographically and is hypothesized to be due to infections with different parasite strains endemic to these regions (Boothroyd, 2009; Grigg et al., 2001; Khan et al., 2006; Sibley and Boothroyd, 1992). More severe ocular toxoplasmosis had been observed in South America compared to other locales (Gilbert et al., 2008; Grigg et al., 2001; Khan et al., 2006; Sauer et al., 2011). Moreover, the genetically diverse "atypical" strains endemic to South America are highly virulent in mice (Fux et al., 2007; Jensen et al., 2015). Among the clonal lineages such as types I, II, and III commonly found in North America and Europe, type I strains are highly virulent, with the lethal dose (LD_{100}) of 1 parasite in laboratory mice (Sibley and Boothroyd, 1992), while types II and III are less virulent with $LD_{100} > 10^3$ and 10^5, respectively (Saeij et al., 2006; Sibley and Boothroyd, 1992). Moreover, *T. gondii* vary in degree of virulence due to their virulence factors that are polymorphic between the different strains. These strains secrete 'effector' proteins that allow *T. gondii* to modulate and escape immune detection in a wide range of infected hosts (Melo et al., 2011). Predation of chronically infected animals leads to host transmission, hence immune evasion is critical to *T. gondii*'s life cycle. Exposure to *T. gondii* infections is primarily detected by serological testing. Currently, there are more than 40 million people infected with *T. gondii* in the United States (CDC, 2018). The high prevalence is

difficult to reduce due to the lack of effective vaccines and/or treatments for the chronic form of infection.

1.2 Parasitophorous Vacuole (PV)

T. gondii forms a parasitophorous vacuole (PV) around itself following an active invasion, invaginating the host cell plasma membrane to create the PV (Coppens et al., 2006). The PV closely associates with the host ER and mitochondria following invasion, though this association is not necessarily maintained once PV formation is complete (Sinai et al., 1997). Upon the initial moments of cell invasion, the parasite releases effectors from specialized secretory organelles directly into the host cytosol, including micronemes (MIC) and rhoptry proteins (ROP), which aid in the invasion process as well as the formation of a PV (Carruthers and Sibley, 1997). Once the PV is formed, *T. gondii* secretes another group of effector proteins from its secretory dense granules, called 'GRAs'. These GRAs help in the structural modification of the PV by forming the intravacuolar network (IVN) (Mercier and Cesbron-Delauw, 2015). Many of these GRAs also associate to the PVM (Mercier and Cesbron-Delauw, 2015); some of this association is mediated by *T. gondii* Golgi aspartyl protease ASP5 (Coffey et al., 2015; Wang et al., 2019). ASP5 is also involved in export of some *T. gondii* proteins out of the PV (Coffey et al., 2018, 2015; Curt-varesano et al., 2016; Hammoudi et al., 2015). Moreover, *T. gondii* utilizes the translocon MYR1 (Myc regulation 1) to export its proteins out of the PV into the host cytosol where they subsequently transit to various cell compartments. For example, dense granule effectors, such as TgIST and GRA24, that travel to host cell nucleus need MYR1 to successfully translocate across the PVM (Franco et al., 2016; Hakimi et al., 2017). Additionally, the PVM contains pores which allow bidirectional diffusion of nutrient exchange (Gold et al., 2015; Schwab et al., 1994). The PV also supports *T. gondii* replication (Blader et al., 2015). *T. gondii* will egress from its PV when there are changes in the environmental cues, such as K^+ efflux within the host cell, usually a result of some host cell damage, or increases in cytosolic Ca^{++}, triggered by calcium ionophores or host cell activation (Blader et al., 2015). Once *T. gondii* egresses from the PV, they find a new cell to infect and this lytic cycle of invasion, replication, and egress repeats. In addition to immune evasion, the PV is the site of many host-parasite interactions, decorated with many host and *T. gondii* proteins. The parasite prioritizes protecting its PV integrity whereas the host aims to destroy it as a means of infection clearance.

1.3 Host controls T. gondii infection through IFNγ and the IRG system at the PV

The host can combat parasitic infection with interferon-gamma (IFNγ), a pro-inflammatory cytokine (Gigley et al., 2009; Suzuki et al., 1988; Suzuki and

Remington, 1990, 1988). Since the PV is able to sequester *T. gondii* allowing it to hide in the host cytosol, the main approach for the host to defend itself is to destroy the PV. The host can effectively achieve this through IFNγ signaling. IFNγ activates the Jak/STAT1 (Janus kinase / signal transducer and activator of transcription 1) signaling pathway, allowing STAT1 dimers to move into the nucleus to induce the transcription of Immunity-Related GTPases (IRGs) (Haldar et al., 2013; Hunn et al., 2008; Kim et al., 2012; Ling et al., 2006). There are two classes of IRGs: 1) regulatory IRGS ('GMS class' or 'IRGM') and 2) effector IRGs ('GKS' class) (Bekpen et al., 2005; Hunn et al., 2008; Maric-Biresev et al., 2016), defined by the amino acid motif in their GTP binding P-loop (Bekpen et al., 2005). In the resting state, regulatory IRGs bind to the effector IRGs and keep them in the inactive GDP-bound state (Hunn et al., 2008). Upon IFNγ signaling, these regulatory IRGs let go of the effector IRGs and localize to the host organelles where they protect them from effector IRG attack (Haldar et al., 2013; Maric-Biresev et al., 2016). The effector IRGs, now in the active GTP-bound state, localize to the unprotected *T. gondii* PV. These effectors IRGs oligomerize at the PV and permeabilize the PVM via GTP hydrolysis (Martens et al., 2005; Pawlowski et al., 2011; Zhao et al., 2009). Mice additionally rely on IFNγ-inducible Guanylate-Binding Proteins (GBP) for *T. gondii* clearance. GBPs also localize to the PV and directly destroy the PVM. Once compromised, GBPs will then attack the parasite's plasma membrane leading to its death (Kravets et al., 2016; Saeij and Frickel, 2017). Other host factors that localize to the PV following IFNγ stimulation include selected members of the autophagy-related proteins (ATG), which in mice prepares the vacuole for being targeted by the IRG and GBP systems (Subauste, 2019). The ATG conjugation system is required to lipidate the vacuole with the ATG8 homologs, the gamma-aminobutyric acid-A-receptor-associated proteins (GABARAPs) and LC3 (Choi et al., 2014; Park et al., 2016). The GABARAP member Gate16 alone is responsible for GBP and IRG localization to the PV and parasite restriction (Sasai et al., 2017). This single LC3 homolog recapitulates the susceptibility of *Ifngr-/-* mice to *T. gondii* infection (Sasai et al., 2017). IFNγ, ATG proteins, and IRGMs are also required for the ubiquitination and recruitment of the p62 sequestosome to the PV (Haldar et al., 2015, 2013; Lee et al., 2015). While p62 is noted as a required substrate for selective autophagy to remove ubiquitinated particulate matter inside the host cell, it is dispensable for the elimination of *T. gondii* infection (Lee et al., 2015). Mice express 23 IRGs while humans express one IRGM and another truncated IRG pseudogene that cannot be induced by IFNγ (Bekpen et al., 2005). Moreover, although mice and humans express GBPs, humans do not rely on GBP pathway for parasite clearance (Ohshima et al., 2014). Instead humans use an ATG-dependent pathway that leads to ubiquitination of the PV, where it is either enveloped by cellular membranes (Selleck et al., 2015), or acidified by fusion with host lysosomes depending on the cell type (Clough et al., 2016). Other IFNγ-mediated activities include tryptophan degradation which limits *T. gondii* replication

(Pfefferkorn, 1984) and induction of reactive nitrogen intermediates though inducible nitric oxide synthase (iNOS). Mice deficient in iNOS are able to survive acute but not chronic *T. gondii* infections (Scharton-Kersten et al., 1997). Overall, the host has several machineries, downstream of IFNγ, that target the PV in attempt to eliminate *T. gondii* infections, with the IRG- and GBP-mediated parasite clearance being the most effective in mice.

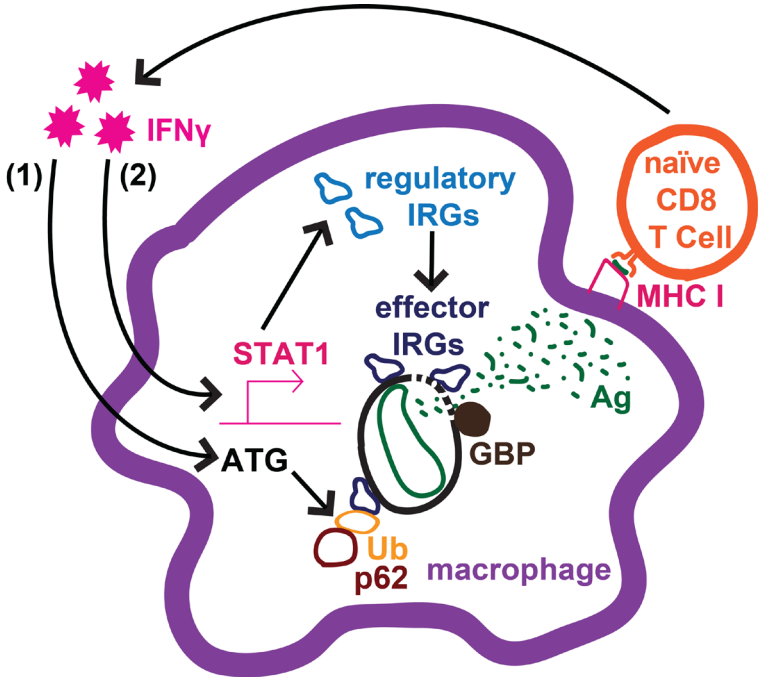

Figure 1-1. CD8 T cell responses are mediated by IFNγ-inducible IRGs and GBPs which localize to the *T. gondii* PV. IFNγ activates the Jak/STAT1 signaling pathway that induces IRG and GBP expression. IRGs and GBPs accumulate at the PV, disrupting the PVM. This process is dependent on the regulatory IRGs, IRGM. *T. gondii* antigens presumably escape into the host cell cytosol and undergo MHC I antigen presentation. Naïve CD8 T cells, upon recognition of the MHC-presented antigen, secrete IFNγ in response. ATG proteins, ubiquitin and p62 are also recruited to the PV, marking it for degradation, through IRG- and GBP-mediated killing—the key mechanism for *T. gondii* clearance. This model is based on observations from parasites expressing the model antigen OVA and data presented in this thesis analyzing the endogenous antigen TGD057.

1.4 T. gondii combats host immune response through virulence factors

T. gondii has evolved to combat host immune responses through its various virulence factors. However, immune suppression is not its only strategy. Several identified virulence factors allow *T. gondii* to 'turn on' or regulate immune signaling pathways, rather than simply suppressing it. In addition, *T. gondii* egress from its PV triggers host cell death (Niedelman et al., 2012; Zhao et al., 2009), hence the parasite must control its replication to not prematurely kill the cell. Therefore, in order to establish infections across a broad host range, it is believed that *T. gondii*

uses its effector proteins to both induce and suppress host immunity to achieve chronic infection and host-to-host transmission (Boothroyd, 2009).

T. gondii ensures its survival in the infected host by protecting its PV. One main virulence factor that helps keep the PV intact is ROP5. ROP5 is a family of pseudokinases injected by *T. gondii* upon invasion. It later re-localizes to the newly formed PV and associates to the PVM (El Hajj et al., 2007). ROP5 binds to host effector IRGs and keeps them in their inactive state at the PV, mimicking the function of host IRGM, thereby preventing the effector IRGs from damaging the PV (Howard et al., 2011; Hunn et al., 2008; Reese et al., 2014). ROP5 becomes more effective working in a complex with ROP18, ROP17, and GRA7, either in a ROP5/ROP18/ROP17 complex (Etheridge et al., 2014) or ROP5/ROP18/GRA7 complex (Alaganan et al., 2014). ROP18 expressed in virulent strains (Etheridge et al., 2014; Niedelman et al., 2012; Saeij et al., 2006; Taylor et al., 2006) as well as ROP17 (Etheridge et al., 2014) are kinases that phosphorylate IRGs in their switch I region (Fentress et al., 2010; Steinfeldt et al., 2010), preventing their activation and GTP hydrolysis (Fleckenstein et al., 2012). GRA7 helps increase the turnover rate of effector IRGs at the PV (Alaganan et al., 2014). In doing so, these virulence factors work together to reduce the accumulation of effector IRGs at the PVM (Alaganan et al., 2014; Hermanns et al., 2016; Reese et al., 2014), thereby rescuing the PV from immune-mediated destruction.

T. gondii also modulates host signaling pathways such as the Jak/STAT and NF-κB signaling cascades to regulate immune function. *T. gondii* uses its effector protein TgIST to block STAT1 activation (Gay et al., 2016; Matta et al., 2019), thereby inhibiting the induction of IFNγ-stimulated genes such as IRGs required for *T. gondii* elimination (Gay et al., 2016). Other STAT transcription factors that *T. gondii* targets include STAT3 and STAT6. The ROP16 tyrosine kinase directly phosphorylates and activates STAT3 and STAT6 (Jensen et al., 2013; Ong et al., 2010; Saeij et al., 2007; Yamamoto et al., 2009), transcription factors that are activated by the immunoregulatory cytokines IL-10 and IL-4, respectively. These cytokines are known for their inhibitory role in Th1 immunity and IFNγ responses. Conversely, *T. gondii* can also induce the production of the Th1 differentiation factor IL-12 with its effector proteins GRA15, which interacts with host TRAF2 and TRAF6 leading NF-κB p65 activation (Rosowski et al., 2011; Sangaré et al., 2019), and GRA24 that shuttles to the host cell nucleus and activates the p38 MAPK pathway (Braun et al., 2013). Polymorphisms in GRA15 and ROP16 determine strain-differences in NF-kB and STAT3/6 activation and whether infected macrophages are either inflammatory and classically activated (M1) or anti-inflammatory and alternatively activated (M2) (Jensen et al., 2011). As such, these effectors profoundly shape the small intestinal inflammation that can occur following oral infection in susceptible mouse strains (Jensen et al., 2013).

To perform their functions, many dense granules must be exported into the host cell and utilize the parasite's export machinery, which includes the MYR1 translocon and Golgi aspartyl protease 5 (ASP5). ASP5 cleaves effector proteins such as GRA16 and TgIST, and this cleavage is required for successful MYR1-dependent export (Gay et al., 2016; Hakimi et al., 2017). Interestingly, GRA24 is not cleaved by ASP5 but GRA24 export is ASP5-dependent (Hammoudi et al., 2015). MYR1 is cleaved by the ASP5 though this cleavage is not required for MYR1 to perform its translocation function (Naor et al., 2018). Overall, these *T. gondii* effectors work together to modulate host immune responses.

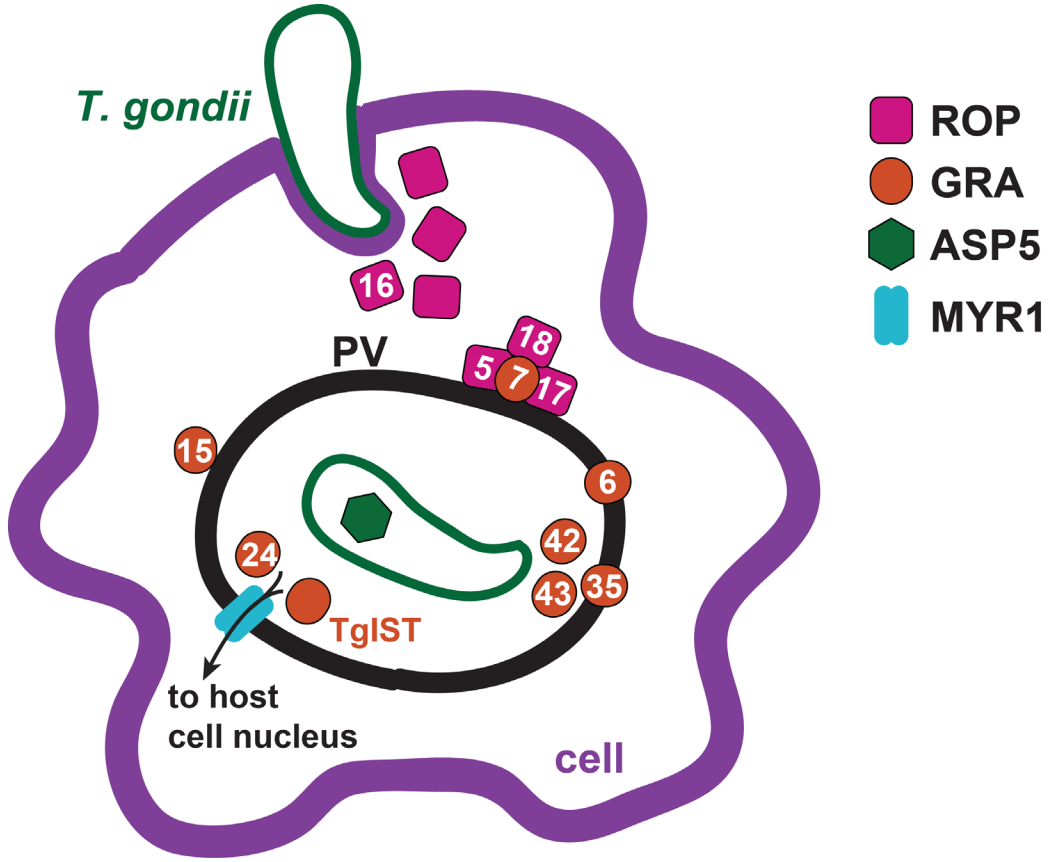

Figure 1-2. *T. gondii* effector proteins. Upon invasion, *T. gondii* secretes rhoptries many of which traffic to the PV including ROP-5, -17 and -18. The parasite sequesters itself in a newly formed PV, in which its structure is modified by GRAs. These GRAs can be found in the PV lumen, including GRA-42 and -43, as well as localized to or integrated within the PVM, including GRA-6 and -35. Some GRAs are exported outside the PV in an ASP5- and MYR1-dependent (i.e. TgIST) or an ASP5-independtent, MYR1-dependent manner (i.e. GRA24). ROP5 and ROP18 work in a complex with either ROP17 or GRA7 to maintain PV integrity by blocking host IRGs. ROP16 and GRA15 induce the alternative vs classical activation state of macrophages through the activation of the STAT6 and NF-kB signaling pathways, respectively.

1.5 Polymorphic virulence factors revealed by genetic mapping

Many of the aforementioned *T. gondii* virulence factors were discovered by genetic mapping experiments utilizing F1 progeny derived from four sexual crosses between type I x type II (F1 IxII), type I x type III (F1 IxIII), type II x type III (F1 IIxIII), and type II x VAND (F1 IIxX) strains. Using these panels, genetic linkage analysis was performed for strain-specific differences in acute virulence. This led to the discovery of ROP16 (from F1 IIxIII) (Saeij et al., 2006), ROP18 (from F1 IIxIII and F1 IxIII) (Saeij et al., 2006; Taylor et al., 2006), and the family of ROP5 psuedokinases (from F1 IxII and F1 IIxX) (Behnke et al., 2015, 2011; Reese et al., 2011). For example, the extreme virulence of type I strains is accounted for by expression of its polymorphic alleles of ROP5 and expression of the ROP18 kinase, where they inhibit the function of the IRGs at the surface of the PV (Reese et al., 2014). In fact, deletion of the *ROP5* locus (*Δrop5*) changes the virulence of the type 1 strain from an LD_{100} of 1 to over 10^6 (Behnke et al., 2011; Reese et al., 2011). Type II strains are less virulent than type I or most atypical strains due to polymorphisms in ROP5, which renders them unable to inhibit the IRGs system (Murillo-León et al., 2019; Niedelman et al., 2012; Reese et al., 2014). Moreover, whereas the type III strain encodes the virulent alleles of ROP5, its relative avirulence is explained by a 2 kb insertion in the promoter of ROP18 rendering it unexpressed (Saeij et al., 2006; Taylor et al., 2006) and unable to phosphorylate and inhibit the function of the IRGs (Lim et al., 2013; Niedelman et al., 2012). Polymorphisms in ROP16 account for some of the virulence differences between the type II and III strains (Saeij et al., 2006). ROP16, expressed in type I and type III ($ROP16_{I/III}$) but not type II ($ROP16_{II}$) strains, allow sustained phosphorylation of STAT3 and STAT6 (Butcher et al., 2011; Jensen et al., 2011; Ong et al., 2010; Yamamoto et al., 2009). $ROP16_{III}$ promotes virulence due to early M2 activation and subsequent suppression of Th1 immunity and CD8 T cell responses (Chen et al., 2020; Tuladhar et al., 2019), presumably due its ability to suppress IL-12 secretion from infected cells (Butcher et al., 2011; Jensen et al., 2011; Rosowski et al., 2011). In contrast, when $ROP16_{I}$ or $ROP16_{III}$ are expressed as a transgene in the type II background, it promotes host resistance (Jensen et al., 2011; Saeij et al., 2006), and parasite killing by a mechanism that is dependent on the genetic background of the parasite (Jensen et al., 2013).

Another QTL analysis of the F1 IxII sexual cross identified nucleoside triphosphate hydrolases (NTPases) as key *T. gondii* polymorphic determinants that regulated cellular levels of ATP during infection (Olias and Sibley, 2016) . The genetic linkage analysis of this strain-specific phenotype identified an NTPase locus which encodes two isoforms: NTPase I and NTPase II (Asai et al., 1995; Olias and Sibley, 2016). Virulent type I *T. gondii* strains express both NTPase I and II isoforms while the NTPase II isoform can be found in all strains (Asai et al., 1995; Olias and

Sibley, 2016). NTPases are secreted by the parasite and localize within the PV lumen (Asai et al., 1995), representing up to 2% of the entire protein content within *T. gondii* (Asai et al., 1983). NTPases hydrolyze ATP and are thought to initiate *T. gondii* egress from its PV (Silverman et al., 1998; Stommel et al., 1997) and promote parasite proliferation (Asai et al., 2002; Nakaar et al., 1999). Olias *et al*. discovered that type I *T. gondii* strains can perform ATP hydrolysis at a higher rate compared to other strains due to NTPase I leading to greater cellular depletion of ATP (Olias and Sibley, 2016; Silverman et al., 1998). Regardless of the site of the host-parasite interaction, *T. gondii* has a wide range of virulence factors, both polymorphic and nonpolymorphic, that can thwart many of the host defense machineries.

1.6 Host CD8 T cells and IFNγ are required for T. gondii elimination

Various host immune cells work together to combat *T. gondii* infections with CD8 T cells being the critical mediators of host immunity to *T. gondii* primary and secondary infections (Gigley et al., 2009). Infected mice that lack CD8 T cells succumb to primary and chronic *T. gondii* infections (Gigley et al., 2009; Hakim et al., 1991; Parker et al., 1991; Splitt et al., 2018). Furthermore, vaccinated mice and/or mice with chronic parasite infections are protected from lethal secondary infections due to CD8 T cells (Gazzinelli et al., 1991; Gigley et al., 2009). CD4 T cells aid in effector CD8 T cell (Casciotti et al., 2002; Khan et al., 2019) and B cell responses (Johnson and Sayles, 2002) against *T. gondii*. However, when vaccine-induced CD4, CD8, and B cells were adoptively transferred into naïve mice, only memory CD8 T cells were able to protect the recipients from a type I RH *T. gondii* infection (Gazzinelli et al., 1991; Gigley et al., 2009). The effector mechanism utilized by CD8 T cells to control *T. gondii* is primarily mediated through IFNγ (Gigley et al., 2011; Nishiyama et al., 2020; Suzuki et al., 1988; Suzuki and Remington, 1990, 1988). This also prevents *T. gondii* dormant cysts form from becoming reactivated (Gazzinelli et al., 1992; Suzuki et al., 1989). Cytolytic functions of CD8 T cells mediated through perforin and FAS-FASL death receptors seem only important for controlling chronic infection and not secondary infections (Jordan et al., 2009; Suzuki et al., 2010; Wang et al., 2004).

Host cytotoxic CD8 T cells respond to intracellular pathogens such as *T. gondii* through recognition of cytosolic-derived peptide antigens presented by MHC I molecules on the surface of an infected cell (Dzierszinski et al., 2007). The CD8 T cell response to *T. gondii* has been largely studied utilizing two antigen-specific experimental models: 1) *T. gondii* strains engineered to express model antigen OVA (Dzierszinski et al., 2007; Gubbels et al., 2005) and 2) *T. gondii* immunodominant PVM-integrated dense granule GRA6, encoded by type II *T. gondii* strains (Blanchard et al., 2008; Feliu et al., 2013), with a C-terminal epitope

being exposed to the host cytosol (Buaillon et al., 2017; Feliu et al., 2013). Studies with these two antigens revealed that the antigen has to be in the parasite's secretory pathway (Dzierszinski et al., 2007; Grover et al., 2014) and that active cell invasion—not the phagocytosis of invasion-blocked or heat-killed *T. gondii*—is required for CD8 T cells activation (Dupont et al., 2014; Dzierszinski et al., 2007; Goldszmid et al., 2009). The antigen is processed by host cytosolic proteasomes (Bertholet et al., 2006; Blanchard et al., 2008) before being transported through ER TAP1/2 translocon (Dzierszinski et al., 2007; Goldszmid et al., 2009; Gubbels et al., 2005) and loaded onto the surface of MHC I molecules. For antigen processing of the PVM integral GRA6, its C-terminal epitope (Feliu et al., 2013) must be targeted to the PVM (Lopez et al., 2015) and exposed to the host cytosol (Buaillon et al., 2017), where it is processed by unknown proteases. For the recognition of transgenic OVA expressed in *T. gondii* PV lumen, two mechanisms of MHC I antigen presentation have been reported. The first mechanism requires fusion of *T. gondii* PVM and either the host ER (Goldszmid et al., 2009) or the ER-derived Golgi Intermediate Compartments (ERGICs) (Cebrian et al., 2011). The fusion of ERGICs and *T. gondii* PVM is dependent on Sec22b SNARE, allowing the host to shuttle *T. gondii* antigens from the PV to host compartments containing antigen processing machinery (Cebrian et al., 2011). The second mechanism for MHC I antigen presentation involves the PVM becoming compromised by the host's effector IRGs and selective ATG systems, leading to OVA antigen escape from the PV (Dzierszinski et al., 2007; Lee et al., 2015). Once the PV is compromised and there is antigen acquisition from the PV, MHC I antigen presentation and CD8 T cell activation can then occur. In this mechanism, IFNγ signaling is critical as it induces the IRG pathway as aforementioned (Haldar et al., 2013; Hunn et al., 2008; Kim et al., 2012; Ling et al., 2006), required for further destruction of PV, more MHC I antigen presentation and activation of CD8 T cells (Lee et al., 2015; Rommereim et al., 2019). Since *T. gondii* evades host immune response by hiding in its PV, the rupture of PVM or complete destruction of the PV would allow *T. gondii* antigen to be exposed and recognized by naïve CD8 T cells. *T. gondii* utilize its effector proteins namely ROP5, ROP18, GRA7 and others to block this IRG-dependent OVA-antigen presentation and CD8 T cell activation (Rommereim et al., 2019).

Naïve CD8 T cells must undergo differentiation steps to become IFNγ producers if they are to control *T. gondii* infection. Following the activation of CD8 T cells, these activated CD8 T cells differentiate into IFNγ through IL-12 signaling (Wilson et al., 2008). Particularly, Wilson *et al.* has shown that there are less IFNγ-producing CD8 T cells in *Il12p35-/-* mice compared to the WT mice in response to *T. gondii* infection (Wilson et al., 2008). Overall, there are various requirements that the host must undergo to successfully eliminate *T. gondii* infections and each of these steps are subjected for manipulation by the parasite to achieve immune evasion.

1.7 Inflammasomes are important for T. gondii control

Another immune mechanism that the host uses to combat *T. gondii* is the inflammasome. *T. gondii* infection leads to the activation of both NLRP3 and NLRP1 inflammasomes (Cirelli et al., 2014; Ewald et al., 2014; Gorfu et al., 2014; Witola et al., 2011). Inflammasome activation requires 1) 'signal 1' (NF-κB-dependent induction of pro-IL-1β and pro-IL-18, 2) priming of the NLR protein (such as deubiquitination of NLRP3), and 3) 'signal 2' that induce oligomerization of NLRP, resulting in the release of mature proinflammatory cytokines IL-1β and IL-18. 'Signal 1' can be induced through various receptors such as the Toll-like receptors (TLR) that trigger signaling cascades culminating inNF-κB activation (Bauernfeind et al., 2009; Elliott and Sutterwala, 2015; He et al., 2016). NOD2 sensors which also activates NF-κB have been shown to be important for *T. gondii* control. There is less IFNγ production as well as increased parasite burden and dissemination in *T. gondii*-infected *Nod2-/-* mice (Heimesaat et al., 2014; Shaw et al., 2009). NOD2 oligomerizes with RIPK2, leading to the activation of NF-κB pathway and production of pro-IL-1β (Keestra-Gounder et al., 2016). Various stimuli can induce NLRP3 inflammasome activation, such as extracellular ATP (eATP). Extracellular ATP acts as damage-associated molecular pattern (DAMP) and interacts with the P2X7 receptor (P2X7R), leading to K^+ efflux. This change in the cytosolic K^+ concentration leads to the assembly of both NLRP1 and NLRP3 inflammasome complexes. Other inflammasome-activating stimuli that drive NLRP3 oligomerization include uric acid crystals and nigericin (Elliott and Sutterwala, 2015). The parasite-derived PAMPs or DAMPs during *T. gondii* infection that activates NLRP3 and NLRP1 are currently unknown.

Following activation, the NLRP3 inflammasome complex assemble with NLRP3 sensor protein, adaptor protein ASC (Apoptosis-associated speck like protein), and a caspase. Canonical inflammasome activation involves caspase 1 while noncanonical inflammasome pathway involves caspase 11 (Broz and Dixit, 2016; Jo et al., 2016). NLRP1 inflammasome activation can occur independent of ASC while other inflammasomes such as NLRP3, NLRP12, NLRC4, and AIM2 inflammasomes associate with ASC to perform their functions (Vanaja et al., 2015). The assembled inflammasome complex cleaves pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18 respectively. Gasdermin D (GSDMD) is also cleaved at this stage; the active N-terminus GSDMD forms pores at the cell membrane, leading to pyroptosis as a response to pathogen infection. Infected *Asc-/-* and *Casp11-/-* exhibited severe symptoms of toxoplasmosis (Coutermarsh-Ott et al., 2016). The production of IL-1β is also reduced if the inflammasome pathway is impaired (Coutermarsh-Ott et al., 2016; Witola et al., 2011). Overall, inflammasomes keep *T. gondii* infections under control in the infected host. *T. gondii* utilizes several factors to modulate signal 1 of inflammasome activation.

Known virulence factors such as GRA15 and GRA24 are able to induce the production of pro-IL-1β and IL-18 (Mukhopadhyay et al., 2020a). Since *T. gondii* aims to achieve chronic infection inside its host, cell death such as inflammasome-induced pyroptosis is not ideal for the parasite.

1.8 Dissecting host-parasite interaction for host CD8 T cell IFNγ response

The overall goal of this dissertation is to unravel the host-parasite interactions that govern CD8 T cell IFNγ responses. Because *T. gondii* is a ubiquitous parasite and can infect nearly all warm-blooded vertebrates, we hypothesize that *T. gondii* has adapted to manipulate this form of adaptive immunity which is central to host protection. The well-studied mechanisms of *T. gondii* elimination associates with disruption of the PVM while many of the parasite virulence effectors work to protect the integrity of the PV. Thus, we ask whether there are novel parasite effectors that protect the PV and prevent antigen escape and entry into the MHC I antigen presentation pathway. As naïve CD8 T cells undergo activation and differentiation prior to IFNγ secretion, we ask what host-parasite interactions determine the outcome of this response. In summary, there are two overall questions that my dissertation aims to investigate: 1) what are the host requirements for the naïve CD8 T cell IFNγ response to *T. gondii*, and 2) how does *T. gondii* modulate the CD8 T cell response to survive in different infected hosts?

To answer these overarching questions, we utilized transnuclear mice that provides a source of naïve CD8 T cells that are specific for a natural endogenous antigen of *T. gondii*. In Chapter 2, through usage of mouse and *T. gondii* gene knockouts, we discovered that naïve CD8 T cell responses are regulated uniquely by *T. gondii* ROP5 and host IRGs. In particular, we describe that even avirulent ROP5 isoforms are potent regulators of the CD8 T cell response. We also found a novel pathway that regulates naïve CD8 T cell differentiation to become IFNγ-producing cells; one that requires the NLRP3 sensor but independent of the inflammasome. In Chapter 3, we performed genetic linkage analysis to identify parasitic Regulators Of the CD8 T cell Response ('ROCTR') that are responsible for strain-specific differences in their ability to induce host CD8 T cell IFNγ responses. Though the identities of these parasite effectors have not yet been revealed, our data suggested that there are multiple ROCTRs. Altogether, our data showed that the host-parasite battles which occur at the PVM dictate the regulation of CD8 T cell responses to *T. gondii* infections.

Chapter 2:

Naïve CD8 T cell IFNγ responses to a vacuolar antigen are regulated by an inflammasome-independent NLRP3 pathway and *Toxoplasma gondii* ROP5

Note: Most of the research presented in chapter 2 has been published in PLOS Pathogens, Kongsomboonvech et al. 2020.

2.1 Introduction

Toxoplasma gondii is a globally spread intracellular parasite that can infect nearly all warm-blooded vertebrates, including humans. Transmission between hosts occurs following ingestion of oocysts shed from the definitive feline host or predation of chronically infected animals harboring infectious 'tissue cysts'. Immune modulation by the parasite during the first weeks of infection is therefore critical for *T. gondii* to establish latency and life cycle progression. The parasite accomplishes this by hiding and manipulating the immune system from within a specialized parasitophorous vacuole (PV) that is created during invasion. *T. gondii* releases 'effector' proteins from secretory organelles, including rhoptry proteins (ROP) that are injected into the host cytosol upon invasion, as well as dense granules (GRA) that are secreted into the lumen of the PV and aid its internal structure and formation. Many of these secreted 'effectors' manipulate host cell signaling pathways and shield the PV from host immune attack (Melo et al., 2011). In mice, *T. gondii* uses several ROP and GRA proteins to antagonize the host's Immunity-Related GTPases (IRGs) which target and compromise the PV (Howard et al., 2011). ROP5 is encoded by a multi-gene variable family of pseudokinases and can directly bind to and induce allosteric changes in host IRGs (Reese et al., 2014), presenting them for phosphorylation by the ROP18 (Behnke et al., 2012; Fleckenstein et al., 2012) and ROP17 parasite kinases (Etheridge et al., 2014). The process of phosphorylation inactivates host IRGs, preventing them from assembling on the surface of the PV, which in turn allows the parasite to replicate (Fentress et al., 2010; Steinfeldt et al., 2010). Genetic variations in ROP5 and ROP18 largely explain parasite strain differences in mouse virulence (Behnke et al., 2015, 2011; Reese et al., 2011; Saeij et al., 2006; Taylor et al., 2006), highlighting the importance of the IRG system in the control of *T. gondii* infection. IRGs also regulate the recruitment of Guanylate-Binding Proteins (GBPs) and autophagy machinery to the PV membrane (PVM), both of which contribute to cell autonomous immunity to *T. gondii* (Kravets et al., 2016; Saeij and Frickel, 2017).

Since IRGs and GBPs are induced transcriptionally following stimulation with IFNγ (Kim et al., 2012), immune cells that produce IFNγ are critically important for resistance to *T. gondii* (Yarovinsky, 2014). CD8 T cell IFNγ responses are required for host survival to *T. gondii* infections (Gigley et al., 2011; Suzuki et al., 1988; Suzuki and Remington, 1990, 1988) and to prevent reactivation of the dormant form (Gazzinelli et al., 1992; Suzuki et al., 1989). In vaccinated or chronically infected mice, IFNγ and CD8 T cells are primarily responsible for protection against lethal secondary infections (Gazzinelli et al., 1991; Gigley et al., 2009). However, most *T. gondii* strains that express virulent alleles of ROP5 and ROP18 evade the host's immunological memory response and superinfect the brains of challenged survivors (Jensen et al., 2015), implicating that sterile immunity to *T. gondii* may be difficult to achieve, as noted for other parasitic pathogens (Sacks, 2014). Whether *T. gondii* manipulates induction of the host's IFNγ response to prolong its survival is unknown, but could represent a general strategy to promote persistence and latency, as noted for numerous viral pathogens in the presence of clonally expanded antigen-specific CD8 T cells (Christiaansen et al., 2015).

In order for naïve CD8 T cells to become IFNγ producers, they must first be activated by peptides derived from the host's MHC I antigen presentation pathway and then receive cues from the environment or other immune cells to differentiate into IFNγ-producing cells. The question of MHC I antigen presentation for *T. gondii* antigens has largely been addressed using two experimental systems. One analyzes antigen-specific CD8 T cell responses to parasite strains expressing the model antigen, chicken ovalbumin (OVA) (Dzierszinski et al., 2007; Gubbels et al., 2005), and the other analyzes responses to *T. gondii* immune-dominant antigen GRA6, encoded by type II strains (Blanchard et al., 2008; Feliu et al., 2013). From these studies, it is appreciated that active cell invasion by *T. gondii*, rather than phagocytosis of invasion-blocked or heat-killed parasites, is required to stimulate host CD8 T cells (Dupont et al., 2014; Dzierszinski et al., 2007; Goldszmid et al., 2009). The antigen must be in the parasite's secretory pathway (Dzierszinski et al., 2007; Grover et al., 2014), degraded by host cytosolic proteasomes (Bertholet et al., 2006; Blanchard et al., 2008), transported via the endoplasmic reticulum (ER) TAP1/2 translocon (Blanchard et al., 2008; Dzierszinski et al., 2007; Goldszmid et al., 2009; Gubbels et al., 2005), and eventually loaded onto MHC I molecules. Although dense granules and rhoptry proteins access the host cytosol where MHC I antigen processing readily occurs, antigens targeted to the dense granule secretory pathway elicit a greater CD8 T cell response (Grover et al., 2014). The PV is therefore a suitable platform for MHC I antigen presentation, which is remarkable given the PV of *T. gondii* does not initially fuse with host organelles (Coppens, 2017; Sinai et al., 1997), nor is contained within the conventional endocytic compartments of the cell.

The mechanism by which the immune system gains access to PV antigens of *T. gondii* has remained an active area of research, notwithstanding for its implication in vaccine development (Jensen, 2016) and the ability of *T. gondii* to elicit anti-tumor responses (Fox et al., 2017). In the case of *T. gondii* GRA6, it must be integrated in the PVM (Lopez et al., 2015), where its C-terminal epitope (Feliu et al., 2013) is exposed to the host cytosol (Buaillon et al., 2017) and degraded by unknown proteases. For the MHC I antigen presentation of transgenic OVA expressed in the PV lumen of *T. gondii*, two general mechanisms have been reported. Fusion between the PVM and the host ER (Goldszmid et al., 2009), or ER-derived Golgi Intermediate Compartments (ERGICs) promotes OVA-specific CD8 T cell activation (Cebrian et al., 2011). In this scenario, through a Sec22b SNARE-dependent mechanism, the host's MHC I antigen-processing machinery gains access to the PV whereby it shuttles parasite proteins into the cytosol for antigen processing. In a second mechanism, though not mutually exclusive, the PVM is compromised by the host's IRGs and selective autophagy systems therefore allowing OVA antigen release (Dzierszinski et al., 2007; Lee et al., 2015). Via ROP5 and ROP18, *T. gondii* can bypass IRGs activity and presumably MHC I antigen presentation by sequestering OVA inside an intact PV (Rommereim et al., 2019). However, several dense granule proteins are also implicated, signifying multiple non-redundant pathways may regulate MHC I antigen presentation of PV antigens (Rommereim et al., 2019). Whether lessons learned from GRA6 and OVA extend to other antigens or parasite genetic backgrounds is unknown.

In addition to activation by antigen, CD8 T cells need proper co-stimulation (Villegas et al., 2002, 1999) and cytokines, in particular IL-12 signaling, to fully commit to IFNγ production during *T. gondii* infection (Gigley et al., 2011; Wilson et al., 2010, 2008). In addition, IL-1 (Chang et al., 1990; Hunter et al., 1995) and IL-18 can promote host survival during acute *T. gondii* infections (Gorfu et al., 2014), and are released following parasite detection and inflammasome activation by the pathogen sensors NLRP3 and NLRP1 (Ewald et al., 2014; Gorfu et al., 2014; Pandori et al., 2019). Depending on the mode and time of infection, IL-1 and IL-18 can induce or repress inflammatory related pathologies in various tissues (Vossenkämper et al., 2004). Inflammasome-matured IL-18 is important for IFNγ production by CD4 T cells but is apparently dispensable for CD8 T cell IFNγ-production during acute *T. gondii* infection (López-Yglesias et al., 2019). Whether the inflammasome contributes to CD8 T cell activation or differentiation in different contexts or stages of *T. gondii* infection is unclear.

Given the parasite's need to establish latency and the host's dependence on CD8 T cells for immunity, we asked whether *T. gondii* has evolved to manipulate CD8 T cell IFNγ responses to an endogenous antigen, and whether certain *T. gondii* genotypes are defined by their ability to induce or repress the

production of this immune-protective cytokine. Through the use of T cell receptor transnuclear and IFNγ reporter mice, host and parasite requirements were defined for the induction of IFNγ-producing CD8 T cells to a conserved vacuolar antigen of *T*. *gondii*, TGD057. Here we report that TGD057-specific CD8 T cell responses are independent of the parasite's PV-export machinery, and like previous findings with OVA-engineered *T*. *gondii* strains, the IRG pathway is required. Multiple ROP5 isoforms suppress this response, including ROP5A which lacks a defined function or interaction with host IRGs. An analysis of parasite strains spanning twelve haplogroups suggests IFNγ production is manipulated by *T*. *gondii* independent of any known IRG-ROP5 interaction or parasite virulence factor. Importantly, an NLRP3-dependent but inflammasome complex-independent pathway is required for inducing maximal CD8 T cell IFNγ responses to *T*. *gondii* infected cells. Our findings point to novel host-parasite interactions by which IRGs and NLRP3 shape CD8 T cell IFNγ responses to an intracellular pathogen.

2.2 Materials and Methods

2.2.1 Parasites

Tachyzoites of *Toxoplasma gondii* strains were passaged in 'Toxo medium' [4.5 g/liter D-glucose in DMEM with GlutaMAX (Gibco, cat# 10566024), 1% heat-inactivated fetal bovine serum (FBS) (Omega Scientific, cat# FB-11, lot#441164), 1% penicillin-streptomycin (Gibco, cat# 15140122)], in confluent monolayers of human foreskin fibroblasts (HFFs). HFFs were cultured in 'HFF medium' [4.5 g/liter D-glucose in DMEM with GlutaMAX (Gibco), 20% heat-inactivated FBS (Omega Scientific), 1% penicillin-streptomycin (Gibco), 0.2% Gentamicin (Gibco, cat# 15710072), 1X L-Glutamine (Gibco, cat# 21051024)]. Strains assayed include GT1 (type I, clade A), BOF (Haplogroup "HG" VI, clade A), FOU (HG VI, clade A), CAST (HG VII, clade A), MAS (HG IV, clade B), TgCatBr5 (HG VIII, clade B), CEP *hxgprt*- (type III, clade C), P89 (HG IX, clade C), ME49 *Δhxgprt*::*Luc* (Tobin and Knoll, 2012) (type II, clade D), Pru *Δhxgprt* (type II, clade D), COUGAR (HG XI, clade D), B73 (HG XII, clade D), GUY-KOE (HG V, clade F), GUY-MAT (HG V, clade F), RUB (HG V, clade F), GUY-DOS (HG X, clade F), and VAND (HG X, clade F). Other strains used include BOF+LC37 (Niedelman et al., 2012), RH *Δhxgprt* (Donald and Roos, 1998), RH *Δhxgprt Δku80* (Huynh and Carruthers, 2009), RH *Δhxgprt Δku80 Δrop5*::*HXGPRT* (RH *Δrop5*) (Reese et al., 2011), RH *Δhxgprt Δku80 Δtgd057*::*HXGPRT* (RH *Δtgd057*) (Jensen et al., 2015), RH *Δhxgprt Δku80 TGD057-HA*::*HXGPRT* (RH_{tgd057}-HA) (generated here), Pru *Δhxgprt Δku80*, Pru *Δhxgprt Δku80 Δtgd057*::*HXGPRT* (Pru *Δtgd057*) (generated here), Pru A7 Δ*hxgprt*::*gra2-GFP*::*tub1-FLUC* (Pru A7) (Kim et al., 2007), Pru A7 *Δhxgprt Δgra15*::*HXGPRT* (Pru *Δgra15*) (Rosowski et al., 2011),

Pru A7 *Δhxgprt Δgra24* (1E9) (Pru *Δgra24)* (generated here), Pru *Δhxgprt Δgra15 Δgra24* (1F8) (Pru *Δgra15 Δgra24*) (generated here), Pru A7 *Δhxgprt +ROP16$_A$*::*HXGPRT* (Pru *+ROP16$_A$*) (Jensen et al., 2011), RH *Δhxgprt Δmyr1*::*HXGPRT* (RH *Δmyr1*) (Wang et al., 2019), and RH *Δku80 Δasp5-ty*::*DHFR* (RH *Δasp5*) (Curt-varesano et al., 2016). ME49 *Δasp5*:: *DHFR* (ME49 *Δasp5*) was a generous gift from Dominique Soldati-Favre (University of Geneva) (Hammoudi et al., 2015). ME49 *Δhxgprt*::*Luc Δmyr1*:: *HXGPRT* (ME49 *Δmyr1*), ME49 *Δhxgprt*::*Luc Δmyr1 +MYR1-HA*:: *HXGPRT* (ME49 *Δmyr1*::*MYR1*) (Franco et al., 2016), and RH *Δhxgprt Δrop17*:: *HXGPRT* (PCRE) (RH *Δrop17*) (Panas et al., 2019a) were generous gifts from John Boothroyd (Stanford University). The ROP5 complementation strains used in this study are as followed: RH *Δhxgprt Δku80 Δrop5 +ROP5A$_{II-ME49}$His6-3x Flag* (C1A6) (RH *Δrop5 +ROP5A$_D$*) (generated here), RH *Δhxgprt Δku80 Δrop5 +ROP5B$_{III-CTG}$-His6-3xFlag* (H2) (RH *Δrop5 +ROP5B*) (generated here), RH *Δhxgprt Δku80 Δrop5 +ROP5A$_{III-CTG}$His6-3xFlag* (C1B1) (RH *Δrop5 + ROP5A)*, RH *Δhxgprt Δku80 Δrop5 Δuprt*::*ROP5A$_{III}$-HA +ROP5B$_{III-CTG}$-His6-3x Flag* (AC11) (RH *Δrop5 +ROP5A+ROP5B)*, RH *Δhxgprt Δku80 Δrop5 Δuprt*:: *ROP5A$_{III}$-HA +ROP5A$_{III-CTG}$-His6-3xFlag* (AA12) (RH *Δrop5 +ROP5A+ROP5A*) (Reese et al., 2011).

2.2.2 Generation of gene knock-in and knockout parasite strains

The Pru *Δhxgprt Δku80 Δtgd057*::*HXGPRT* strain was generated using the same primers and strategy as previously described (Jensen et al., 2015). The RH *Δhxgprt Δku80 TGD057-HA*::*HXGPRT* endotagged strain was generated as previously described (Camejo et al., 2014). In brief, for endogenous tagging (Huynh and Carruthers, 2009) of TGD057 with an HA tag, the gene (*TGGT1_215980*) was amplified with a forward primer internal to the ATG start site of *TGD057* [“AC_tgd057endoF2” 5’-CACCAACTACGTCGGAGCGCCTGTACG-3’], containing a 5′-CACC-3′ sequence required for directional TOPO cloning in pENTR/D-TOPO (Invitrogen), and a reverse primer [“Tgd057 R HA stop” 5’-TTACGCGTAGTCCGGGACGTCGTACGGGTACTCGACCTCAATGTTGTATTC-3’], containing the hemagglutinin (HA) tag sequence (underlined) followed by a stop codon. The resulting TGD057 HA-tagged DNA fragment was then cloned into the pTKO-att parasite expression vector (Rosowski et al., 2011) by Gateway Recombination Cloning Technology (Invitrogen). The resulting vector was linearized and transfected into RH *Δhxgprt Δku80* parasites by electroporation in a 2 mm cuvette (Bio-Rad Laboratories) with 2 mM ATP (MP Biomedicals) and 5 mM glutathione (EMD) in a Gene Pulser Xcell (Bio-Rad Laboratories), with the following settings: 25 µFD, 1.25 kV, ∞ Ω. Stable integrants were selected in media with 50 µg/ml of mycophenolic acid (Axxora) and 50 µg/ml of xanthine (Alfa Aesar) and cloned by limiting dilution. The correct tagging was confirmed by PCR, using

a primer upstream of the plasmid integration site and a primer specific for the HA tag (5'-CGCGTAGTCCGGGACGTCGTACGGGTA-3'), and by an immune-fluorescence assay (IFA) using an HA-specific antibody (Sigma, clone 3F10).

For the generation of RH *Δrop5 +ROP5A$_D$* and RH *Δrop5 +ROP5B* complementation strains, RH *Δhxgprt Δku80 Δrop5*::*HXGPRT* tachyzoites were transfected by electroporation with linearized pTKO-"ROP5KO" -*ROP5A$_{II-ME49}$-His6-3xFlag* plasmid (Reese et al., 2011), or a similarly generated pTKO -*ROP5B$_{III-CTG}$-His6-3xFlag* plasmid as described in (Reese et al., 2011). In brief, for both strains the complementation allele, *ROP5-His6-3xFlag*, is flanked by homology arms to the *Δrop5*::*HXGPRT* locus, whereby the transfected population is selected for removal of *HXGPRT* and replacement with the complementation allele in 6-thioxanthine selection medium [177 µg/mL of 6-thioxanthine (TRC, cat# T385800) in 4.5 g/liter D-glucose in GlutaMAX DMEM (Gibco), with 1% dialyzed FBS (Omega Scientific, cat# FB-03, lot#463304)]. Post-selection and limiting dilution cloning, *ROP5-His6-3xFlag* complementation strains were assessed by IFA with a mouse anti-Flag primary antibody (Sigma, clone M2) at 1:500 dilution and Alexa Flour 594 goat anti-mouse IgG secondary antibody (Life Technologies) at 1:3000 dilution. Both clones expressed the ROP5-HF in the expected rhoptry organelles by IFA.

For generating the Pru A7 *Δhxgprt Δgra24* strain, Pru A7 *Δhxgprt* parasites were transfected with a NotI-linearized plasmid expressing a loxP-flanked pyrimethamine selectable cassette (*loxP-DHFR-mCherry-loxP*) (Addgene plasmid #70147, was a gift from David Sibley, Washington University in St. Louis) and a CRISPR-CAS9 construct targeting *GRA24* (*TGME49_230180*). Transfectants were selected and cloned in medium containing pyrimethamine, and screened for the disruption of *GRA24*. For generating the Pru A7 *Δhxgprt Δgra15 Δgra24* strain, first the Pru A7 *Δhxgprt Δgra15*::*HXGPRT* strain (Rosowski et al., 2011) was gene-edited with CRISPR-CAS9 targeting *HXGPRT* and selected against its expression with 6-thioxanthine. Then, a Pru A7 *Δhxgprt Δgra15*::*hxgprt*- clone was used to make a Pru A7 *Δhxgprt Δgra15 Δgra24* double knockout strain using the same method as described above. Finally, the *loxP-DHFR-mCherry-loxP* cassette was removed from both *Δgra24* and *Δgra15*/*Δgra24* strains by transfection with a Cre recombinase parasite-expression plasmid (Heaslip et al., 2011), and immediately cloned by limiting dilution. The details of which can be found in the manuscript by Mukhopadhyay *et al.* (Mukhopadhyay et al., 2020a).

2.2.3 Immunofluorescence assay

For TGD057 visualization, HFFs were seeded on coverslips with HFF medium in 24-well tissue culture-treated plates. The confluent monolayer HFFs were infected

with *T. gondii* and incubated at 37°C, 5% CO_2 overnight. For Irgb6- and p62-PV localization, BMDMs were plated on coverslips with 'BMDM medium' [4.5 g/liter D-glucose in DMEM with GlutaMAX (Gibco), 20% heat-inactivated FBS (Omega Scientific), 1% penicillin-streptomycin (Gibco), 1X non-essential amino acids (Gibco, cat# 11140076), 1mM sodium pyruvate (Gibco, cat# 11360070)] supplemented with 20% L929 conditioned medium in 24-well tissue culture-treated plates. The BMDMs were treated with 20 ng/ml of IFNγ overnight. The cells were then infected with *T. gondii* and incubated at 37°C, 5% CO_2 for 3–4 hours. The samples were fixed with 3% formaldehyde in phosphate buffered saline (PBS) for 20 minutes and blocked with blocking buffer (3% BSA, 5% normal goat serum or fetal bovine serum depending the species of antibody used, 0.2% Triton X-100, 0.1% sodium azide in PBS). To visualize TGD057-HA, RH or RH_{tgd057}-HA were stained with rat anti-HA primary antibody (Sigma, clone 3F10) at 1:500 dilution, followed by Alexa Fluor 594 goat anti-rat IgG (Life Technologies) secondary antibody (1:3000 dilution). To visualize p62, infected cells were stained with mouse anti-p62 (anti-SQSTM1) primary monoclonal antibody (Abnova, clone 2C11) at 1:50 or 1:100 dilutions, followed by Alexa Fluor 594 goat anti-mouse IgG (Life Technologies) secondary antibody at 1:3000 dilution. To visualize Irgb6, infected BMDMs were stained with TGTP goat polyclonal primary antibody (Santa Cruz Biotechnology, sc-11079) at 1:100 dilution, followed by Alexa Fluor 594 donkey anti-goat IgG (Life Technologies) at 1:3000 dilution. *T. gondii* PVM was stained with polyclonal rabbit anti-GRA7 primary antibody (gift from John Boothroyd, Stanford University) and Alexa Fluor 488 anti-rabbit IgG (Life Technologies) at 1:3000 dilution. Host nuclei were visualized with DAPI (Thermo Fisher, cat# 62248) at 1:10,000 dilution or Hoechst (Life Technologies, cat# H3075) at 1:3000 dilution.

2.2.4 Mice and generation of bone marrow-derived macrophages

All animal protocols were approved by UC Merced's Committee on Institutional Animal Care and Use Committee (IACUC) (AUP17-0013). All mouse work was performed in accordance with the recommendations in the *Guide to the Care and Use of Laboratory Animals* of the National Institutes of Health and the Animal Welfare Act (assurance number A4561-1). Inhalation of CO_2 to effect of 1.8 liters per minute was used for euthanasia of mice.

Six-week-old female *Stat1-/-* (colony 012606), *Ifngr-/-* (colony 003288), *Nlrp1-/-* (colony 021301), *Nlrp3-/-* (colony 021302), *Casp1/11-/-* (colony 016621), *Il18-/-* (colony 004130), *Il12b-/-* (colony 002693), *P2x7r-/-* (colony 005576), *NOD2-/-* (colony 005763), *Ripk2-/-* (colony 007017), and wildtype C57BL/6J (B6) (colony 000664) mice were purchased from Jackson Laboratories, and all of the C57BL/6 background. C57BL/6 *Asc-/-* mice were generous gifts from Vishva Dixit (Genentech). Hind bones from C57BL/6 *Gsdmd-/-* mice (Rauch et al., 2017) were

generous gifts from Igor Brodsky (University of Pennsylvania). *Irgm1-/-* and *Irgm1/m3-/-* hind bones were provided from Gregory Taylor (Duke University). *GBP^{chr3}-/-* hind bone marrow cells were provided by Masahiro Yamamoto (Osaka University). Bone marrow cells were obtained and cultured in BMDM medium supplemented with 20% L929 conditioned medium. After 6–7 days of differentiation, BMDMs were harvested and were 98% pure CD11b+ CD11c- macrophages by FACS. *Asc-/-*, *Gsdmd-/-*, and *Nlrp3*-/- BMDMs were stained with anti-mouse MHC I K^b-PE labeled antibodies (BioLegend, clone AF6-88.5) and they were positive by FACS analysis.

Transnuclear T57 mice (Kirak et al., 2010) were bred in-house under specific pathogen free (SPF) conditions. 'T-GREAT' mice were generated by back- and inter-crossing between T57 and IFNγ-stop-IRES:eYFP- endogenous poly-A tail reporter mice (GREAT mice) (Cebrián et al., 1988), such that breeders obtained from F3 intercrossed mice were homozygous at three alleles: T57 TCRα (*TRAV6-4 TRAJ12* rearrangement), T57 TCRβ (*TRBV13-1 TRBJ2-7* rearrangement), and the GREAT reporter. T-GREAT mice were then maintained in our SPF facility with no overt fitness defects observed. Genotyping primers were as followed: GREAT allele (FW 5'-CCATGGTGAGCAAGGGCGAGG-3'; RV 5'-TTACTTGTACAGC TCGTCCAT-3'); wildtype *Ifng* allele (FW 5'-CAGGAAGCGGAAAAGGAGTCG-3'; RV 5'-GTCACTGCAGCTCTGAATGTT-3'); T57 TCRα (*TRAV6-4 TRAJ12* rearrangement: "146-alpha" FW 5'-GATAAGGGATGCTTCAATCTGATGG-3'; "108-alpha" RV 5'-CTTCCTTAGCTCACTTACCAGGGCTTAC-3'); endogenous non-rearranged *TRAV6-4* and *TRAJ12* loci ("191-alpha" FW 5'-GAGGCTTTACG TTAGTGATCTAAAC-3'; "108-alpha" RV); T57 TCRβ (*TRBV13-1 TRBJ2-7* rearrangement: "91-beta" FW 5'- CTTGGTCGCGAGATGGGCTCCAG-3'; "103-beta" RV 5'- GTGGAAGCGAGAGATGTGAATCTTAC-3'); endogenous non-rearranged *TCRBV13-1* and *TRBJ2-7* loci ("142-beta" FW 5'-GCACTCGGCTCCT CGTGTTAGGTG-3'; "103-beta" RV).

2.2.5 T cell activation assay

2x10^5 BMDMs cells were plated per well in a 96-well tissue culture-treated plate, in BMDM medium supplemented with 10% L929 conditioned medium. The following day, these BMDMs were infected with *T. gondii* tachyzoites in 'T cell medium' [(RPMI 1640 with GlutaMAX (Gibco, cat# 61870127), 20% heat-inactivated FBS (Omega Scientific, cat# FB-11, lot#441164), 1% penicillin-streptomycin (Gibco, cat# 15140122), 1 mM sodium pyruvate (Gibco, cat# 11360070), 10 mM HEPES (Gibco), 1.75 µl of β-mercaptoethanol (Gibco, cat# 21985023) per 500 mL RPMI 1640 with GlutaMAX]. The infections were performed in triplicates, at MOI 0.6, 0.2, and 0.07. Then, lymph nodes and spleens were obtained from either T57 or T-GREAT transnuclear mice. The lymph node cells

and splenocytes were combined and red blood cells were lysed with ammonium chloride-potassium (ACK) lysis buffer. $5x10^5$ cells were added into each well of the infected BMDMs (approximately 2 hours post-infection). For IL-1R neutralization, 50 µg/mL of anti-mouse IL-1R antibody (BioXCell, clone JAMA-147) or 50 µg/mL of isotype control (BioXCell, cat# BE0091) were added when BMDMs were infected.

2.2.6 Correction for relative viability between parasites

Confluent monolayer HFFs, seeded in 24-well plates, were infected with 100 and 300 parasites. Plaques were counted 4–6 days after infection. Displayed results are from MOIs with similar viability, the equivalent of ~MOI 0.2 was chosen for most assays.

2.2.7 ELISA

The concentration of cytokines in the 24h and 48h supernatants from the T57 T cell activation assay was measured by ELISA according to the manufacturer's instructions (IFNγ: Invitrogen eBioscience, cat# 88731477, IL-2: Invitrogen eBioscience, cat# 88702477, IL-17A: Invitrogen eBioscience, cat# 88737188, IL-1β: R&D Systems, cat# DY401-05, IL-18: Invitrogen, cat# BMS618-3). The supernatants were analyzed at various dilutions (1:2, 1:20, and 1:200) to obtain values within the linear range of the manufacture's ELISA standards.

2.2.8 Flow cytometry

At 18h after T-GREAT T cell activation, samples were harvested for FACS analysis. With preparations all done on ice, cells were washed with 'FACS buffer' [PBS pH 7.4 (Gibco, cat# 10010049), 2% heat-inactivated FBS (Omega Scientific)] and blocked with 'blocking buffer' [FACS buffer with 5% normal Syrian hamster serum (Jackson Immunoresearch, cat# 007-000-120), 5% normal mouse serum (Jackson Immunoresearch, cat# 015-000-120), and anti-mouse CD16/CD32 FcBlock (BD Biosciences, clone 2.4G2) at 1:100 dilution)]. Then, the samples were stained at 1:120 dilution with fluorophore-conjugated anti-mouse monoclonal antibodies against CD8α PE (eBioscience, clone 53–6.7), CD3ε APC-eFlour780 (eBioscience, clone 17A2), CD62L eFlour450 (eBioscience, clone MEL-14), and CD69 APC (BioLegend, clone H1.2F3). For analysis of GFP+ Pru A7-infected BMDMs, cells were harvested at 18h, washed and blocked as previously described, and stained at 1:100 dilution with PE-labeled anti-mouse antibodies against CD40 (BioLegend, clone 3/23), CD70 (eBioscience, clone FR70), CD80 (eBioscience, clone 16-10A1), CD86 (eBioscience, clone GL1), CD252 (eBioscience, OX40L clone RM134L), CD274 (eBioscience, PD-L1 clone MIH5),

CD275 (eBioscience, ICOSL clone HK5.3), or rat IgG2a kappa isotype control antibodies (eBioscience, clone eBR2a). Tissue culture plates containing the infected BMDMs were placed on ice prior to harvesting and washing as described above. All samples were then stained with propidium iodide (PI) at 1:1000 dilution (Sigma, cat# P4170). Flow cytometry was performed on an LSRII (Becton Dickinson) and analyzed with FlowJo software; PI+ cells were excluded from analysis.

2.2.9 Western Blot

T. gondii lysates were prepared through syringe-lysis, resuspended in Laemmli buffer (125 mM Tris HCl, 30% glycerol, 2% SDS, 0.2% bromophenol blue), and denatured at 90-100°C for 5 minutes. The lysates were separated on 4-20% Mini-PROTEAN TGX pre-cast 540 gels (cat# 4561096, Bio-Rad) or 5-15% polyacrylamide gels and then transferred to nitrocellulose membranes. The membranes were blocked with 10% fortified bovine milk in 'TBS-T 0.1%' [Tris-Buffered Saline and 0.1% Tween] at room temperature for 1h, and then incubated with α-TGD057 antibody at 1:4000 dilution (gift from Nicolas Blanchard, INSERM) in TBS-T 0.1% overnight at 4°C. Membranes were washed with TBS-T 0.1% three times and later incubated with goat α-rabbit horseradish peroxidase (HRP)-conjugated antibodies (cat# 4030-05, Southern Biotech) at 1:4000 dilution for 1h at room temperature. Membranes were then washed again TBS-T 0.1% three times and developed with Immobilon® Forte Western HRP Substrate (WBLUF0500). All blots were imaged via chemiluminescence on a ChemiDoc Touch (cat# 12003153, Bio-Rad).

2.2.10 Statistical analysis and normalization between experiments

For all bar graphs, dots represent values obtained from an individual experiment. Results between parasite strains were often expressed relative to the response elicited by the type II strain (equal 1), or the response to infected knockout macrophages normalized to infected wildtype macrophages (equal 1). All statistical analyses (one-way or two-way ANOVA with Bonferroni's correction, Kruskal-Wallis test with Dunn's correction for non-parametric data in **Fig 2-9**, and unpaired two-tailed t-test) were performed with GraphPad Prism version 8.3.0.

2.3 Results

2.3.1 Naïve CD8 T cells respond to the vacuolar antigen TGD057 with a robust IFNγ response

To determine host and parasite requirements for eliciting antigen-specific CD8 T cell responses to *T*. *gondii*, we took advantage of 'T57' transnuclear mice which were cloned from the nucleus of a single tetramer-positive *T*. *gondii*-specific CD8 T cell. T57 T cells from these mice have a single T cell receptor (TCR) specificity for the $TGD057_{96\text{-}103}$ epitope presented by H-$2K^b$ MHC I (Kirak et al., 2010; Wilson et al., 2010), and when adoptively transferred, confer resistance to infection with a type II strain (Kirak et al., 2010). TGD057 is a protein with unknown function but is predicted to be in the parasite's secretory pathway (Wan and Ajioka, 2004), and when deleted does not negatively impact parasite fitness, as inferred from a genome-wide CRISPR-CAS9 loss-of-function screen (phenotype score 2.1) (Sidik et al., 2016), and from similar growth rates in tissue culture and plaque sizes observed between *Δtgd057* and parental strains. Importantly, *TGD057* (TG_215980) is highly expressed (ToxoDB.org) and the peptide epitope is conserved between strains (**Fig 2-1**), facilitating comparative analyses of naïve CD8 T cell responses to parasite strains which may differ in immune modulation. In our experimental setup (i.e. the T cell activation 'T57 assay') (**Fig 2-2A**), bone marrow-derived macrophages (BMDMs) are infected with *T*. *gondii*, co-cultured with splenocytes and lymph node cells from naïve transnuclear T57 mice, and CD8 T cell activation markers or effector cytokines in the supernatant are measured. Reflecting early T cell activation events culminating in calcium-dependent NFAT activation of the IL-2 gene (Northrop et al., 1994), this cytokine is produced as early as 24 hours post addition of T cells to parasite-infected BMDMs (**Fig 2-2B**). In contrast and consistent with their naïve state, the T57 IFNγ response develops with time, and is maximally detected at 48 hours (**Fig 2-2C**). Its magnitude is influenced by the parasite genotype, as evidenced by consistently high cytokine responses to the ME49 and MAS strains, and low responses to the RH strain (**Fig 2-2B and 2-2C**). *T*. *gondii* infection elicits strong CD8 T cell IFNγ responses to TGD057 (Wilson et al., 2010) and other antigens (Tsitsiklis et al., 2019), as such IL-17 is only marginally detected in this system (**Fig 2-3A**). The measured phenotypes are antigen-specific, because the cytokine response is abolished in response to *Δtgd057* strains which do not express the antigen (**Fig 2-2D and 2-2E**). Additionally, we noted that T57 CD8 T cells fail to upregulate the early activation marker CD69 in response to *Δtgd057* (not shown). Previous observations from sub-cellular fractionation and immunofluorescence studies have identified TGD057 to be both within the PV of infected cells (Lopez et al., 2015) and to the cytoskeleton region of the parasite (Wilson et al., 2008). Three-dimensional mass spectrometry LOPIT analysis (Location of Organelle Proteins by Isotype Tagging) posits TGD057 to dense granules but lacks a strict assignment to any one organelle, the latter observation being consistent with most cytoskeleton network associated proteins (Crook et al., 2018). An endotagged RH_{tgd057}-HA strain was generated and TGD057 is always found within PVM defined by GRA7 staining vacuoles (**Fig 2-2F**), demonstrating TGD057, in its natural state,

stays inside the PV. In summary, the T57 system allows analysis of naïve CD8 T cell responses to an endogenous vacuolar antigen of *T. gondii*.

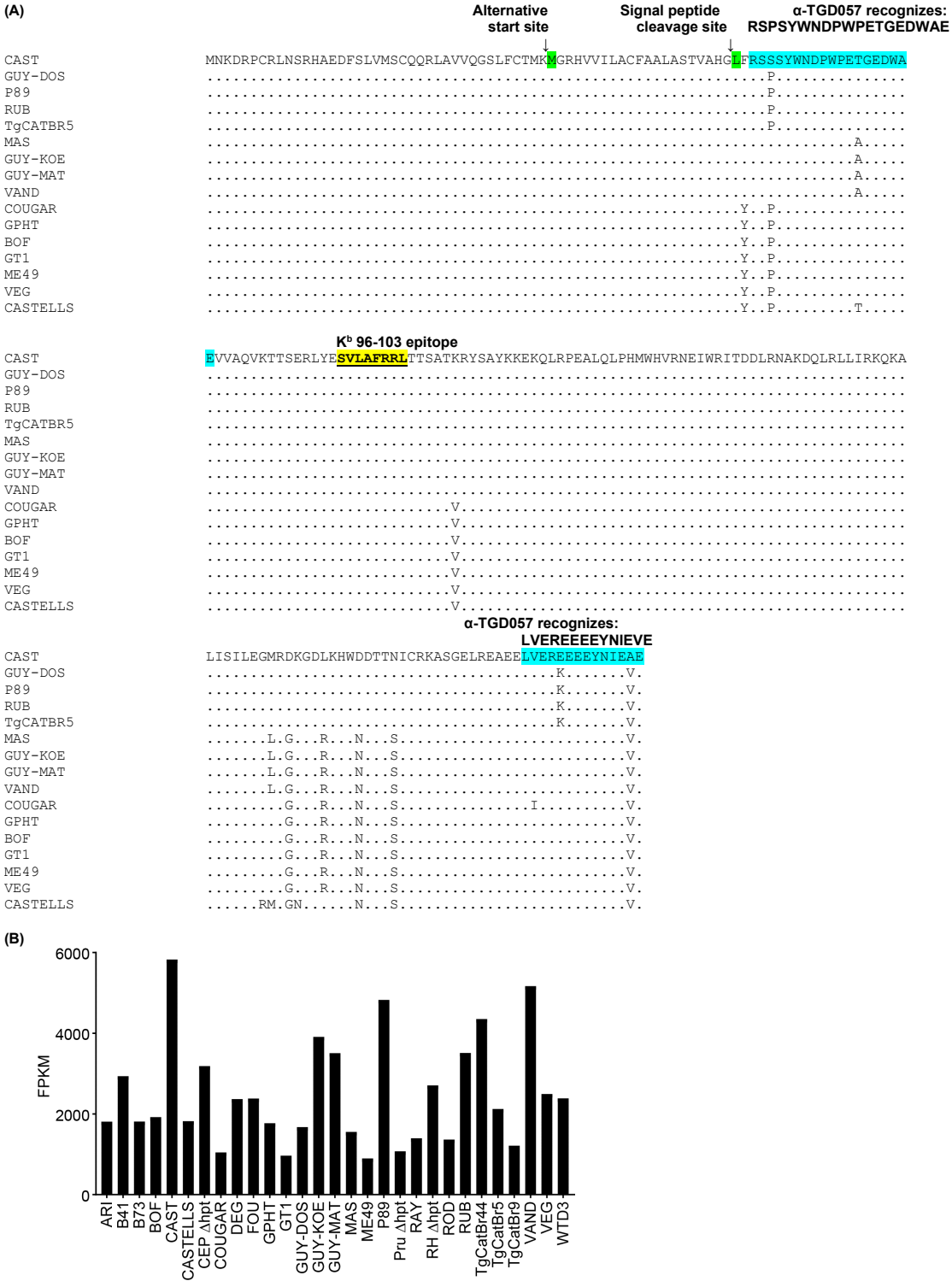

Figure 2-1. Conservation of the TGD057 96–103 peptide epitope and high *TGD057* gene expression between *T. gondii* strains. **(A)** Multiple protein alignment of TGD057 (215980) encoded by various *T. gondii* strains; the 96–103 MHC I K^b T57 T cell epitope is highlighted in yellow. The putative TEXEL sequence, RRL, is within this epitope. Dots represent amino acid conservation with TGD057 from the CAST strain. The predicted signal peptide cleavage site and an alternative translational start site (Wan et al., 2004) is indicated with an arrow and highlighted in green. Peptide fragments recognized by a rabbit polyclonal antibody directed against TGD057 (gift from Nicholas Blanchard, INSERM) are indicated and highlighted in blue, and correspond to the peptide fragments of the clonal strains (i.e. GT1 and ME49). **(B)** *TGD057* gene ex-pression (*TG_215980*) for 29 parasite strains following 20–22 hours post-infection in BMDMs (C57BL/6) is plotted from data previously reported (Minot et al., 2012); expression values are in fragments per kilobase of exon model, per million mapped reads (FPKM).

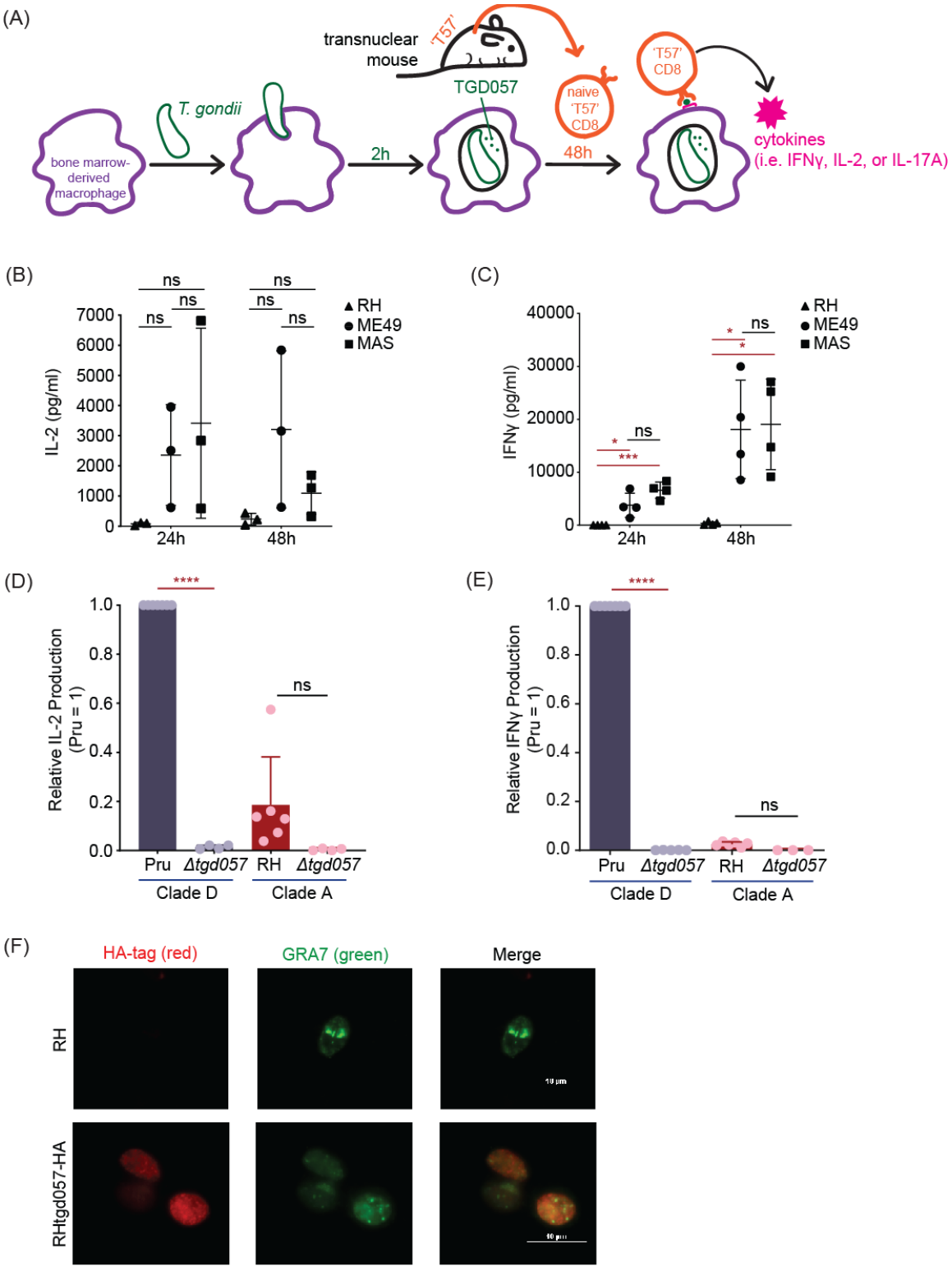
(A)
transnuclear mouse
'T57'
TGD057
naive 'T57' CD8
'T57' CD8
cytokines (i.e. IFNγ, IL-2, or IL-17A)
T. gondii
bone marrow-derived macrophage
2h
48h
(B)
IL-2 (pg/ml)
RH
ME49
MAS
24h
48h
ns
(C)
IFNγ (pg/ml)
(D)
Relative IL-2 Production (Pru = 1)
(E)
Relative IFNγ Production (Pru = 1)
Pru
Δtgd057
RH
Clade D
Clade A
****
(F)
HA-tag (red)
GRA7 (green)
Merge
RH
RHtgd057-HA

Figure 2-2. A model to study naïve CD8 T cell responses to a *T. gondii* vacuolar antigen, TGD057. **(A)** Schematic of the 'T57 assay'. Bone marrow-derived macrophages (BMDMs) are infected with *T. gondii* and 2h later, naïve T57 CD8 T cells obtained from transnuclear mice are added to the infected macrophages. T57 T cells bear antigen receptor specificity for a natural *T. gondii* antigen, the processed $TGD057_{96\text{-}103}$ peptide in complex with MHC I K^b. Supernatant from the co-culture is then harvested and cytokine concentrations are analyzed by ELISA. **(B-C)** TGD057-specific CD8 T cell responses to *T. gondii*-infected macrophages were measured over time to the indicated parasite strains. At 24h and 48h time points, IFNγ and IL-2 was measured by ELISA. Average of 3–4 experiments + SD (standard deviation) is plotted; each dot represents the result from an individual experiment. Statistical analysis comparing parasite strain differences were performed by two-way ANOVA with Bonferroni's correction; * $p \leq 0.05$, *** $p \leq 0.001$, ns non-significant. **(D-E)** Parental and *Δtgd057 T. gondii* strains were assayed for the CD8 T cell response as described in Fig 1A. T57 IFNγ and IL-2 responses at 48h, analyzed by ELISA, are normalized to that of the clade D wildtype strain (Pru). Average of 3–5 experiments + SD is shown, each dot represents the results from an individual experiment. Statistical analysis between parental and knockout strains is performed by an unpaired t-test; **** $p \leq 0.0001$, ns non-significant. Clade assignments (clades A-F) are indicated for each strain as previously determined by population based genome-wide SNP comparisons and similar clustering of *T. gondii* strains (Lorenzi et al., 2016). **(F)** Human foreskin fibroblasts (HFFs) were infected with RH or an RH_{tgd057}-HA endotagged strain. After 20 hours of infection, the samples were fixed, permeabilized and the tagged TGD057-HA was visualized in rat anti-HA antibodies, visualized in red. PVM is indicated by presence of the PVM integral and PV luminal dense granule protein, GRA7, visualized in green. A representative immunofluorescence image is shown.

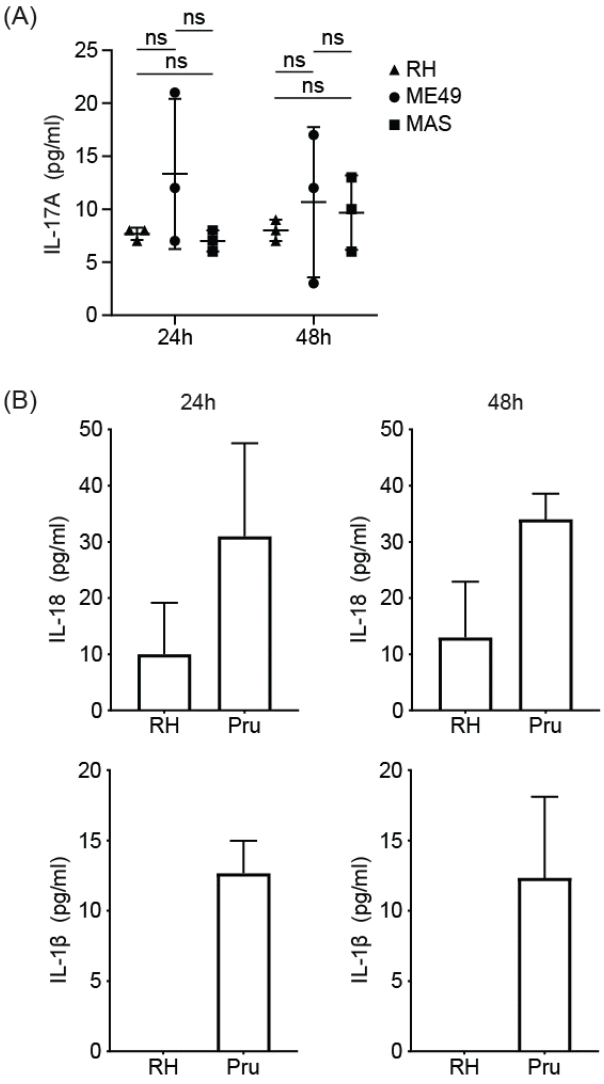

Figure 2-3. Negligible amounts of IL-17A, IL-1β and IL-18 are detected in co-cultures of *T. gondii*-infected BMDMs and T57 CD8 T cells. (A) BMDMs were infected with the indicated parasite strains and IL-17A was measured in the supernatant at 48h post addition of naïve T57 CD8 T cells. Average of 3 experiments + SD are plotted; each dot represents the result from an individual experiment. Statistical analysis was performed with two-way ANOVA with Bonferroni's correction, ns non-significant. **(B)** BMDMs were infected with RH or Pru strains, and IL-1β or IL-18 was measured in the supernatant at 24h or 48h post addition of naïve T57 CD8 T cells. Plotted is the average + SD of 3 technical replicates. Results obtained at 48 h are representative of two experiments, and a single experiment at 24h was performed.

2.3.2 TGD057 antigen acquisition is not dependent on the parasite's protein export pathway

To understand how *T. gondii* vacuolar antigens might escape from the vacuole and enter the host's MHC I antigen presentation pathway, the parasite's export machinery was explored. One way for vacuolar proteins to enter the host cytosol is through parasite-mediated export across the PV membrane (PVM). Dense granule proteins that reside within the PV can leave the vacuole through *T. gondii*'s export machinery, which includes the Golgi-resident protein aspartyl protease, ASP5 (Coffey et al., 2015; Hammoudi et al., 2015), and the PVM-integrated translocon protein MYR1 (Franco et al., 2016). *T. gondii* ASP5 is an orthologue of *Plasmodium* protease Plasmepsin V which recognizes a *Plasmodium* export element (PEXEL) motif (RxLxE/Q/D) (Boddey and Cowman, 2013) and cleaves after the leucine (RxL↓xE/Q/D) preparing PEXEL-bearing proteins for export across the PVM into the host erythrocyte (Russo et al., 2010). Like Plasmepsin V, *T. gondii* ASP5 recognizes and cleaves a PEXEL-like motif (RRL↓XX) (*Toxoplasma* export element or 'TEXEL' motif) (Coffey et al., 2015), and its protease function is necessary for the export of all known exported PV proteins (Rastogi et al., 2019). For example, GRA16 contains an RRL↓XX sequence, is cleaved by ASP5, and utilizes the MYR1 translocon complex for protein export (Coffey et al., 2015; Curt-varesano et al., 2016). Other GRAs including GRA24 (Franco et al., 2016; Hammoudi et al., 2015) and TEEGR/HCE1 (Braun et al., 2019; Panas et al., 2019b), while being fully dependent on ASP5 and MYR1 for their export, lack a functional TEXEL sequence. As TGD057 contains an RRL sequence, we hypothesized ASP5 and/or MYR1 may be involved in the export of TGD057 from the PV, leading to MHC I antigen presentation and T57 antigen-specific CD8 T cell responses. To test this, the TGD057-specific CD8 T cell response to *Δasp5* and *Δmyr1* strains was measured as previously described in **Fig 2-2A**. In contrast to the hypothesis, ME49 *Δasp5* induced a higher CD8 T cell response compared to that of wildtype (WT) ME49 (**Fig 2-4**). Moreover, T57 responses to ME49, ME49 *Δmyr1* and *MYR1* complementation strains (ME49 *Δmyr1*::*MYR1*) were comparable (**Fig 2-4**). Since the T57 cytokine response to the type I RH strain was uniformly low (**Fig 2-2B and 2-2C**), inferring requirements for export machinery using these parasite strains was uninformative. Additionally, we looked for evidence of ASP5 cleavage of TGD057 by western blot using a polyclonal α-TGD057 antibody (gift from Nicolas Blanchard, INSERM) which recognizes two peptide sequences indicated in **Fig 2-1A**, flanking the potential ASP5 cleavage site of TGD057 (**Fig 2-1A**). The western blot analysis revealed one band around the size of 18 kDa representing the full length TGD057 protein present in parental and *Δasp5 T.gondii* strains. No peptide fragments of 5 and 13 kDa were observed, which would be generated if TGD057 were to be cleaved at its putative TEXEL sequence (**Fig 2-5**). These observations suggest

that TGD057 is not cleaved by ASP5. Nonetheless, CD8 T cell responses to the type II strain do not require ASP5 and MYR1, suggesting protein export from the PV is not necessary for MHC I antigen presentation of the vacuolar TGD057 antigen.

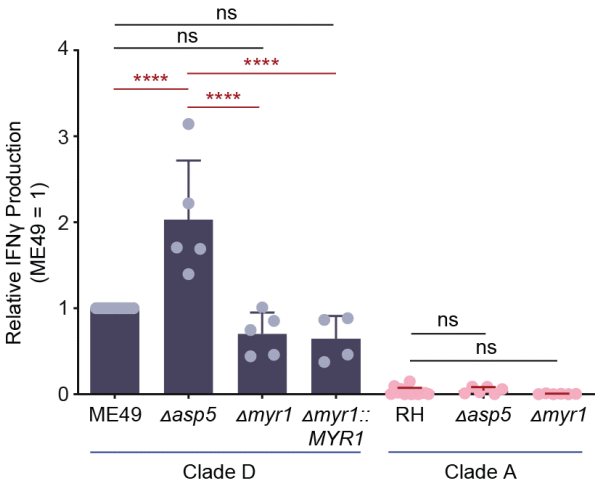

Figure 2-4. TGD057-speicifc CD8 T cell IFNγ responses do not require the parasite's export machinery. The *Δasp5* and *Δmyr1* *T. gondii* strains listed were assayed for host CD8 T cell responses as previously described in Fig 2-2A. The IFNγ response at 48h, as analyzed by ELISA, is normalized to that of the clade D wildtype strain (ME49). Average of 4–6 experiments + SD is shown, each dot represents the results from one experiment. Statistical analysis was performed using one-way ANOVA with Bonferroni's correction comparing mutant to parental strains; **** $p \leq 0.0001$, ns non-significant.

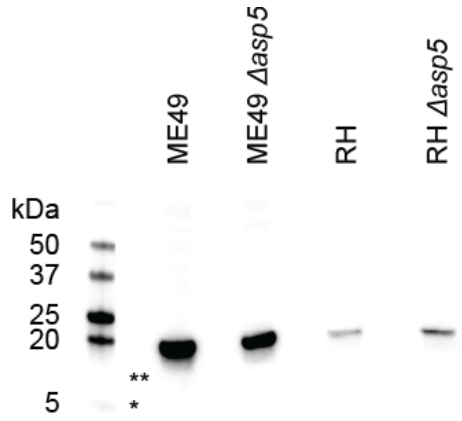

Figure 2-5. TGD057 is not cleaved by ASP5. TGD057 from lysates of *T. gondii* parental and *Δasp5* strains were detected by western blot analysis using an α-TGD057 polyclonal antibody. TGD057 is 18 kDa. Representative blot of two experiments is shown. Asterisks indicate 13 kDa (**) and 5 kDa (*) regions on the gel that would correspond to the predicted peptide fragment sizes generated if ASP5 cleaved TGD057 at its putative TEXEL sequence.

2.3.3 CD8 T cell IFNγ responses to TGD057 require host machinery downstream of the regulatory IRGs

Instead, we hypothesized the T57 CD8 T cell response requires PV disruption and is IRG-mediated, as implicated from studies of OT1 CD8 T cell responses, or hybridoma derivatives, to parasite strains that express the model OVA antigen in the PV lumen (Gregg et al., 2011; Rommereim et al., 2019; Yamamoto et al., 2012). To this end, T57 assays were performed with various BMDMs that are defective in IRG function. In the experimental setup, IFNγ is derived from activated

T57 cells and is predicted to induce IRG expression in WT but not *Stat1-/-* or *Ifngr -/-* macrophages. Consistent with this supposition, the TGD057-specific CD8 T cell response to the ME49 strain was nearly abolished in the absence of IFNγ-STAT1 signaling (**Fig 2-6A**). In mice, there are 23 IRGs that can be separated into two subfamilies: 1) the effector IRGs (or 'GKS class' based on an amino acid motif in their GTP binding P-loop) and 2) the regulatory IRGs ('GMS class') (Bekpen et al., 2005). Whereas effector IRGs bind the vacuolar membrane of the PV (Martens et al., 2005; Zhao et al., 2009) and mediate membrane destruction via GTP hydrolysis (Pawlowski et al., 2011), regulatory IRGs (or 'IRGMs') localize to host cellular organelles preventing effector IRGs from destroying host membranes (Haldar et al., 2013; Maric-Biresev et al., 2016). Regulatory IRGMs bind to effector IRGs keeping them in their GDP-bound inactive state (Hunn et al., 2008), in a manner similar to *T. gondii* ROP5 (Fleckenstein et al., 2012; Niedelman et al., 2012; Reese et al., 2014). In the absence of IRGMs, effector IRGs fail to localize to pathogen PVs (Henry et al., 2009; Hunn et al., 2008) and pathogen restriction is lost (Taylor et al., 2000). In mice there are three regulatory IRGs (IRGM-1, -2 and -3) and *Irgm1-/-* or *Irgm1/3-/-* double knockout macrophages were analyzed. Similar to the OVA system, the TGD057-specific CD8 T cell response to stimulatory parasite strains, such as type II ME49 and the atypical strain MAS, require the activity of regulatory IRGMs (**Fig 2-6A and 2-6B**). In addition, IFNγ-inducible Guanylate-Binding Proteins (GBPs) localize to the PV in an IRGM-dependent manner (Haldar et al., 2013) and mediate *T. gondii* resistance (Saeij and Frickel, 2017). GBPs encoded on murine chromosome 3 (GBP^{chr3}) are involved in PV disruption, promote effector IRG recruitment to the PV of *T. gondii* (Selleck et al., 2013; Yamamoto et al., 2012), and once compromised will attack the parasite's plasma membrane, decreasing its fitness (Kravets et al., 2016). To test whether GBPs are required for TGD057-specific CD8 T cell responses, GBP^{chr3}-deficient BMDMs lacking *Gbp1*, *Gbp2*, *Gbp3*, *Gbp5*, and *Gbp7* were screened (Yamamoto et al., 2012). GBP^{chr3} were not significantly involved in promoting TGD057-specific CD8 T cell IFNγ responses to the ME49 strain (**Fig 2-6C**), but were partially required for the response to the atypical strain MAS (**Fig 2-6D**). Altogether, these observations are consistent with a model in which the PVM is compromised by host machinery downstream of regulatory IRGs, including GBPs (Haldar et al., 2013) and likely other immunity genes, that mediate vacuolar antigen escape from the PV and entry into the host's MHC I antigen-presentation pathway.

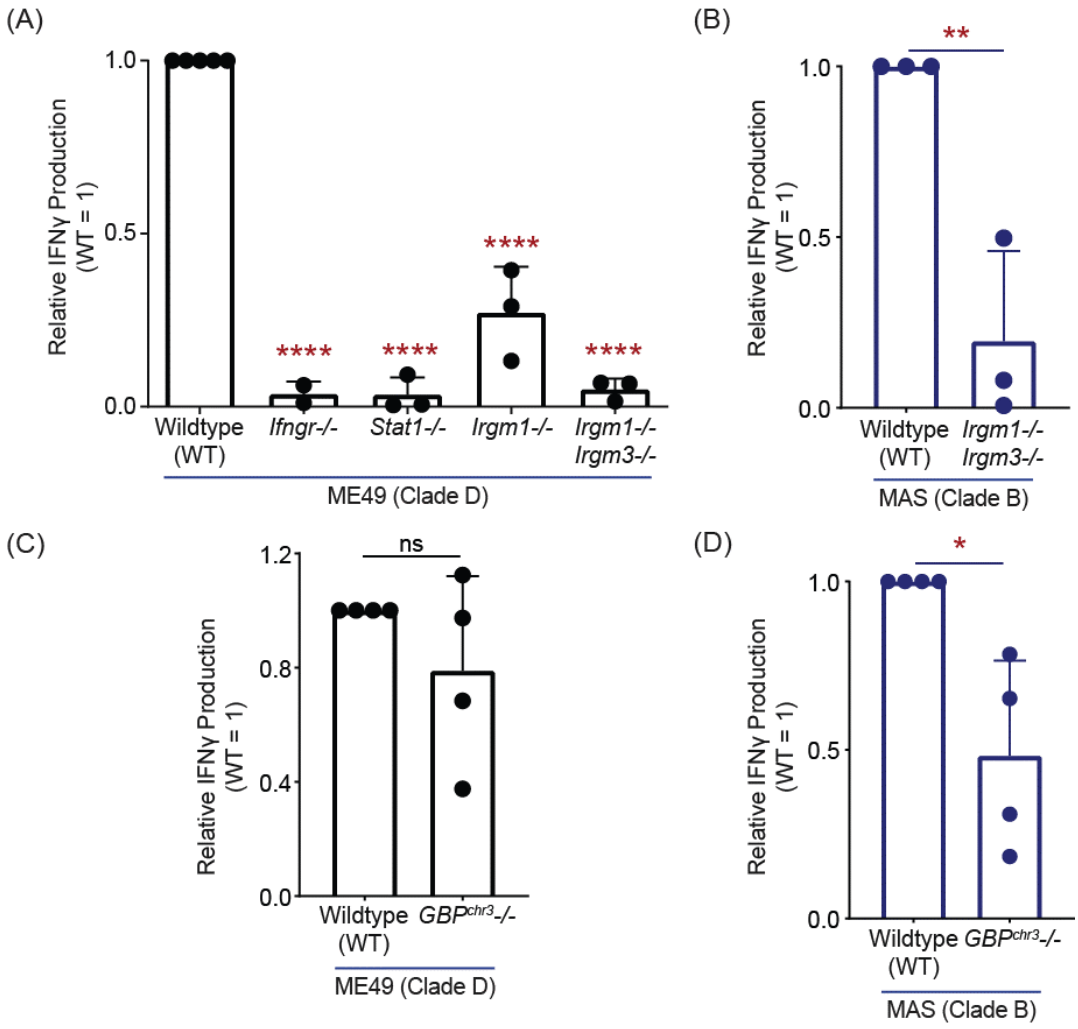

Figure 2-6. TGD057-specific CD8 T cell IFNγ responses are partially dependent on host GBPs but entirely dependent on regulatory IRGs. (A,C) BMDMs with indicated gene deletion (-/-) were infected with the clade D ME49, or **(B,D)** clade B MAS strain. T57 T cell IFNγ responses were analyzed by ELISA at 48h and normalized to the response elicited by infected wildtype (WT) BMDMs. Average of 2–4 experiments + SD is shown, each dot represents the result from an individual experiment. Statistical analyses were performed by one-way ANOVA with Bonferroni's correction **(A)** or unpaired two-tailed t-tests **(B-D)**; * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$, ns non-significant.

2.3.4 Multiple ROP5 isoforms of T. gondii suppress the CD8 T cell response to TGD057

Previous studies have shown that *T. gondii* virulence in mice is determined by parasite effectors that protect the PV from host immune attack. Specific alleles and isoforms of the rhoptry pseudokinase ROP5 (Behnke et al., 2015, 2012, 2011; Reese et al., 2014, 2011; Reese and Boothroyd, 2011), ROP18 (Fentress et al., 2010; Fleckenstein et al., 2012; Niedelman et al., 2012; Saeij et al., 2006; Taylor et al., 2007) and ROP17 kinases (Fleckenstein et al., 2012; Niedelman et al., 2012)

encoded by virulent parasite strains, are noted for their ability to inhibit the destructive functions of IRGs at the PVM, including Irgb6 and Irga6 (Fleckenstein et al., 2012; Howard et al., 2011; Hunn et al., 2008; Niedelman et al., 2012; Reese et al., 2014). These rhoptry proteins also impact the association of GBPs with the PV (Selleck et al., 2013; Virreira Winter et al., 2011). Thus, we reasoned the host CD8 T cell response to TGD057 would be antagonized by some or all of these secreted effectors. Indeed, when ROP5 is not expressed in the type I RH strain (clade A, RH *Δrop5*), the CD8 T cell IFNγ and IL-2 response is robust (**Fig 2-7 and Fig 2-8A**), and on average, the IFNγ response is half of that elicited by the stimulatory type II strains (**Fig 2-7**). The *ROP5* locus consists of *ROP5A*, *ROP5B* and *ROP5C* genes, which differ in copy number between strains, and is under diversifying selection (Behnke et al., 2015, 2011; Niedelman et al., 2012; Reese et al., 2014, 2011; Reese and Boothroyd, 2011). ROP5B and ROP5C isoforms of virulent strains bind to and prevent accumulation of effector IRGs on the parasite's PVM, including Irga6 and Irgb6 (Fleckenstein et al., 2012; Niedelman et al., 2012; Reese et al., 2014), which are required for host resistance to primary infection (Lee et al., 2020; Liesenfeld et al., 2011). In contrast, ROP5A lacks a defined host binding partner and function, but does promote virulence by an unknown mechanism that is sensitive to copy number. For example, increasing copy number of *ROP5A* from one to two copies promotes virulence of RH *Δrop5* complementation strains, whereas RH *Δrop5+ROP5B+ROP5A* phenocopies the virulence of the parental RH strain (Reese et al., 2011). Therefore, a series of RH Δ*rop5* strains complemented with one or two copies of *ROP5B* or *ROP5A* isoforms from clade A, or a single *ROP5A* isoform from the type II genetic background (clade D) was analyzed. Importantly, all RH *Δrop5 +ROP5* complementation strains phenocopied the T57 response to the parental RH strain (**Fig 2-7**). Since ROP5A inhibits the T57 IFNγ response (**Fig 2-7**), we infer Irgb6 and Irga6 are likely not responsible for this phenotype because the ROP5A isoform does not inhibit Irgb6 or Irga6 coating of the PVM (Fleckenstein et al., 2012), nor Irga6 oligomerization (Reese et al., 2014). Consistent with this supposition, all *ROP5A* complementation strains failed to inhibit Irgb6-PV association (**Fig 2-7B and 2-7C**). Of note, the sequestosome-1 (p62) associates with the PV in an IFNγ-induced IRGM-dependent manner to promote OT1 T cell responses to OVA-expressing *T. gondii* strains (Rommereim et al., 2019). However, p62-PV association was not inhibited by ROP5A but instead phenocopied the PV-association patterns of Irgb6 (**Fig 2-7D and 2-7E**). Irgb6 has recently been shown to be required for p62-PV association in IFNγ-stimulated cells, which is consistent with these observations (Lee et al., 2020). The ROP18 (Fleckenstein et al., 2012) and ROP17 kinases (Etheridge et al., 2014) phosphorylate and inactivate Irga6 and Irgb6. Although there were slight increases in the T57 IFNγ response to the RH *Δrop17* and RH *Δrop18* strains, these responses were not significantly different compared to that of the parental RH

strain (**Fig 2-7**). Altogether, the data show that multiple ROP5 alleles and isoforms can suppress the T57 response to the TGD057 antigen, and this most likely by a mechanism independent of host Irgb6, Irga6 and p62 localization to the PV.

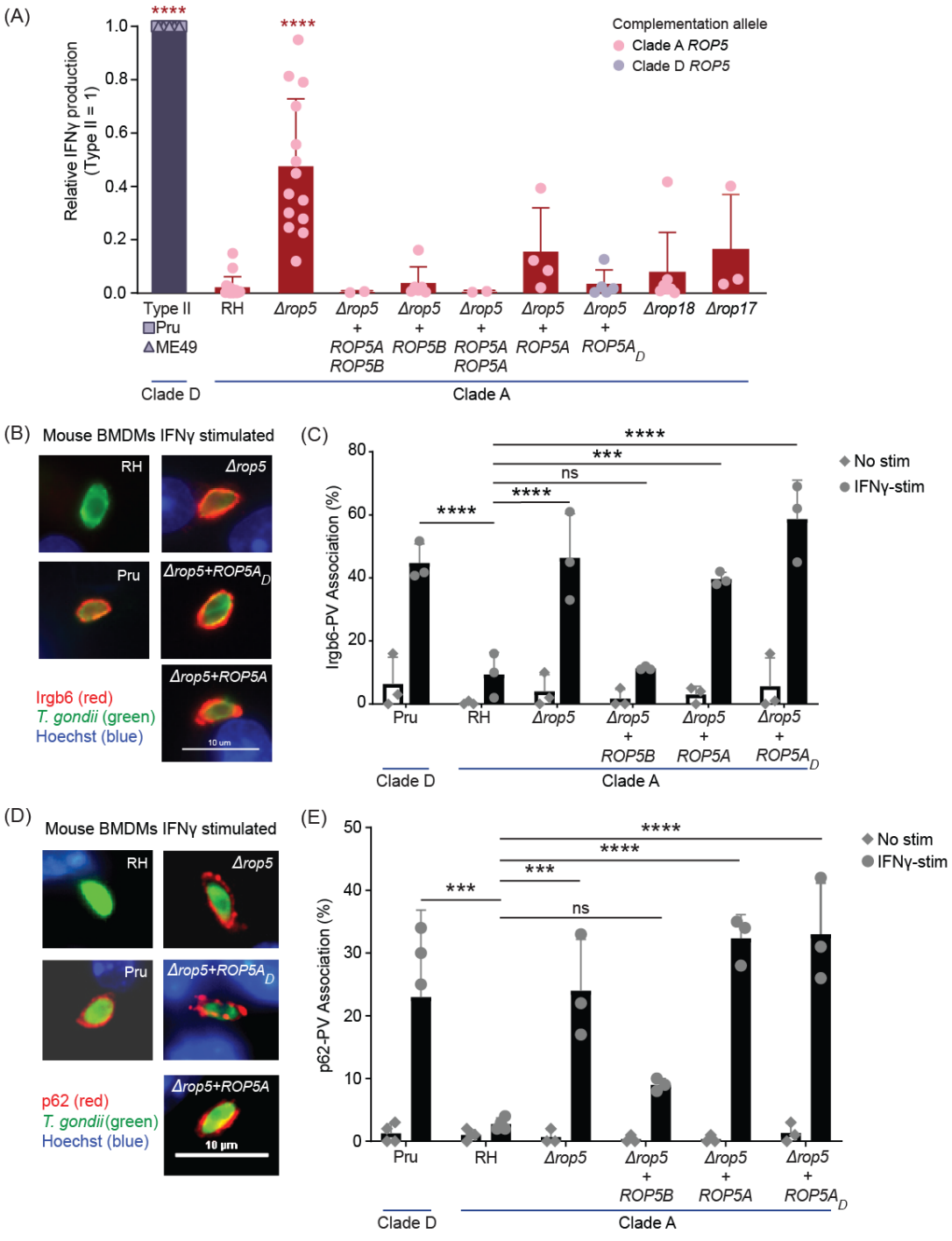

Figure 2-7. Multiple ROP5 isoforms inhibit TGD057-specific CD8 T cell IFNγ responses to *T. gondii*. (A) T57 CD8 T cell IFNγ responses to the RH and RH *Δrop5* strains, including various ROP5A and/or ROP5B complementation strains from clade A or from clade D (RH *Δrop5* +$ROP5A_D$), were analyzed as described in Fig 2-2A. Additionally, T57 IFNγ responses to RH *Δrop17* and RH *Δrop18* strains were determined. IFNγ was detected by ELISA at 48h and normalized to the response elicited by clade D strains, Pru (□) or ME49 (Δ). Average of 2–14 experiments + SD is shown, each dot represents the result from an individual experiment. Statistical analysis was performed using one-way ANOVA with Bonferroni's correction comparing all strains to the RH strain, only type II and RH *Δrop5* strains proved significantly different from RH over multiple experiments; **** $p \leq 0.0001$. **(B-E)** Untreated or IFNγ-stimulated BMDMs were infected with the indicated *T. gondii* strains. After 3–4 hours of infection, the samples were fixed and analyzed by fluorescence microscopy. *T. gondii* PVM was visualized in green with anti-GRA7 polyclonal rabbit antibodies and 100 vacuoles were quantified for each condition. **(B)** Representative images are shown of Irgb6-PV localization in parasite-infected IFNγ-stimulated BMDMs. Irgb6-PV localization was visualized in red with anti-Irgb6 polyclonal goat antibodies, and **(C)** plotted as percent of total GRA7+ *T. gondii* vacuoles. Plotted is the average + SD of 3 experiments. Statistical analysis was performed using two-way ANOVA with Bonferroni's correction; *** $p \leq 0.001$, **** $p \leq 0.0001$, ns non-significant; shown are statistical comparisons to the RH parental strain. **(D)** Representative images are shown of p62-PV localization in parasite-infected IFNγ-stimulated BMDMs. P62-PV localization was visualized in red with a mouse anti-p62 monoclonal antibody, and **(E)** plotted as percent of total $GRA7^+$ *T. gondii* vacuoles. Plotted is the average + SD of 3–4 experiments. Statistical analysis was performed using two-way ANOVA with Bonferroni's correction; *** $p \leq 0.001$, **** $p \leq 0.0001$, ns non-significant; shown are statistical comparisons to the RH parental strain.

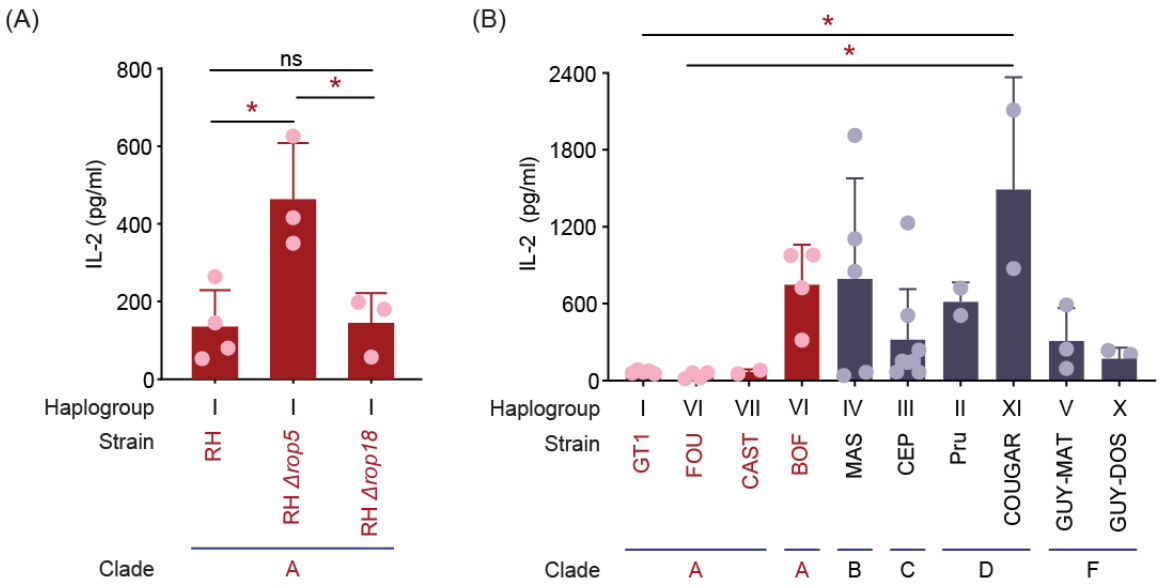

Figure 2-8. TGD057-specific CD8 T cell IL-2 responses to various strains of *T. gondii*. (A) BMDMs were infected with the indicated clade A RH, RH *Δrop5* and *Δrop18* strains and IL-2 was measured in the supernatant at 48h post addition of naïve T57 CD8 T cells. Plotted is the average + SD of 3 experiments. Statistical analysis was performed using one-way ANOVA with Bonferroni's correction; * $p \leq 0.05$. **(B)** BMDMs were infected with *T. gondii* strains—clonal (types I-III), atypical (HG IV-X), and HG XI—representative of various clades and haplogroups. Infected BMDMs were incubated with naïve T57 CD8 T cells for 48 hours and IL-2 concentration in supernatant was measured by ELISA. Each dot represents the result from an individual experiment and the averages + SD of 2–8 experiments per strain are shown. Statistical analysis was performed using one-way ANOVA with Bonferroni's correction; * $p \leq 0.05$.

2.3.5 ROP5-expressing clade A strains confer low TGD057-specific CD8 T cell IFNγ responses

Due to the conserved nature of the TGD057 peptide epitope (**Fig 2-1**), a unique opportunity arose to explore the development of CD8 T cell IFNγ responses to multiple parasite strains spanning the genetic diversity of *T. gondii* (Lorenzi et al., 2016). Any observed trend between *T. gondii* virulence, genetic background, and the T cell response may offer clues to possible parasite immune modulation and adaptation to immune pressure incurred by CD8 T cells. Among the Eurasian clonal strains (types I, II, III) and North American isolates from haplogroups (HG) XI and XII, the virulent type I strains (GT1, RH) induced the lowest CD8 T cell response while intermediate virulent types II (Pru, ME49), XI (COUGAR) and XII (B73) and low virulent type III (CEP) strains, induced relatively high CD8 T cell IFNγ (**Fig 2-2C and Fig 2-9A**) and IL-2 responses (**Fig 2-2B and Fig 2-8B**). 'Atypical' strains, many of which are endemic to South America and highly virulent in laboratory mice (FOU, CAST, MAS, TgCatBr5, P89, GUY-MAT, GUY-DOS, GUY-KOE, RUB, and VAND), differed dramatically in eliciting T57 cytokine responses (**Fig 2-9A and Fig 2-8B**), signifying that parasite virulence is not a sole predictor of CD8 T cell activation. Instead a unique phenotypic pattern emerged, in which clade A strains (type I, HG VI and VII) (Lorenzi et al., 2016) conferred low T57 cytokine responses while most other strains from clades B, C, D and F had potential to induce high cytokine responses (**Fig 2-9A and Fig 2-8B**). Consistent with previous results, T57 IFNγ responses did not correlate with known Irgb6- and/or Irga6-PV associations of these strains. For example, a low percentage (~10%) of Irgb6 recruitment to the PV is observed for the MAS strain, yet a high CD8 T cell IFNγ response is induced (**Fig 2-9A**), similar in magnitude to that of type II clade D strains (**Fig 2-2C and Fig 2-9A**) whose PVs are highly decorated with Irgb6 (~45%) (Niedelman et al., 2012). Even among highly virulent type I GT1 and Guyanan strains (i.e. GUY-KOE, GUY-MAT, GUY-DOS, VAND), where approximately 25% or less Irgb6-PV coating is observed (Niedelman et al., 2012), the CD8 T cell responses to these strains differ dramatically (**Fig 2-9A and Fig 2-8B**). Furthermore, one notable outlier among clade A strains is the relatively high T57 response to BOF (**Fig 2-9B and Fig 2-8B**). BOF encodes a single copy of *ROP5B* that is marginally expressed (Niedelman et al., 2012). When BOF is complemented with the LC37 cosmid, which encodes the entire ROP5 locus from the clade A type I genetic background, the CD8 T cell IFNγ response is largely reduced (**Fig 2-8B**). We infer from these assays the clade A genetic background inhibits T57 IFNγ responses, but the identity of the ROP5-host interacting partner and why this genetic background leads to repressed responses is currently unknown.

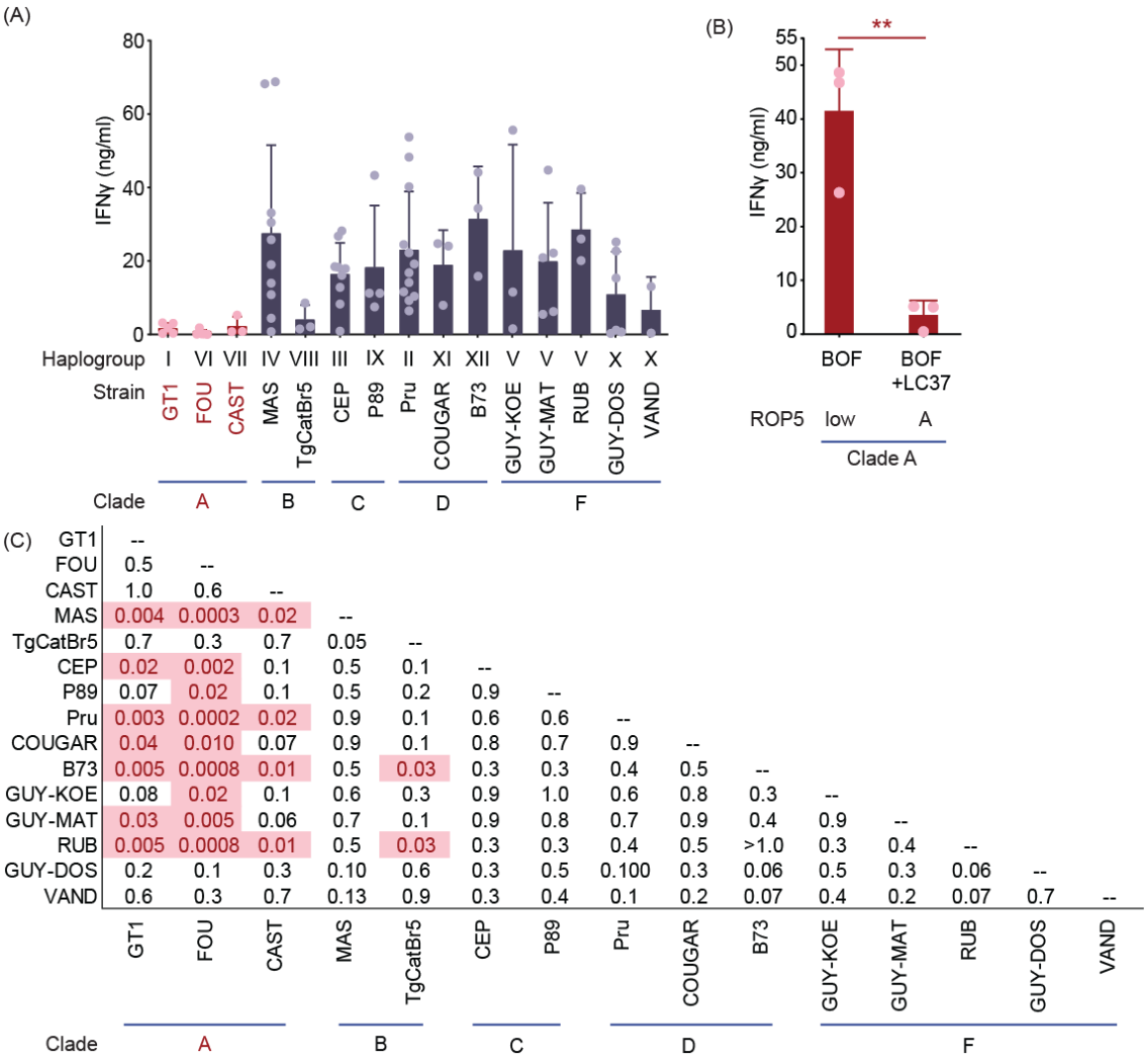

Figure 2-9. Low CD8 T cell IFNγ responses to ROP5-expressing clade A strains of *T. gondii*. **(A)** The T57 CD8 T cell IFNγ response to BMDMs infected with various *T. gondii* strains from most haplogroups (HG) were analyzed, including the clonal (types I-III), atypical (HG IV-X), and HG XI and XII strains. IFNγ in the supernatant was measured by ELISA at 48h. Average of 2–12 experiments + SD for each strain is shown, each dot represents a single experiment. **(B)** The clade A BOF strain, which encodes a lowly expressed single *ROP5B* gene ('low') and BOF complemented with an LC37 cosmid that expresses the entire clade A *ROP5* locus ('A') were assayed as described in Fig 2-2A. Average IFNγ detected in the supernatant at 48h of 3 experiments + SD is shown. Statistical analysis was performed by an unpaired two-tailed t-test; ** $p \leq 0.01$. **(C)** Statistical analysis of the T57 CD8 T cell IFNγ response differences observed to parasite strains from clades A-F was performed using a Kruskal-Wallis non-parametric test with Dunn's correction. Calculated p-values are shown for each strain by strain comparison; p-values ≤ 0.05 are highlighted in red and considered significant. As low inducers of IFNγ, all clade A strains, as well as TgCatBr5 from clade B, produced statistically significant differences with at least two other parasite strains.

2.3.6 Activation and IFNγ differentiation of CD8 T cells are only partially inhibited by T. gondii ROP5

Following antigen-driven TCR stimulation (or 'signal 1'), early activated T cells receive secondary cues from the environment including co-stimulation ('signal 2') and cytokines ('signal 3') to commit to the production of cytokines such as IFNγ. Whether clade A strains, through ROP5 or other effectors, intersect one or several of these activation steps to lower T57 IFNγ responses is unclear. To explore this issue further, we generated a 'T-GREAT' IFNγ reporter mouse line by crossing T57 with GREAT mice (Reinhardt et al., 2015). GREAT mice report IFNγ transcription with an internal ribosomal entry site (IRES)-eYFP reporter cassette inserted between the stop codon and endogenous 3'UTR with the poly-A tail of the *Ifng* gene. Without abrogating translation of IFNγ, GREAT mice allow faithful detection of *Ifng* transcription via YFP fluorescence and flow cytometry (Reinhardt et al., 2015). In addition, surface expression of the activation marker CD69 is a proxy for early TCR signaling events, and is one of the first markers expressed by naïve T lymphocytes after activation (Cebrián et al., 1988). In this way, the relative amount of TGD057 that has escaped the PV and ultimately presented by MHC I molecules can be inferred by T cell upregulation of CD69, and this can be measured independently of IFNγ transcription in T-GREAT cells. Naïve T-GREAT cells were co-cultured with BMDMs infected with RH (clade A), RH *Δrop5*, and ME49 (clade D) strains (**Fig 2-10A**), and the frequency of activated CD8 T cells (CD62L-CD69+ CD8+ T cells) (**Fig 2-10B**) as well as YFP levels (*Ifng*:YFP+ of CD62L-CD69+ CD8+ T cells) (**Fig 2-10C**) were measured by flow cytometry at 18 hours. Without parasite, few CD69+ or *Ifng*:YFP+ T-GREAT CD8 T cells were detected in the co-culture, consistent with their naïve beginnings (**Fig 2-10B and Fig 2-10C**). In contrast and over a range of multiplicity of infections (MOIs), there was approximately three-fold more CD69+ CD8 T cells elicited by the ME49 compared to the RH strains (**Fig 2-10B and Fig 2-10D**). Among T cells that have been activated (CD69+), a comparison of the *Ifng* transcript level revealed a six-fold increase in response to the ME49 compared to the RH strain (**Fig 2-10 and Fig 2-10E**). Although removal of ROP5 enhances the activation and differentiation of T-GREAT cells to the RH *Δrop5* strain, *Ifng* transcript levels never equaled that elicited by ME49, especially at lower MOIs (**Fig 2-10E**). Therefore, the data show T57 cells do in-fact recognize the RH strain, but once activated this genetic background fails to elicit other signals necessary for full induction of the T57 IFNγ response. Moreover, the data implicate *T. gondii* genetic determinants other than ROP5 intersect CD8 T cell IFNγ responses at the activation and likely differentiation steps.

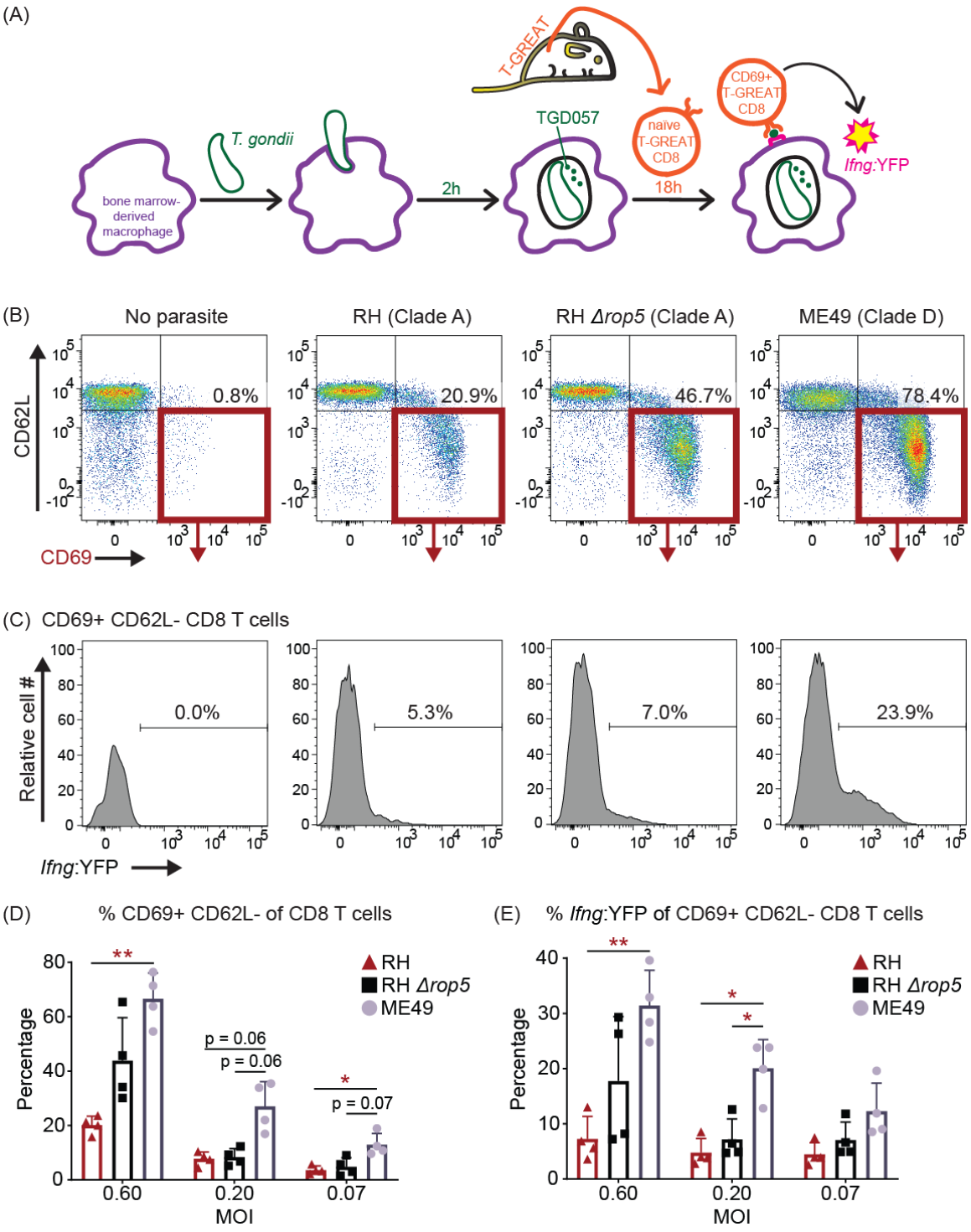
(A)
T-GREAT
T. gondii
TGD057
naïve T-GREAT CD8
18h
CD69+ T-GREAT CD8
Ifng:YFP
bone marrow-derived macrophage
2h
(B)
No parasite
RH (Clade A)
RH Δrop5 (Clade A)
ME49 (Clade D)
0.8%
20.9%
46.7%
78.4%
CD62L
CD69
(C) CD69+ CD62L- CD8 T cells
Relative cell #
0.0%
5.3%
7.0%
23.9%
Ifng:YFP
(D) % CD69+ CD62L- of CD8 T cells
Percentage
MOI
RH
RH Δrop5
ME49
p = 0.06
p = 0.07
(E) % Ifng:YFP of CD69+ CD62L- CD8 T cells

Figure 2-10. In the absence of ROP5, clade A strains still inhibit TGD057-specific CD8 T cell activation and IFNγ production. (A) 'T-GREAT' reporter mice were generated by crossing T57 mice with an IFNγ (IRES)-eYFP reporter GREAT mouse line, which allows IFNγ transcript levels to be measured by flow cytometry as a function of YFP expression. T-GREAT cells were analyzed for activation (CD69+) and IFNγ-differentiation (*Ifng*:YFP+) in response to parasite-infected BMDMs at 18h. **(B)** The frequency of activated CD8 T cells (CD69+ CD62L- CD8+ T cells), as well as **(C)** the frequency of YFP+ (*Ifng*:YFP+) cells among activated CD69+ CD62L- CD8 T cells were compared between clade A RH, RH *Δrop5*, and clade D ME49 strains. Representative flow plots with indicated gates and percentages are shown. **(D)** Percent CD69+ CD62L- of total CD8 T cells, and **(E)** percent *Ifng*:YFP+ of CD69+ CD62L- CD8 T cells are shown. Each dot represents the results of an individual experiment and plotted is the average + SD of 4 experiments. Statistical analyses were performed using two-way ANOVA with Bonferroni corrections; * $p \leq 0.05$, ** $p \leq 0.01$.

2.3.7 IFNγ-production by TGD057-specific CD8 T cells does not solely depend on IL-12

IL-12 signaling is essential for IFNγ-mediated control of *T. gondii* (Ely et al., 1999; Scharton-Kersten et al., 1996; Shaw et al., 2003), and is required for full induction of IFNγ-producing KLRG1+ effector CD8 T cells following *T. gondii* type II infections *in vivo* (Wilson et al., 2010, 2008). Moreover, the RH strain fails to induce robust IL-12 secretion in infected macrophages (Kim et al., 2006; Robben et al., 2004), perhaps underpinning the low T57 IFNγ responses to clade A strains observed in this system. To understand what extent IL-12 influences IFNγ-production, the T57 assay was performed with IL-12p40 deficient *Il12b-/-* BMDMs. The T57 IFNγ response to ME49- (**Fig 2-11A**), or MAS-infected *Il12b-/-* BMDMs (**Fig 2-11B**) was reduced but not entirely abrogated compared to that of infected WT BMDMs. A partial reduction was also reported for adoptively transferred *Il-12rβ2-/-* CD8 T cells that specifically lack IL-12 signaling during primary infection (Wilson et al., 2008). Next, the T57 assay was performed with parasite strains known to regulate host IL-12 production. Three *T. gondii* effector proteins—GRA15, GRA24 and ROP16—modulate IL-12 production in infected BMDMs (Braun et al., 2013; Butcher et al., 2011; Jensen et al., 2011; Rosowski et al., 2011). Polymorphisms in GRA15 and ROP16 largely account for parasite strain differences in alternative (M2) and classical activation (M1) of macrophages (Jensen et al., 2011). Specifically, polymorphisms in GRA15 render type II strains able to activate the NF-κB pathway through direct association with TRAF2 and TRAF6 (Sangaré et al., 2019), and its expression is an absolute requirement for IL-12p70 (Jensen et al., 2011) and largely responsible for IL-12p40 production by type II-infected BMDMs (Rosowski et al., 2011). Through activation of host p38 MAPK, GRA24 promotes IL-12p40 and chemokine secretion by *T. gondii*-infected BMDMs (Braun et al., 2013). Although no consistent difference between parental type II (Pru) and GRA15-deficient or GRA24-deficient strains was observed, T57

IFNγ production was decreased but not abolished in response to a double deletion Pru *Δgra15 Δgra24* strain (**Fig 2-11C**). With respect to ROP16, in all *T. gondii* strains except those of clade D (Melo et al., 2013), the ROP16 kinase activates host STAT3, STAT5, STAT6 transcription factors (Jensen et al., 2013; Ong et al., 2010; Saeij et al., 2007; Yamamoto et al., 2009), leading to the suppression of NF-κB signaling by an unknown mechanism (Jensen et al., 2013; Saeij et al., 2007). When activating alleles of ROP16 are expressed as a transgene within the type II strain (Pru *+ROP16*$_A$), it reduces IL-12 production in *T. gondii*-infected BMDMs and induces the expression of many M2 associated genes (Jensen et al., 2011). The T57 IFNγ response to the Pru *+ROP16*$_A$ was reduced to half that of the parental strain (**Fig 2-11D**). Thus, although IL-12 and *T. gondii* GRA15, GRA24, and ROP16 have some impact in regulating TGD057-specific CD8 T cell IFNγ-production, the IL-12 axis does not fully account for IFNγ commitment in this system.

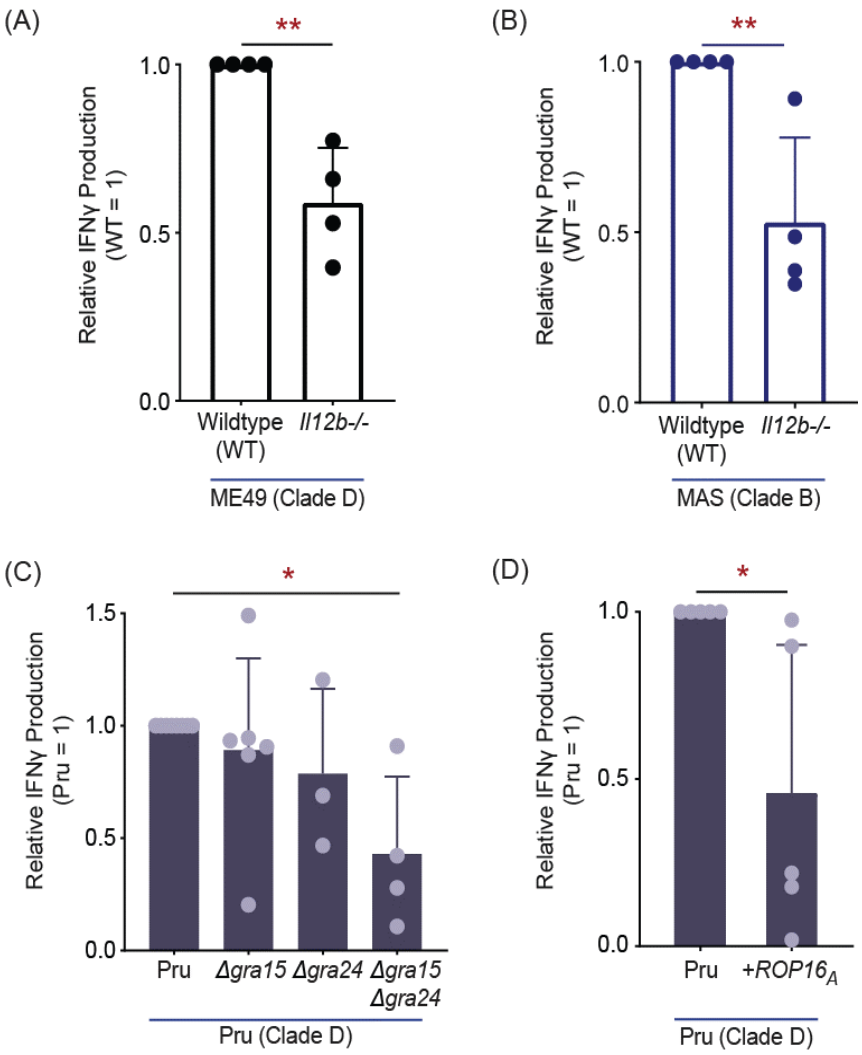

Figure 2-11. IL-12 signaling is partially required for TGD057-specifc CD8 T cell IFNγ responses. **(A)** *Il12b-/-* (IL-12p40) BMDMs were infected with clade D ME49, or **(B)** clade B MAS and TGD057-specific CD8 T cell IFNγ responses were measured as described in Fig 2-2A. Each dot represents the result of an individual experiment and the average of 4 experiments + SD is shown; statistical analysis was performed with an unpaired two-tailed t-test; ** $p \leq 0.01$. **(C)** Various Pru (clade D) gene deletion strains, *Δgra15*, *Δgra24*, and *Δgra15 Δgra24*, or **(D)** a Pru strain transgenically expressing clade A ROP16 (Pru +$ROP16_A$) were assayed for TGD057-specific CD8 T cell IFNγ responses. The IFNγ response, as analyzed by ELISA, is normalized to that induced by the wildtype Pru strain. Each dot represents the result of an individual experiment and the average of 3–6 experiments + SD is shown. Statistical analysis was performed using one-way ANOVA with Bonferroni's correction for (C), and an unpaired two-tailed t-test for (D); * $p \leq 0.05$.

2.3.8 An inflammasome-independent NLRP3 pathway is required for maximal CD8 T cell IFNγ responses to T. gondii

Both NLRP3 and NLRP1 inflammasome activation occur following *T. gondii* infection (Cirelli et al., 2014; Ewald et al., 2014; Gorfu et al., 2014), and IL-1 and IL-18 are known regulators of IFNγ production in a variety of cell types (Afonina et al., 2015), including cells of the adaptive immune system (Evavold and Kagan, 2018). Therefore, BMDMs deficient at various steps in the inflammasome activation cascade were analyzed. In brief, NLRP proteins undergo ASC-driven oligomerization, causing auto-activation of caspase-1, that in turn lead to the cleavage and maturation of IL-1β and IL-18 (Broz and Dixit, 2016). Inflammasome activated caspase-1 and -11 also activate Gasdermin D, a key pore forming protein responsible for pyroptosis and extracellular release of IL-1/18 in several biological contexts (Ding et al., 2016; Evavold et al., 2018; He et al., 2015; Kayagaki et al., 2015; Liu et al., 2016; Shi et al., 2015). Therefore, BMDMs deficient at various steps in the inflammasome activation cascade were analyzed. The T57 IFNγ response was largely reduced to parasite-infected *Nlrp1-/-* BMDMs, and completely absent to infected *Nlrp3-/-* BMDMs (**Fig 2-12A and Fig 2-12B**). In contrast, the IFNγ response was only partially decreased to *Asc-/-*, *Casp1/11-/-*, and *Gsdmd-/-* BMDMs infected with ME49 (**Fig 2-12A**), and no consistent difference was observed between knockout and WT BMDMs infected with MAS (**Fig 2-12B**). These results indicate CD8 T cell IFNγ differentiation, though entirely dependent on NLRs, only partially involves inflammasome matured IL-1 and/or IL-18. Consistent with this supposition, the CD8 T cell IFNγ response to parasite-infected macrophages is reduced but not abrogated in the absence of IL-18 and IL-1R-signaling, as assessed with neutralizing antibodies that block IL-1β and IL-1α engagement with its receptor, IL-1R, and *Il18-*/- BMDMs (**Fig 2-12C**). Low levels of IL-1β and IL-18 detected in the co-culture may underscore the limited role these cytokines play in promoting IFNγ responses in this system (**Fig 2-3B**).

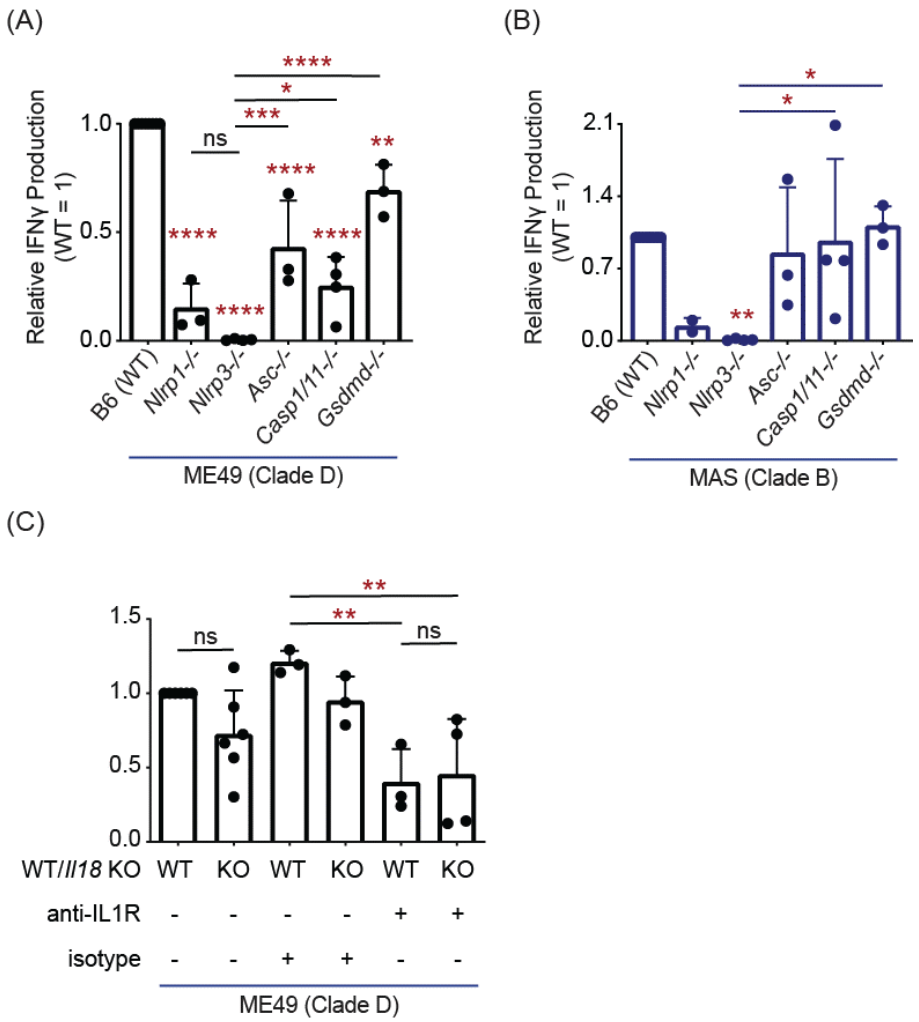

Figure 2-12. An inflammasome-independent NLR pathway promotes CD8 T cell IFNγ responses to *T. gondii*. **(A)** BMDMs with indicated gene deletion (-/-) were infected with the clade D ME49, or **(B)** clade B MAS strain. The T57 CD8 T cell IFNγ response to TGD057 was analyzed by ELISA and normalized to that of wildtype (WT) BMDMs. Each dot represents the result of an individual experiment, and the average of 2–4 experiments + SD is shown. Statistical analysis was performed using one-way ANOVA with Bonferroni's corrections compared to infected WT or *Nlrp3*-/- BMDMs, the latter comparisons are indicated with a line; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, ns non-significant. **(C)** WT and *Il18*-/- BMDMs were infected with the clade D ME49 and assayed for CD8 T cell T57 IFNγ production. Additionally, co-cultures were treated with either anti-IL-1R neutralization or isotype control antibodies. The IFNγ level was measured by ELISA and normalized to that of untreated WT BMDMs. Each dot represents the results from an individual experiment and the average of 3–6 experiments + SD is shown. Statistical analysis was performed with one-way ANOVA and Bonferroni's corrections compared to untreated WT BMDMs; ** $p \leq 0.01$, ns non-significant.

Since the inflammasome pathway regulates the T57 CD8 T cell IFNγ response, we explored additional host components that intersect the inflammasome including the upstream activator of NLRP3, the P2X7 receptor (P2X7R), as well as the cytosolic NOD1/2 sensors which triggers NF-κB activation and pro-IL-1 induction. P2X7R induces potassium (K^+) efflux from the cytosol when extracellular ATP (eATP) is present (Amores-Iniesta et al., 2017; Eltzschig et al., 2012; Jo et al., 2016). K^+ efflux is a requirement for NLRP3 oligomerization following stimuli with several known NLRP3 agonists and is the main mechanism for P2X7R-dependent NLRP3 inflammasome activation (Elliott and Sutterwala, 2015; He et al., 2016). Signaling through P2X7R can also induce NLRP1 inflammasome activation (Silverman et al., 2009; Yap et al., 2019). Our results show that the T57 IFNγ response to ME49-infected WT and *P2x7r-/-* BMDMs are similar (**Fig 2-13**), suggesting that P2X7R signaling is not a requirement for the CD8 IFNγ response. Inflammasome activation also requires 'signal 1', or the NF-κB mediated priming step. The priming step upregulates NLRP3 as well as pro-IL-1β expression (Bauernfeind et al., 2009). NOD2 can oligomerize with RIPK2 and form the NODosome (Keestra and Bäumler, 2014; Tattoli et al., 2007), leading to the activation of the NF-κB pathway and production of NF-κB-dependent factors such as pro-IL-1β, pro-IL-18 and others. NOD2 had been shown to be important for *T. gondii* control; there is less IFNγ production as well as increased parasite burden and dissemination in *T.gondii*-infected *Nod2-/-* mice (Heimesaat et al., 2014; Shaw et al., 2009). To examine whether NOD-RIPK2 pathway plays a role in CD8 T cell-mediated IFNγ response, we assessed this response for ME49-infected *Nod2-/-* and *Ripk2-/-* BMDMs. The T57 IFNγ response to infected *Nod2-/-* BMDMs is reduced compare to the infected WT BMDMs, while the response to infected *Ripk2-/-* BMDMs is entirely abrogated (**Fig 2-14**). This suggests that there may be a RIPK2-NF-κB axis required for the T57 CD8 IFNγ response that is different from the induction of pro-IL-1β and pro-IL-18, consistent with the low IL-1β and IL-18 detected in this system (**Fig 2-3B**).The identity of the RIPK2-dependent host factor required for T57 CD8 IFNγ responses is unknown.

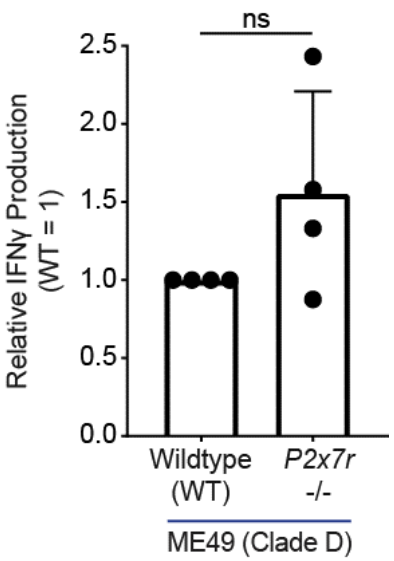

Figure 2-13. P2X7R is not required for CD8 T cell-mediated IFNγ responses. *P2x7r-/-* BMDMs were infected with clade D ME49 and TGD057-specific CD8 T cell IFNγ responses was assessed as described in Fig 2-2A. The IFNγ response was normalized to the response to ME49-infected WT BMDMs. Each dot represents the result of an individual experiment. The average of 4 experiments + SD is shown; statistical analysis was performed with an unpaired two-tailed t-test; ns.

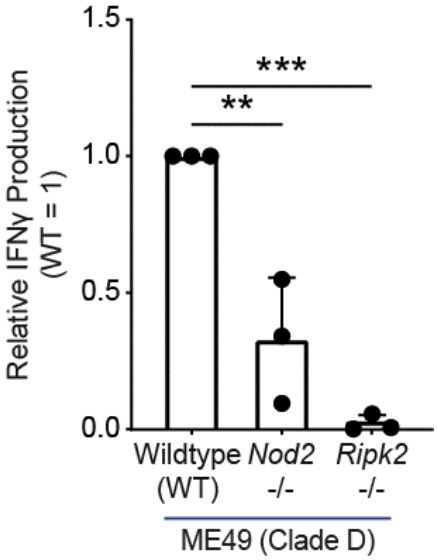

Figure 2-14. NOD-RIPK2 pathway is important for CD8 T cell-mediated IFNγ responses. *Nod2-/- and Ripk2-/-* BMDMs were infected with clade D ME49. The TGD057-specific CD8 T cell IFNγ responses were measured as previously described in Fig 2-2A. The IFNγ response was normalized to that of ME49-infected WT BMDMs. Each dot represents the result of an individual experiment and the average of 3 experiments + SD is shown. Statistical analysis was performed using one-way ANOVA with Bonferroni's correction; ** $p \leq 0.01$, *** $p \leq 0.001$.

Finally, to test whether NLRP3 impacts T cell activation or differentiation, T-GREAT CD8 T cell activation profiles to parasite-infected *Nlrp3-/-* BMDMs were explored. Whereas the percentage of early activated CD69+ T-GREAT cells in response to ME49 and MAS infections was slightly decreased to *Nlrp3-/-* compared to WT BMDMs (**Fig 2-15A**), IFNγ transcript levels in the activated CD69+ population dropped by 50–70% (**Fig 2-15B**), suggesting NLPR3 induces a macrophage-derived signal required for IFNγ transcription in activated CD8 T cells. Co-stimulation determines transcriptional regulation of IFNγ in tumor-infiltrating T cells (Salerno et al., 2019), and in its absence, enforces post-transcriptional silencing of IFNγ in anergic self-reactive T cells (Villarino et al., 2011), perhaps underscoring the blunted transcriptional (**Fig 2-15B**) and translational CD8 T cell

IFNγ response to parasite-infected *Nlrp3*-/- BMDMs (**Fig 2-12**). To determine whether NLRP3 regulates co-stimulatory pathways, a variety of co-stimulatory ligands and the PD-L1 inhibitory receptor were measured on infected BMDMs. As previously demonstrated for B7 family members CD80, CD86 (Morgado et al., 2014), PD-L1 (Jensen et al., 2011), and as demonstrated here, ICOSL, are readily induced on the surface of infected BMDMs (**Fig 2-15C**). Receptors and ligands of the TNF superfamily are also expressed transcriptionally following infection in BMDMs including CD70, CD40, OX40L and 41BBL (Jensen et al., 2011), of which surface expression of OX40L appears most sensitive to induction by *T. gondii* infection (**Fig 2-15C and Fig 2-16B**). However, none of the measured receptors or ligands bore evidence for NLRP3-dependent regulation (**Fig 2-15D and Fig 2-16C**), nor does loss of NLRP3 influence MHC I K^b expression (**Fig 2-16C**), as has been described for NLRC5-dependent induction of MHC class 1 genes (Meissner et al., 2010). To summarize, although inflammasome matured cytokines may play some role in promoting T57 IFNγ production, there is no substitute for NLRP3, and to a similar extent NLRP1 in this system. Importantly, our data identify a novel NLR-dependent but NLR-ASC inflammasome complex-independent pathway that regulates CD8 T cell IFNγ responses to an intracellular pathogen.

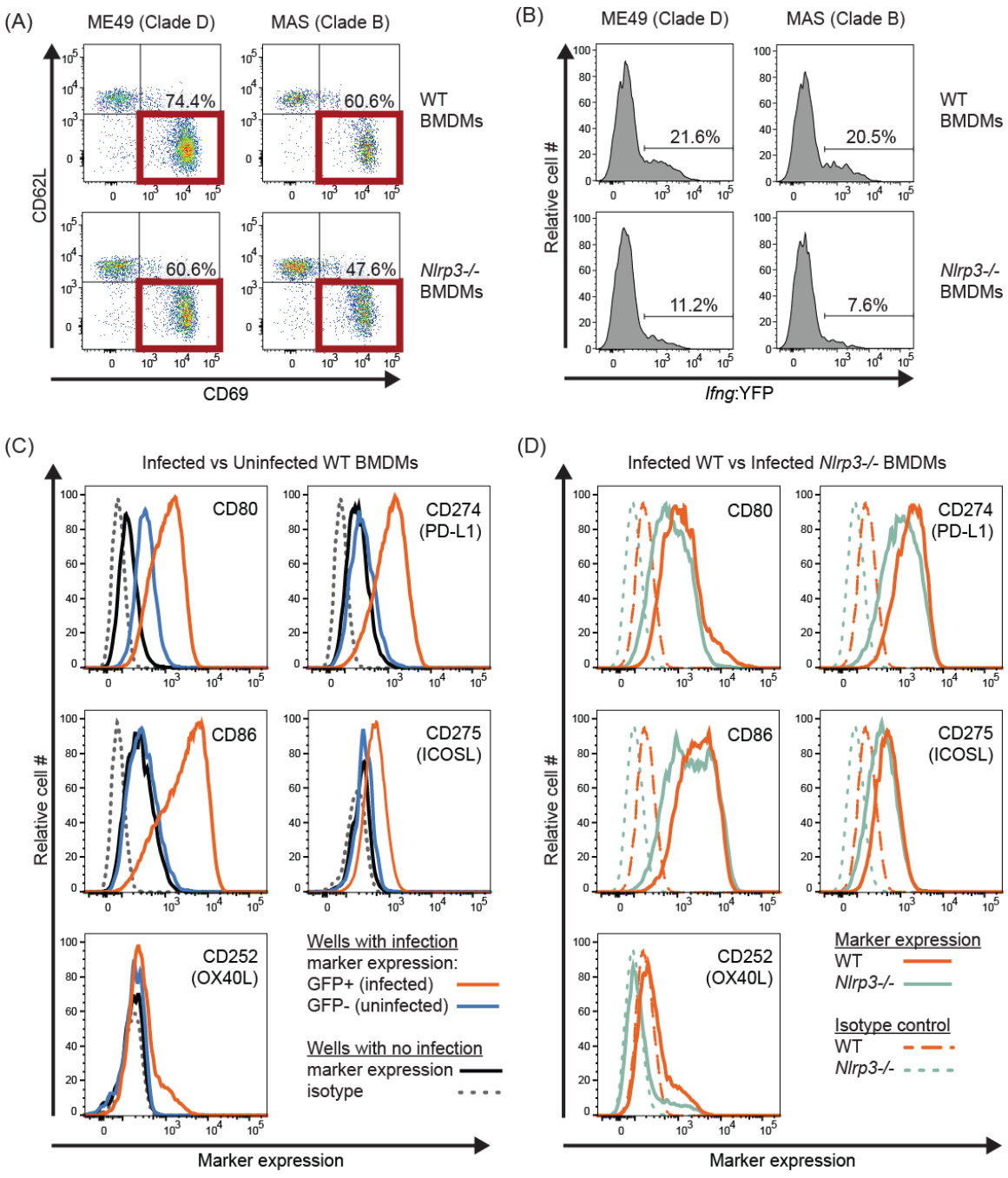
(A)
ME49 (Clade D)
MAS (Clade B)
74.4%
60.6%
WT BMDMs
60.6%
47.6%
Nlrp3-/- BMDMs
CD62L
CD69
(B)
ME49 (Clade D)
MAS (Clade B)
21.6%
20.5%
WT BMDMs
11.2%
7.6%
Nlrp3-/- BMDMs
Relative cell #
Ifng:YFP
(C)
Infected vs Uninfected WT BMDMs
CD80
CD274 (PD-L1)
CD86
CD275 (ICOSL)
CD252 (OX40L)
Relative cell #
Wells with infection
marker expression:
GFP+ (infected)
GFP- (uninfected)
Wells with no infection
marker expression
isotype
Marker expression
(D)
Infected WT vs Infected Nlrp3-/- BMDMs
CD80
CD274 (PD-L1)
CD86
CD275 (ICOSL)
CD252 (OX40L)
Relative cell #
Marker expression
WT
Nlrp3-/-
Isotype control
WT
Nlrp3-/-
Marker expression

Figure 2-15. Low IFNγ transcriptional CD8 T cell responses to *T. gondii*-infected *Nlrp3-/-* BMDMs are not due to dysregulated co-stimulatory pathways. (A-B) Wildtype (WT) and *Nlrp3-/-* BMDMs were infected with clade D ME49 or clade B MAS strains. T-GREAT CD8 T cell responses to parasite-infected BMDMs were analyzed as described in Fig 2-10. **(A)** The frequency of activated CD8 T cells (CD69+ CD62L- CD8+ T cells), and **(B)** the frequency of YFP+ (*Ifng*:YFP+) cells among activated CD69+ CD62L- CD8 T cells were determined. Representative flow plots with indicated gates and frequencies are shown from 3–4 experiments. **(C-D)** WT and *Nlrp3-/-* BMDMs were infected with a GFP-expressing Pru strain (Pru A7) and stained for the indicated co -stimulatory and -inhibitory molecules at 18h. **(C)** Surface marker expression of infected (GFP+) and uninfected (GFP-) WT BMDMs from an infected well, and of BMDMs from uninfected control wells are all compared in a single histogram plot; the isotype staining control is also indicated. A representative histogram plot from 2–3 independent experiments is shown for each marker. The gating strategy is depicted in Fig 2-16A. **(D)** As in C, but infected WT and *Nlrp3-/-* BMDMs (GFP+) are compared; isotype staining control of infected cells is also indicated. A representative histogram plot of 2–3 independent experiments is shown for each marker.

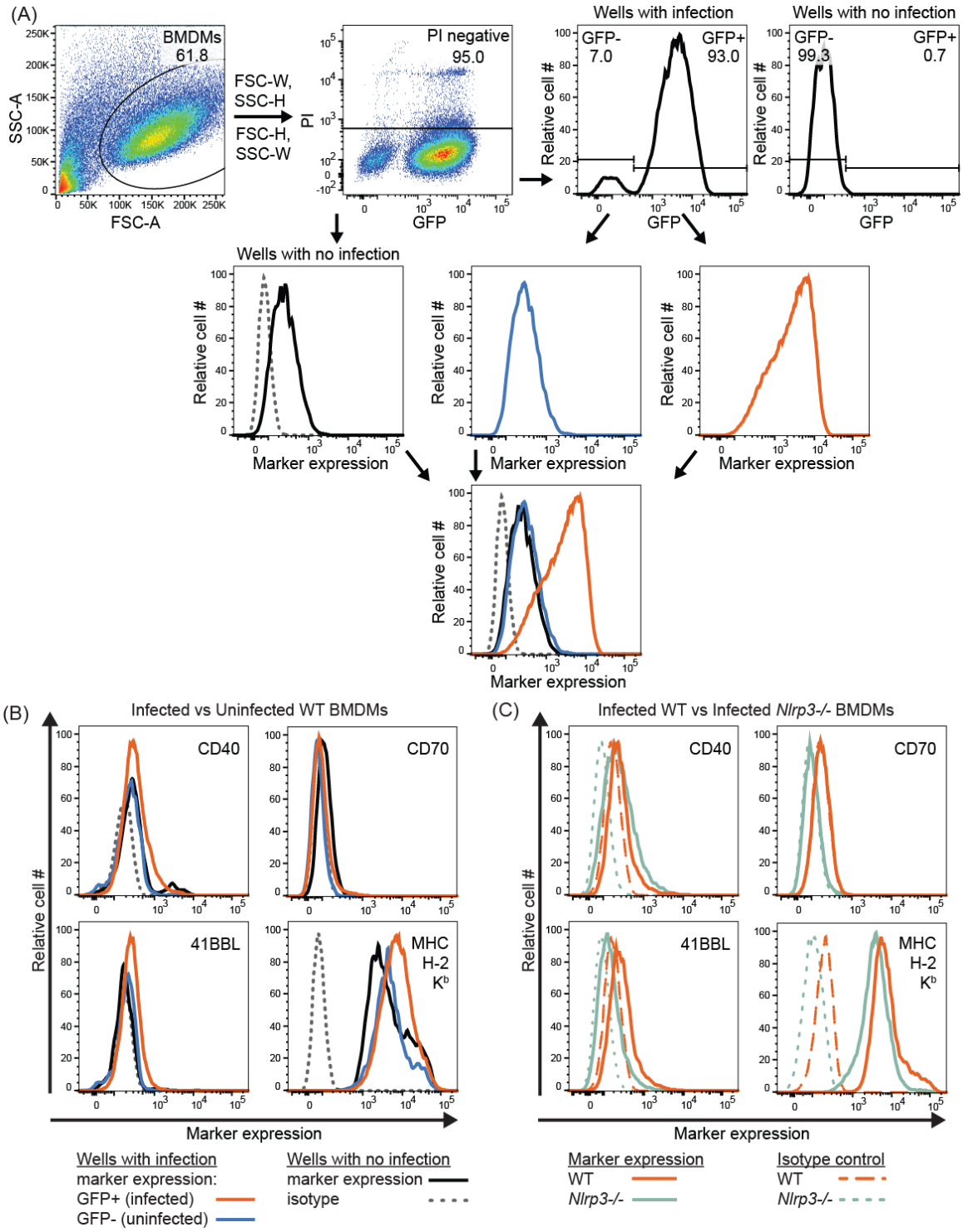
(A)
BMDMs
61.8
SSC-A
FSC-A
FSC-W,
SSC-H
FSC-H,
SSC-W
PI negative
95.0
PI
GFP
Wells with infection
GFP-
7.0
GFP+
93.0
Wells with no infection
GFP-
99.3
GFP+
0.7
Relative cell #
Wells with no infection
Marker expression
(B)
Infected vs Uninfected WT BMDMs
CD40
CD70
41BBL
MHC
H-2
Kb
Marker expression
Wells with infection
marker expression:
GFP+ (infected)
GFP- (uninfected)
Wells with no infection
marker expression
isotype
(C)
Infected WT vs Infected Nlrp3-/- BMDMs
Marker expression
WT
Nlrp3-/-
Isotype control
WT
Nlrp3-/-

Figure 2-16. Surface expression of MHC I and several co-stimulatory molecules are not impaired in *T. gondii*-infected *Nlrp3-/-* BMDMs. **(A)** Gating strategy for flow cytometry analysis of co-stimulatory molecules expressed by infected BMDMs. BMDMs were infected with a GFP-expressing *T. gondii* strain or left uninfected, and later stained with fluorescently labeled marker-specific antibodies. The BMDMs were gated on forward and side scatter, and infected (GFP+) or uninfected (GFP-) live (PI-) BMDMs, shown with indicated frequencies, were then analyzed for the expression of co-stimulatory molecules. **(B-C)** The surface expression of co-stimulatory molecules and MHC I K^b were analyzed as described in Fig 2-15C and Fig 2-15D, and compared **(B)** between infected (GFP+) and uninfected (GFP-) BMDMs, as well as **(C)** between infected *Nlrp3-/-* and WT BMDMs (GFP+). Histogram plots are representative of 2–3 experiments.

2.4 Discussion

In this work, we set out to explore the role of parasite virulence factors, and host pathways that regulate CD8 T cell IFNγ responses to an endogenous antigen. Given the close association between T cell activation and parasite-infected cells *in vivo* (Dupont et al., 2014), and the immune pressure incurred by CD8 T cells, we reasoned there may be unidentified mechanisms governing host-pathogen interactions between *T. gondii* and this cell type. To this end, we used T57 transnuclear mice to develop a system to study this interaction, which has several advantages including the conserved nature of the TGD057 epitope and the ability to analyze the first moments of cytokine induction by an antigen-specific clonal CD8 T cell population with a normally expressed TCR. Without need for further antigen cloning, certain matters surrounding CD8 T cell IFNγ differentiation and MHC I antigen presentation of TGD057 were revealed, including a fundamental role for *T. gondii* ROP5 and host NLRP3 in regulating this response. Our observations are both similar and divergent from results obtained in other systems, pointing to the contextual nature of immune responses to live pathogens, but also to new host mechanisms that possibly promote CD8 T cell immunity to *T. gondii*.

First, many similarities exist between the CD8 T cell response to TGD057 and OVA. For example, CD8 T cell activation to both antigens utilize the host's IRG system for optimal responses (Dzierszinski et al., 2007; Lee et al., 2015; Rommereim et al., 2019). Such results indicate a need for the host to actively acquire antigens sequestered inside a PV (Jensen, 2016). We lend credence to this hypothesis, in that the parasite's export machinery appears dispensable for inducing TGD057-specific CD8 T cell responses (**Fig 2-4**). One curious observation in this regard, is that the TGD057 peptide epitope, SVLAFRRL, encodes the lone putative ASP5 recognition TEXEL motif in this protein (underlined) (**Fig 2-1**). In fact, ASP5 cleavage would preferentially produce parasite peptides with a terminal leucine, which is the preferred anchor residue of the P8/9 peptide binding pocket of MHC I K^b (Koch et al., 2013) and many other murine MHC and human HLA alleles. We initially thought ASP5 might

prepare *T. gondii* antigens for binding host MHC I molecules, such that in the absence of ASP5, a blunted CD8 T cell response would ensue. Rather, the opposite occurred (**Fig 2-4**), ruling against parasite-assisted antigen processing of TGD057. However, beyond containing an RRL sequence, there is no evidence that TGD057 is actually cleaved by ASP5. In the original characterization of TGD057, using polyclonal rabbit serum generated against recombinant TGD057, only one band of predicted size (21 kDa) was observed (Wan and Ajioka, 2004). For known dense granules cleaved by ASP5, the protein migrates at a lower molecular weight than predicted and often two or more species can be observed by western blot analysis, including that of GRA7, GRA16, GRA19 (Hammoudi et al., 2015), IST (Gay et al., 2016), MYR1 (Coffey et al., 2015), LCAT, GRA44, GRA45, GRA46, and WNG2 (Coffey et al., 2018). Studies that utilized unbiased mass-spectrometry approaches to discover the repertoire of *T. gondii* proteins cleaved by ASP5 have failed to detect TGD057 as a substrate (Coffey et al., 2018). Moreover, of the over 300 mass spectra counts assigned to TGD057 from several proteomics studies (ToxoDB.org), not a single TGD057 peptide contains an intact RRL or TTSA sequence (C-terminal to the RRL). Our own western blot analysis, using a polyclonal α-TGD057 antibody (gift from Nicolas Blanchard, INSERM) which recognizes two peptide sequences of TGD057 (**Fig 2-1A**) flanking the potential ASP5 cleavage site (**Fig 2-1A**), demonstrated a single 20 kDa species of TGD057 (**Fig 2-5**), a result consistent to that of the uncleaved TGD057 previously shown (Lopez et al., 2015; Wan and Ajioka, 2004). In addition to its role in protein export, ASP5 is required for targeting dense granules to the PVM (Coffey et al., 2015; Hammoudi et al., 2015; Wang et al., 2019). GRA6 association with the PVM significantly enhances its entry into the host's MHC I antigen presentation pathway, yet TGD057 is detected only in the non-membranous and soluble fraction of the PV (Lopez et al., 2015). Whether TGD057 sub-localization inside the PV impacts entry into the host's MHC I antigen presentation pathway is currently unknown. The enhanced response to the type II *Δasp5* strain (**Fig 2-4**) may also reflect decreased parasite fitness observed for this strain (Hammoudi et al., 2015), or the role of an unidentified ASP5-targeted and PVM-associated GRA that inhibits CD8 T cell activation. Such possibilities await experimental validation.

Second, since *T. gondii* defends itself from immune attack by its virulence factor ROP5, this strategy affords a second benefit, the hiding of its vacuolar antigens from the host's MHC I antigen processing machinery. For TGD057, this battle is uniquely defined by ROP5A that has no known interacting partner, but suppresses the CD8 T cell response to TGD057. We predict this occurs by a mechanism independent from any known ROP5-IRG interaction. For example, ROP5B and ROP5C from clade A strains are able to prevent accumulation of effector IRGs on the parasite's PVM, including Irgb10, Irga6 and Irgb6, but this is not a function of clade A ROP5A (Fleckenstein et al., 2012; Reese et al., 2014), nor is this a known

function for any ROP5 isoforms from clade D. Although ROP5C was not tested here, recently an OVA-expressing RH *Δrop5 +ROP5C* strain was generated and CD8 T cell activation was partially inhibited by this strain (Rommereim et al., 2019). Whatever mechanism underlies the ability of ROP5 to inhibit CD8 T cell activation, we hypothesize that it is controlled by ROP5A and ROP5B isoforms, and less so by ROP5C. Amino-acids in the 346–370 region in the IRG binding interface of ROP5 (Reese et al., 2014) can be found that distinguish clade A ROP5C from ROP5A and ROP5B of clades A and D. Whether these amino acids define a novel interaction with a less-studied effector IRG, of which there are 13 functional effector IRGs encoded in the C57BL/6 genome (Lilue et al., 2013, p. 201), or another host protein is currently unknown.

It is likely there is no single host-parasite interaction that determines antigen presentation for all *T. gondii* vacuolar antigens. For example, one notable difference between CD8 T cell responses to the vacuolar TGD057 and OVA antigens, is the role of the *T. gondii* ROP18 kinase. The CD8 T cell response to OVA appears largely inhibited by ROP18 (Rommereim et al., 2019), but at best, ROP18 plays a marginal role in this system (**Fig 2-7 and Fig 2-8A**). These observations imply effector IRG modulation or the ATF6β ER-stress response, which is known to regulate CD8 T cell IFNγ responses to *T. gondii* and is directly antagonized by the kinase activity of ROP18 (Yamamoto et al., 2011), might be more important for CD8 T cell detection of OVA than TGD057. Furthermore, p62 is required for OVA-specific OT1 CD8 T cell activation by a mechanism that includes binding to ubiquitin-tagged PVs in IFNγ-stimulated cells (Rommereim et al., 2019). P62 recruits GBPs to the vacuole of *T. gondii* (Haldar et al., 2015), suggesting GBPs may also assist in MHC I antigen presentation, for which we found some evidence (**Fig 2-6C and 2-6D**). Although the role of p62 using mouse knockout cells was not directly tested, a comparison of p62-PV localization patterns between stimulatory and non-stimulatory parasites strains (**Fig 2-7D and 2-7E**) argues against a dominant role for this pathway in our system. Other differences include lessons learned from the GRA6 antigen. When the C-terminal GRA6 epitope is facing the host cytosol it is highly stimulatory to CD8 T cells (Buaillon et al., 2017). The protruding nature of the GRA6 epitope into the host cytosol may bypass need for host recruitment of IFNγ-induced IRG/GBP machinery, thus facilitating its immuno-dominance. However, TGD057 is not an integral membrane protein nor is it associated with the membranous fractions of the PV (Lopez et al., 2015). It is therefore unclear how the initial antigen is first detected to start T57 IFNγ responses, which paradoxically require IFNγ signaling to begin with (**Fig 2-6**). A clue may come from the OVA system. Host derived ERGICs fuse with the PVM in a Sec22b SNARE-dependent process to initiate MHC I presentation of *T. gondii* expressed OVA (Cebrian et al., 2011). Whether this pathway seeds the initial antigen-specific response to TGD057 is unknown.

Yet even in response to clade A strains, which are poor inducers of TGD057-specific CD8 T cell IFNγ and IL-2 responses (**Fig 2-9 and Fig 2-8B**), the early activation marker CD69 was readily detected on T57 CD8 T cells (**Fig 2-10**). The immune system is therefore robust in its ability to perceive *T. gondii* antigens, which employs multiple non-redundant pathways to acquire antigens from vacuolated pathogens (Blanchard and Shastri, 2010).

Third, our studies demonstrate an absolute requirement for the pathogen sensor NLRP3, and to a similar extent NLRP1, for promoting naïve TGD057-specific CD8 T cell IFNγ responses to parasite-infected cells. Moreover, it appears NLR-mediated regulation can occur in the absence of other components of the inflammasome cascade. This is inferred because T57 IFNγ production was still detected in response to parasite-infected P2X7R, ASC, caspase-1/11, and gasdermin D deficient cells, or when IL-1/18 cytokine signaling was inhibited (**Fig 2-13 and Fig 2-12**). In contrast, when NLRP3 is removed, there was no IFNγ response (**Fig 2-12**), even though the CD8 T cells were activated (**Fig 2-15**). NLRs have several inflammasome independent functions, including the ability to form a bridge between ER and mitochondria to initiate inflammasome signaling (Liu et al., 2018). NLRs also bind to and directly activate transcription factors, such as IRF4 (Bruchard et al., 2015) and the RFX complex (Meissner et al., 2010), the latter induces MHC I expression (Zhao and Shao, 2012). Our results diverge from a recent report exploring the role of inflammasome components in promoting CD8 T cell IFNγ responses during primary *T. gondii* infection. NLRP3, ASC and caspase 1/11 deficiency had no bearing on the frequency of peritoneal or splenic IFNγ+ CD8 T cells during primary *T. gondii* infection (López-Yglesias et al., 2019). Certainly, CD8 T cells receive environmental cues *in vivo* that compensate for the lack of the inflammasome pathway, but are missing in our system. Given the diminished IFNγ-transcriptional response to infected NLRP3-deficient cells (**Fig 2-15**) and the role co-stimulation plays in regulating T cell IFNγ responses, we hypothesized co-stimulatory pathways were dysregulated in *Nlrp3-/-* infected cells, but this was not the case. Current studies are underway to understand the mechanism by which NLRP3 deficiency impacts transcriptional and translation regulation of IFNγ in activated CD8 T cells.

ER stress has been shown to activate the NLRP3 inflammasome (Bronner et al., 2015; Elliott and Sutterwala, 2015; Menu et al., 2012), resulting in IL-1β production (Elliott and Sutterwala, 2015; Menu et al., 2012). The unfolded protein response (UPR) of ER stress generally involves ATF6, PERK, and IRE1 signaling (Li et al., 2020). When initially described, ER stress-induced NLRP3 inflammasome activation did not seem to be the effect of any of these signaling pathways triggered by UPR (Menu et al., 2012). Based on their observations, Menu *et al.* suggested that there is a fourth pathway of the ER stress that modulates NLRP3

inflammasome activation, and it is not dependent on UPR but dependent on reactive oxygen species (ROS) production (Menu et al., 2012). In the work by Bronner *et al.*, IRE1 signaling mediated by ER stress following *Brucella abortus* infection lead to mitochondrial ROS (mtROS) production and membrane damage that is dependent on NLRP3 but not other components of the inflammasome complex such as ASC and caspase 1 (Bronner et al., 2015). In this study, the NLRP3 which initially associates on the ER (Zhou et al., 2011) re-localized to the mitochondria following ER stress, and induced caspase 2-BID-dependent destruction of the mitochondria, releasing mitochondrial DNA (Bronner et al., 2015). In addition to NLRP3 activation, ER stress has been shown to also activate NOD2/RIPK2 via IRE1 signaling (Keestra-Gounder et al., 2016), leading to NF-κB activation (Keestra-Gounder et al., 2016). Though there is no known interaction between NLRP3 and RIPK2 (Wagner et al., 2009), both NLRP3 and RIPK2 seem to be crucial for the CD8 T cell IFNγ response to *T. gondii* infections (**Fig 2-14**). Whether NLRP3 and RIPK2 work together or in compensatory manner requires further investigation. As alluded to above, one connection may be ER stress; though it is not yet known whether ER stress or UPR signaling are required for the CD8 IFNγ response to *T. gondii* in our system. Host ATF6β ER stress response has been shown to modulate the CD8 T cell IFNγ response to *T. gondii* infection, and that the parasite is able to combat this through the kinase activity of the virulence factor ROP18 (Yamamoto et al., 2011). It should be noted that following *T. gondii* PV formation, the host ER closely associates with the PV (Sinai et al., 1997); this may lead to ER stress and the UPR response. Studies using BMDMs deficient in IRE1, caspase 2, or defective in mtROS production would clarify whether these or related ER stress pathways are required for T57 CD8 T cell IFNγ production.

Finally, we present evidence that *T. gondii* strains differ in their ability to modulate CD8 T cell IFNγ responses, which in theory might aid the parasite's survival in a broad host range. For example, T57 IFNγ cell responses were low to *ROP5*-expressing clade A strains, which we hypothesized might be due to polymorphisms unique to clade A *ROP5* alleles. However, both clade D and A *ROP5* alleles were equally able to repress the CD8 T cell IFNγ response (**Fig 2-7**), indicating another polymorphic regulator is in effect. Our current work searching for this polymorphic modulator of host CD8 T cell IFNγ response has revealed no genotype-phenotype correlation at the *ROP5* locus. Instead, the polymorphic regulator could intersect CD8 T cell differentiation, for example through modulation of the host's NLRs, or antigen release, possibly by assisting the function of ROP5A. Given the enhanced T cell response to the ME49 *Δasp5* strain, ASP5 may target a polymorphic dense granule to the PVM that represses the response. Recently, Rommereim *et al.* has shown that OVA-specific CD8 T cell activation is regulated by multiple GRA(s)

(Rommereim et al., 2019). Whether any such GRA is responsible for the observed strain differences in T57 activation require further investigation.

In summary, since any warm-blooded animal can serve as an intermediate host for *T*. *gondii*, the parasite may have difficulty achieving stable chronic infections in every animal to promote its transmission. This is evidenced by the observation that *T*. *gondii* strains differ dramatically in virulence in laboratory mice (Howe and Sibley, 1995) and correlate with the severity of human toxoplasmosis (Gilbert et al., 2008; Grigg et al., 2001; Khan et al., 2006; McLeod et al., 2012). This led to the hypothesis that parasite strains have adapted to certain intermediate host niches (Boothroyd, 2009), defined by host genetics, including that of the murine IRG locus (Lilue et al., 2013). This adaptation may also necessitate the manipulation of host adaptive immune responses. Here, we present evidence that *T*. *gondii* may direct CD8 T cell IFNγ response for its advantage. Perhaps NLRP3 and the MHC I antigen presentation pathway serve as two distinct sites for immune pressure, leading to the evolution of novel parasite virulence factors. Nevertheless, a closer and detailed understanding of the interactions between *T*. *gondii* and host CD8 T cells will eventually help us find potential therapeutic targets for toxoplasmosis, as well as to understand why *T*. *gondii* has spread so extensively.

Chapter 3:

Variation in the CD8 T cell IFNγ response to *Toxoplasma gondii* is characterized by small effect QTLs

3.1 Introduction

Toxoplasma gondii is an intracellular pathogen responsible for toxoplasmosis, an underdiagnosed and neglected parasitic disease. *T. gondii* is considered one of the most successful parasites as it can accommodate a wide host range, infecting nearly all warm-blooded vertebrates including an estimated one-third of the world's human population. Upon invasion, *T. gondii* forms and sequesters itself in a parasitophorous vacuole (PV) that does not initially fuse with host organelles (Coppens, 2017; Sinai et al., 1997), shielding itself from the cytosolic immune sensing mechanisms and defense machinery aimed at its elimination. Host cytotoxic CD8 T cells respond to intracellular pathogens such as *T. gondii* through recognition of cytosolic-derived peptide antigens presented by MHC I molecules on the surface of infected cells (Dzierszinski et al., 2007). If detected, CD8 T cells will secrete the pro-inflammatory cytokine, interferon-gamma (IFNγ) to combat protozoan infections. This CD8 T cell-mediated IFNγ response is required for the elimination of *T. gondii* (Gazzinelli et al., 1992; Gigley et al., 2011; Nishiyama et al., 2020; Suzuki et al., 1988; Suzuki and Remington, 1990, 1988). In turn, IFNγ activates the Janus kinase-signal transducer and activator of transcription 1 (Jak/STAT1) signaling pathway, inducing the Immunity-Related GTPases (IRGs) (Haldar et al., 2013; Hunn et al., 2008; Kim et al., 2012; Ling et al., 2006). IRGs then bind to the parasite's PV membrane (PVM) and disrupt it through GTPase-driven IRG oligomerization on the PVM (Hunn et al., 2008). The IRG pathway is necessary for the CD8 T cell response to antigens that are sequestered inside the PV of *T. gondii* (Jensen, 2016), which includes the model antigen OVA engineered to be secreted into the lumen of the PV (Gregg et al., 2011; Gubbels et al., 2005; Lee et al., 2015; Rommereim et al., 2019) and the PV-associated TGD057 antigen (Kongsomboonvech et al., 2020). Inflammasomes, particularly NLRP3 and NLRP1 inflammasome complexes, have also been shown to recognize and control *T. gondii* infections in a variety of species (Cirelli et al., 2014; Ewald et al., 2014; Gorfu et al., 2014; Gov et al., 2017; Wang et al., 2019; Witola et al., 2011). We have recently shown that NLRP sensors, but not the inflammasome complexes are required for full induction of the naïve CD8 T cell IFNγ response to parasite-infected cells (Kongsomboonvech et al., 2020).

T. gondii utilize various virulence factors to combat host immune responses. If the host succumbs to an infection, the parasite will lose the resources required for its

own survival and die. Thus, *T. gondii* must achieve a 'balance' where it can establish a chronic infection and produce tissue cysts, an infectious form that allows transmission between hosts following oral consumption. However, the parasite cannot achieve this balance in every host. For example, *T. gondii* strains differ dramatically in virulence within laboratory mice (Howe and Sibley, 1995) and with the severity of human toxoplasmosis (Gilbert et al., 2008; Grigg et al., 2001; Khan et al., 2006; McLeod et al., 2012). Of the clonal lineages endemic to North America and Europe, type I strains are highly virulent. Its lethal dose (LD_{100}) is 1 parasite for laboratory mouse infections (Sibley and Boothroyd, 1992), and kills the mice prior to establishing chronic infection. Types II and III are less virulent than type I, with a LD_{100} of approximately 10^3 and 10^5, respectively (Saeij et al., 2006; Sibley and Boothroyd, 1992). The genetically diverse "atypical" strains commonly found in South America are also extremely virulent in mice (Fux et al., 2007; Jensen et al., 2015; Khan et al., 2011), and human ocular toxoplasmosis is more severe in South America compared to other locales (Gilbert et al., 2008; Grigg et al., 2001; Khan et al., 2006; Sauer et al., 2011). In contrast, type I strains fail to cause disease in Lewis rats (Cavaillès et al., 2006; Wang et al., 2019), other subspecies of *Mus musculus* (Lilue et al., 2013) and certain farm animals, including pigs and poultry which are relatively refractory to toxoplasmosis across the globe (Stelzer et al., 2019). Whether *T. gondii* requires unique virulence strategies to infect the various hosts they encounter in nature is unknown. Various species differ with respect to the exact mechanism by which *T. gondii* is killed and immune pathways available for parasitic resistance (Gazzinelli et al., 2014; Mukhopadhyay et al., 2020b; Saeij and Frickel, 2017; Wang et al., 2020). Hence, manipulation of CD8 T cell responses by *T. gondii* might represent one of a diverse set of strategies needed to achieve balanced infections across a broad host range.

The use of genetic crosses has proven fruitful for discovery of polymorphic virulence factors that intersect host-parasite interactions relevant to *T. gondii* and its murine host. The F1 progeny of the clonal lineages (from type I x type II, type I x type III, and type II x type III sexual crosses) differ greatly in virulence phenotypes (Behnke et al., 2011; Saeij et al., 2006; Taylor et al., 2006). Through quantitative trait loci (QTL) mapping, a number of *T. gondii* proteins contributing to virulence has been identified, namely three polymorphic rhoptry proteins (ROPs): ROP16 (Saeij et al., 2006, 2007), ROP18 (Saeij et al., 2006; Taylor et al., 2006), and a family of ROP5 pseudokinases (Behnke et al., 2011; Reese et al., 2011). Polymorphisms in the tyrosine kinase ROP16, expressed in type I and type III ($ROP16_{I/III}$) but not type II ($ROP16_{II}$) strains, allow sustained phosphorylation of STAT3 and STAT6, inducing alternative activation (M2) of macrophages and dampening of host IL-12 production (Butcher et al., 2011; Jensen et al., 2011; Ong et al., 2010; Yamamoto et al., 2009). $ROP16_{III}$ promotes virulence due to early M2 activation and subsequent suppression of Th1 immunity and CD8 T cell responses

(Chen et al., 2020; Tuladhar et al., 2019). In contrast, when $ROP16_{I}$ or $ROP16_{III}$ are expressed as a transgene in the type II background, it promotes host resistance (Jensen et al., 2011; Saeij et al., 2006) and parasite killing by a mechanism that is dependent on the genetic background of the parasite (Jensen et al., 2013).The serine threonine kinase ROP18 phosphorylates and inactivates IRGs, protecting the PV from destruction (Fentress et al., 2010; Fleckenstein et al., 2012; Niedelman et al., 2012; Steinfeldt et al., 2010). However, ROP18 is not expressed in type III *T. gondii* strains (Saeij et al., 2006; Taylor et al., 2006), explaining its lesser degree of virulence compared to types I and II *T. gondii* strains. The pseudokinase ROP5 binds to IRGs, preventing them from oligomerizing and accumulating at the PV (Howard et al., 2011; Hunn et al., 2008; Reese et al., 2014). In contrast, the ROP5 variants expressed by type II *T. gondii* strains fail to perform this function rendering type II strains less virulent (Niedelman et al., 2012; Reese et al., 2014). F1 progeny from a cross between the highly virulent atypical strain VAND and a type II strain further demonstrate the importance of ROP5 polymorphisms in regulating parasite virulence (Behnke et al., 2015). Altogether, these polymorphic rhoptries are largely responsible for the strain-specific differences in murine virulence of *T. gondii*. Recently, we observed strain differences in host CD8 T cell responses to *T. gondii* infections (Kongsomboonvech et al., 2020) and found that both ROP5 and ROP16 play a role in modulating the CD8 T cell IFNγ response to *T. gondii*. However, there may still be other unidentified polymorphic regulators that modulate this CD8 T cell IFNγ response. For example, type I strains and other clade A isolates induced relatively low amounts of IFNγ secretion from naïve CD8 T cells, but most other strains induced relatively high CD8 T cell IFNγ responses (Kongsomboonvech et al., 2020), a phenotypic pattern that currently matches no known biology of parasite virulence in laboratory mice.

Here, we aim to identify polymorphic *T. gondii* Regulator(s) Of CD8 T cell Responses ('ROCTR') that are responsible for strain differences in eliciting CD8 T cell IFNγ responses. We utilized an experimental approach by which naïve antigen-specific CD8 T cells that bear specificity to a conserved *T. gondii* endogenous antigen, TGD057, were analyzed for IFNγ responses to parasite-infected bone marrow-derived macrophages (BMDMs). This phenotype was measured for F1 progeny of the types IxII, IxIII, and IIxIII sexual crosses and analyzed by genome-wide QTL mapping. QTL analysis of the F1 IxII cross suggested that ROCTRs may be encoded on *T. gondii* chromosomes X and XII with very weak effect, while no discernable QTLs were generated from the F1 IIxIII and F1 IxIII crosses. Our attempts to identify ROCTRs on chromosomes X and XII through generation of gene deletion strains proved uninformative, as the knockout (KO) strains were not different from their parental strain. The failure to detect QTLs with large effect after analyzing each of the genetic crosses of the clonal lineage

strains suggest that there may be multiple genetic loci controlling host CD8 T cell responses to *T. gondii* with small effect. However, we do present further evidence that *T. gondii* effectors known to regulate delivery of GRAs to the PVM appear to regulate the response.

3.2 Materials and Methods

3.2.1 Parasites

Toxoplasma gondii strains were serially passaged in 'Toxo medium' [4.5 g/liter D-glucose in DMEM with GlutaMAX (Gibco, cat# 10566024), 1% heat-inactivated fetal bovine serum (FBS) (Omega Scientific, cat# FB-11, lot#441164), 1% penicillin-streptomycin (Gibco, cat# 15140122)], in confluent flasks of monolayers of human foreskin fibroblasts (HFFs) and cultured at 37°C, 5% CO_2. HFFs were cultured in 'HFF medium' [4.5 g/liter D-glucose in DMEM with GlutaMAX (Gibco), 20% heat-inactivated FBS (Omega Scientific), 1% penicillin-streptomycin (Gibco), 0.2% Gentamicin (Gibco, cat# 15710072), 1X L-Glutamine (Gibco, cat# 21051024)]. Strains assayed included RH *Δhxgprt* (Donald and Roos, 1998), RH *Δhxgprt Δku80* (Huynh and Carruthers, 2009), GT1, ME49 *Δhxgprt*::*Luc* (Tobin and Knoll, 2012), Pru *Δhxgprt,* CEP *hxgprt*-, RH *Δku80 Δhxgprt Δasp5-ty*::*HXPRT*::*DHFR* (RH *Δasp5*) (Curt-varesano et al., 2016), ME49 *Δasp5*::*DHFR* (ME49 *Δasp5*) (generous gift from Dominique Soldati-Favre, University of Geneva) (Hammoudi et al., 2015). RH *Δgra35::HXGPRT::GFP* (RH *Δgra35*), RH *Δgra42::HXGPRT::GFP* (RH *Δgra42*), RH *Δgra43::HXGPRT::GFP* (*RH Δgra43*), ME49-RFP *Δgra35::HXGPRT::GFP* (ME49 *Δgra35*), ME49-RFP *Δgra42::HXGPRT::GFP* (ME49 *Δgra42*), and ME49-RFP *Δgra43::HXGPRT::GFP* (ME49 *Δgra43*) are generous gift from Jeroen Saeij (University of California, Davis) (Wang et al., 2019). F1 strains assayed are listed in the Table 3-1, some of which are generous gifts form Jeroen Saeij (University of California, Davis) and David Sibley (Washington University); others were obtained from BEI Resources.

3.2.2 Mice and generation of bone marrow-derived macrophages

Transnuclear T57 mice (Kirak et al., 2010) were bred in-house under specific pathogen free (SPF) conditions. C57BL/6J (B6) (colony 000664) mice were purchased from Jackson Laboratories or bred in-house under SPF conditions. Bone marrow cells from 6-8 weeks old female wildtype B6 mice were obtained and cultured in 'BMDM medium' [4.5 g/liter D-glucose in DMEM with GlutaMAX (Gibco), 20% heat-inactivated FBS (Omega Scientific), 1% penicillin-streptomycin (Gibco), 1X non-essential amino acids (Gibco, cat# 11140076), 1mM sodium pyruvate (Gibco, cat# 11360070)] supplemented with 20% L929 conditioned medium, and then harvested after 6-7 days of differentiation. All animal protocols

were approved by UC Merced's Committee on Institutional Animal Care and Use Committee (AUP17-0013). All mouse work was performed in accordance to the *Guide to the Care and Use of Laboratory Animals* of the National Institutes of Health and the Animal Welfare Act (assurance number A4561-1). Euthanasia of mice was performed by inhalation of CO_2 to effect of 1.8 liters per minute.

3.2.3 T cell activation assay

B6 BMDMs were plated at $2x10^5$ cells per well in a 96-well tissue culture-treated plate, in BMDM medium supplemented with 10% L929 conditioned medium, and incubated overnight. The next day, these BMDMs were infected with *T. gondii* tachyzoites, in triplicates, in 'T cell medium' [RPMI 1640 with GlutaMAX (Gibco, cat# 61870127), 20% heat-inactivated FBS (Omega Scientific, cat# FB-11, lot# 441164), 1% penicillin-streptomycin (Gibco, cat#15140122), 1 mM sodium pyruvate (Gibco, cat# 11360070), 10 mM HEPES (Gibco), 1.75 µl of β-mercaptoethanol (Gibco, cat# 21985023) per 500 mL RPMI 1640 with GlutaMAX]. The infections were done at multiplicity of infection (MOI) of 0.6, 0.2, and 0.07. Approximately 2 hours post-infection, $5x10^5$ naïve lymph node cells and splenocytes obtained from a naïve T57 transnuclear mouse were added into all wells of infected BMDMs. The lymph nodes and spleens were processed and combined, and red blood cells were lysed with ammonium chloride-potassium (ACK) lysis buffer, prior to being added to the co-culture. The supernatants of the co-cultures were harvested 48 hours later for further analysis by ELISA.

3.2.4 Correction for relative viability between parasites

HFFs were plated in 24-well tissue culture-treated plates in HFF medium. Confluent monolayer HFFs were infected with 100 and 300 parasites. Plaques were quantified 4-6 days after infection. Displayed results are from MOIs with similar viability, the equivalent of ~MOI 0.2 was chosen for most assays.

3.2.5 ELISA

The IFNγ concentration in the 48h supernatants of the T57 T cell activation assays were determined by ELISA according to the manufacturer's instructions (Invitrogen eBioscience, cat# 88731477). The supernatants were analyzed at 1:2, 1:20, and 1:200 dilutions to obtain values within the linear range of the manufacture's ELISA standards.

3.2.6 Genetic linkage analysis and Quantitative Trait Loci (QTL) mapping

The QTL analysis for the CD8 T cell IFNγ response phenotype, including both 1D (scanone) and 2D scans (scantwo) as well as the effect plots, was performed using 'R' version 3.6.1 and 'R/qtl' package (Broman et al., 2003). The concentration of CD8 T cell-secreted IFNγ that was obtained for each F1 IxII progeny was normalized to that of type II strain, and for the F1 IxIII progeny, the values were normalized to the type III strain. The average of normalized values obtained over multiple experiments was then Log10 transformed and used for the QTL analysis. For the F1 IIxIII progeny, the Log10 of the CD8 T cell IFNγ concentration from a single experiment was used. A thousand permutations were calculated to obtain significant threshold values ($p \leq 0.05$ and $p \leq 0.1$). Genetic markers and the allelic assignments for each F1 progeny were obtained from previous studies (Behnke et al., 2011; Su et al., 2002) and publicly available databases (http://toxomap.wustl.edu/IxIII_Typing_Table.html). The genetic maps of chromosomes VIIb and VIII were stitched into a single chromosome in which we renamed as "VIIb-VIII". Recent evidence suggests these are a single chromosome (Bunnik et al., 2019; Xia et al., 2020), and our revised genetic map reflects the correct genetic marker orientation in the stitched VIIb-VIII *T. gondii* chromosome.

3.2.7 Generation of gene knockout parasite strains

For ROCTR candidate knockout strains, individual candidates were targeted. To generate a knockout strain, Cas9-expressing pSS013 plasmid (generous gift from Jeroen Saeij, University of California, Davis) containing single guide RNAs (sgRNAs) targeting the candidate gene (**Table 3-3**) were co-transfected, via electroporation, with selectable marker hypoxanthine-guanine phosphoribosyl transferase (HXGPRT) or dihydrofolate reductase (DHFR) into RH *Δhxgprt* (RH *Δhpt*) or ME49 *Δhxgprt* (ME49 *Δhpt*) at a 5:1 ratio of sgRNAs/plasmid to an PCR amplicon of the selectable marker. The HXGPRT amplicon was generated using the pTKO-att plasmid as the template DNA and the DHFR amplicon was generated using the pLoxP-DHFR-mCherry plasmid as the template DNA, with primers indicated in **Table 3-3**. The transfection bulk populations were then selected with 'MPA xanthine' medium [4.5 g/liter D-glucose in DMEM with GlutaMAX (Gibco, cat# 10566024), 1% heat-inactivated fetal bovine serum (FBS) (Omega Scientific, cat# FB-11, lot#441164), 1% penicillin-streptomycin (Gibco, cat# 15140122), 0.05 mg/ml mycophenolic acid (MPA) (Millipore Sigma, cat# 475913), 0.05 mg/ml xanthine (Alfa Aesar; cat# AAA11077-22)] or in Toxo medium containing 3 μM pyrimethamine (Millipore Sigma, cat# 46706) to select for the presence of HXGPRT or DHFR insertion, respectively. The transfection bulk with successful targeting as confirmed by PCR are then cloned through limiting dilution in a 96-

well plate in MPA xanthine or pyrimethamine selection medium. Wells with only one clone are isolated and candidate gene disruption is confirmed by PCR.

Further subcloning was performed for ME49 *Δhpt Δ278882 Δ278878*::DHFR 1D8 which represents two distinct *Δ278878* population. This was done by limiting dilution in a 96-well plate in Toxo medium. Wells that contain a single plaque of *T. gondii* were isolated and then confirmed for candidate gene disruption by PCR.

3.2.8 Confirmation of gene disruption

T. gondii strains and ROCTR candidate KO clones were grown to confluency in HFF flask with Toxo media. Parasite genomic DNA was isolated using DNAzol (Invitrogen, cat# 10503027) and ethanol precipitation. Presence or absence of selectable marker at the candidate gene of interest, as determined by PCR, is used to indicate successful targeting. PCR was performed with MangoMix, which contains MangoTaq DNA polymerase (Bioline, cat# BIO-25-33), along with primers that flank the entire locus of the targeted gene as well as primers specific to an internal site of the selectable marker, as illustrated in **Fig 3-4 and Fig 3-5**. The primers used are listed in **Table 3-3**. Sequencing of PCR products were performed by UC Berkeley DNA Sequencing Facility and samples were prepared according to their protocol. Sequencing results were analyzed using Sequencher 5.4.6 and SnapGene Viewer 5.2.1.

3.2.9 Immunofluorescence assay

HFFs were plated on coverslips with HFF medium in 24-well tissue culture-treated plates. Once confluent, the monolayer HFFs were infected with *T. gondii* and incubated 37°C, 5% CO_2 for 16h. The samples were then fixed with 3% formaldehyde in phosphate buffered saline (PBS) for 20 minutes. After the fixation, these were blocked with blocking buffer (3% BSA, 5% normal goat serum, 0.2% Triton X-100, 0.1% sodium azide in PBS). To visualize GRA5, the infected cells were stained with mouse anti-GRA5 primary monoclonal antibody (BioVision, clone TG 17.113) at 1:500 dilution, followed by Alexa Fluor 488 goat anti-mouse IgG (Life Technologies) secondary antibody at 1:3000 dilution. To visualize TGD057, the infected samples were stained with rabbit anti-TGD057 polyclonal antibody (gift from Nicolas Blanchard) at 1:2000 dilution, followed by Alexa Fluor 594 goat anti-rabbit IgG (Life Technologies) secondary antibody at 1:3000 dilution.

3.2.10 Statistical analysis and normalization between experiments

Bar graphs represent the average with standard deviations. For these, values from individual experiments are also represented as dots. Results between parasites

strains were at times represented as relative to the response elicited by type II or type III strains (equal 1). All statistical analyses (unpaired t-test and one-way ANOVA with Bonferroni's correction) were performed with GraphPad Prism version 8.3.0.

3.3 Results

3.3.1 QTL analysis suggests ROCTRs are encoded on T. gondii chromosomes X and XII

Polymorphic *T. gondii* rhoptries (ROP) that are responsible for the strain differences in virulence during acute infection have been identified through genome-wide quantitative trait loci (QTL analysis). Similarly, strain differences have also been observed in CD8 T cell-mediated IFNγ responses to *T. gondii* (Kongsomboonvech et al., 2020), suggesting the existence of a polymorphic *T. gondii* virulence factors that modulates host CD8 T cell responses, termed "ROCTR".

To identify *T. gondii* genetic loci responsible for the strain differences in CD8 T cell responses to infections, and ultimately ROCTR, we assessed 29 strains of F1 progeny from type I x type II cross (F1 IxII), 34 strains of F1 progeny from type II x type III cross (F1 IIxIII), and 32 strains of F1 progeny from type I x type III cross (F1 IxIII) (**Table 3-1**) in an antigen-specific T cell activation assay, the 'T57 assay'. Wildtype (WT) C57BL/6 bone marrow-derived macrophages (BMDMs) were infected with *T. gondii* and co-cultured with splenocytes and lymph node cells from naïve transnuclear 'T57' mice. These mice were cloned from the nucleus of a single tetramer-positive *T. gondii*-specific CD8 T cell which have a single T cell receptor specific for the $TGD057_{96\text{-}103}$ epitope (T57 epitope) presented by $H\text{-}2K^b$ MHC I (Kirak et al., 2010; Wilson et al., 2010). The supernatant was harvested at 48 hours of the co-culture and analyzed for IFNγ concentration by ELISA. As previously demonstrated (Kongsomboonvech et al., 2020), type I *T. gondii* strains (RH, GT1) induce low T57-specific CD8 T cell-mediated IFNγ responses, while type II (ME49, Pru) and type III strains (CEP) induce a much higher response (**Fig 3-1A**). The CD8 T cell response differs greatly amongst the infections of F1 IxII *T. gondii* strains. For example, the CD8 T cell IFNγ response to SF46 is quite low, unlike the high IFNγ level elicited in response to SF20 infections. (**Fig 3-1A**). These results suggest the potential to genetically map loci responsible for strain differences in T57 IFNγ responses (**Fig 3-1B**).

Strain	From	Reference
F1 IxII		
C7B1	BEI Resources	Behnke *et al.*, 2011

C7E4	BEI Resources	Behnke *et al.*, 2011
C7K4	BEI Resources	Behnke *et al.*, 2011
2C10A3	BEI Resources	Behnke *et al.*, 2011
2C10B5	BEI Resources	Behnke *et al.*, 2011
SF3	BEI Resources	Behnke *et al.*, 2011
SF8	BEI Resources	Behnke *et al.*, 2011
SF9	BEI Resources	Behnke *et al.*, 2011
SF14	Sibley Lab	Behnke *et al.*, 2011
SF15	BEI Resources	Behnke *et al.*, 2011
SF17	BEI Resources	Behnke *et al.*, 2011
SF19	BEI Resources	Behnke *et al.*, 2011
SF20	BEI Resources	Behnke *et al.*, 2011
SF26	BEI Resources	Behnke *et al.*, 2011
SF28	Sibley Lab	Behnke *et al.*, 2011
SF34	Sibley Lab	Behnke *et al.*, 2011
SF35	BEI Resources	Behnke *et al.*, 2011
SF36	Sibley Lab	Behnke *et al.*, 2011
SF37	BEI Resources	Behnke *et al.*, 2011
SF39	BEI Resources	Behnke *et al.*, 2011
SF40	BEI Resources	Behnke *et al.*, 2011
SF41	BEI Resources	Behnke *et al.*, 2011
SF42	BEI Resources	Behnke *et al.*, 2011
SF44	BEI Resources	Behnke *et al.*, 2011
SF46	BEI Resources	Behnke *et al.*, 2011
SF47	BEI Resources	Behnke *et al.*, 2011
SF50	BEI Resources	Behnke *et al.*, 2011
SF58	BEI Resources	Behnke *et al.*, 2011
SF61	BEI Resources	Behnke *et al.*, 2011
F1 IxIII		
c96 A5	Saeij Lab	Khan *et al.*, 2005
c96 B4	Saeij Lab	Khan *et al.*, 2005
c96 C12	Saeij Lab	Khan *et al.*, 2005
c96 E7	Saeij Lab	Khan *et al.*, 2005
c96 H6	Saeij Lab	Khan *et al.*, 2005
c96 STC7	Saeij Lab	Khan *et al.*, 2005
c96 STC8	Saeij Lab	Khan *et al.*, 2005
c96 STD3	Saeij Lab	Khan *et al.*, 2005
c96 STE1	Saeij Lab	Khan *et al.*, 2005
c96 STF3	Saeij Lab	Khan *et al.*, 2005
c96 STG10	Saeij Lab	Khan *et al.*, 2005
c96 STH1	Saeij Lab	Khan *et al.*, 2005
c96 STH5	Saeij Lab	Khan *et al.*, 2005

c96 STH10	Saeij Lab	Khan *et al.*, 2005
c96 STH11	Saeij Lab	Khan *et al.*, 2005
CL11	Saeij Lab	Sibley *et al.*, 1992
CL12	Saeij Lab	Sibley *et al.*, 1992
CL13	Saeij Lab	Sibley *et al.*, 1992
CL16	Saeij Lab	Sibley *et al.*, 1992
CL17	Saeij Lab	Sibley *et al.*, 1992
CL18	Saeij Lab	Sibley *et al.*, 1992
CL19	Saeij Lab	Sibley *et al.*, 1992
S1T	Saeij Lab	Sibley *et al.*, 1992
S2T	Saeij Lab	Sibley *et al.*, 1992
S21	Saeij Lab	Sibley *et al.*, 1992
S22	Saeij Lab	Sibley *et al.*, 1992
S23	Saeij Lab	Sibley *et al.*, 1992
S25	Saeij Lab	Sibley *et al.*, 1992
S26	Saeij Lab	Sibley *et al.*, 1992
S27	Saeij Lab	Sibley *et al.*, 1992
S28	Saeij Lab	Sibley *et al.*, 1992
S29	Saeij Lab	Sibley *et al.*, 1992
S30	Saeij Lab	Sibley *et al.*, 1992
STG5	Saeij Lab	Shastri *et al.*, 2014
F1 IIxIII		
c285-1	BEI Resources	Su *et al.*, 2002
c285-11	BEI Resources	Su *et al.*, 2002
c285-13	BEI Resources	Su *et al.*, 2002
c285-31	BEI Resources	
c285-38	BEI Resources	
c285-4	BEI Resources	Su *et al.*, 2002
c285-42	BEI Resources	
c285-50	BEI Resources	
c285-51	BEI Resources	
c285-62	BEI Resources	
c295-2	BEI Resources	Su *et al.*, 2002
c295-27	BEI Resources	Su *et al.*, 2002
c295-29	BEI Resources	Su *et al.*, 2002
c295-31	BEI Resources	Su *et al.*, 2002
c295-31	BEI Resources	Su *et al.*, 2002
c295-5	BEI Resources	Su *et al.*, 2002
c295-P1A5	BEI Resources	
c295-P1D7	BEI Resources	
A6AF	BEI Resources	Su *et al.*, 2002
A9AF	BEI Resources	Su *et al.*, 2002

A9SF	BEI Resources	Su *et al.*, 2002
B10AF	BEI Resources	Su *et al.*, 2002
B4SF	BEI Resources	Su *et al.*, 2002
C11AF	BEI Resources	Su *et al.*, 2002
C7SF	BEI Resources	Su *et al.*, 2002
D9AF	BEI Resources	Su *et al.*, 2002
E6AF	BEI Resources	Su *et al.*, 2002
E7SF	BEI Resources	Su *et al.*, 2002
E8SF	BEI Resources	Su *et al.*, 2002
G2AF	BEI Resources	Su *et al.*, 2002
H7AF	BEI Resources	Su *et al.*, 2002
M12AF	BEI Resources	

Table 3-1. Parasite strains. The F1 *T. gondii* strains used in this study are listed in various categories: F1 IxII (F1 progeny from the type I GT1 x type II ME49 sexual cross), F1 IIxIII (F1 progeny from the type II ME49 x type III CEP sexual cross), and F1 IxIII (F1 progeny from the type I GT1 x type III CEP sexual cross). Sources of the strains used for the experiments and primary references, when known, are indicated.

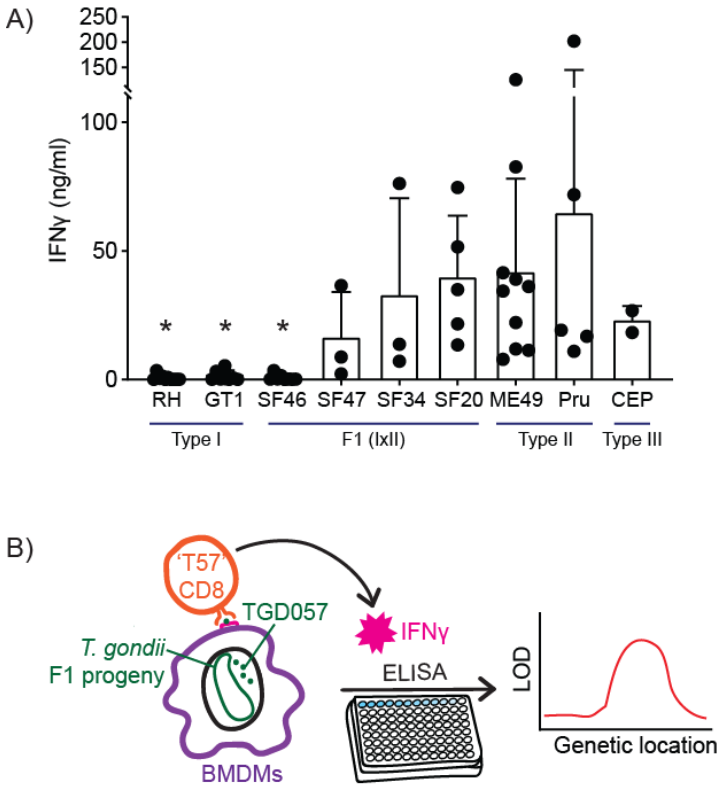

Figure 3-1. Variation of CD8 T cell IFNγ responses to *T. gondii* strains. (A) T57 IFNγ responses to the indicated strains were obtained, average of 2-5 experiments +SD (standard deviation) is plotted; each dot represents the result from an individual experiment. Statistical analysis was performed using one-way ANOVA with Bonferroni's correction comparing to Pru; * $p \leq 0.05$. **(B)** Schematic of the strategy to identify 'ROCTR'. TGD057-specific CD8 T cell IFNγ responses to *T. gondii*-infected bone marrow-derived macrophages (BMDMs) were measured. Two hours post-infection, naïve antigen-specific TGD057 ('T57') CD8 T cells obtained from transnuclear mice were added to the infected BMDMs. Supernatant from the co-culture was harvested 48h later and then analyzed for IFNγ concentration by ELISA. Genetic linkage analyses were performed with IFNγ response values obtained from the F1 progeny to find the quantitative trait loci (QTL) responsible for the phenotype.

A genome-wide QTL scan was performed to detect genotype-phenotype correlations using F1 progeny derived from all three sexual crosses previously mentioned. QTL analysis of the F1 IxII cross (**Fig 3-2A**) revealed three suggestive peaks with an LOD value greater than 2 on *T. gondii* chromosomes X (chrX) and XII (chrXII) (**Fig 3-2B**). The QTL on chromosome XII (PEAK 1) with the logarithm of odds (LOD) score of 4.4 reaches the threshold value of p = 0.10 (**Fig 3-2B**), but none of the other QTLs surpass genome-wide permutation testing. Polymorphic candidate genes within the 1.5 LOD interval of PEAK1, a 106 kb region spanning 13 genes (TGME49_chrX:5754579.5860469), include most notably two tandem uncharacterized NTPases, *TG_278878* and *TG_278882* (ToxoDB.org). The genetic marker corresponding to the maximal LOD score within this PEAK1 (65.m01195_at9) is a SNP within *TG_278878*. On chromosome X, there are two

noticeable peaks. The major peak towards the right end of chromosome X (PEAK2) is a large 1.1 Mb locus (TGME49_chrX:3782766.4930776) encoding 147 genes and includes the genetic marker 46.m03675_at7, which produced the highest LOD score of 3.6 within this chromosome (**Fig 3-2B**). The minor peak towards the left end of chromosome X (PEAK3) corresponds to a ~419 kb region of 48 genes (TGME49_chrX:1383691.1802907) and encompasses the genetic marker 42.m03493_at7 with a LOD score of 2.3 (**Fig 3-2B**); this marker defines a SNP within the *T. gondii* dense granule *GRA35*. Notably, the *ROP5* locus, which is a known virulence determinant encoded on chromosome XII and identified using this panel of F1 IxII progeny (Behnke et al., 2011), did not produce an identifiable QTL in our screen. Thus, while ROP5 can inhibit the T57 response (Kongsomboonvech et al., 2020; Rommereim et al., 2019), polymorphisms in ROP5 do not account for strain-specific differences observed for this phenotype, as previously suggested (Kongsomboonvech et al., 2020). The antigen encoded by *TGD057* is similarly expressed and entirely conserved between clonal strains, and no phenotype-genotype correlation was observed at this site. Based on the effect plots, F1 IxII progeny that express type II alleles at the chromosome X genetic markers 46.m03675_at7 and 42.m03493_at7 induce higher CD8 T cell-mediated IFNγ responses compared to those that express type I alleles at these loci, respectively (**Fig 3-2C**). In contrast, F1 IxII progeny that express a type I allele at the chromosome XII genetic marker 65.m01195_at9 induce higher CD8 T cell responses compared to those that express the type II allele (**Fig 3-2C**), suggesting the genetic background of type I strains may mask the effect of the putative chrXII ROCTR. In search of loci that potentially modify the function of the chrXII ROCTR, we investigated epistatic interactions or 'interactive' QTLs, but this was not detected between PEAKS 1, 2, and 3 or other loci within the F1 IxII genetic cross (**Fig 3-3**). Other sexual crosses examined included the F1 progeny derived from the IxIII and IIxIII crosses, but QTL mapping revealed no statistically significant peak for this phenotype (**Fig 3-4 and Fig 3-5**). It can be concluded that the polymorphic rhoptries responsible for strain differences in virulence previously identified through QTL analyses of these same panels of F1 progeny, such as ROP16 (from F1 IIxIII cross) encoded on chromosome VIIb-VIII (Saeij et al., 2006) and ROP18 (from both F1 IIxIII and F1 IxIII crosses) encoded on chromosome VIIa (Saeij et al., 2006; Taylor et al., 2006), do not seem to account for the strain differences in host CD8 T cell responses to *T. gondii* infections (**Fig 3-4 and Fig 3-5**). Moreover, the small effect QTLs suggest that the T57 CD8 IFNγ response may be controlled by multiple gene loci of *T. gondii* and subject to environmental input during the 48 hours co-culture of the CD8 T cells and infected macrophages. Top polymorphic ROCTR candidates are included in **Table 3-2**.

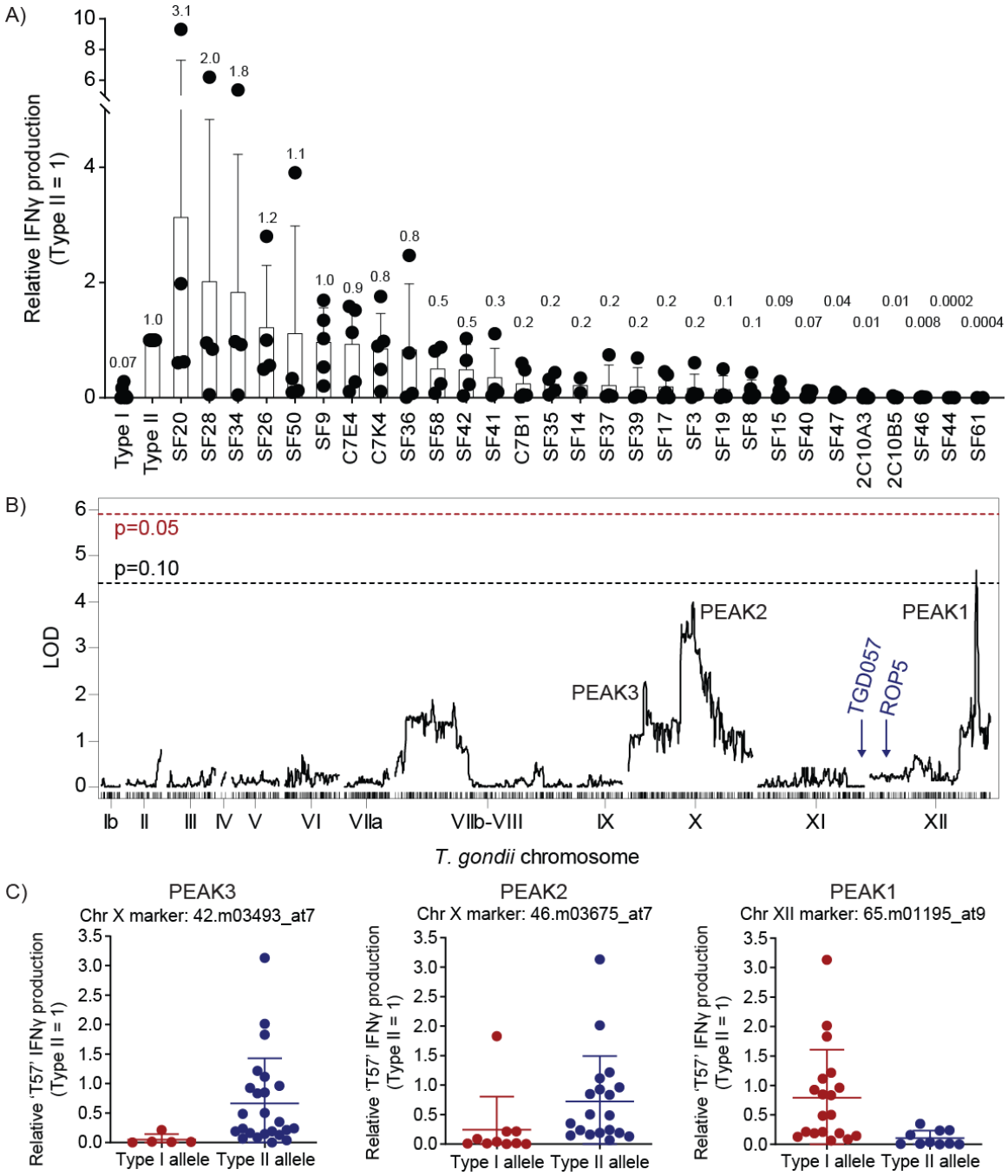
A)
Relative IFNγ production (Type II = 1)
Type I
Type II
SF20
SF28
SF34
SF26
SF50
SF9
C7E4
C7K4
SF36
SF58
SF42
SF41
C7B1
SF35
SF14
SF37
SF39
SF17
SF3
SF19
SF8
SF15
SF40
SF47
2C10A3
2C10B5
SF46
SF44
SF61
B)
p=0.05
p=0.10
LOD
PEAK1
PEAK2
PEAK3
TGD057
ROP5
Ib
II
III
IV
V
VI
VIIa
VIIb-VIII
IX
X
XI
XII
T. gondii chromosome
C)
PEAK3
Chr X marker: 42.m03493_at7
PEAK2
Chr X marker: 46.m03675_at7
PEAK1
Chr XII marker: 65.m01195_at9
Relative 'T57' IFNγ production (Type II = 1)
Type I allele
Type II allele

Figure 3-2. *T. gondii* genetic loci on chromosomes X and XII correlate with parasite strain differences in inducing CD8 T cell IFNγ responses. (A) TGD057-specific CD8 T cell responses to *T. gondii*-infected BMDMs were assayed, as previously described in Fig 3-1, with F1 progeny of the type I x type II cross (F1 IxII). The IFNγ concentration was measured by ELISA and normalized to that of the type II strain. Average of 2-5 experiments +SD (standard deviation) is plotted and values are indicated above the bar graphs; each dot represents the result from an individual experiment. **(B)** Genetic linkage analysis of TGD057-specific CD8 T cell-mediated IFNγ responses to F1 IxII *T. gondii* strains were analyzed. The running LOD score for each genetic marker is plotted. The quantitative trait loci (QTL) map reveals regions of interest on *T. gondii* chromosomes X and XII. QTLs with LOD scores >2 are labeled as PEAK1, PEAK2, and PEAK3. The LOD significant threshold values are calculated from 1,000 permutations, indicated with dashed lines and p-values (p = 0.05 in red, p = 0.10 in black). The genetic locations of *ROP5* and the *TGD057* antigen are shown for reference. **(C)** Effect plots for the genetic markers corresponding to PEAK3, PEAK2, and PEAK1 are shown. The IFNγ responses are normalized to that induced by the wildtype (WT) type II strain. Each dot represents the average value obtained for each F1 IxII strain.

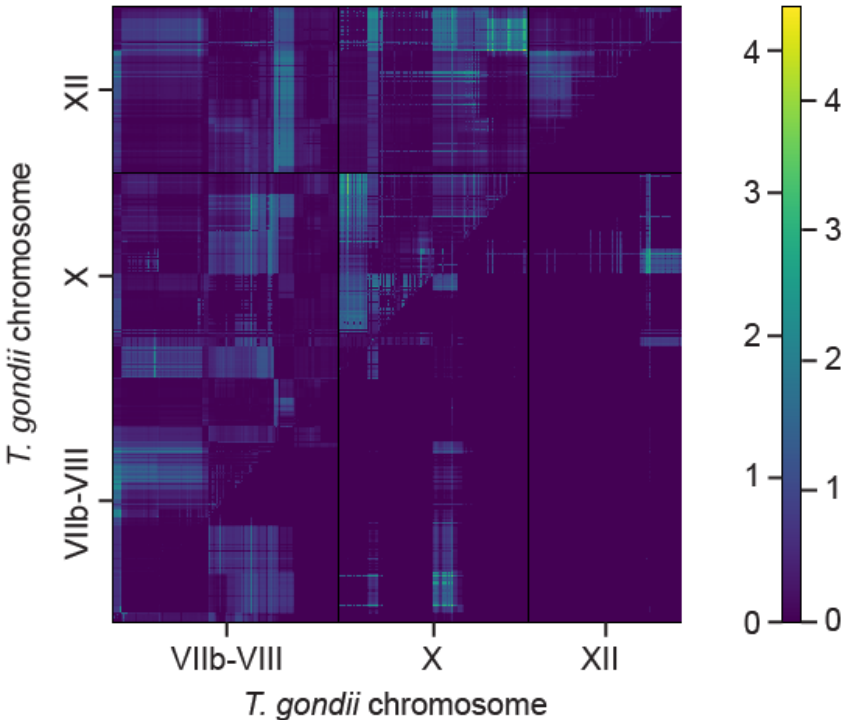

Figure 3-3. The two-dimensional (2D) QTL genome-wide scan does not detect interactive effects between ROCTR loci in modulating CD8 T cell IFNγ responses to *T. gondii* infections. A 2D QTL analysis of CD8 T cell IFNγ responses to *T. gondii* F1 IxII infections. The selected *T. gondii* chromosomes are labeled on both axes. The LOD scores in the 2D plot are represented on a color scale indicated on the right. The LOD score scale from 0 to 4.2 corresponds to the upper left triangle which compares the full two-locus or "interactive QTL" model, in which all possible combinations of two markers are calculated for epistasis, to an additive-QTL model. The LOD score scale from 0 to 4.8 corresponds to the lower right triangle of the 2D plot, which compares the two-locus to a single-locus QTL model. No two pairs of loci in the two-locus model surpass a p-value of p = 0.1, and no advantage is gained over a single-QTL model (R/qtl).

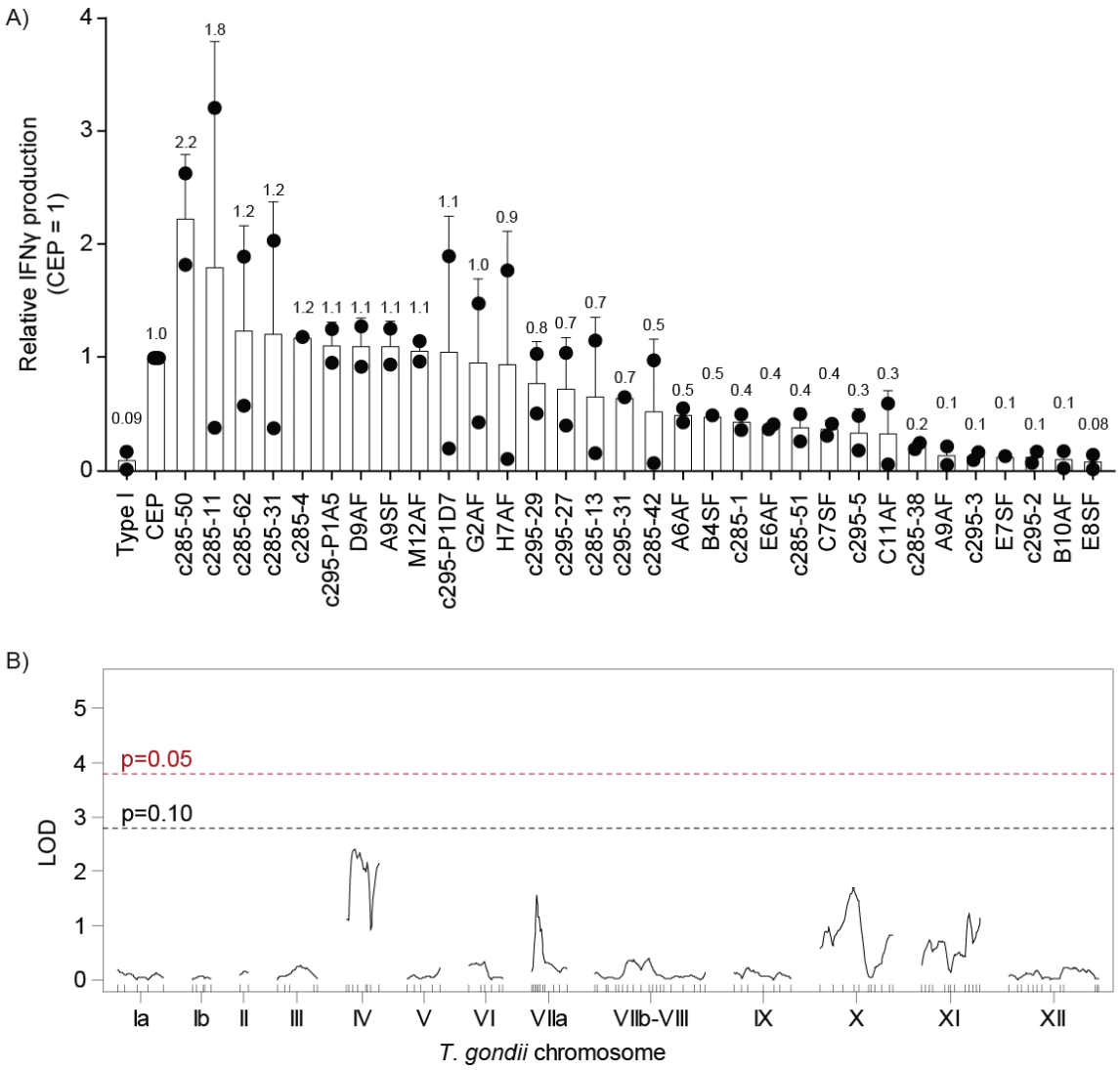

Figure 3-4. Genetic linkage analysis of TGD057-specific CD8 T cell IFNγ responses to *T. gondii* F1 progeny of the type I x type III cross reveals no significant QTLs. (A) The host CD8 T cell responses to infections with F1 IxIII *T. gondii* strains were measured as previously described and normalized to that induced by the CEP strain (WT type III strain). The normalized values are indicated above the bar graphs. Average of 2 experiments +SD are shown, each dot represents the results from one experiment. **(B)** A genome-wide QTL scan of the CD8 T cell response to F1 IxIII *T. gondii*-infected BMDMs was performed. The running LOD score for each genetic marker is shown; *T. gondii* chromosomes are indicated. Significant threshold LOD values of p = 0.05 and p = 0.10 following 1,000 permutations are indicated in red and black, respectively.

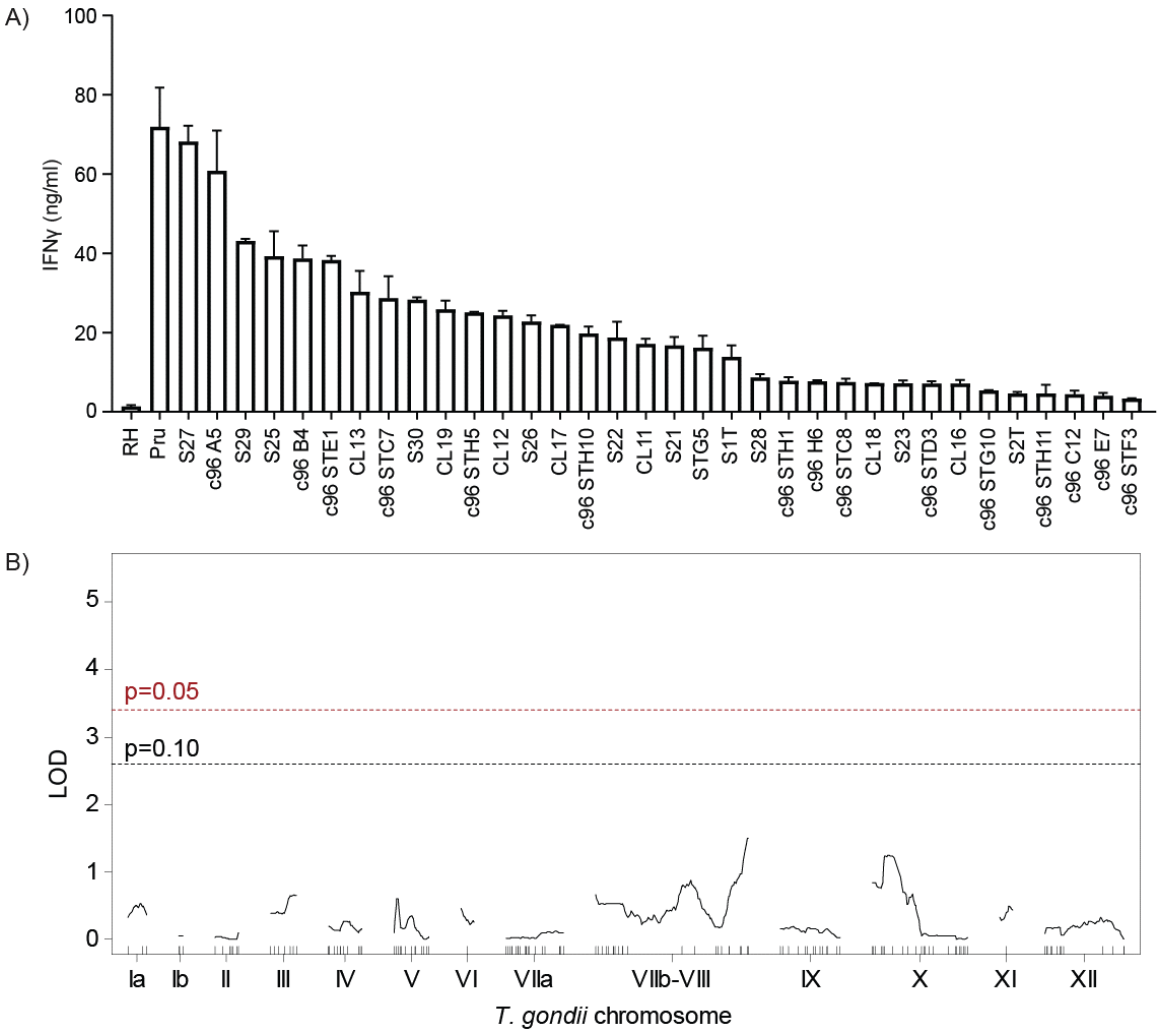

Figure 3-5. Genetic linkage analysis of TGD057-specific CD8 T cell IFNγ responses to *T. gondii* F1 progeny of the type II x type III cross reveals no significant QTLs. (A) F1 IIxIII *T. gondii* strains were assayed for host CD8 T cell IFNγ responses as previously described. Plotted is the average IFNγ concentration in the supernatant at 48h post addition of T57 CD8 T cells +SD of 3 technical replicates from a single experiment. **(B)** A genome-wide QTL scan of the CD8 T cell response to F1 IIxIII *T. gondii* infections was performed and the running LOD score for each *T. gondii* genetic marker is shown. The significant threshold LOD values of p = 0.05 and p = 0.10 following 1,000 permutations are indicated in red and black, respectively.

Gene ID	Coordinates	Product description	NonSyn/Syn SNP Ratio All Strains	Number of Amino Acid Differences between type I & type II strains	Avg Expression of RH/GT1	Average Expression of Pru/ME49	Single exon	Predicted signal peptide	Predicted transmembrane domain	fitness score*
PEAK1: Chromosome X - minor peak										
TGME49_226470	TGME49_chrX: 1,607,575 - 1,618,478 (+)	hypothetical protein	2.42	55	9.98	21.37	N	N	N	1.25
TGME49_226390	TGME49_chrX: 1,665,302 - 1,670,080 (+)	hypothetical protein	2.92	17	5.00	5.16	**Y**	N	N	0.04
TGME49_226380*	TGME49_chrX: 1,674,639 - 1,678,229 (+)	GRA35	8.67	11	86.41	135.17	**Y**	**Y**	**Y**	1.98
PEAK2: Chromosome X - major peak										
TGME49_223485	TGME49_chrX: 3,784,432 - 3,788,334 (+)	hypothetical protein	10.0	155	46.38	44.97	N	N	N	-1.49
TGME49_223430	TGME49_chrX: 3,850,738 - 3,854,319 (-)	hypothetical protein with putative oxidoreductase activity	0.84	2	5.00	13.29	N	Y	Y	0.66
TGME49_212280	TGME49_chrX: 3,942,555 - 3,944,845 (+)	hypothetical protein	1.5	1	47.82	21.45	Y	Y	N	0.69
TGME49_212140	TGME49_chrX: 4,023,907 - 4,035,947 (+)	hypothetical protein	1.01	72	6.97	7.58	N	Y	N	-0.1
TGME49_234230	TGME49_chrX: 4,207,706 - 4,228,971 (-)	hypothetical protein	1.07	40	5.00	10.24	N	N	N	-3.19
TGME49_234270	TGME49_chrX: 4,254,283 - 4,268,067 (+)	hypothetical protein, localizes to the apical complex	1.32	223	16.53	40.60	N	N	N	-0.44
TGME49_234300	TGME49_chrX: 4,285,787 - 4,297,019 (-)	hypothetical protein	2.01	138	5.00	12.86	Y	N	N	-0.33
TGME49_234350	TGME49_chrX: 4,305,901 - 4,308,402 (-)	hypothetical protein	1.44	14	10.56	22.59	Y	Y	N	-0.73
TGME49_234590*	TGME49_chrX: 4,485,141 - 4,486,247 (-)	hypothetical protein	6.0	24	5.00	5.00	Y	N	N	-0.14
TGME49_235140	TGME49_chrX: 4,633,596 - 4,638,849 (+)	hypothetical protein	6.7	166	46.36	52.18	N	N	N	1.41
PEAK3: Chromosome XII										
TGME49_278930	TGME49_chrXII: 5,750,428 - 5,757,826 (+)	Tubulin-tyrosine ligase family protein	0.53	2	23.5875	9.9775	N	N	N	0.5
TGME49_278882	TGME49_chrXII: 5,785,062 - 5,788,158 (-)	GDA1/CD39 (nucleoside phosphatase) family protein	0.52	0	6.3475	5	**Y**	**Y**	**Y**	0.71
TGME49_278878*	TGME49_chrXII: 5,790,682 - 5,793,202 (-)	GDA1/CD39 (nucleoside phosphatase) family protein	2.47	1	7.4125	8.26	N	**Y**	N	0.6
TGME49_278840	TGME49_chrXII: 5,828,465 - 5,832,264 (+)	hypothetical protein	1.53	6	5	11.83	N	N	N	1.39
TGME49_278815	TGME49_chrXII: 5,852,666 - 5,861,034 (+)	Putative F-box protein	1.63	71	9.6325	19.0975	N	N	N	-1.59

Table 3-2. ROCTR candidates. Top ROCTR candidates within the boundaries of each QTL peak (PEAK1, PEAK2, and PEAK3) are listed with genetic coordinates. The information regarding each gene was obtained from the Toxoplasma informatics database (ToxoDB.org). The list includes ROCTR candidates that exhibited a high degree of amino acid polymorphisms between all strains deposited in ToxoDB, and/or number of amino acid substitutions or gene expression differences between type I and II strains. Genes with single exons/and or predicted to have a signal peptide were favored for inclusion as a ROCTR candidate. The average expression of RH and GT1 (type I strains) as well as the average expression of Pru and ME49 (type II strains) were calculated from values provided by Minot *et al.*, 2012. The fitness score was obtained from Sidik *et al.*, 2016. Genes investigated by CRISPR inactivation in this study are highlighted in red. Genes encoding the genetic marker that produced the highest LOD score at each QTL peak are denoted with an asterisk.

3.3.2 Interrogation of ROCTR candidates on chromosomes X and XII

To confirm whether our candidates are the genetic loci responsible for the parasite strain differences observed in eliciting host CD8 T cell responses to *T. gondii* infections, candidate genes were inactivated with CRISPR-Cas9 and the gene disruption was confirmed by PCR (**Fig 3-6A and Fig 3-9A**) and DNA sequencing. During this process, a selectable marker was added as a DNA template for *T. gondii* to potentially use for repair via the non-homologous end joining (NHEJ) repair pathway. Thus, the presence of the selectable marker at the targeted gene locus would be indicative of a successfully disrupted gene, or knockout (KO).

The candidate genes on *T. gondii* chromosome XII that correspond to PEAK1 are *TG_278878* and its adjacent gene *TG_278882,* which are nucleoside triphosphate hydrolases (NTPases) of the GDA1/CD39 family of ecto-ATPases with apyrase activity (ToxoDB.org). In general, NTPases are secreted following invasion and localize to the PV lumen and PVM (Bermudes et al., 1994; Sibley et al., 1994). The related NTPases, NTPase I and NTPase II (Bermudes et al., 1994), do not contribute to type I strain virulence in mice but deplete cellular ATP (Olias and Sibley, 2016) and are thought to be important for tachyzoite replication (Asai et al., 2002; Nakaar et al., 1999) and possibly egress (Stommel et al., 1997). Large quantities of parasite-derived NTPase can be detected in the serums of infected mice (Asai et al., 1987) and represents up to 2-3% of the entire protein in the tachyzoite (Asai et al., 1983). NTPases may therefore impact the host response in another way. It is possible that the ATP hydrolysis activity of NTPases may dampen host inflammasome activation in response to *T. gondii* infections (Melo et al., 2011). Inflammasome activation, for both NLRP1 and NLRP3, has been shown to be important for *T. gondii* control (Cirelli et al., 2014; Ewald et al., 2014; Gorfu et al., 2014) and can be triggered through the binding of exogenous ATP to the purinergic receptor P2X7 (Amores-Iniesta et al., 2017; Jo et al., 2016). It is possible that *T. gondii* NTPases deplete the amount of cytosolic ATP, thus preventing inflammasome activation either by lowering available exogenous ATP required for

P2X7 receptor (P2X7R) activation following egress, or by thwarting NLR-oligomerization which is an ATP-dependent process (Duncan et al., 2007). In our recent studies, we discovered that there is an NLRP3-dependent pathway to induce CD8 T cell responses to *T. gondii* infections (Kongsomboonvech et al., 2020). For these reasons, the *TG_278878* and *TG_278882* NTPases were pursued as candidates in our search of ROCTR.

We have attempted to generate double mutant *Δ278878 Δ278882 T. gondii* parasites, in both type I and type II WT parental strains but have come across a few challenges. Our strategy is to allow *T. gondii* to repair CRISPR/Cas9-edited site with a co-transfected selectable marker and confirm by diagnostic PCR and growth in selection medium (**Fig 3-6A**). In the type I RH strain, *TG_278878* was successfully disrupted by insertion with the dihydrofolate reductase (DHFR) selectable marker (Δ*278878::DHFR*) as confirmed by PCR (**Fig 3-6B**) and sequencing of the edited locus (not shown). However, the type I RH *TG_278882* gene locus seems to differ from those of type I GT1 and type II ME49 strains. PCR performed with several primer pairs flanking and within *TG_278882* consistently yield no PCR product for RH (**Fig 3-6B and Fig 3-6C**) though PCR products of expected sizes were consistently observed with GT1 and ME49 strains (**Fig 3-6C**). The primers have been designed based on the available annotated information in the Toxoplasma database (ToxoDB.org), which includes the GT1 and ME49 genomes but not the RH genome. Although our PCR results suggest that the *TG_278882* gene locus of RH is unique, possibly lost or mutated, the 278882 locus of the mutant RH strain bore evidence for integration of the selectable marker using a reverse primer flanking the 278882 gene and a forward primer internal to the DHFR repair cassette, albeit of an unexpected larger size (**Fig 3-6B**). Regardless, the RH *Δ278878* and possibly *Δ278882* clone (2G9) was assayed and no difference in the CD8 T cell IFNγ response to the RH *Δ278878* and its parental strain was observed (**Fig 3-8**).

We faced another challenge in our attempt to generate a ME49 *Δ278878 Δ278882 T. gondii* strain. Here, PCR confirmations demonstrated that both genes of interest were inactivated in the double mutant 1D8 line, as the presence of the inserted selectable marker was observed at both gene loci (*Δ278878::DHFR Δ278882::DHRF)* (**Fig 3-7A**). However, diagnostic PCR for the wildtype locus *TG_278878* was also observed for the 1D8 line (**Fig 3-7A**). Our hypothesis is that there is more than one population of *Δ278878 Δ278882* parasites in the 1D8 line. Currently, as of this dissertation submission, we have confirmed that the 1D8 line is indeed a mixed population and are in the process subcloning a ME49 *Δ278878::DHFR Δ278882::DHRF* clonal strain from the 1D8 line. Even though 1D8 is a mixed population, PCR and sequencing results confirmed both populations contain disrupted *TG_278878* gene, one through DHFR insertion and

the other a single nucleotide deletion resulting in a frameshift mutation (**Fig 3-7B**). The T57-specific CD8 T cell responses to the ME49 *Δ278878 Δ278882* 1D8 line was assayed and the response was comparable to its ME49 parental line (**Fig 3-8**). Thus far, our data suggest that these NTPases may not be ROCTR.

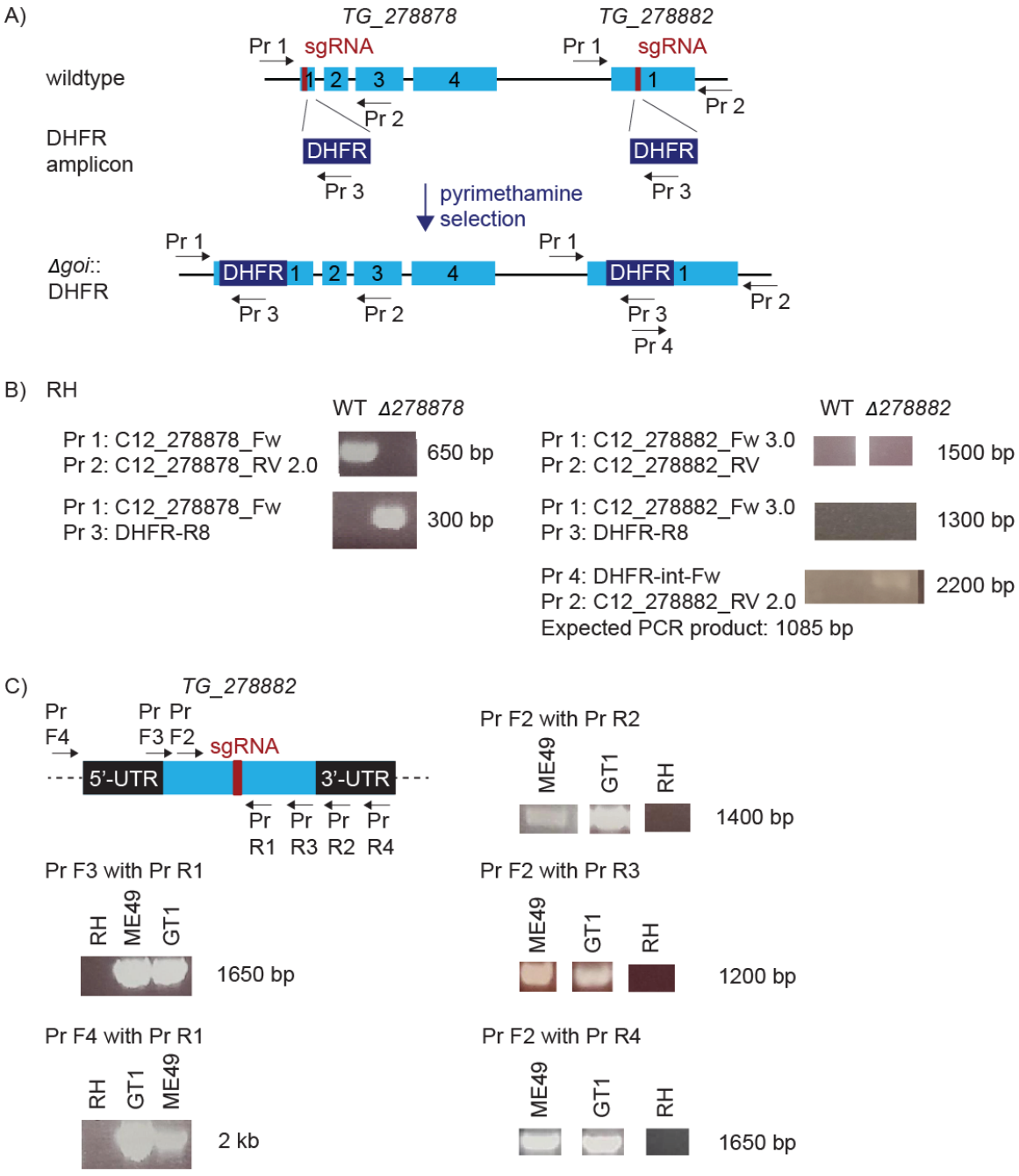

Figure 3-6. Gene disruption of NTPase genes in the RH *T. gondii* strain. (A) Schematic diagram of the gene knockout strategy. The genes, *TG_278887* and *TG_278882*, were CRISPR/Cas9-targeted. A DHFR amplicon was used as a template for DNA repair and inserted at the disrupted gene site. Primers flanking the CRISPR/Cas9 target site, Pr 1 and Pr 2, were used to indicate the wildtype locus. Pr 1 and an internal primer targeting the selectable marker DHFR, Pr 3 or Pr 4, were used to confirm selectable marker insertion at the disrupted gene site. **(B)** Left: PCR results confirming successful disruption of the 278878 gene (*Δ278878::DHFR*) in the RH *T. gondii* clone 2G9, with the indicated primer pairs and expected PCR product sizes. Right: PCR results for diagnosing 278882 gene disruption in RH *T. gondii* clone 2G9. No PCR product could be obtained with the forward primer, Pr 1. Instead, PCR was performed with Pr 4 and reverse primer Pr2 which yielded a PCR product of larger than expected size, suggesting the presence of the DHFR selectable marker at or near the 278882 gene site. **(C)** The *TG_278882* gene locus in RH may differ from that of GT1 and ME49 strains. PCR was performed with various combinations of primers flanking and within the *TG_278882* gene for RH, GT1, and ME49 WT strains. Each primer pair is indicated along with the expected sizes for the PCR product. A PCR product was never obtained for RH regardless of the primer pairs.

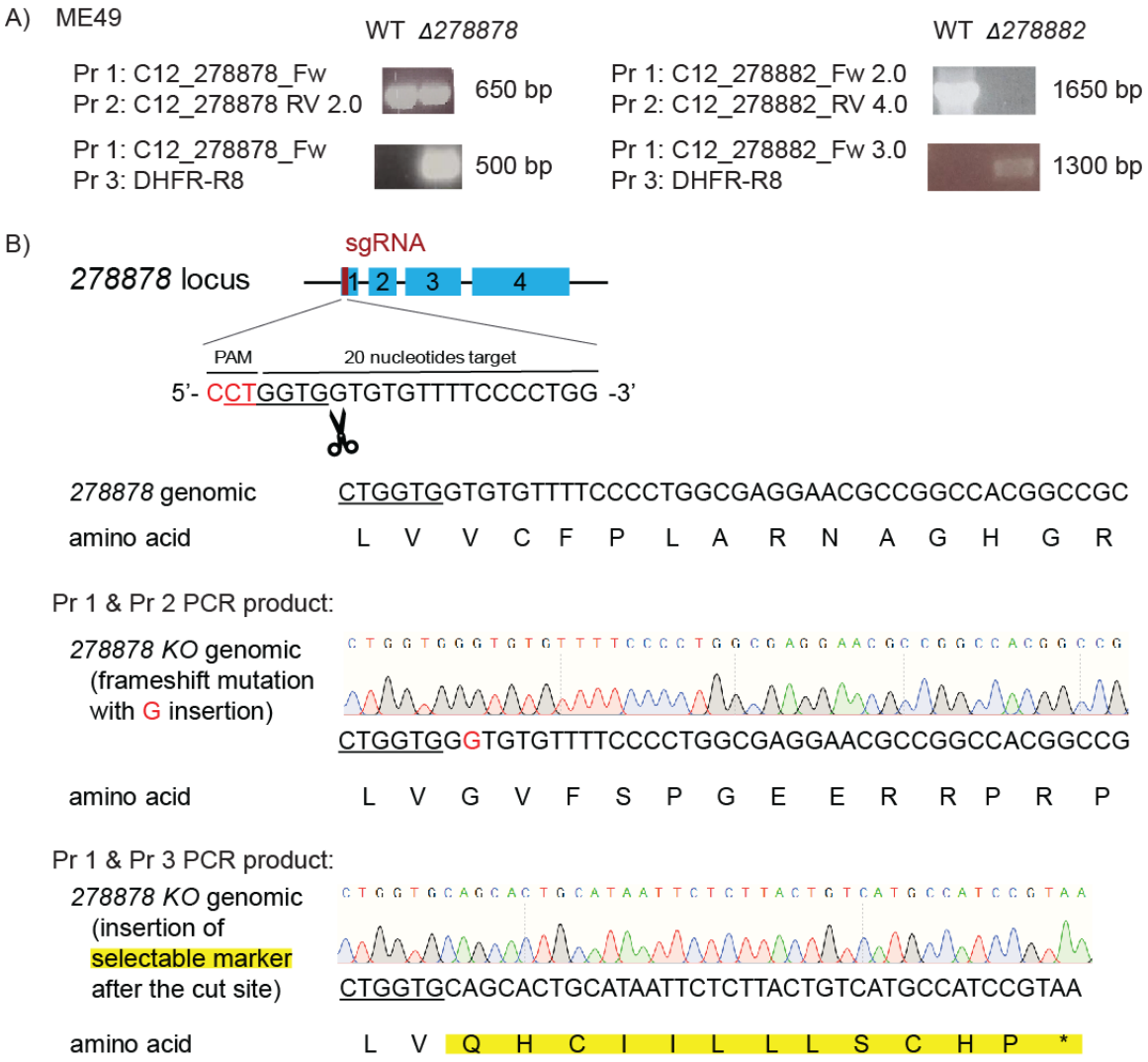

Figure 3-7. Gene disruption of NTPase genes in the ME49 *T. gondii*. (A) The same approach as in Fig 3-6 was used to generate a ME49 *Δ278878 Δ278882* strain. Left: PCR results confirming gene disruption of *TG_278878* (*Δ278878::DHFR*) for ME49 *T. gondii* line 1D8, with indicated primer pairs and expected PCR product sizes. The 1D8 line also showed an unexpected PCR product with the flanking primers, similar to that of the WT parental ME49 strain. Right: PCR results confirming successful disruption of *TG_278882* (*Δ278878::DHFR*) for the ME49 *T. gondii* line 1D8, with indicated primer pairs and expected PCR product sizes. **(B)** Sequencing results of ME49 1D8 line from indicated PCR reactions. For the PCR product from Pr 1 and Pr 2, there is a nucleotide insertion indicated in red, resulting in a frameshift and missense mutation. For the PCR product from Pr 1 and Pr 3, there is a premature stop codon. Both suggested the successful disruption of the *TG_278878*, and that 1D8 represents a mixed population as only one *TG_278878* gene is present in the ME49 genome.

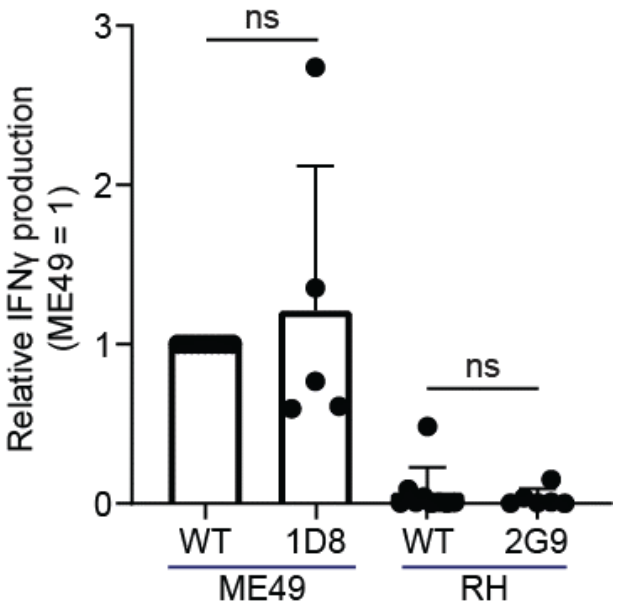

Figure 3-8. NTPases may not modulate the CD8 T cell IFNγ response. TGD057-specific CD8 T cell IFNγ secretion was analyzed at 48h in response to BMDMs infected with the RH *Δ278878 Δ278882* clone 2G9 and ME49 *Δ278878 Δ278882* line 1D8. The average value normalized to the type II strain for 5 experiments +SD is shown; each dot represents the results from an individual experiment. Statistical analysis was performed using one-way ANOVA with Bonferroni's correction; ns non-significant.

We next turned to ROCTR candidates on chromosome X. Within PEAK 2, the gene candidate *TG_234590* was disrupted in both type I and type II WT *T. gondii* strains and assayed for their ability to induce antigen-specific CD8 T cell IFNγ responses. This gene was selected as it encodes the SNP responsible for the genetic marker (46.m03675_at7) with the highest LOD score. Both RH and ME49 *Δ234590* clones bore evidence for integration of the selectable marker at the gene locus (*Δ234590::HXGPRT*) (**Fig 3-9**). However, both RH *Δ234590* and ME49 *Δ234590* strains elicited comparable CD8 T cell IFNγ responses compared to their WT parental strains (**Fig 3-9C**), suggesting that *TG_234590* is not ROCTR. Other ROCTR candidates within PEAK 2 are indicated in **Table 3-2** but were not pursued further.

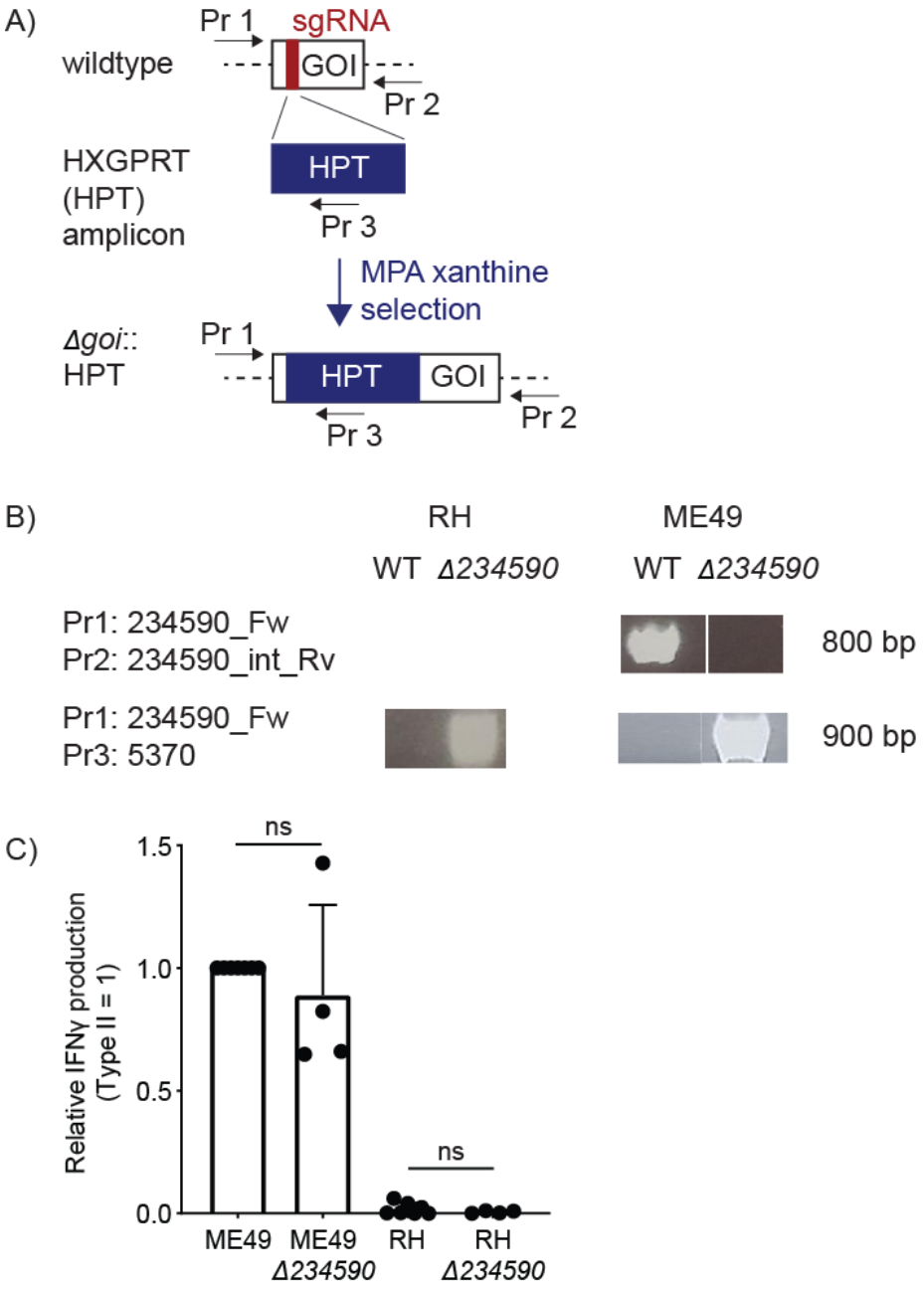

Figure 3-9. Chromosome X *TG_234590* is not ROCTR. (A) Schematic diagram of the gene knockout strategy. The gene of interest (GOI) was disrupted at the CRISPR/Cas9-targeted site. The selectable marker HXGPRT amplicon was used as a template for DNA repair and insertion at the Cas9-cut site. Primers flanking the targets gene locus, Pr 1 and Pr 2, were used to identify the wildtype locus. Pr 1 and an internal primer targeting the selectable marker, Pr 3, were used to confirm selectable marker insertion at the disrupted gene site. **(B)** PCR results confirming 234590 KO (*Δ234590::HXGPRT*) for the type I RH and type II ME49 *T. gondii* strains, with indicated primer pairs and expected PCR product sizes are shown. **(C)** The CD8 T cell responses were analyzed to infection with *Δ234590* clones. Average of 4 experiments +SD is shown with each dot representing the results from an individual experiment, and the responses were normalized to that obtained for the type II strain. Statistical analysis was performed using one-way ANOVA with Bonferroni's correction; ns non-significant.

Primer name	Sequence (5' to 3')	Gene	Comments	Generated by
234590_2_sg25855_F	AAGTTCGGCGAGGCGCAAAGAACCGG	TG_234590	Forward oligo for gRNA to disrupt 234590 on Chr X (ME49 strain)	Jensen Lab
234590_2_sg25855_R	AAAACCGGTTCTTTGCGCCTCGCCGA	TG_234590	Reverse oligo for gRNA to disrupt 234590 on Chr X (ME49 strain)	Jensen Lab
234590_4_sg25857_F	AAGTTGCAGATCTTCGACTACGCCGG	TG_234590	Forward oligo for gRNA to disrupt 234590 on Chr X (RH strain)	Jensen Lab
234590_4_sg25857_R	AAAACCGGCGTAGTCGAAGATCTGCA	TG_234590	Reverse oligo for gRNA to disrupt 234590 on Chr X (RH strain)	Jensen Lab
234590_Fw	TCCGCTTTTGAGGGAGAGGAAG	TG_234590	Forward primer for 234590 to determine KO, used as Pr 1	Jensen Lab
234590_Int_Rv	TGCCAGCACAGGAGAACTCTCTTATTT	TG_234590	Reverse primer for 234590 to determine KO, used as Pr 2	Jensen Lab
5370	GCCGTAGTCTTCAATGGGTTTGG	HXGPRT	Reverse primer targeting the HXGPRT selectable marker, used as Pr 3	Hammoudi *et al.*, 2015
DHFR-HXGPRT Forward	CAGCACGAAACCTTGCATTCAAACC	HXGPRT	Forward primer for HXGPRT amplicon generation (with DHFR 5' and 3' UTR) from the pTKO-att	Jensen Lab
DHFR-HXGPRT Reverse	GTGTCACTGTAGCCTGCCAGAAC	HXGPRT	Reverse primer for HXGPRT amplicon generation (with DHFR 5' and 3' UTR) from the pTKO-att	Jensen Lab
C12_278878_gRNA Fw	AAGTTCCAGGGGAAAACACACCACCG	TG_278878	Forward oligo for gRNA to disrupt 278878 on Chr XII, exon 1	Jensen Lab
C12_278878_gRNA Rv	AAAACGGTGGTGTGTTTTCCCCTGGA	TG_278878	Reverse oligo for gRNA to disrupt 278878 on Chr XII, exon 1	Jensen Lab
C12_278878_Fw	ACGATGTGTCATCTCGGTCTC	TG_278878	Forward primer for 278878 to determine KO, used as Pr 1	Jensen Lab
C12_278878_Rv 2.0		TG_278878	Reverse primer for 278878 to determine KO, used as Pr 2	Jensen Lab
C12_278882_gRNA Fw	AAGTTTGCAGTCTCGATCAACCACGG	TG_278882	Forward oligo for gRNA to disrupt 278882 on Chr XII, exon 1	Jensen Lab
C12_278882_gRNA Rv	AAAACCGTGGTTGATCGAGACTGCAA	TG_278882	Reverse oligo for gRNA to disrupt 278882 on Chr XII, exon 1	Jensen Lab
C12_278882_Fw 2.0	GTGGCGTGCGTGATTTC	TG_278882	Forward primer for 278882 to determine KO, used as Pr F2 and Pr 1	Jensen Lab
C12_278882_Fw 3.0	ATCCACACTATCGGCTACCAGT	TG_278882	Forward primer for 278882 to determine KO, used as Pr F3	Jensen Lab
C12_278882_RV	ACTCAACACTCCTGCTTTCACA	TG_278882	Reverse primer for 278882 to determine KO, used as Pr R1 and Pr 2	Jensen Lab
C12_278882_RV 2.0	CCGTTCAAGGACAGTG	TG_278882	Reverse primer for 278882 to determine KO, used as Pr R2	Jensen Lab
C12_278882_RV 3.0	GCCAACCCAATTCTGCAACTTG	TG_278882	Reverse primer for 278882 to determine KO, used as Pr R3	Jensen Lab
C12_278882_RV 4.0	CTAAGGCGGAATGGACAC	TG_278882	Reverse primer for 278882 to determine KO, used as Pr R4 and Pr 2	Jensen Lab
DHFR-internal_FW	GCCCCGTAATGCAGAAGAAGACCAT	DHFR	Forward primer targetting DHFR, used as Pr 4	Jensen Lab
DHFR-R8	GGATTTACAGCCTGGCGAAG	DHFR	Reverse primer targeting the DHFR selectable marker, used as Pr 3	Jensen lab
DHFR_For_LoxP2	TTAAGTTGGGTAACGCCAGG	DHFR	Forward primer for DHFR amplicon generatoin from the pLoxp-DHFR-mCherry	Gas-Pascual *et al.*, 2019
DHFR-Rv	CATCCTGCAAGTGCATAGAAG	DHFR	Reverse primer for DHFR amplicon generatoin from the pLoxp-DHFR-mCherry	Gas-Pascual *et al.*, 2019

Table 3-3. Primers and oligos. The sequences of single-guided RNAs (sgRNAs) targeting ROCTR candidate genes (*TG_234590*, *TG_278878*, *TG_278882*) are listed. The sequences of diagnostic primers used to confirm gene knockouts are also included. The sgRNAs and primers for specific gene candidates are grouped. The primers used to generate amplicons of selectable markers are also included.

3.3.3 A minor QTL peak on T. gondii chromosome X identifies a group of dense granules that regulate host CD8 T cell responses

The genetic marker 42.m03493_at7 that corresponds to PEAK3 is within the gene encoding the *T. gondii* dense granule GRA35. GRA35 was previously identified as an NLRP1 inflammasome activator in *T. gondii* infections of Lewis rat macrophages (Wang et al., 2019). GRA35 localizes at the PVM with the aid of GRA42 and GRA43 and remains in PV lumen in absence of GRA42 or GRA43 (Wang et al., 2019). Therefore, we examined all three dense granules. To test whether GRA35, GRA42, or GRA43 modulates the CD8 T cell response, we assayed *Δgra35*, *Δgra42*, and *Δgra35 T. gondii* strains and measured the T57-specific CD8 T cell IFNγ response as previously described. Compared to the parental RH strain, CD8 T cell IFNγ responses were slightly elevated to BMDMs infected with RH *Δgra35* and RH *Δgra42* strains, but significant differences were only observed in response to RH *Δgra43* infections (**Fig 3-10**). A similar trend was observed with the type II ME49 genetic background, but none of the differences between deletion and wildtype strains were of statistical significance (**Fig 3-10**). Whether GRA35 is responsible for some of the strain differences in *T. gondii*-induced CD8 T cell responses is unknown. Given the relatively small effect that GRA35 had on the phenotype, we did not pursue the generation of complementation strains to address this question. Regardless, it appears that upstream regulators of GRA35, in particular GRA43, seem quite important for modulating the T57 IFNγ response to *T. gondii*.

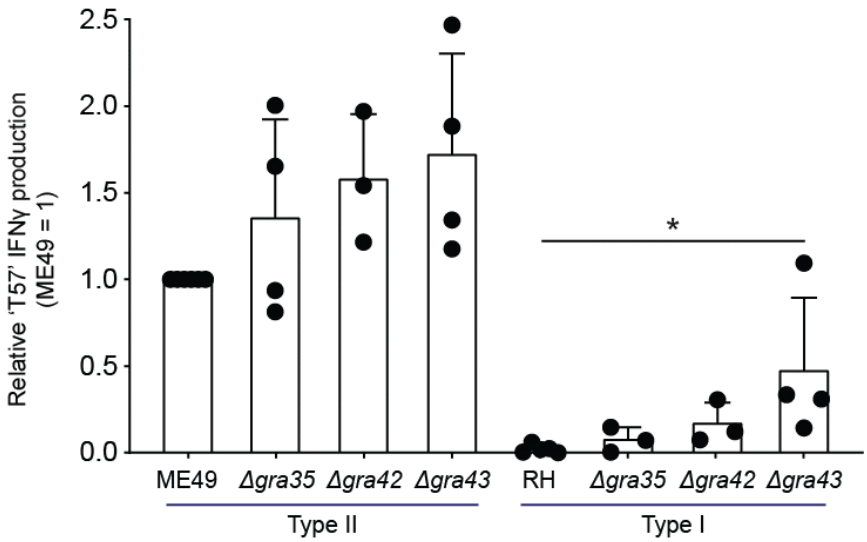

Figure 3-10. *T. gondii* GRA43 regulates host CD8 T cell IFNγ responses. BMDMs were infected with WT, *Δgra35*, *Δgra42*, or *Δgra43 T. gondii* strains as indicated. TGD057-specific CD8 T cell IFNγ responses were measured in the supernatant at 48h post addition of naïve T57 CD8 T cells. Results were normalized to the type II ME49 strain. Average +SD of 3-4 experiments is plotted; each dot represents the result from an individual experiment. Statistical analysis was performed using one-way ANOVA with Bonferroni's correction comparing to the WT Type I RH; * $p \leq 0.05$.

3.3.4 The localization of TGD057 is not affected by the PVM-targeting pathway mediated by ASP5 and GRA43

Since members of the PVM-targeting pathway, including ASP5 (Kongsomboonvech et al., 2020) and GRA43, modulate the host CD8 T cell response (**Fig 3-10**), we asked whether ASP5 or GRA43 affect where TGD057 localizes to within the PV. Thus far, the Blanchard and Yap groups and our studies have shown through immunofluorescence assays (IFA) and/or differential centrifugation techniques that TGD057 is a PV resident protein within the PV lumen (Kongsomboonvech et al., 2020; Lopez et al., 2015) and within insoluble structural elements of the tachyzoite (Wilson et al., 2010). Here, we investigated the localization of TGD057 in *Δasp5* and *Δgra43 T. gondii* strains. TGD057 was detected with a rabbit polyclonal α-TGD057 (gift from Nicolas Blanchard, INSERM) and the PVM and dense granules were marked by α-GRA5. RH *Δgra43* expresses GFP hence GRA5 was not assessed in this manner. Regardless, TGD057 appeared as puncta and was distributed throughout the PV and tachyzoite in all strain types analyzed (**Fig 3-11**). These results imply GRA43 and ASP5 regulate the T57 IFNγ response independently of where TGD057 localizes within the vacuole.

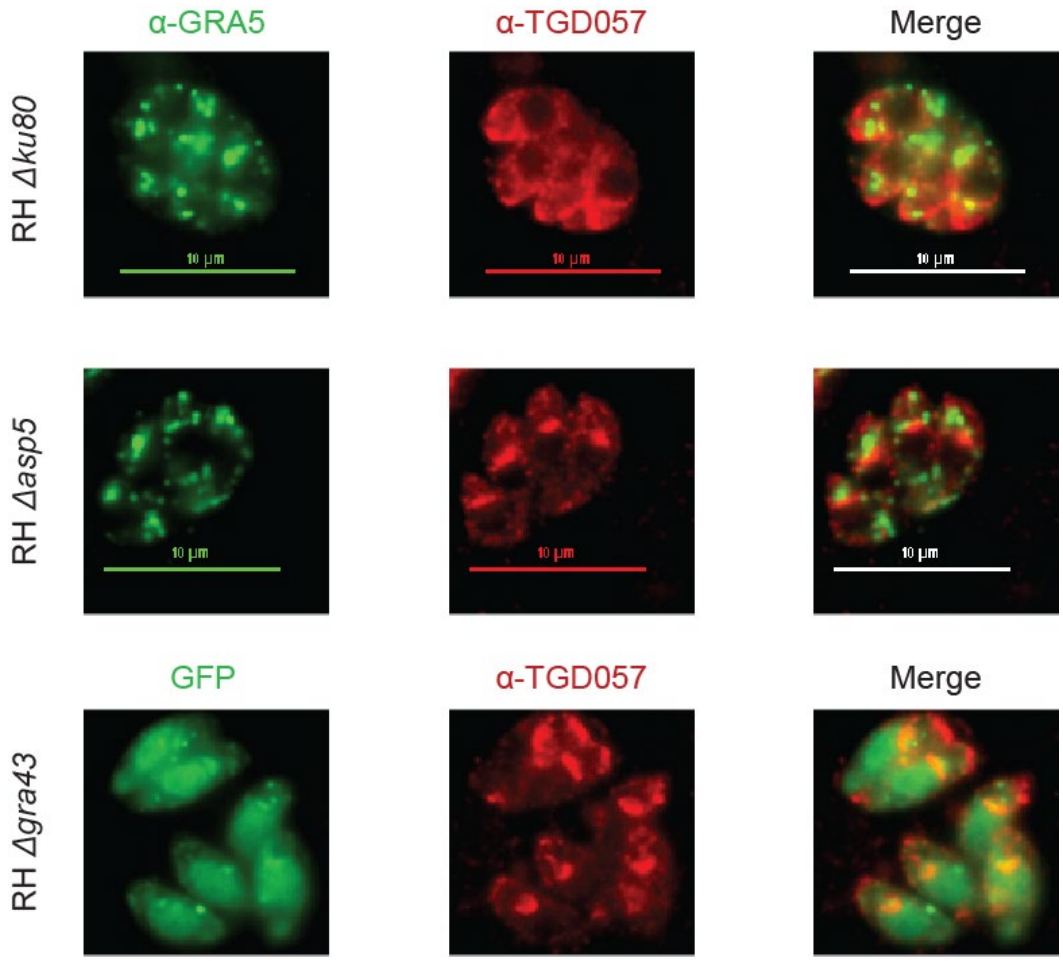

Figure 3-11. ASP5 and GRA43 do not affect the localization pattern of TGD057 within the PV. Human foreskin fibroblasts were infected with indicated *T. gondii* strains. After 16 hours of infection, the samples were fixed and visualized. TGD057 was detected with a rabbit polyclonal anti-TGD057 antibody, visualized in red. The PVM is indicated by the presence of the PVM integral and PV luminal dense granule GRA5. This was detected with an anti-GRA5 antibody, visualized in green, for WT RH and RH *Δasp5* strains. RH *Δgra43* is GFP+. A representative immunofluorescence image from 2 experiments is shown.

3.4 Discussion

Toxoplasma gondii is considered a successful parasite because it can infect nearly all warm-blooded vertebrates. To accommodate this broad host range, the parasite co-evolves with its host to be able to modulate host immune responses. Among various host immune cells, CD8 T cells are critical for the elimination of parasites. Therefore, we hypothesized the existence of *T. gondii* virulence factors, or ROCTRs, responsible for strain-specific differences in inducing CD8 T cell responses to infection. Broadly, the identity of ROCTR would help us better understand how *T. gondii* can survive in a variety of hosts that it infects.

In our genetic mapping experiments, we identified two loci with small effects on *T. gondii* chromosomes X and XII, which we will refer to as ROCTR-X and ROCTR-XII, respectively. A probable ROCTR-XII candidate is an NTPase. We speculated that these NTPases could modulate T cell function through hydrolysis of ATP and inhibition of the inflammasome activation process during parasitic infection. Cytosolic or extracellular ATP (eATP) induces signaling through P2X7 receptors (P2X7R), leading to potassium (K^+) efflux and NLRP3 inflammasome activation (Amores-Iniesta et al., 2017; Jo et al., 2016; Wang et al., 2019). Exogenous ADP has also been shown to mediate NLRP3 inflammasome activation through P2Y2 purinergic receptors (Baron et al., 2015). If the amount of eATP or eADP is depleted by *T. gondii*, then there would be no formation of inflammasome complex nor inflammasome activation via the extracellular purinergic receptor signaling pathway. In addition, depletion of cytosolic ATP by *T. gondii*, as previously shown for the related NTPase I (Olias and Sibley, 2016), could potentially thwart NLR-oligomerization and inflammasome activation which is an ATP-dependent process (Duncan et al., 2007). This possible modulation of NLRP3 activity by ROCTR-XII would coincide with our previous finding that an NLRP-dependent pathway is required for the commitment of activated CD8 T cells to differentiate into IFNγ-producing cells (Kongsomboonvech et al., 2020). However, no difference was observed between CD8 T cell responses induced by the NTPase KO strains compared to their parental counterparts. Interestingly, the effect plot for this ROCTR-XII candidate (**Fig 3-2C**) revealed that strains which expressed the type I allele of this gene induced high CD8 T cell responses while the opposite was observed with strains that expressed the type II allele. Given that the highly virulent type I *T. gondii* strain, with its effective ROP5, consistently induce low CD8 T cell IFNγ responses, then it is possible that the effect of the ROCTR-XII NTPase may not be observed when ROP5 is present or by another locus, currently unidentified, encoded in the type I genetic background.

Although the ROCTR-X candidate associated with the major QTL peak encoded on *T. gondii* chromosome X (PEAK2, **Fig 3-2B**) is yet to be identified, in analyzing the smaller peak on chromosome X (PEAK3, **Fig 3-2B**), we discovered that three *T. gondii* dense granules—GRA35, GRA42, and GRA43—affect the CD8 T cell IFNγ response. The *T. gondii* chromosome X PEAK3 (**Fig 3-2B**) corresponds to the dense granule GRA35 which localizes to the PVM with aid of ASP5, GRA42, or GRA43 (Wang et al., 2019). ASP5 is crucial to *T. gondii* PV integrity as it aids delivery of various dense granules, such as GRA3 and GRA7 (Hammoudi et al., 2015) and MAF1 (Coffey et al., 2015), to the PVM. Similarly, GRA42 and GRA43 are important for the targeting of other dense granules to the PVM including GRA17 and GRA23 which promote a normal PV structure (Gold et al., 2015), (Wang et al., 2019). We noted a general increase in the CD8 T cell response to *T.*

gondii strains with defects in the PVM-targeting pathway, which included *Δasp5* (Kongsomboonvech et al., 2020), *Δgra42*, and *Δgra43 T. gondii* strains (**Fig 3-10**). Several possible mechanisms may explain these observations. First, the PVM-targeting pathway, mediated by ASP5 and GRA43, maintain PV integrity and prevent TGD057 antigen escape; hence impairments in this process would induce host CD8 T cell responses. Second, PVM-targeted proteins are themselves ROCTRs, thereby modulating host CD8 T cell responses. The PVM-associated GRA35 was shown as an NLRP1 inflammasome activator in infected Lewis rat BMDMs (Wang et al., 2019). Together with our previous findings that NLRP1 sensor proteins are required for CD8 T cell responses (Kongsomboonvech et al., 2020), we hypothesized that GRA35 may interact with murine NLRP1 to regulate the CD8 T response during *T. gondii* infection. However, GRA35 had only a minor effect on CD8 T cell IFNγ responses (**Fig 3-10**), and whether GRA35 interacts with murine NLPR1 is unknown. Finally, we explored whether ASP5 and GRA43 deficiency would alter TGD057 association to the PVM and potentially expose it to host cytosolic defense machinery, as PVM association is required for optimal MHC I antigen presentation of the GRA6 antigen (Lopez et al., 2015). However, this does not seem to be the case; TGD057 remains in the PV lumen regardless of ASP5 or GRA43 (**Fig 3-11**).

ROP5 is an important suppressor of the response to *T. gondii* and it is highly polymorphic between strains. The *ROP5* locus consists of *ROP5A*, *ROP5B*, and *ROP5C* genes and the copy number of these genes vary among strains (Reese et al., 2011). However, these polymorphisms are not responsible for the strain differences in regulation of the CD8 T cell IFNγ response (Kongsomboonvech et al., 2020). Though ROP5 was discovered though the usage of F1 progeny sexual crosses as a polymorphic determinant responsible for strain-specific difference in *T. gondii* virulence during mouse infections (Behnke et al., 2011), it was not detected in our QTL mapping. ROP5 works independently or in complexes, including the ROP5/ROP18/ROP17 complex (Etheridge et al., 2014) or ROP5/ROP18/GRA7 complex (Alaganan et al., 2014; Hermanns et al., 2016), to keep the PV free from attack by host IRGs. It is possible that the polymorphic ROCTRs also assist ROP5 in an undisclosed pathway that regulate vacuolar antigen escape. Whether there are other parasite effectors, including ROCTRs, that associate with ROP5 remains to be determined, but this would be consistent with a general role for pseudokinases as scaffolds that facilitate protein-protein interactions (Boudeau et al., 2006; Langeberg and Scott, 2015). Previously, we demonstrated that *Δrop5* strains complemented with avirulent type I and II *ROP5A* alleles, which are unable to antagonize known effector IRGs (Hunter and Sibley, 2012; Reese et al., 2014), were able to equally inhibit the CD8 T cell response compared to the wildtype type I strain (Kongsomboonvech et al., 2020). This finding suggests that all ROP5 alleles are functional, but likely requires assistance

from a genetic determinant within type I genetic background to perform its function, presumably a ROCTR. Perhaps ROP5A which has no known function or interacting partner requires ROCTR to effectively inhibit MHC I antigen presentation, thereby inhibiting CD8 T cell IFNγ responses.

Overall, the CD8 T cell-mediated IFNγ response from a naïve state is an intricate process which requires several steps: antigen presentation on the surface via peptide-MHC (pMHC) I molecules to naïve CD8 T cells, activation of CD8 T cells which requires both detection of the pMHC and co-stimulation through co-receptor-ligand interactions, and finally differentiation of activated CD8 T cells to become IFNγ producers. This complexity perhaps contributes to the challenge in identifying a ROCTR, as there could be one ROCTR modulating each step or numerous ROCTRs working collectively to modulate the CD8 T cell response. *T. gondii* must protect its PV, not be caught in the host cytosol, avoid having its peptide antigen presented on the cell surface by MHC I molecules, all this to ensure its own survival. Hence, any *T. gondii* dense granules and rhoptries that mediate PV integrity, and thus antigen release from the PV, or regulate cytokine secretion and thus modulate differentiation, could generally contribute to the strain differences in CD8 T cell IFNγ responses. Further compounding our search for ROCTR is the relative variability in the magnitude of the CD8 T cell response. For example, we have observed a range of 4,000 pg/ml to 120,000 pg/ml of IFNγ secreted by CD8 T cells in response to type II ME49 *T. gondii* strain from our antigen-specific T cell activation assay. Consequently, these experiment-to-experiment variations may dampen a consistent signal that would assist genetic linkage analysis.

In summary, the CD8 T cell response to *T. gondii* infections is a complex phenotype that can be altered by multiple processes including antigen presentation, CD8 T cell activation, and the differentiation of activated CD8 T cells into IFNγ-producing cells. To avoid being eliminated by host cytotoxic CD8 T cells and ensure its survival, it is possible *T. gondii* manipulates all or one of the steps required for this response, likely through ROCTRs, as well as maintain its intact PV. Recently, a novel requirement for the CD8 T cell response against *T. gondii* infections has been identified; the cytosolic pathogen sensor NLRP3 protein, but not its associated inflammasome complex, is crucial for the CD8 T cell response for parasitic infections (Kongsomboonvech et al., 2020). Moreover, previous studies from our lab and others have shown the importance of the IRG pathway in initiating CD8 T cell response (Kongsomboonvech et al., 2020; Lee et al., 2015; Rommereim et al., 2019). We hypothesize that ROCTR(s) may intersect these pathways, providing insights to novel host-parasite interactions that control CD8 T cell IFNγ responses. Altogether, we propose a model in which *T. gondii* utilize more than one ROCTR, including but not limited to ROCTR-X and ROCTR-XII, to regulate the host CD8 T cell IFNγ response in a multilayered manner.

Chapter 4:

Conclusion

4.1 Contributions to the field

4.1.1 Experimental model for T. gondii endogenous antigen-specific CD8 T cell IFNγ responses

In the proposed studies of this dissertation, the CD8 T cell response for a specific *T. gondii* vacuolar antigen TGD057 was analyzed. Following the invasion of a host cell, *T. gondii* resides in its parasitophorous vacuole (PV) and hides from the host immune response. Previous studies pertaining to CD8 T cell responses to *T. gondii* utilize OVA-engineered expressing *T. gondii*, which express OVA antigen in the PV lumen, and OT-I CD8 T cells or hybridomas (Gregg et al., 2011; Lee et al., 2015; Rommereim et al., 2019). In addition to the model OVA antigen, CD8 T cell responses to *T. gondii* had been studied in respect to the endogenous *T. gondii* dense granule GRA6, only encoded by type II *T. gondii* strains, which is integrated into the PV membrane (PVM) and exposed to the host cytosol (Buaillon et al., 2017; Lopez et al., 2015). Therefore, we were able to explore the CD8 T cell response as well as the MHC I antigen presentation pathway to an endogenous *T. gondii* antigen whose peptide epitope is conserved among all parasite strains. For this reason our system is advantageous as it allowed the analyses of the CD8 T cell response to a natural antigen of ~150 parasite strains; as we were not limited by the genetic background of the parasite nor did we need to express a model antigen as a transgene in multiple strain types. This allowed us to determine that the CD8 T cell response is genetically controlled by multiple parasite effectors of *T. gondii*. Since we obtained naïve CD8 T cells from transnuclear mice (Kirak et al., 2010), we were able to observe how functional responses develop overtime in a co-culture system with parasite-infected macrophages. This afforded several unique insights into the requirements for CD8 T cell differentiation and commitment to IFNγ production from a naïve state.

4.1.2 All avirulent of T. gondii effector pseudokinase ROP5 are able to inhibit CD8 T cell response

Among parasite effectors analyzed, we found that the CD8 T cell response is critically regulated by the family of parasite effector pseudokinases, ROP5, which are known to antagonize host ‘effector’ IRG proteins, thereby preventing PV disruption. There are three isoforms of *T. gondii* ROP5: ROP5A, ROP5B, and

ROP5C. It was previously demonstrated that only some isoforms of ROP5, ROP5B and ROP5C, expressed by virulent *T. gondii* strains are functional antagonists of host IRGs (Fleckenstein et al., 2012; Liesenfeld et al., 2011; Niedelman et al., 2012; Reese et al., 2014). Specifically, it was shown that the ROP5A isoform of virulent *T. gondii* strains and all ROP5 isoforms of avirulent *T. gondii* strains fail to antagonize the host IRGs (Hunter and Sibley, 2012; Reese et al., 2011). However, our data illustrated that avirulent alleles of *ROP5* (ROP5A isoform of avirulent and virulent strains) can repress the CD8 T cell IFNγ response, which is a novel finding (**Chapter 2, Fig 2-7**). ROP5A has no known interacting partner in the host cell and likely functions by a mechanism independent from any known IRG-ROP5 interaction. We favor the hypothesis that ROP5 prevents *T. gondii* antigen escape from the PV by antagonizing an unknown host mechanism for vacuolar antigen access. The identity of this pathway would have implications for vaccine development for vacuolar pathogens.

4.1.3 NLRP sensor proteins and RIPK2 are absolutely required for the CD8 T cell-mediated IFNγ responses to T. gondii infections

Both NLRP3 and NLRP1 inflammasome activation occur following *T. gondii* infection (Cirelli et al., 2014; Ewald et al., 2014; Gorfu et al., 2014). The NLRP3, and to a similar extent the NLRP1 sensors are absolutely required for the antigen-specific CD8 T cell IFNγ response to TGD057 (Kongsomboonvech et al., 2020; **Chapter 2, Fig 2-12**); which had not been described before. We further investigated whether the loss of CD8 T cell IFNγ responses was due to an impairment of T cell activation or differentiation. To test this, we generated a 'T-GREAT' IFNγ reporter mouse line by crossing T57 with GREAT mice (Reinhardt et al., 2015) as described in Chapter 2. We observed that the percentage of activated CD69+ CD8 T cells in response to *T. gondii*-infected *Nlrp3-/-* BMDMs was slightly reduced in comparison to *T. gondii*-infected WT BMDMs (Kongsomboonvech et al., 2020; **Chapter 2, Fig 2-15**) implicating the that MHC I antigen presentation was intact in *Nlrp3-/-* BMDMs. Interestingly, the IFNγ transcript levels of these activated CD69+ CD8 T cells dropped significantly in response to parasite-infected *Nlrp3-/-* BMDMs compared to WT BMDMs (Kongsomboonvech et al., 2020; **Chapter 2, Fig 2-15**). Our results suggest that NLRP3 is critically important for activated CD8 T cells to differentiate into IFNγ-producing cells when responding to *T. gondii* infections (**Fig 4-1**). In follow-up experiments, we discovered unexpectedly that the NLR-mediated regulation of the CD8 T cell responses occurred in the absence of other components of the inflammasome cascade. This is inferred because the T57 CD8 T cell IFNγ product was still detected in response to *T. gondii*-infected *Asc-/-*, *Casp1/11-/-*, *and Gsdmd-/-* BMDMs, or when IL-1 and IL-18 cytokine signaling was inhibited (Kongsomboonvech et al., 2020; **Chapter 2, Fig 2-12**). Thus, IL-1 and IL-18 are

not the primary cytokines responsible for the differentiation of activated CD8 T cell to become IFNγ producers. Altogether, this suggests a novel NLRP3 protein-dependent but NLRP3 inflammasome complex-independent pathway for CD8 T cell IFNγ responses to *T. gondii*. It is currently unknown the nature of the NLRP3-dependent signal that is required for CD8 T cell IFNγ differentiation. There are very few reports of NLRP3 protein-dependent but inflammasome complex-independent phenotypes, hence a novel biology for NLRP3 is expected in our system. We have also screened components that intersect the inflammasome pathway, particularly those involved in the priming step such as NOD2 and RIPK2, which induces the transcription of *NLRP3* and *IL1B* via NF-kB activation, as previously described in Chapter 2. Interestingly, the CD8 T cell IFNγ response to infected *Nod2-/-* BMDMs is reduced and completely diminished in response to infected *Ripk2-/-* BMDMs, compared to parasite-infected WT BMDMs (**Fig 2-14**). The role of RIPK2 in the CD8 T cell IFNγ response has yet to be explored and the future directions will be discussed in Section 4.2.

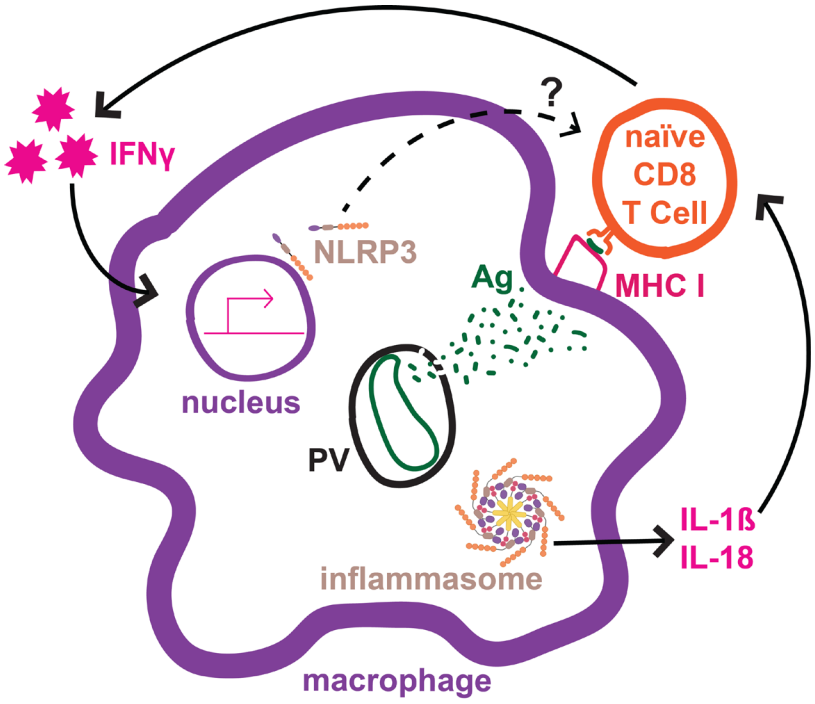

Figure 4-1. NLRP3 sensor protein promotes CD8 T cell-mediated IFNγ response to *T. gondii* infections. NLRP3 likely intersects the CD8 T cell IFNγ response at the differentiation step, including both the IL-1/18 pathway through conventional inflammasome activation, and via an NLRP3-inflammasome independent pathway through an unknown mechanism.

4.1.4 There are likely multiple genetic loci with small effect that determine strain-specific differences in the host CD8 T cell response to T. gondii

CD8 T cell IFNγ production was analyzed in response to multiple atypical *T. gondii* strains (haplogroups IV-X), which are not commonly studied, in additional to the less virulent North American strains (types I, II, III, and haplogroups XI-XII) (**Fig 2-8 and Fig 2-9**). The analysis of these atypical strains led to the observation of the CD8 T cell response's unique phenotypic pattern associated with only clade A strains. Thus, we hypothesize that there is at least one *T. gondii* polymorphic factor modulating the CD8 T cell response that accounts for the strain differences, which we termed Regulator Of CD8 T cell Response (ROCTR). To identify ROCTR, we performed quantitative trait loci (QTL) analysis of the CD8 T cell IFNγ response induced by 29 *T. gondii* F1 progeny strains derived from a cross between a type I (GT1, low CD8 T cell IFNγ response inducer) and type II (ME49, high CD8 T cell IFNγ response inducer) *T. gondii* strains. The genetic analysis of this strain differences phenotype reveals two QTLs of small effect on *T. gondii* chromosomes X and XII (**Fig 3-1**). Our attempts to identity ROCTR(s) have not been successful, nor was our search assisted by screening other F1 progeny from the IIxIII and IxIII genetic crosses. For these reasons, we argue there are multiple polymorphic ROCTRs, each with small effect, that possibly account for the clade A-specific strain differences in CD8 T cell activation. We also believe our search for ROCTR was inhibited by the complexity of the phenotype measured—the CD8 T cell IFNγ response—which requires intricate host processes related to MHC I antigen presentation, co-stimulation by co-receptors and ligands, and differentiation steps. Because the CD8 T cell response to *T. gondii* infections requires host expression of NLR sensor proteins as well as the IRG pathway, our observations offer clues as to where ROCTRs may function. For example, we speculate that ROCTRs could modulate PVM integrity, perhaps working in conjunction with ROP5. Additionally, ROCTRs may intersect the NLRP3-dependent, inflammasome-independent pathway described in Section 4.1.3. Identifying ROCTR and its function may further elucidate how CD8 T cell commit to engaging with a parasite and the rules governing anti-parasitic immunity.

4.1.5 T. gondii PVM-targeting effectors modulate host CD8 T cell IFNγ responses

Clues as to where ROCTRs might function come from studies of parasites that are defective in the PVM-targeting pathway. First, genetic mapping revealed a weak association at the locus encoding the dense granule GRA35, an NLRP1 inflammasome activator in *T. gondii*-infected Lewis rat macrophages (Wang et al., 2019), as a potential ROCTR candidate. The PV luminal GRA35 associates to the PVM with the aid of GRA42 and GRA43 (Wang et al., 2019). We examined all three dense granules and their impact on host CD8 T cell responses and observed that all three dense granules GRA35, GRA42, and GRA43 were able to modulate the CD8 T cell response. Overall, there were increased CD8 T cell responses to *Δgra35*, *Δgra42*, and *Δgra43* infections compared to the parental strains (**Fig 3-9**).

GRA43 had the largest effect of the three GRAs. Similarly, ASP5 seemed to be able to suppress CD8 T cell responses (**Fig 2-4**). ASP5, GRA42, and GRA43 aid various dense granules in their PVM association, as described and discussed in Chapter 2 and Chapter 3. Altogether, we hypothesize that PVM-targeting effectors of *T. gondii* (ASP5, GRA42, GRA43) may regulate the CD8 T cell response by either, 1) targeting ROCTRs to the PVM where they interact with the host cell compartments, and/or, by 2) modulating the localization of PVM-targeted proteins that maintain PVM integrity. For example, GRA17 and GRA23 that localize to the PVM and form pores, thereby facilitating movement of molecules in and out of the PV (Gold et al., 2015), cannot localize to the PVM in absence of GRA43 (Wang et al., 2019).

4.2 Future directions

4.2.1 The role of RIPK2 and inflammasome-independent NLRP3 in CD8 T cell responses to T. gondii infections

In our attempt to dissect the components of the inflammasome pathway required for host CD8 T cell IFNγ responses to *T. gondii* infections, we discovered that RIPK2 is an absolute requirement for this response (**Fig 2-14**). ER stress has been shown to induce inflammasome activation (Bronner et al., 2015; Elliott and Sutterwala, 2015; Menu et al., 2012) as well as NOD2/RIPK2 through IRE1 signaling (Keestra-Gounder et al., 2016). ER stress-mediated IRE1 signaling leads to NLRP3-dependent yet inflammasome-independent mitochondrial ROS (mtROS) production and caspase2-mediated damage of the mitochondria (Bronner et al., 2015). IRE1 signaling and mitochondrial damage via caspase 2 and inflammasome-independent NLRP3 may be at the core of the CD8 T cell IFNγ response to *T. gondii* infections and should be investigated. This can be addressed by analyzing the CD8 IFNγ response to *T. gondii*-infected caspase2-deficient BMDMs or BMDMs treated with specific IRE-1 inhibitors such as 4μ8C (Cross et al., 2012) and KIRA6 (Ghosh et al., 2014), or the IRE1α RNase inhibitor STF-083010 (Papandreou et al., 2011). Similarly, the mtROS generated by IRE1 was mediated by the thioredoxin-interacting protein (TXNIP) and can be silenced in BMDMs (Bronner et al., 2015). Another intriguing possibility is whether NOD/RIPK2 is the missing link between IRE1 signaling and the NLRP3-dependent mitochondrial damage pathway, which could be validated in our system. Since the host ER is closely associated with the PV (Sinai et al., 1997), it would not be unexpected if ER stress plays a major role in regulating CD8 T cell immunity to *T. gondii* (Goldszmid et al., 2009; Yamamoto et al., 2011).

4.2.2 The search for ROCTR responsible for strain differences

By definition, ROCTR is a polymorphic parasite effector that is responsible for the observed strain-specific differences in eliciting a CD8 T cell IFNγ response to *T. gondii*. However, the search for ROCTR has been challenging. This is likely because the CD8 T cell IFNγ response is the product of several steps that CD8 T cells must undergo to combat *T. gondii* infections. First, naïve CD8 T cells will need to find the *T. gondii* antigen presented on the surface of MHC I molecule by an infected cell. Then, these naïve CD8 T cells undergo activation. Only some of the activated CD8 T cells will differentiate into IFNγ-producing CD8 T cells. Perhaps an investigation at each of these steps will provide more obvious clues to the identity of ROCTR, for which there is likely many. Based on our data in which the level of CD8 T cell activation is regulated by the IFNγ-IRG pathway (**Fig 2-10**) and that the differentiation of activated CD8 T cells is greatly impacted in absence of host *Nlrp3* (**Fig 2-15**), perhaps these two steps should be individually investigated when searching for ROCTR. For example, genome-wide mapping analysis could first be performed by screening a simpler phenotype or readout for the inflammasome or IRG pathways. Then, following the discovery of putative ROCTRs, the T cell activation assay would be performed followed by flow cytometry as described in Chapter 2 to confirm their identity as a bona fide ROCTR.

4.3 Summary

T. gondii is capable of hiding from the immune system by forming a PV around itself. If detected, host cytotoxic CD8 T cells will respond by secreting IFNγ. This CD8 T cell-mediated IFNγ response is required for parasite elimination. Though the role of CD8 T cells in parasitic infections have been well-studied, there are remaining knowledge gaps in the field of host-parasite interactions including 1) the host requirements for naïve CD8 T cells to respond to parasitic infections, 2) the requirements for CD8 T cell differentiation to become IFNγ-producing CD8 T cells in response to parasitic infections, and 3) parasite effectors that intersect CD8 T cell activation and/or differentiation. The goal of this doctoral dissertation was to unravel interactions between the host and *T. gondii* that influence the CD8 T cell response in hope that we can provide insights for novel immunotherapeutic strategies against *T. gondii* as well as other parasites in the Apicomplexa phylum, including the related *Plasmodium* species which also utilize a PV to hide from the immune system. Here, we present a novel NLRP3-dependent, inflammasome-independent host requirement for IFNγ differentiation of CD8 T cells. We have also shown that RIPK2 is another absolute requirement for host CD8 T cell-mediated IFNγ response to *T. gondii* infections. We have demonstrated that even so-called ‘avirulent’ isoforms of ROP5 work as inhibitors by an unknown mechanism, and

have confirmed that the *T. gondii* PVM-targeting pathway mediated by ASP5, and GRA43 are able to modulate this phenotype. This further supports what is known in the field that PV integrity is important for *T. gondii* to evade host CD8 T cell immunity. Altogether, we hypothesize that there are multiple parasitic ROCTRs that influence the variation of host CD8 T cell IFNγ responses to *T. gondii*. We propose a working model in which CD8 T cell IFNγ responses to *T. gondii* are determined by 1) the host-parasite battle that occurs at the PVM, and 2) differentiation factors produced by a novel inflammasome pathway. ROCTRs, though their identities remain unknown, may intersect the CD8 T cell response at any of these steps to allow different *T. gondii* strains to "turn up" or "turn down" the response to achieve a balanced and life-long chronic infection across a wide host range.

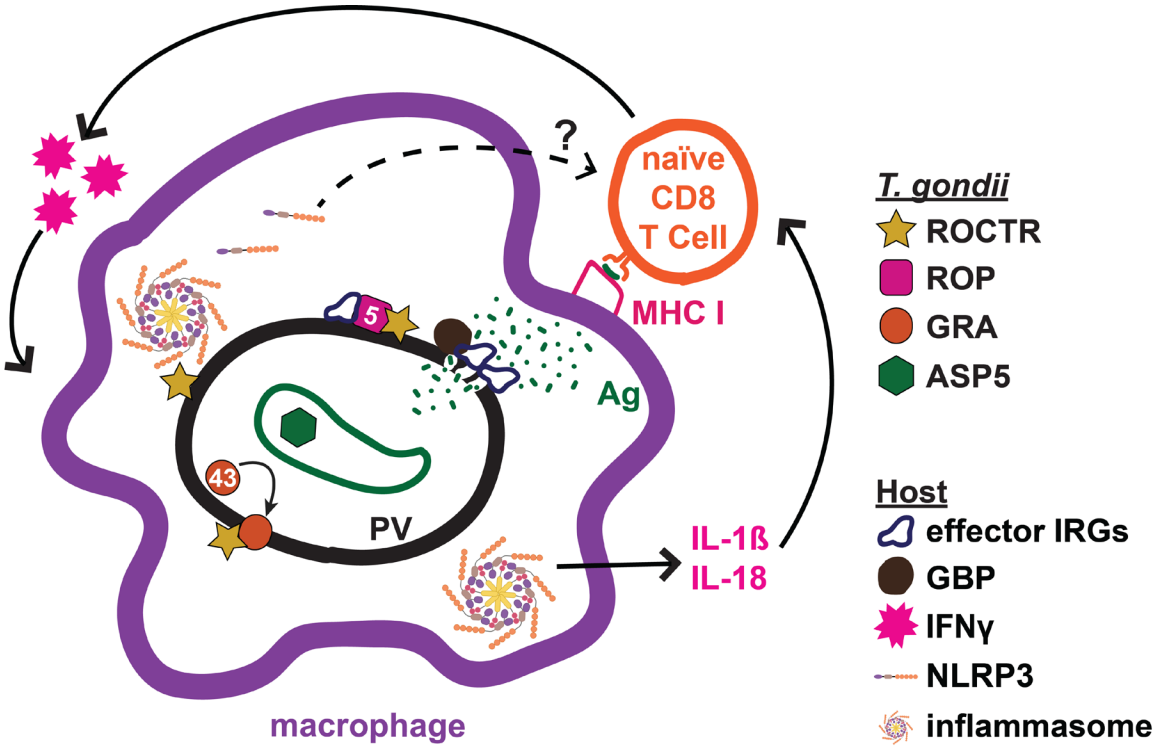

Figure 4-2. The CD8 T cell responses to parasite infections may be regulated at multiple processes by *T. gondii*. We propose a model in which *T. gondii* modulates the host CD8 T cell response in a multilayered manner, utilizing more than one effector to regulate CD8 T cell activation. We hypothesize that ROCTRs could modulate the CD8 T cell response in various ways including possibly 1) interacting with ROP5, allowing all alleles of ROP5 to properly function, or 2) interfering with inflammasome activation, or with the NLRP3 sensor when it functions independently of the inflammasome complex. We also speculate that ROCTR may be directly targeted to the PVM by ASP5 and GRA43 where it can serve to help the function of other *T. gondii* effector proteins at the PVM, either allowing or preventing antigen escape from the PV.

References

Afonina, I.S., Müller, C., Martin, S.J., Beyaert, R., 2015. Proteolytic Processing of Interleukin-1 Family Cytokines: Variations on a Common Theme. Immunity 42, 991–1004. https://doi.org/10.1016/j.immuni.2015.06.003

Alaganan, A., Fentress, S.J., Tang, K., Wang, Q., Sibley, L.D., 2014. Toxoplasma GRA7 effector increases turnover of immunity-related GTPases and contributes to acute virulence in the mouse. Proceedings of the National Academy of Sciences of the United States of America 111, 1126–1131. https://doi.org/10.1073/pnas.1313501111

Amores-Iniesta, J., Barberà-Cremades, M., Martínez, C.M., Pons, J.A., Revilla-Nuin, B., Martínez-Alarcón, L., Di Virgilio, F., Parrilla, P., Baroja-Mazo, A., Pelegrín, P., 2017. Extracellular ATP Activates the NLRP3 Inflammasome and Is an Early Danger Signal of Skin Allograft Rejection. Cell Reports 21, 3414–3426. https://doi.org/10.1016/j.celrep.2017.11.079

Asai, T., Kim, T.J., Kobayashi, M., Kojima, S., 1987. Detection of nucleoside triphosphate hydrolase as a circulating antigen in sera of mice infected with Toxoplasma gondii. Infect Immun 55, 1332–1335. https://doi.org/10.1128/IAI.55.5.1332-1335.1987

Asai, T., Miura, S., Sibley, L.D., Okabayashi, H., Takeuchi, T., 1995. Biochemical and molecular characterization of nucleoside triphosphate hydrolase isozymes from the parasitic protozoan Toxoplasma gondii. J Biol Chem 270, 11391–11397. https://doi.org/10.1074/jbc.270.19.11391

Asai, T., O'Sullivan, W.J., Tatibana, M., 1983. A potent nucleoside triphosphate hydrolase from the parasitic protozoan Toxoplasma gondii. Purification, some properties, and activation by thiol compounds. J Biol Chem 258, 6816–6822.

Asai, T., Takeuchi, T., Diffenderfer, J., Sibley, L.D., 2002. Identification of Small-Molecule Inhibitors of Nucleoside Triphosphate Hydrolase in Toxoplasma gondii. Antimicrobial Agents and Chemotherapy 46, 2393–2399. https://doi.org/10.1128/AAC.46.8.2393-2399.2002

Baron, L., Gombault, A., Fanny, M., Villeret, B., Savigny, F., Guillou, N., Panek, C., Le Bert, M., Lagente, V., Rassendren, F., Riteau, N., Couillin, I., 2015. The NLRP3 inflammasome is activated by nanoparticles through ATP, ADP and adenosine. Cell Death &Amp; Disease 6, e1629.

Bauernfeind, F.G., Horvath, G., Stutz, A., Alnemri, E.S., MacDonald, K., Speert, D., Fernandes-Alnemri, T., Wu, J., Monks, B.G., Fitzgerald, K.A., Hornung, V., Latz, E., 2009. Cutting Edge: NF-κB Activating Pattern Recognition and Cytokine

Receptors License NLRP3 Inflammasome Activation by Regulating NLRP3 Expression. The Journal of Immunology 183, 787–791. https://doi.org/10.4049/jimmunol.0901363

Behnke, M.S., Fentress, S.J., Mashayekhi, M., Li, L.X., Taylor, G.A., Sibley, L.D., 2012. The Polymorphic Pseudokinase ROP5 Controls Virulence in Toxoplasma gondii by Regulating the Active Kinase ROP18. PLoS Pathogens 8. https://doi.org/10.1371/journal.ppat.1002992

Behnke, M.S., Khan, A., Lauron, E.J., Jimah, J.R., Wang, Q., Tolia, N.H., Sibley, L.D., 2015. Rhoptry Proteins ROP5 and ROP18 Are Major Murine Virulence Factors in Genetically Divergent South American Strains of Toxoplasma gondii. PLoS genetics 11. https://doi.org/10.1371/journal.pgen.1005434

Behnke, M.S., Khan, A., Wootton, J.C., Dubey, J.P., Tang, K., Sibley, L.D., 2011. Virulence differences in Toxoplasma mediated by amplification of a family of polymorphic pseudokinases. Proceedings of the National Academy of Sciences 108, 9631–9636. https://doi.org/10.1073/pnas.1015338108

Bekpen, C., Hunn, J.P., Rohde, C., Parvanova, I., Guethlein, L., Dunn, D.M., Glowalla, E., Leptin, M., Howard, J.C., 2005. The interferon-inducible p47 (IRG) GTPases in vertebrates: loss of the cell autonomous resistance mechanism in the human lineage. Genome biology 6. https://doi.org/gb-2005-6-11-r92

Bermudes, D., Peck, K.R., Afifi, M.A., Beckers, C.J., Joiner, K.A., 1994. Tandemly repeated genes encode nucleoside triphosphate hydrolase isoforms secreted into the parasitophorous vacuole of Toxoplasma gondii. J Biol Chem 269, 29252–29260.

Bertholet, S., Goldszmid, R., Morrot, A., Debrabant, A., Afrin, F., Collazo-Custodio, C., Houde, M., Desjardins, M., Sher, A., Sacks, D., 2006. Leishmania Antigens Are Presented to CD8+ T Cells by a Transporter Associated with Antigen Processing-Independent Pathway In Vitro and In Vivo. J. Immunol. 177, 3525. https://doi.org/10.4049/jimmunol.177.6.3525

Blader, I.J., Coleman, B.I., Chen, C.-T., Gubbels, M.-J., 2015. Lytic Cycle of Toxoplasma gondii: 15 Years Later. Annual Review of Microbiology 69, 463–485. https://doi.org/10.1146/annurev-micro-091014-104100

Blanchard, N., Gonzalez, F., Schaeffer, M., Joncker, N.T., Cheng, T., Shastri, A.J., Robey, E.A., Shastri, N., 2008. Immunodominant, protective response to the parasite Toxoplasma gondii requires antigen processing in the endoplasmic reticulum. Nature immunology 9, 937–944. https://doi.org/10.1038/ni.1629

Blanchard, N., Shastri, N., 2010. Topological journey of parasite-derived antigens for presentation by MHC class I molecules. Trends in Immunology 31, 414–421. https://doi.org/10.1016/j.it.2010.08.004

Boddey, J.A., Cowman, A.F., 2013. Plasmodium Nesting: Remaking the Erythrocyte from the Inside Out. Annual Review of Microbiology 67, 243–269. https://doi.org/10.1146/annurev-micro-092412-155730

Boothroyd, J.C., 2009. Expansion of host range as a driving force in the evolution of Toxoplasma. Memorias do Instituto Oswaldo Cruz 104, 179–184. https://doi.org/S0074-02762009000200009

Boudeau, J., Miranda-Saavedra, D., Barton, G.J., Alessi, D.R., 2006. Emerging roles of pseudokinases. Trends in Cell Biology 16, 443–452. https://doi.org/10.1016/j.tcb.2006.07.003

Braun, L., Brenier-Pinchart, M.-P., Hammoudi, P.-M., Cannella, D., Kieffer-Jaquinod, S., Vollaire, J., Josserand, V., Touquet, B., Couté, Y., Tardieux, I., Bougdour, A., Hakimi, M.-A., 2019. The Toxoplasma effector TEEGR promotes parasite persistence by modulating NF-κB signalling via EZH2. Nature Microbiology 4, 1208–1220. https://doi.org/10.1038/s41564-019-0431-8

Braun, L., Brenier-Pinchart, M.-P., Yogavel, M., Curt-Varesano, A., Curt-Bertini, R.-L., Hussain, T., Kieffer-Jaquinod, S., Coute, Y., Pelloux, H., Tardieux, I., Sharma, A., Belrhali, H., Bougdour, A., Hakimi, M.-A., 2013. A Toxoplasma dense granule protein, GRA24, modulates the early immune response to infection by promoting a direct and sustained host p38 MAPK activation. J Exp Med 210, 2071. https://doi.org/10.1084/jem.20130103

Broman, K.W., Wu, H., Sen, Ś., Churchill, G.A., 2003. R/qtl: QTL mapping in experimental crosses. Bioinformatics 19, 889–890.

Bronner, D.N., Abuaita, B.H., Chen, X., Fitzgerald, K.A., Nuñez, G., He, Y., Yin, X.-M., O'Riordan, M.X.D., 2015. Endoplasmic Reticulum Stress Activates the Inflammasome via NLRP3- and Caspase-2-Driven Mitochondrial Damage. Immunity 43, 451–462. https://doi.org/10.1016/j.immuni.2015.08.008

Broz, P., Dixit, V.M., 2016. Inflammasomes: mechanism of assembly, regulation and signalling. Nature Reviews Immunology 16, 407–420. https://doi.org/10.1038/nri.2016.58

Bruchard, M., Rebé, C., Derangère, V., Togbé, D., Ryffel, B., Boidot, R., Humblin, E., Hamman, A., Chalmin, F., Berger, H., Chevriaux, A., Limagne, E., Apetoh, L., Végran, F., Ghiringhelli, F., 2015. The receptor NLRP3 is a transcriptional regulator of TH2 differentiation. Nature Immunology 16, 859–870. https://doi.org/10.1038/ni.3202

Buaillon, C., Guerrero, N.A., Cebrian, I., Blanié, S., Lopez, J., Bassot, E., Vasseur, V., Santi-Rocca, J., Blanchard, N., 2017. MHC I presentation of Toxoplasma gondii immunodominant antigen does not require Sec22b and is regulated by antigen orientation at the vacuole membrane. European Journal of Immunology 47, 1160–1170. https://doi.org/10.1002/eji.201646859

Bunnik, E.M., Venkat, A., Shao, J., McGovern, K.E., Batugedara, G., Worth, D., Prudhomme, J., Lapp, S.A., Andolina, C., Ross, L.S., Lawres, L., Brady, D., Sinnis, P., Nosten, F., Fidock, D.A., Wilson, E.H., Tewari, R., Galinski, M.R., Ben Mamoun, C., Ay, F., Le Roch, K.G., 2019. Comparative 3D genome organization in apicomplexan parasites. Proceedings of the National Academy of Sciences 116, 3183–3192. https://doi.org/10.1073/pnas.1810815116

Butcher, B.A., Fox, B.A., Rommereim, L.M., Kim, S.G., Maurer, K.J., Yarovinsky, F., Herbert, D.R., Bzik, D.J., Denkers, E.Y., 2011. Toxoplasma gondii Rhoptry Kinase ROP16 Activates STAT3 and STAT6 Resulting in Cytokine Inhibition and Arginase-1-Dependent Growth Control. PLOS Pathogens 7. https://doi.org/10.1371/journal.ppat.1002236

Camejo, A., Gold, D.A., Lu, D., McFetridge, K., Julien, L., Yang, N., Jensen, K.D., Saeij, J.P., 2014. Identification of three novel Toxoplasma gondii rhoptry proteins. International journal for parasitology 44, 147–160. https://doi.org/10.1016/j.ijpara.2013.08.002

Carruthers, V.B., Sibley, L.D., 1997. Sequential protein secretion from three distinct organelles of Toxoplasma gondii accompanies invasion of human fibroblasts. Eur J Cell Biol 73, 114–123.

Casciotti, L., Ely, K.H., Williams, M.E., Khan, I.A., 2002. CD8+-T-Cell Immunity against Toxoplasma gondii Can Be Induced but Not Maintained in Mice Lacking Conventional CD4+ T Cells. Infection and Immunity 70, 434–443. https://doi.org/10.1128/IAI.70.2.434-443.2002

Cavaillès, P., Sergent, V., Bisanz, C., Papapietro, O., Colacios, C., Mas, M., Subra, J.-F., Lagrange, D., Calise, M., Appolinaire, S., Faraut, T., Druet, P., Saoudi, A., Bessieres, M.-H., Pipy, B., Cesbron-Delauw, M.-F., Fournié, G.J., 2006. The rat Toxo1 locus directs toxoplasmosis outcome and controls parasite proliferation and spreading by macrophage-dependent mechanisms. Proc Natl Acad Sci U S A 103, 744–749. https://doi.org/10.1073/pnas.0506643103

CDC, 2018. Parasites - Neglected Parasitic Infections (NPIs) in the United States.

Cebrian, I., Visentin, G., Blanchard, N., Jouve, M., Bobard, A., Moita, C., Enninga, J., Moita, L.F., Amigorena, S., Savina, A., 2011. Sec22b regulates

phagosomal maturation and antigen crosspresentation by dendritic cells. Cell 147, 1355–1368. https://doi.org/10.1016/j.cell.2011.11.021

Cebrián, M., Yagüe, E., Rincón, M., López-Botet, M., de Landázuri, M.O., Sánchez-Madrid, F., 1988. Triggering of T cell proliferation through AIM, an activation inducer molecule expressed on activated human lymphocytes. The Journal of Experimental Medicine 168, 1621–1637. https://doi.org/10.1084/jem.168.5.1621

Chang, H.R., Grau, G.E., Pechère, J.C., 1990. Role of TNF and IL-1 in infections with Toxoplasma gondii. Immunology 69, 33–37.

Chen, L., Christian, D.A., Kochanowsky, J.A., Phan, A.T., Clark, J.T., Wang, S., Berry, C., Oh, J., Chen, X., Roos, D.S., Beiting, D.P., Koshy, A.A., Hunter, C.A., 2020. The Toxoplasma gondii virulence factor ROP16 acts in cis and trans, and suppresses T cell responses. Journal of Experimental Medicine 217. https://doi.org/10.1084/jem.20181757

Choi, J., Park, S., Biering, S.B., Selleck, E., Liu, C.Y., Zhang, X., Fujita, N., Saitoh, T., Akira, S., Yoshimori, T., Sibley, L.D., Hwang, S., Virgin, H.W., 2014. The Parasitophorous Vacuole Membrane of Toxoplasma gondii Is Targeted for Disruption by Ubiquitin-like Conjugation Systems of Autophagy. Immunity 40, 924–935. https://doi.org/10.1016/j.immuni.2014.05.006

Christiaansen, A., Varga, S.M., Spencer, J.V., 2015. Viral manipulation of the host immune response. Current Opinion in Immunology 36, 54–60. https://doi.org/10.1016/j.coi.2015.06.012

Cirelli, K.M., Gorfu, G., Hassan, M.A., Printz, M., Crown, D., Leppla, S.H., Grigg, M.E., Saeij, J.P.J., Moayeri, M., 2014. Inflammasome Sensor NLRP1 Controls Rat Macrophage Susceptibility to Toxoplasma gondii. PLoS Pathogens. https://doi.org/10.1371/journal.ppat.1003927

Clough, B., Wright, J.D., Pereira, P.M., Hirst, E.M., Johnston, A.C., Henriques, R., 2016. K63-linked ubiquitination targets Toxoplasma gondii for endo-lysosomal destruction in IFNγ-stimulated human cells. PLoS Pathogens 12. https://doi.org/10.1371/journal.ppat.1006027

Coffey, M.J., Dagley, L.F., Seizova, S., Kapp, E.A., Infusini, G., Roos, D.S., Boddey, J.A., Webb, A.I., Tonkin, C.J., 2018. Aspartyl Protease 5 Matures Dense Granule Proteins That Reside at the Host-Parasite Interface in Toxoplasma gondii. mBio 9. https://doi.org/10.1128/mBio.01796-18

Coffey, M.J., Sleebs, B.E., Uboldi, A.D., Garnham, A., Franco, M., Marino, N.D., Panas, M.W., Ferguson, D.J.P., Enciso, M., O'Neill, M.T., Lopaticki, S., Stewart, R.J., Dewson, G., Smyth, G.K., Smith, B.J., Masters, S.L., Boothroyd, J.C.,

Boddey, J.A., Tonkin, C.J., O'Neill, M.T., Lopaticki, S., Stewart, R.J., Dewson, G., Smyth, G.K., Smith, B.J., Masters, S.L., Boothroyd, J.C., Boddey, J.A., Tonkin, C.J., 2015. An aspartyl protease defines a novel pathway for export of Toxoplasma proteins into the host cell. eLife 4, 1–34. https://elifesciences.org/lookup/doi/10.7554/eLife.10809.001

Coppens, I., 2017. How Toxoplasma and malaria parasites defy first, then exploit host autophagic and endocytic pathways for growth. Current Opinion in Microbiology 40, 32–39. https://doi.org/10.1016/J.MIB.2017.10.009

Coppens, I., Dunn, J.D., Romano, J.D., Pypaert, M., Zhang, H., Boothroyd, J.C., Joiner, K.A., 2006. Toxoplasma gondii Sequesters Lysosomes from Mammalian Hosts in the Vacuolar Space. Cell 125, 261–274. https://doi.org/10.1016/j.cell.2006.01.056

Coutermarsh-Ott, S.L., Doran, J.T., Campbell, C., Williams, T.M., Lindsay, D.S., Allen, I.C., 2016. Caspase-11 Modulates Inflammation and Attenuates Toxoplasma gondii Pathogenesis. Mediators of Inflammation 2016. https://doi.org/10.1155/2016/9848263

Crook, O.M., Mulvey, C.M., Kirk, P.D.W., Lilley, K.S., Gatto, L., 2018. A Bayesian mixture modelling approach for spatial proteomics. PLoS Computational Biology 14, 1–29. https://doi.org/10.1371/journal.pcbi.1006516

Cross, B.C.S., Bond, P.J., Sadowski, P.G., Jha, B.K., Zak, J., Goodman, J.M., Silverman, R.H., Neubert, T.A., Baxendale, I.R., Ron, D., Harding, H.P., 2012. The molecular basis for selective inhibition of unconventional mRNA splicing by an IRE1-binding small molecule. Proceedings of the National Academy of Sciences 109, E869–E878. https://doi.org/10.1073/pnas.1115623109

Curt-varesano, A., Braun, L., Ranquet, C., Hakimi, M., Bougdour, A., 2016. The aspartyl protease TgASP5 mediates the export of the Toxoplasma GRA16 and GRA24 effectors into host cells. Cell Microbiol. 18, 151–167. https://doi.org/10.1111/cmi.12498

Ding, J., Wang, K., Liu, W., She, Y., Sun, Q., Shi, J., Sun, H., Wang, D.-C., Shao, F., 2016. Pore-forming activity and structural autoinhibition of the gasdermin family. Nature 535, 111–116. https://doi.org/10.1038/nature18590

Donald, R.G.K., Roos, D.S., 1998. Gene knock-outs and allelic replacements in Toxoplasma gondii: HXGPRT as a selectable marker for hit-and-run mutagenesis. Molecular and Biochemical Parasitology 91, 295–305. https://doi.org/10.1016/S0166-6851(97)00210-7

Dubey, J.P., Lindsay, D.S., Speer, C.A., 1998. Structures of Toxoplasma gondii tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. Clin Microbiol Rev 11, 267–299.

Duncan, J.A., Bergstralh, D.T., Wang, Y., Willingham, S.B., Ye, Z., Zimmermann, A.G., Ting, J.P.-Y., 2007. Cryopyrin/NALP3 binds ATP/dATP, is an ATPase, and requires ATP binding to mediate inflammatory signaling. Proceedings of the National Academy of Sciences 104, 8041–8046. https://doi.org/10.1073/pnas.0611496104

Dupont, C.D., Christian, D.A., Selleck, E.M., Pepper, M., Leney-Greene, M., Pritchard, G.H., Koshy, A.A., Wagage, S., Reuter, M.A., Sibley, L.D., Betts, M.R., Hunter, C.A., 2014. Parasite fate and involvement of infected cells in the induction of CD4+ and CD8+ T cell responses to Toxoplasma gondii. PLoS Pathogens 10, e1004047. https://doi.org/10.1371/journal.ppat.1004047

Dzierszinski, F., Pepper, M., Stumhofer, J.S., LaRosa, D.F., Wilson, E.H., Turka, L.A., Halonen, S.K., Hunter, C.A., Roos, D.S., 2007. Presentation of Toxoplasma gondii antigens via the endogenous major histocompatibility complex class I pathway in nonprofessional and professional antigen-presenting cells. Infection and Immunity 75, 5200–5209. https://doi.org/10.1128/IAI.00954-07

El Hajj, H., Lebrun, M., Fourmaux, M.N., Vial, H., Dubremetz, J.F., 2007. Inverted topology of the Toxoplasma gondii ROP5 rhoptry protein provides new insights into the association of the ROP2 protein family with the parasitophorous vacuole membrane. Cellular Microbiology 9, 54–64. https://doi.org/10.1111/j.1462-5822.2006.00767.x

Elliott, E.I., Sutterwala, F.S., 2015. Initiation and perpetuation of NLRP3 inflammasome activation and assembly. Immunological reviews 265, 35–52. https://doi.org/10.1111/imr.12286

Eltzschig, H.K., Sitkovsky, M.V., Robson, S.C., 2012. Purinergic Signaling during Inflammation. New England Journal of Medicine 367, 2322–2333. https://doi.org/10.1056/NEJMra1205750

Ely, K.H., Kasper, L.H., Khan, I.A., 1999. Augmentation of the CD8+ T Cell Response by IFN-γ in IL-12-Deficient Mice During Toxoplasma gondii Infection. The Journal of Immunology 162, 5449–5454.

Etheridge, R.D., Alaganan, A., Tang, K., Lou, H.J., Turk, B.E., Sibley, L.D., 2014. The Toxoplasma pseudokinase ROP5 forms complexes with ROP18 and ROP17 kinases that synergize to control acute virulence in mice. Cell Host & Microbe 15, 537–550. https://doi.org/10.1016/j.chom.2014.04.002

Evavold, C.L., Kagan, J.C., 2018. How Inflammasomes Inform Adaptive Immunity. Journal of Molecular Biology 430, 217–237. https://doi.org/10.1016/j.jmb.2017.09.019

Evavold, C.L., Ruan, J., Tan, Y., Xia, S., Wu, H., Kagan, J.C., 2018. The Pore-Forming Protein Gasdermin D Regulates Interleukin-1 Secretion from Living Macrophages. Immunity 48, 35-44.e6. https://doi.org/10.1016/j.immuni.2017.11.013

Ewald, S.E., Chavarria-Smith, J., Boothroyd, J.C., 2014. NLRP1 Is an Inflammasome Sensor for Toxoplasma gondii. Infection and Immunity 82, 460–468. https://doi.org/10.1128/IAI.01170-13

Feliu, V., Vasseur, V., Grover, H.S., Chu, H.H., Brown, M.J., Wang, J., Boyle, J.P., Robey, E.A., Shastri, N., Blanchard, N., 2013. Location of the CD8 T cell epitope within the antigenic precursor determines immunogenicity and protection against the Toxoplasma gondii parasite. PLoS Pathogens 9, e1003449. https://doi.org/10.1371/journal.ppat.1003449

Fentress, S.J., Behnke, M.S., Dunay, I.R., Mashayekhi, M., Rommereim, L.M., Fox, B.A., Bzik, D.J., Taylor, G.A., Turk, B.E., Lichti, C.F., Townsend, R.R., Qiu, W., Hui, R., Beatty, W.L., Sibley, L.D., 2010. Phosphorylation of immunity-related GTPases by a Toxoplasma gondii-secreted kinase promotes macrophage survival and virulence. Cell Host & Microbe 8, 484–495. https://doi.org/10.1016/j.chom.2010.11.005

Fleckenstein, M.C., Reese, M.L., Konen-Waisman, S., Boothroyd, J.C., Howard, J.C., Steinfeldt, T., 2012. A Toxoplasma gondii pseudokinase inhibits host IRG resistance proteins. PLoS Biology 10, e1001358. https://doi.org/10.1371/journal.pbio.1001358

Fox, B.A., Butler, K.L., Guevara, R.B., Bzik, D.J., 2017. Cancer therapy in a microbial bottle: Uncorking the novel biology of the protozoan Toxoplasma gondii. PLOS Pathogens 13, e1006523. https://doi.org/10.1371/journal.ppat.1006523

Franco, M., Panas, M.W., Marino, N.D., Lee, M.-C.W., Buchholz, K.R., Kelly, F.D., Bednarski, J.J., Sleckman, B.P., Pourmand, N., Boothroyd, J.C., 2016. A Novel Secreted Protein, MYR1, Is Central to Toxoplasma's Manipulation of Host Cells. mBio 7, e02231–15. https://doi.org/10.1128/mBio.02231-15

Fux, B., Nawas, J., Khan, A., Gill, D.B., Su, C., Sibley, L.D., 2007. Toxoplasma gondii strains defective in oral transmission are also defective in developmental stage differentiation. Infection and immunity 75, 2580–2590. https://doi.org/IAI.00085-07

Gay, G., Braun, L., Brenier-Pinchart, M.-P., Vollaire, J., Josserand, V., Bertini, R.-L., Varesano, A., Touquet, B., De Bock, P.-J., Coute, Y., Tardieux, I., Bougdour, A., Hakimi, M.-A., 2016. Toxoplasma gondii TgIST co-opts host chromatin repressors dampening STAT1-dependent gene regulation and IFN-γ–mediated host defenses. The Journal of Experimental Medicine 213. https://doi.org/10.1084/jem.20160340

Gazzinelli, R., Xu, Y., Hieny, S., Cheever, A., Sher, A., 1992. Simultaneous depletion of CD4+ and CD8+ T lymphocytes is required to reactivate chronic infection with Toxoplasma gondii. Journal of immunology (Baltimore, Md.: 1950) 149, 175–180.

Gazzinelli, R.T., Hakim, F.T., Hieny, S., Shearer, G.M., Sher, A., 1991. Synergistic role of CD4+ and CD8+ T lymphocytes in IFN-gamma production and protective immunity induced by an attenuated Toxoplasma gondii vaccine. Journal of immunology (Baltimore, Md.: 1950) 146, 286–292.

Gazzinelli, R.T., Mendon??a-Neto, R., Lilue, J., Howard, J., Sher, A., 2014. Innate resistance against Toxoplasma gondii: An evolutionary tale of mice, cats, and men. Cell Host & Microbe 15, 132–138. https://doi.org/10.1016/j.chom.2014.01.004

Ghosh, R., Wang, L., Wang, E.S., Perera, B.G.K., Igbaria, A., Morita, S., Prado, K., Thamsen, M., Caswell, D., Macias, H., Weiberth, K.F., Gliedt, M.J., Alavi, M.V., Hari, S.B., Mitra, A.K., Bhhatarai, B., Schürer, S.C., Snapp, E.L., Gould, D.B., German, M.S., Backes, B.J., Maly, D.J., Oakes, S.A., Papa, F.R., 2014. Allosteric Inhibition of the IRE1α RNase Preserves Cell Viability and Function during Endoplasmic Reticulum Stress. Cell 158, 534–548. https://doi.org/10.1016/j.cell.2014.07.002

Gigley, J.P., Bhadra, R., Khan, I.A., 2011. CD8 T Cells and Toxoplasma gondii: A New Paradigm. Journal of parasitology research 2011, 243796. https://doi.org/10.1155/2011/243796

Gigley, J.P., Fox, B.A., Bzik, D.J., 2009. Cell-mediated immunity to Toxoplasma gondii develops primarily by local Th1 host immune responses in the absence of parasite replication. Journal of immunology (Baltimore, Md.: 1950) 182, 1069–1078. https://doi.org/10.4049/jimmunol.182.2.1069

Gilbert, R.E., Freeman, K., Lago, E.G., Bahia-Oliveira, L.M.G., Tan, H.K., Wallon, M., Buffolano, W., Stanford, M.R., Petersen, E., Toxoplasmosis (EMSCOT), for T.E.M.S. on C., 2008. Ocular Sequelae of Congenital Toxoplasmosis in Brazil Compared with Europe. PLoS Neglected Tropical Diseases 2, 1–7. https://doi.org/10.1371/journal.pntd.0000277

Gold, D.A., Kaplan, A.D., Rasmusson, R.L., Saeij, J.P.J., Gold, D.A., Kaplan, A.D., Lis, A., Bett, G.C.L., Rosowski, E.E., Cirelli, K.M., Hakimi, M., Rasmusson, R.L., Saeij, J.P.J., 2015. The Toxoplasma Dense Granule Proteins GRA17 and GRA23 Mediate the Movement of Small Molecules between the Host and the Parasitophorous Vacuole. Cell Host & Microbe.

Goldszmid, R.S., Coppens, I., Lev, A., Caspar, P., Mellman, I., Sher, A., 2009. Host ER-parasitophorous vacuole interaction provides a route of entry for antigen cross-presentation in Toxoplasma gondii-infected dendritic cells. The Journal of experimental medicine 206, 399–410. https://doi.org/10.1084/jem.20082108

Gorfu, G., Cirelli, K.M., Melo, M.B., Mayer-Barber, K., Crown, D., Koller, B.H., Masters, S., Sher, A., Leppla, S.H., Moayeri, M., Saeij, J.P.J., Grigg, M.E., 2014. Dual Role for Inflammasome Sensors NLRP1 and NLRP3 in Murine Resistance to Toxoplasma gondii. mBio 5, e01117–13. https://doi.org/10.1128/mBio.01117-13

Gov, L., Schneider, C.A., Lima, T.S., Pandori, W., Lodoen, M.B., 2017. NLRP3 and Potassium Efflux Drive Rapid IL-1β Release from Primary Human Monocytes during Toxoplasma Infection. The Journal of Immunology. https://doi.org/10.4049/jimmunol.1700245

Gregg, B., Dzierszinski, F., Tait, E., Jordan, K.A., Hunter, C.A., Roos, D.S., 2011. Subcellular antigen location influences T-Cell activation during acute infection with Toxoplasma gondii. PLoS One 6, 1–7. https://doi.org/10.1371/journal.pone.0022936

Grigg, M.E., Ganatra, J., Boothroyd, J.C., Margolis, T.P., 2001. Unusual Abundance of Atypical Strains Associated with Human Ocular Toxoplasmosis. The Journal of infectious diseases 184, 633–639. https://doi.org/10.1086/322800

Grover, H.S., Chu, H.H., Kelly, F.D., Yang, S.J., Reese, M.L., Blanchard, N., Gonzalez, F., Chan, S.W., Boothroyd, J.C., Shastri, N., Robey, E.A., 2014. Impact of regulated secretion on antiparasitic CD8 T cell responses. Cell reports 7, 1716–1728. https://doi.org/10.1016/j.celrep.2014.04.031

Gubbels, M.J., Striepen, B., Shastri, N., Turkoz, M., Robey, E.A., 2005. Class I major histocompatibility complex presentation of antigens that escape from the parasitophorous vacuole of Toxoplasma gondii. Infection and Immunity 73, 703–711. https://doi.org/10.1128/IAI.73.2.703-711.2005

Hakim, F.T., Gazzinelli, R.T., Denkers, E., Hieny, S., Shearer, G.M., Sher, A., 1991. CD8+ T cells from mice vaccinated against Toxoplasma gondii are cytotoxic for parasite-infected or antigen-pulsed host cells. The Journal of Immunology 147, 2310–2316.

Hakimi, M.-A., Olias, P., Sibley, L.D., 2017. Toxoplasma Effectors Targeting Host Signaling and Transcription. Clin. Microbiol. Rev. 30, 615. https://doi.org/10.1128/CMR.00005-17

Haldar, A.K., Foltz, C., Finethy, R., Piro, A.S., Feeley, E.M., Pilla-Moffett, D.M., Komatsu, M., Frickel, E.-M., Coers, J., 2015. Ubiquitin systems mark pathogen-containing vacuoles as targets for host defense by guanylate binding proteins. Proceedings of the National Academy of Sciences 112, E5628–E5637. https://doi.org/10.1073/pnas.1515966112

Haldar, A.K., Saka, H.A., Piro, A.S., Dunn, J.D., Henry, S.C., Taylor, G.A., Frickel, E.M., Valdivia, R.H., Coers, J., 2013. IRG and GBP host resistance factors target aberrant, “non-self” vacuoles characterized by the missing of “self” IRGM proteins. PLoS Pathogens 9, e1003414. https://doi.org/10.1371/journal.ppat.1003414

Hammoudi, P.M., Jacot, D., Mueller, C., Cristina, M.D., Dogga, S.K., Marq, J.B., Romano, J., Tosetti, N., Dubrot, J., Emre, Y., Lunghi, M., Coppens, I., Yamamoto, M., Sojka, D., Pino, P., Soldati-Favre, D., 2015. Fundamental Roles of the Golgi-Associated Toxoplasma Aspartyl Protease, ASP5, at the Host-Parasite Interface. PLoS Pathogens 11. https://doi.org/10.1371/journal.p/ppat.1005211

He, W., Wan, H., Hu, L., Chen, P., Wang, X., Huang, Z., Yang, Z.-H., Zhong, C.-Q., Han, J., 2015. Gasdermin D is an executor of pyroptosis and required for interleukin-1β secretion. Cell Research 25, 1285–1298. https://doi.org/10.1038/cr.2015.139

He, Y., Hara, H., Núñez, G., 2016. Mechanism and Regulation of NLRP3 Inflammasome Activation. Trends in Biochemical Sciences 41, 1012–1021. https://doi.org/10.1016/j.tibs.2016.09.002

Heaslip, A.T., Nishi, M., Stein, B., Hu, K., 2011. The Motility of a Human Parasite, Toxoplasma gondii, Is Regulated by a Novel Lysine Methyltransferase. PLoS Pathogens 7, 1–21. https://doi.org/10.1371/journal.ppat.1002201

Heimesaat, M.M., Dunay, I.R., Alutis, M., Fischer, A., Möhle, L., Göbel, U.B., Kühl, A.A., Bereswill, S., 2014. Nucleotide-Oligomerization-Domain-2 Affects Commensal Gut Microbiota Composition and Intracerebral Immunopathology in Acute Toxoplasma gondii Induced Murine Ileitis. PLOS ONE 9, 1–11. https://doi.org/10.1371/journal.pone.0105120

Henry, S.C., Daniell, X.G., Burroughs, A.R., Indaram, M., Howell, D.N., Coers, J., Starnbach, M.N., Hunn, J.P., Howard, J.C., Feng, C.G., Sher, A., Taylor, G.A., 2009. Balance of Irgm protein activities determines IFN-gamma-induced host

defense. Journal of Leukocyte Biology 85, 877–885. https://doi.org/10.1189/jlb.1008599

Hermanns, T., Müller, U.B., Könen-waisman, S., Howard, J.C., Steinfeldt, T., 2016. The Toxoplasma gondii rhoptry protein ROP18 is an Irga6-specific kinase and regulated by the dense granule protein GRA7 18, 244–259. https://doi.org/10.1111/cmi.12499

Howard, J.C., Hunn, J.P., Steinfeldt, T., 2011. The IRG protein-based resistance mechanism in mice and its relation to virulence in Toxoplasma gondii. Current Opinion in Microbiology 14, 414–421. https://doi.org/10.1016/j.mib.2011.07.002

Howe, D.K., Sibley, L.D., 1995. Toxoplasma gondii comprises three clonal lineages: correlation of parasite genotype with human disease. The Journal of infectious diseases 172, 1561–1566.

Hunn, J.P., Koenen-Waisman, S., Papic, N., Schroeder, N., Pawlowski, N., Lange, R., Kaiser, F., Zerrahn, J., Martens, S., Howard, J.C., 2008. Regulatory interactions between IRG resistance GTPases in the cellular response to Toxoplasma gondii. EMBO 27, 2495–2509. https://doi.org/10.1038/emboj.2008.176

Hunter, C.A., Chizzonite, R., Remington, J.S., 1995. IL-1 beta is required for IL-12 to induce production of IFN-gamma by NK cells. A role for IL-1 beta in the T cell-independent mechanism of resistance against intracellular pathogens. The Journal of Immunology 155, 4347–4354.

Hunter, C.A., Sibley, L.D., 2012. Modulation of innate immunity by Toxoplasma gondii virulence effectors. Nature Reviews Microbiology 10, 766–778. https://doi.org/10.1038/nrmicro2858

Huynh, M.H., Carruthers, V.B., 2009. Tagging of endogenous genes in a Toxoplasma gondii strain lacking Ku80. Eukaryotic cell 8, 530–539. https://doi.org/10.1128/EC.00358-08

Jensen, K.D., Hu, K., Whitmarsh, R.J., Hassan, M.A., Julien, L., Lu, D., Chen, L., Hunter, C.A., Saeij, J.P., 2013. Toxoplasma gondii rhoptry 16 kinase promotes host resistance to oral infection and intestinal inflammation only in the context of the dense granule protein GRA15. Infection and Immunity 81, 2156–2167. https://doi.org/10.1128/IAI.01185-12

Jensen, K.D., Wang, Y., Wojno, E.D., Shastri, A.J., Hu, K., Cornel, L., Boedec, E., Ong, Y.C., Chien, Y.H., Hunter, C.A., Boothroyd, J.C., Saeij, J.P., 2011. Toxoplasma polymorphic effectors determine macrophage polarization and intestinal inflammation. Cell Host & Microbe 9, 472–483. https://doi.org/10.1016/j.chom.2011.04.015

Jensen, K.D.C., 2016. Antigen Presentation of Vacuolated Apicomplexans–Two Gateways to a Vaccine Antigen. Trends in parasitology 32, 88–90. https://doi.org/10.1016/j.pt.2015.12.011

Jensen, K.D.C., Camejo, A., Melo, M.B., Cordeiro, C., Julien, L., Grotenbreg, G.M., Frickel, E.M., Ploegh, H.L., Young, L., Saeij, J.P.J., 2015. Toxoplasma gondii superinfection and virulence during secondary infection correlate with the exact ROP5/ROP18 allelic combination. mBio 6. https://doi.org/10.1128/mBio.02280-14

Jo, E.-K., Kim, J.K., Shin, D.-M., Sasakawa, C., 2016. Molecular mechanisms regulating NLRP3 inflammasome activation. Cellular & Molecular Immunology 13, 148–159. https://doi.org/10.1038/cmi.2015.95

Johnson, L.L., Sayles, P.C., 2002. Deficient Humoral Responses Underlie Susceptibility to Toxoplasma gondii in CD4-Deficient Mice. Infection and Immunity 70, 185–191. https://doi.org/10.1128/IAI.70.1.185-191.2002

Jordan, K.A., Wilson, E.H., Tait, E.D., Fox, B.A., Roos, D.S., Bzik, D.J., Dzierszinski, F., Hunter, C.A., 2009. Kinetics and Phenotype of Vaccine-Induced CD8+ T-Cell Responses to Toxoplasma gondii. Infection and Immunity 77, 3894–3901. https://doi.org/10.1128/IAI.00024-09

Kayagaki, N., Stowe, I.B., Lee, B.L., O'Rourke, K., Anderson, K., Warming, S., Cuellar, T., Haley, B., Roose-Girma, M., Phung, Q.T., Liu, P.S., Lill, J.R., Li, H., Wu, J., Kummerfeld, S., Zhang, J., Lee, W.P., Snipas, S.J., Salvesen, G.S., Morris, L.X., Fitzgerald, L., Zhang, Y., Bertram, E.M., Goodnow, C.C., Dixit, V.M., 2015. Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. Nature 526, 666–671. https://doi.org/10.1038/nature15541

Keestra, A.M., Bäumler, A.J., 2014. Detection of enteric pathogens by the nodosome. Trends in Immunology 35, 123–130. https://doi.org/10.1016/j.it.2013.10.009

Keestra-Gounder, A.M., Byndloss, M.X., Seyffert, N., Young, B.M., Chávez-Arroyo, A., Tsai, A.Y., Cevallos, S.A., Winter, M.G., Pham, O.H., Tiffany, C.R., de Jong, M.F., Kerrinnes, T., Ravindran, R., Luciw, P.A., McSorley, S.J., Bäumler, A.J., Tsolis, R.M., 2016. NOD1 and NOD2 signalling links ER stress with inflammation. Nature 532, 394–397. https://doi.org/10.1038/nature17631

Khan, A., Dubey, J.P., Su, C., Ajioka, J.W., Rosenthal, B.M., Sibley, L.D., 2011. Genetic analyses of atypical Toxoplasma gondii strains reveal a fourth clonal lineage in North America. International Journal for Parasitology 41, 645–655. https://doi.org/10.1016/j.ijpara.2011.01.005

Khan, A., Jordan, C., Muccioli, C., Vallochi, A.L., Rizzo, L.V., Jr, R.B., Vitor, R.W., Silveira, C., Sibley, L.D., 2006. Genetic divergence of Toxoplasma gondii strains associated with ocular toxoplasmosis, Brazil. Emerging infectious diseases 12, 942–949.

Khan, I.A., Hwang, S., Moretto, M., 2019. Toxoplasma gondii: CD8 T Cells Cry for CD4 Help. Frontiers in Cellular and Infection Microbiology 9, 136. https://doi.org/10.3389/fcimb.2019.00136

Kim, B.H., Shenoy, A.R., Kumar, P., Bradfield, C.J., MacMicking, J.D., 2012. IFN-inducible GTPases in host cell defense. Cell Host & Microbe 12, 432–444. https://doi.org/10.1016/j.chom.2012.09.007

Kim, L., Butcher, B.A., Lee, C.W., Uematsu, S., Akira, S., Denkers, E.Y., 2006. Toxoplasma gondii Genotype Determines MyD88-Dependent Signaling in Infected Macrophages. The Journal of Immunology 177, 2584–2591. https://doi.org/10.4049/jimmunol.177.4.2584

Kim, S.-K., Karasov, A., Boothroyd, J.C., 2007. Bradyzoite-Specific Surface Antigen SRS9 Plays a Role in Maintaining Toxoplasma gondii Persistence in the Brain and in Host Control of Parasite Replication in the Intestine. Infect. Immun. 75, 1626. https://doi.org/10.1128/IAI.01862-06

Kirak, O., Frickel, E.M., Grotenbreg, G.M., Suh, H., Jaenisch, R., Ploegh, H.L., 2010. Transnuclear mice with predefined T cell receptor specificities against Toxoplasma gondii obtained via SCNT. Science 328, 243–248. https://doi.org/10.1126/science.1178590

Koch, C.P., Perna, A.M., Pillong, M., Todoroff, N.K., Wrede, P., Folkers, G., Hiss, J.A., Schneider, G., 2013. Scrutinizing MHC-I Binding Peptides and Their Limits of Variation. PLoS Computational Biology 9, 1–9. https://doi.org/10.1371/journal.pcbi.1003088

Kongsomboonvech, A.K., Rodriguez, F., Diep, A.L., Justice, B.M., Castallanos, B.E., Camejo, A., Mukhopadhyay, D., Taylor, G.A., Yamamoto, M., Saeij, J.P.J., Reese, M.L., Jensen, K.D.C., 2020. Naïve CD8 T cell IFNγ responses to a vacuolar antigen are regulated by an inflammasome-independent NLRP3 pathway and Toxoplasma gondii ROP5. PLOS Pathogens 16, 1–35. https://doi.org/10.1371/journal.ppat.1008327

Kravets, E., Degrandi, D., Ma, Q., Peulen, T.-O., Klümpers, V., Felekyan, S., Kühnemuth, R., Weidtkamp-Peters, S., Seidel, C.A., Pfeffer, K., 2016. Guanylate binding proteins (GBPs) directly attack T. gondii via supramolecular complexes. eLife 5, 1–30. https://doi.org/10.7554/eLife.11479

Langeberg, L.K., Scott, J.D., 2015. Signalling scaffolds and local organization of cellular behaviour. Nature Reviews Molecular Cell Biology 16, 232–244. https://doi.org/10.1038/nrm3966

Lee, Y., Sasai, M., Ma, J.S., Sakaguchi, N., Ohshima, J., Bando, H., 2015. p62 plays a specific role in interferon-γ-induced presentation of a Toxoplasma vacuolar antigen. Cell Rep. 13. https://doi.org/10.1016/j.celrep.2015.09.005

Lee, Y., Yamada, H., Pradipta, A., Ma, J.S., Okamoto, M., Nagaoka, H., Takashima, E., Standley, D.M., Sasai, M., Takei, K., Yamamoto, M., 2020. Initial phospholipid-dependent Irgb6 targeting to Toxoplasma gondii vacuoles mediates host defense. Life Science Alliance 3. https://doi.org/10.26508/lsa.201900549

Li, W., Cao, T., Luo, C., Cai, J., Zhou, X., Xiao, X., Liu, S., 2020. Crosstalk between ER stress, NLRP3 inflammasome, and inflammation. Applied Microbiology and Biotechnology 104, 6129–6140. https://doi.org/10.1007/s00253-020-10614-y

Liesenfeld, O., Parvanova, I., Zerrahn, J., Han, S.-J., Heinrich, F., Muñoz, M., Kaiser, F., Aebischer, T., Buch, T., Waisman, A., Reichmann, G., Utermöhlen, O., von Stebut, E., von Loewenich, F.D., Bogdan, C., Specht, S., Saeftel, M., Hoerauf, A., Mota, M.M., Könen-Waisman, S., Kaufmann, S.H.E., Howard, J.C., 2011. The IFN-γ-Inducible GTPase, Irga6, Protects Mice against Toxoplasma gondii but Not against Plasmodium berghei and Some Other Intracellular Pathogens. PLoS One 6, 1–12. https://doi.org/10.1371/journal.pone.0020568

Lilue, J., enedikt Muller, U.B., Steinfeldt, T., Howard, J.C., 2013. Reciprocal virulence and resistance polymorphism in the relationship between Toxoplasma gondii and the house mouse. eLife 2. https://doi.org/10.7554/eLife.01298

Lim, D., Gold, D.A., Julien, L., Rosowski, E.E., Niedelman, W., Yaffe, M.B., Saeij, J.P.J., 2013. Structure of the Toxoplasma gondii ROP18 kinase domain reveals a second ligand binding pocket required for acute virulence. J Biol Chem 288, 34968–34980. https://doi.org/10.1074/jbc.M113.523266

Ling, Y.M., Shaw, M.H., Ayala, C., Coppens, I., Taylor, G.A., Ferguson, D.J.P., Yap, G.S., 2006. Vacuolar and plasma membrane stripping and autophagic elimination of Toxoplasma gondii in primed effector macrophages. The Journal of experimental medicine 203, 2063–2071. https://doi.org/jem.20061318

Liu, E., Corcino, Y.L., Portillo, J.A.C., Miao, Y., Subaustea, C.S., 2016. Identification of signaling pathways by which CD40 stimulates autophagy and antimicrobial activity against toxoplasma gondii in macrophages. Infection and Immunity. https://doi.org/10.1128/IAI.00101-16

Liu, Q., Zhang, D., Hu, D., Zhou, X., Zhou, Y., 2018. The role of mitochondria in NLRP3 inflammasome activation. Molecular Immunology 103, 115–124. https://doi.org/10.1016/j.molimm.2018.09.010

Lopez, J., Bittame, A., Massera, C., Vasseur, V., Effantin, G., Valat, A., Buaillon, C., Allart, S., Fox, B.A., Rommereim, L.M., Bzik, D.J., Schoehn, G., Weissenhorn, W., Dubremetz, J.-F.F., Gagnon, J., Mercier, C., Cesbron-Delauw, M.-F.F., Blanchard, N., 2015. Intravacuolar Membranes Regulate CD8 T Cell Recognition of Membrane-Bound Toxoplasma gondii Protective Antigen. Cell Reports 13, 2273–2286. https://doi.org/10.1016/j.celrep.2015.11.001

López-Yglesias, A.H., Camanzo, E., Martin, A.T., Araujo, A.M., Yarovinsky, F., 2019. TLR11-independent inflammasome activation is critical for CD4+ T cell-derived IFN-γ production and host resistance to Toxoplasma gondii. PLoS Pathogens 15, 1–20. https://doi.org/10.1371/journal.ppat.1007872

Lorenzi, H., Khan, A., Behnke, M.S., Namasivayam, S., Swapna, L.S., Hadjithomas, M., Karamycheva, S., Pinney, D., Brunk, B.P., Ajioka, J.W., Ajzenberg, D., Boothroyd, J.C., Boyle, J.P., Darde, M.L., Diaz-Miranda, M.A., Dubey, J.P., Fritz, H.M., Gennari, S.M., Gregory, B.D., Kim, K., Saeij, J.P., Su, C., White, M.W., Zhu, X.Q., Howe, D.K., Rosenthal, B.M., Grigg, M.E., Parkinson, J., Liu, L., Kissinger, J.C., Roos, D.S., Sibley, L.D., 2016. Local admixture of amplified and diversified secreted pathogenesis determinants shapes mosaic Toxoplasma gondii genomes. Nature Communications 7, 10147. https://doi.org/10.1038/ncomms10147

Maric-Biresev, J., Hunn, J.P., Krut, O., Helms, J.B., Martens, S., Howard, J.C., 2016. Loss of the interferon-gamma-inducible regulatory immunity-related GTPase (IRG), Irgm1, causes activation of effector IRG proteins on lysosomes, damaging lysosomal function and predicting the dramatic susceptibility of Irgm1-deficient mice to infection. BMC Biology 14, 33–34. https://doi.org/10.1186/s12915-016-0255-4

Martens, S., Parvanova, I., Zerrahn, J., Griffiths, G., Schell, G., Reichmann, G., Howard, J.C., 2005. Disruption of Toxoplasma gondii parasitophorous vacuoles by the mouse p47-resistance GTPases. PLoS Pathogens 1, e24. https://doi.org/10.1371/journal.ppat.0010024

Matta, S.K., Olias, P., Huang, Z., Wang, Q., Park, E., Yokoyama, W.M., Sibley, L.D., 2019. Toxoplasma gondii effector TgIST blocks type I interferon signaling to promote infection. Proceedings of the National Academy of Sciences 116, 17480–17491. https://doi.org/10.1073/pnas.1904637116

McLeod, R., Boyer, K.M., Lee, D., Mui, E., Wroblewski, K., Karrison, T., Noble, A.G., Withers, S., Swisher, C.N., Heydemann, P.T., Sautter, M., Babiarz, J.,

Rabiah, P., Meier, P., Grigg, M.E., Group, T.S., 2012. Prematurity and severity are associated with Toxoplasma gondii alleles (NCCCTS, 1981-2009). Clinical infectious diseases: an official publication of the Infectious Diseases Society of America 54, 1595–1605. https://doi.org/10.1093/cid/cis258

Meissner, T.B., Li, A., Biswas, A., Lee, K.-H., Liu, Y.-J., Bayir, E., Iliopoulos, D., van den Elsen, P.J., Kobayashi, K.S., 2010. NLR family member NLRC5 is a transcriptional regulator of MHC class I genes. Proceedings of the National Academy of Sciences of the United States of America 107, 13794–13799. https://doi.org/10.1073/pnas.1008684107

Melo, M.B., Jensen, K.D.C., Saeij, J.P.J., 2011. Toxoplasma gondii effectors are master regulators of the inflammatory response. Trends in parasitology 27, 487–495. https://doi.org/10.1016/j.pt.2011.08.001

Melo, M.B., Nguyen, Q.P., Cordeiro, C., Hassan, M.A., Yang, N., McKell, R., Rosowski, E.E., Julien, L., Butty, V., Dardé, M.-L., Ajzenberg, D., Fitzgerald, K., Young, L.H., Saeij, J.P.J., 2013. Transcriptional Analysis of Murine Macrophages Infected with Different Toxoplasma Strains Identifies Novel Regulation of Host Signaling Pathways. PLOS Pathogens 9, e1003779. https://doi.org/10.1371/journal.ppat.1003779

Menu, P., Mayor, A., Zhou, R., Tardivel, A., Ichijo, H., Mori, K., Tschopp, J., 2012. ER stress activates the NLRP3 inflammasome via an UPR-independent pathway. Cell Death & Disease 3, e261–e261. https://doi.org/10.1038/cddis.2011.132

Mercier, C., Cesbron-Delauw, M.-F., 2015. Toxoplasma secretory granules: one population or more? Trends Parasitol 31, 60–71. https://doi.org/10.1016/j.pt.2014.12.002

Minot, S., Melo, M.B., Li, F., Lu, D., Niedelman, W., Levine, S.S., Saeij, J.P., 2012. Admixture and recombination among Toxoplasma gondii lineages explain global genome diversity. Proceedings of the National Academy of Sciences of the United States of America 109, 13458–13463. https://doi.org/10.1073/pnas.1117047109

Montoya, J.G., Liesenfeld, O., 2004. Toxoplasmosis. Lancet 363, 1965–1976. https://doi.org/10.1016/S0140-6736(04)16412-X

Morgado, P., Sudarshana, D.M., Gov, L., Harker, K.S., Lam, T., Casali, P., Boyle, J.P., Lodoen, M.B., 2014. Type II Toxoplasma gondii induction of CD40 on infected macrophages enhances interleukin-12 responses. Infection and Immunity 82. https://doi.org/10.1128/IAI.01615-14

Mukhopadhyay, D., Arranz-Solís, D., Saeij, J.P.J., 2020a. Toxoplasma GRA15 and GRA24 are important activators of the host innate immune response in the absence of TLR11. PLOS Pathogens 16, 1–23. https://doi.org/10.1371/journal.ppat.1008586

Mukhopadhyay, D., Arranz-Solís, D., Saeij, J.P.J., 2020b. Influence of the Host and Parasite Strain on the Immune Response During Toxoplasma Infection. Frontiers in Cellular and Infection Microbiology 10, 586. https://doi.org/10.3389/fcimb.2020.580425

Murillo-León, M., Müller, U.B., Zimmermann, I., Singh, S., Widdershooven, P., Campos, C., Alvarez, C., Könen-Waisman, S., Lukes, N., Ruzsics, Z., Howard, J.C., Schwemmle, M., Steinfeldt, T., 2019. Molecular mechanism for the control of virulent Toxoplasma gondii infections in wild-derived mice. Nature Communications 10, 1233. https://doi.org/10.1038/s41467-019-09200-2

Nakaar, V., Samuel, B.U., Ngo, E.O., Joiner, K.A., 1999. Targeted reduction of nucleoside triphosphate hydrolase by antisense RNA inhibits Toxoplasma gondii proliferation. J Biol Chem 274, 5083–5087. https://doi.org/10.1074/jbc.274.8.5083

Naor, A., Panas, M.W., Marino, N., Coffey, M.J., Tonkin, C.J., Boothroyd, J.C., 2018. MYR1-dependent effectors are the major drivers of a host cell's early response to Toxoplasma, including counteracting MYR1-independent effects. mBio 9. https://doi.org/10.1128/mBio.02401-17

Niedelman, W., Gold, D.A., Rosowski, E.E., Sprokholt, J.K., Lim, D., Farid Arenas, A., Melo, M.B., Spooner, E., Yaffe, M.B., Saeij, J.P.J., 2012. The Rhoptry Proteins ROP18 and ROP5 Mediate Toxoplasma gondii Evasion of the Murine, But Not the Human, Interferon-Gamma Response. PLoS Pathogens 8, e1002784. https://doi.org/10.1371/journal.ppat.1002784

Nishiyama, S., Pradipta, A., Ma, J.S., Sasai, M., Yamamoto, M., 2020. T cell-derived interferon-γ is required for host defense to Toxoplasma gondii. Parasitology International 75, 102049. https://doi.org/10.1016/j.parint.2019.102049

Northrop, J.P., Ho, S.N., Chen, L., Thomas, D.J., Timmerman, L.A., Nolan, G.P., Admon, A., Crabtree, G.R., 1994. NF-AT components define a family of transcription factors targeted in T-cell activation. Nature 369, 497–502. https://doi.org/10.1038/369497a0

Ohshima, J., Lee, Y., Sasai, M., Saitoh, T., Su Ma, J., Kamiyama, N., Matsuura, Y., Pann-Ghill, S., Hayashi, M., Ebisu, S., Takeda, K., Akira, S., Yamamoto, M., Ma, J.S., Kamiyama, N., Matsuura, Y., Pann-Ghill, S., Hayashi, M., Ebisu, S., Takeda, K., Akira, S., Yamamoto, M., 2014. Role of mouse and human

autophagy proteins in IFN-gamma-induced cell-autonomous responses against Toxoplasma gondii. Journal of immunology (Baltimore, Md.: 1950) 192, 3328–3335. https://doi.org/10.4049/jimmunol.1302822

Olias, P., Sibley, L.D., 2016. Functional Analysis of the Role of Toxoplasma gondii Nucleoside Triphosphate Hydrolases I and II in Acute Mouse Virulence and Immune Suppression. Infection and Immunity 84, 1994 LP – 2001.

Ong, Y.C., Reese, M.L., Boothroyd, J.C., 2010. Toxoplasma rhoptry protein 16 (ROP16) subverts host function by direct tyrosine phosphorylation of STAT6. The Journal of biological chemistry 285, 28731–28740. https://doi.org/10.1074/jbc.M110.112359

Panas, M.W., Ferrel, A., Naor, A., Tenborg, E., Lorenzi, H.A., Boothroyd, J.C., 2019a. Translocation of Dense Granule Effectors across the Parasitophorous Vacuole Membrane in Toxoplasma-Infected Cells Requires the Activity of ROP17, a Rhoptry Protein Kinase. mSphere 4. https://doi.org/10.1128/mSphere.00276-19

Panas, M.W., Naor, A., Cygan, A.M., Boothroyd, J.C., 2019b. Toxoplasma Controls Host Cyclin E Expression through the Use of a Novel MYR1-Dependent Effector Protein, HCE1. mBio 10. https://doi.org/10.1128/mBio.00674-19

Pandori, W.J., Lima, T.S., Mallya, S., Kao, T.H., Gov, L., Lodoen, M.B., 2019. Toxoplasma gondii activates a Syk-CARD9-NF-κB signaling axis and gasdermin D-independent release of IL-1β during infection of primary human monocytes. PLoS Pathogens 15, 1–24. https://doi.org/10.1371/journal.ppat.1007923

Papandreou, I., Denko, N.C., Olson, M., Van Melckebeke, H., Lust, S., Tam, A., Solow-Cordero, D.E., Bouley, D.M., Offner, F., Niwa, M., Koong, A.C., 2011. Identification of an Ire1alpha endonuclease specific inhibitor with cytotoxic activity against human multiple myeloma. Blood 117, 1311–1314. https://doi.org/10.1182/blood-2010-08-303099

Park, S., Choi, J., Biering, S.B., Dominici, E., Williams, L.E., Hwang, S., 2016. Targeting by AutophaGy proteins (TAG): Targeting of IFNG-inducible GTPases to membranes by the LC3 conjugation system of autophagy. Autophagy 12, 1153–1167. https://doi.org/10.1080/15548627.2016.1178447

Parker, S.J., Roberts, C.W., Alexander, J., 1991. CD8+ T cells are the major lymphocyte subpopulatlon involved in the protective immune response to Toxoplasma gondii in mice. Clinical & Experimental Immunology 84, 207–212. https://doi.org/10.1111/j.1365-2249.1991.tb08150.x

Pawlowski, N., Khaminets, A., Hunn, J.P., Papic, N., Schmidt, A., Uthaiah, R.C., Lange, R., Vopper, G., Martens, S., Wolf, E., Howard, J.C., 2011. The activation

mechanism of Irga6, an interferon-inducible GTPase contributing to mouse resistance against Toxoplasma gondii. BMC Biology 9, 7. https://doi.org/10.1186/1741-7007-9-7

Pfefferkorn, E.R., 1984. Interferon gamma blocks the growth of Toxoplasma gondii in human fibroblasts by inducing the host cells to degrade tryptophan. Proceedings of the National Academy of Sciences 81, 908–912. https://doi.org/10.1073/pnas.81.3.908

Rastogi, S., Cygan, A.M., Boothroyd, J.C., 2019. Translocation of effector proteins into host cells by Toxoplasma gondii. Current Opinion in Microbiology 52, 130–138. https://doi.org/10.1016/j.mib.2019.07.002

Rauch, I., Deets, K.A., Ji, D.X., Moltke, J. von, Tenthorey, J.L., Lee, A.Y., Philip, N.H., Ayres, J.S., Brodsky, I.E., Gronert, K., Vance, R.E., 2017. NAIP-NLRC4 Inflammasomes Coordinate Intestinal Epithelial Cell Expulsion with Eicosanoid and IL-18 Release via Activation of Caspase-1 and -8. Immunity 46, 649–659. https://doi.org/10.1016/j.immuni.2017.03.016

Reese, M.L., Boothroyd, J.C., 2011. A conserved non-canonical motif in the pseudoactive site of the ROP5 pseudokinase domain mediates its effect on Toxoplasma virulence. The Journal of biological chemistry 286, 29366–29375. https://doi.org/10.1074/jbc.M111.253435

Reese, M.L., Shah, N., Boothroyd, J.C., Shah, Boothroyd, J.C., 2014. The Toxoplasma pseudokinase ROP5 is an allosteric inhibitor of the immunity-related GTPases. The Journal of Biological Chemistry 289, 27849–27858. https://doi.org/10.1074/jbc.M114.567057

Reese, M.L., Zeiner, G.M., Saeij, J.P.J., Boothroyd, J.C., Boyle, J.P., 2011. Polymorphic family of injected pseudokinases is paramount in Toxoplasma virulence. Proceedings of the National Academy of Sciences of the United States of America 108, 9625–9630. https://doi.org/10.1073/pnas.1015980108

Reinhardt, R.L., Liang, H.-E., Bao, K., Price, A.E., Mohrs, M., Kelly, B.L., Locksley, R.M., 2015. A Novel Model for IFN-γ–Mediated Autoinflammatory Syndromes. The Journal of Immunology 194, 2358–2368. https://doi.org/10.4049/jimmunol.1401992

Robben, P.M., Mordue, D.G., Truscott, S.M., Takeda, K., Akira, S., Sibley, L.D., 2004. Production of IL-12 by Macrophages Infected with Toxoplasma gondii Depends on the Parasite Genotype. The Journal of Immunology 172, 3686–3694. https://doi.org/10.4049/jimmunol.172.6.3686

Rommereim, L.M., Fox, B.A., Butler, K.L., Cantillana, V., Taylor, G.A., Bzik, D.J., 2019. Rhoptry and Dense Granule Secreted Effectors Regulate CD8+ T Cell

Recognition of Toxoplasma gondii Infected Host Cells. Frontiers in Immunology 10, 2104. https://doi.org/10.3389/fimmu.2019.02104

Rosowski, E.E., Lu, D., Julien, L., Rodda, L., Gaiser, R.A., Jensen, K.D., Saeij, J.P., 2011. Strain-specific activation of the NF-kappaB pathway by GRA15, a novel Toxoplasma gondii dense granule protein. The Journal of Experimental Medicine 208, 195–212. https://doi.org/10.1084/jem.20100717

Russo, I., Babbitt, S., Muralidharan, V., Butler, T., Oksman, A., Goldberg, D.E., 2010. Plasmepsin V licenses Plasmodium proteins for export into the host erythrocyte. Nature 463, 632–636. https://doi.org/10.1038/nature08726

Sacks, D.L., 2014. Vaccines against tropical parasitic diseases: a persisting answer to a persisting problem. Nature immunology 15, 403–405. https://doi.org/10.1038/ni.2853

Saeij, J.P., Boyle, J.P., Coller, S., Taylor, S., Sibley, L.D., Brooke-Powell, E.T., Ajioka, J.W., Boothroyd, J.C., 2006. Polymorphic secreted kinases are key virulence factors in toxoplasmosis. Science 314, 1780–1783. https://doi.org/10.1126/science.1133690

Saeij, J.P., Frickel, E.M., 2017. Exposing Toxoplasma gondii hiding inside the vacuole: a role for GBPs, autophagy and host cell death 40. https://doi.org/10.1016/j.mib.2017.10.021

Saeij, J.P.J., Coller, S., Boyle, J.P., Jerome, M.E., White, M.W., Boothroyd, J.C., 2007. Toxoplasma co-opts host gene expression by injection of a polymorphic kinase homologue. Nature 445, 324–327. https://doi.org/10.1038/nature05395

Salerno, F., Guislain, A., Freen-Van Heeren, J.J., Nicolet, B.P., Young, H.A., Wolkers, M.C., 2019. Critical role of post-transcriptional regulation for IFN-γ in tumor-infiltrating T cells. Oncoimmunology 8, e1532762. https://doi.org/10.1080/2162402X.2018.1532762

Sangaré, L.O., Yang, N., Konstantinou, E.K., Lu, D., Mukhopadhyay, D., Young, L.H., Saeij, J.P.J., 2019. Toxoplasma GRA15 Activates the NF-κB Pathway through Interactions with TNF Receptor-Associated Factors. mBio 10, e00808-19. https://doi.org/10.1128/mBio.00808-19

Sasai, M., Sakaguchi, N., Ma, J.S., Nakamura, S., Kawabata, T., Bando, H., Lee, Y., Saitoh, T., Akira, S., Iwasaki, A., Standley, D.M., Yoshimori, T., Yamamoto, M., 2017. Essential role for GABARAP autophagy proteins in interferon-inducible GTPase-mediated host defense. Nature immunology 18. https://doi.org/10.1038/ni.3767

Sauer, A., de la Torre, A., Gomez-Marin, J., Bourcier, T., Garweg, J., Speeg-Schatz, C., Candolfi, E., 2011. Prevention of Retinochoroiditis in Congenital

Toxoplasmosis: Europe Versus South America. The Pediatric Infectious Disease Journal 30.

Scharton-Kersten, T.M., Wynn, T.A., Denkers, E.Y., Bala, S., Grunvald, E., Hieny, S., Gazzinelli, R.T., Sher, A., 1996. In the absence of endogenous IFN-gamma, mice develop unimpaired IL-12 responses to Toxoplasma gondii while failing to control acute infection. J. Immunol. 157, 4045.

Scharton-Kersten, T.M., Yap, G., Magram, J., Sher, A., 1997. Inducible Nitric Oxide Is Essential for Host Control of Persistent but Not Acute Infection with the Intracellular Pathogen Toxoplasma gondii. Journal of Experimental Medicine 185, 1261–1274. https://doi.org/10.1084/jem.185.7.1261

Schwab, J.C., Beckers, C.J., Joiner, K.A., 1994. The parasitophorous vacuole membrane surrounding intracellular Toxoplasma gondii functions as a molecular sieve. Proceedings of the National Academy of Sciences 91, 509–513. https://doi.org/10.1073/pnas.91.2.509

Selleck, E.M., Fentress, S.J., Beatty, W.L., Degrandi, D., Pfeffer, K., 4th, H.W.V., Macmicking, J.D., Sibley, L.D., 2013. Guanylate-binding protein 1 (Gbp1) contributes to cell-autonomous immunity against Toxoplasma gondii. PLoS Pathogens 9, e1003320. https://doi.org/10.1371/journal.ppat.1003320

Selleck, E.M., Orchard, R.C., Lassen, K.G., Beatty, W.L., Xavier, R.J., Levine, B., 2015. A noncanonical autophagy pathway restricts Toxoplasma gondii growth in a strain-specific manner in IFN-γ-activated human cells. MBio 6. https://doi.org/10.1128/mBio.01157-15

Shaw, M.H., Boyartchuk, V., Wong, S., Karaghiosoff, M., Ragimbeau, J., Pellegrini, S., Muller, M., Dietrich, W.F., Yap, G.S., 2003. A natural mutation in the Tyk2 pseudokinase domain underlies altered susceptibility of B10.Q/J mice to infection and autoimmunity. Proceedings of the National Academy of Sciences of the United States of America 100, 11594–11599. https://doi.org/10.1073/pnas.1930781100

Shaw, M.H., Reimer, T., Sánchez-Valdepeñas, C., Warner, N., Kim, Y.-G., Fresno, M., Nuñez, G., 2009. T cell–intrinsic role of Nod2 in promoting type 1 immunity to Toxoplasma gondii. Nature Immunology 10, 1267–1274. https://doi.org/10.1038/ni.1816

Shi, J., Zhao, Y., Wang, K., Shi, X., Wang, Y., Huang, H., Zhuang, Y., Cai, T., Wang, F., Shao, F., 2015. Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. Nature 526, 660.

Sibley, L.D., Boothroyd, J.C., 1992. Virulent strains of Toxoplasma gondii comprise a single clonal lineage. Nature 359, 82–85. https://doi.org/10.1038/359082a0

Sibley, L.D., Niesman, I.R., Asai, T., Takeuchi, T., 1994. Toxoplasma gondii: Secretion of a Potent Nucleoside Triphosphate Hydrolase into the Parasitophorous Vacuole. Experimental Parasitology 79, 301–311. https://doi.org/10.1006/expr.1994.1093

Sidik, S.M., Huet, D., Ganesan, S.M., Carruthers, V.B., Niles, J.C., Lourido, S., Sidik, S.M., Huet, D., Ganesan, S.M., Huynh, M., Wang, T., Nasamu, A.S., 2016. A Genome-wide CRISPR Screen in Toxoplasma Identifies Essential Apicomplexan Genes. Cell 166, 1423–1430.e12. https://doi.org/10.1016/j.cell.2016.08.019

Silverman, J.A., Qi, H., Riehl, A., Beckers, C., Nakaar, V., Joiner, K.A., 1998. Induced activation of the Toxoplasma gondii nucleoside triphosphate hydrolase leads to depletion of host cell ATP levels and rapid exit of intracellular parasites from infected cells. J Biol Chem 273, 12352–12359. https://doi.org/10.1074/jbc.273.20.12352

Silverman, W.R., de Rivero Vaccari, J.P., Locovei, S., Qiu, F., Carlsson, S.K., Scemes, E., Keane, R.W., Dahl, G., 2009. The pannexin 1 channel activates the inflammasome in neurons and astrocytes. J Biol Chem 284, 18143–18151. https://doi.org/10.1074/jbc.M109.004804

Sinai, A.P., Webster, P., Joiner, K.A., 1997. Association of host cell endoplasmic reticulum and mitochondria with the Toxoplasma gondii parasitophorous vacuole membrane: a high affinity interaction. Journal of Cell Science 110, 2117–2128.

Splitt, S.D., Souza, S.P., Valentine, K.M., Castellanos, B.E., Curd, A.B., Hoyer, K.K., Jensen, K.D.C., 2018. PD-L1, TIM-3, and CTLA-4 Blockade Fails To Promote Resistance to Secondary Infection with Virulent Strains of Toxoplasma gondii. Infection and Immunity 86. https://doi.org/10.1128/IAI.00459-18

Steinfeldt, T., Konen-Waisman, S., Tong, L., Pawlowski, N., Lamkemeyer, T., Sibley, L.D., Hunn, J.P., Howard, J.C., 2010. Phosphorylation of mouse immunity-related GTPase (IRG) resistance proteins is an evasion strategy for virulent Toxoplasma gondii. PLoS Biology 8. https://doi.org/10.1371/journal.pbio.1000576

Stelzer, S., Basso, W., Silván, J.B., Ortega-Mora, L.M., Maksimov, P., Gethmann, J., Conraths, F.J., Schares, G., 2019. Toxoplasma gondii infection and toxoplasmosis in farm animals: Risk factors and economic impact. Food and Waterborne Parasitology 15, e00037. https://doi.org/10.1016/j.fawpar.2019.e00037

Stommel, E.W., Ely, K.H., Schwartzman, J.D., Kasper, L.H., 1997. Toxoplasma gondii: Dithiol-Induced Ca2+Flux Causes Egress of Parasites from the Parasitophorous Vacuole. Experimental Parasitology 87, 88–97. https://doi.org/10.1006/expr.1997.4187

Su, C., Howe, D.K., Dubey, J.P., Ajioka, J.W., Sibley, L.D., 2002. Identification of quantitative trait loci controlling acute virulence in Toxoplasma gondii. Proceedings of the National Academy of Sciences 99, 10753–10758. https://doi.org/10.1073/pnas.172117099

Subauste, C.S., 2019. Interplay Between Toxoplasma gondii, Autophagy, and Autophagy Proteins. Frontiers in Cellular and Infection Microbiology 9, 139. https://doi.org/10.3389/fcimb.2019.00139

Suzuki, Y., Conley, F.K., Remington, J.S., 1989. Importance of endogenous IFN-gamma for prevention of toxoplasmic encephalitis in mice. Journal of immunology (Baltimore, Md.: 1950) 143, 2045–2050.

Suzuki, Y., Orellana, M.A., Schreiber, R.D., Remington, J.S., 1988. Interferon-gamma: the major mediator of resistance against Toxoplasma gondii. Science 240, 516–518.

Suzuki, Y., Remington, J.S., 1990. The effect of anti-IFN-gamma antibody on the protective effect of Lyt-2+ immune T cells against toxoplasmosis in mice. J. Immunol. 144, 1954.

Suzuki, Y., Remington, J.S., 1988. Dual regulation of resistance against Toxoplasma gondii infection by Lyt-2+ and Lyt-1+, L3T4+ T cells in mice. Journal of immunology (Baltimore, Md.: 1950) 140, 3943–3946.

Suzuki, Y., Wang, X., Jortner, B.S., Payne, L., Ni, Y., Michie, S.A., Xu, B., Kudo, T., Perkins, S., 2010. Removal of Toxoplasma gondii Cysts from the Brain by Perforin-Mediated Activity of CD8+ T Cells. The American Journal of Pathology 176, 1607–1613. https://doi.org/10.2353/ajpath.2010.090825

Tattoli, I., Travassos, L.H., Carneiro, L.A., Magalhaes, J.G., Girardin, S.E., 2007. The Nodosome: Nod1 and Nod2 control bacterial infections and inflammation. Seminars in Immunopathology 29, 289. https://doi.org/10.1007/s00281-007-0083-2

Taylor, G.A., Collazo, C.M., Yap, G.S., Nguyen, K., Gregorio, T.A., Taylor, L.S., Eagleson, B., Secrest, L., Southon, E.A., Reid, S.W., Tessarollo, L., Bray, M., McVicar, D.W., Komschlies, K.L., Young, H.A., Biron, C.A., Sher, A., Woude, G.F.V., 2000. Pathogen-specific loss of host resistance in mice lacking the IFN-gamma-inducible gene IGTP. Proceedings of the National Academy of Sciences of the United States of America 97, 751–755.

Taylor, G.A., Feng, C.G., Sher, A., 2007. Control of IFN-g-mediated host resistance to intracellular pathogens by immunity-related GTPases (p47 GTPases). Microbes and Infection 9, 1644–1651. https://doi.org/10.1016/j.micinf.2007.09.004

Taylor, S., Barragan, A., Su, C., Fux, B., Fentress, S.J., Tang, K., Beatty, W.L., Hajj, H.E., Jerome, M., Behnke, M.S., White, M., Wootton, J.C., Sibley, L.D., 2006. A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen Toxoplasma gondii. Science 314, 1776–1780. https://doi.org/10.1126/science.1133643

Tobin, C.M., Knoll, L.J., 2012. A Patatin-Like Protein Protects Toxoplasma gondii from Degradation in a Nitric Oxide-Dependent Manner. Infect. Immun. 80, 55. https://doi.org/10.1128/IAI.05543-11

Tsitsiklis, A., Bangs, D.J., Robey, E.A., 2019. CD8+ T Cell Responses to Toxoplasma gondii: Lessons from a Successful Parasite. Trends in Parasitology 35. https://doi.org/10.1016/j.pt.2019.08.005

Tuladhar, S., Kochanowsky, J.A., Bhaskara, A., Ghotmi, Y., Chandrasekaran, S., Koshy, A.A., 2019. The ROP16III-dependent early immune response determines the subacute CNS immune response and type III Toxoplasma gondii survival. PLOS Pathogens 15, 1–32. https://doi.org/10.1371/journal.ppat.1007856

Vanaja, S.K., Rathinam, V.A.K., Fitzgerald, K.A., 2015. Mechanisms of inflammasome activation: recent advances and novel insights. Trends in Cell Biology 25, 308–315. https://doi.org/10.1016/J.TCB.2014.12.009

Villarino, A.V., Katzman, S.D., Gallo, E., Miller, O., Jiang, S., McManus, M.T., Abbas, A.K., 2011. Posttranscriptional Silencing of Effector Cytokine mRNA Underlies the Anergic Phenotype of Self-Reactive T Cells. Immunity 34, 50–60. https://doi.org/10.1016/j.immuni.2010.12.014

Villegas, E.N., Elloso, M.M., Reichmann, G., Peach, R., Hunter, C.A., 1999. Role of CD28 in the Generation of Effector and Memory Responses Required for Resistance to Toxoplasma gondii. The Journal of Immunology 163, 3344–3353.

Villegas, E.N., Lieberman, L.A., Mason, N., Blass, S.L., Zediak, V.P., Peach, R., Horan, T., Yoshinaga, S., Hunter, C.A., 2002. A Role for Inducible Costimulator Protein in the CD28- Independent Mechanism of Resistance to Toxoplasma gondii. The Journal of Immunology 169, 937–943. https://doi.org/10.4049/jimmunol.169.2.937

Virreira Winter, S., Niedelman, W., Jensen, K.D., Rosowski, E.E., Julien, L., Spooner, E., 2011. Determinants of GBP recruitment to Toxoplasma gondii

vacuoles and the parasitic factors that control it. PLoS One 6. https://doi.org/10.1371/journal.pone.0024434

Vossenkämper, A., Struck, D., Alvarado-Esquivel, C., Went, T., Takeda, K., Akira, S., Pfeffer, K., Alber, G., Lochner, M., Förster, I., Liesenfeld, O., 2004. Both IL-12 and IL-18 contribute to small intestinal Th1-type immunopathology following oral infection with Toxoplasma gondii, but IL-12 is dominant over IL-18 in parasite control. European Journal of Immunology 34, 3197–3207. https://doi.org/10.1002/eji.200424993

Wagner, R.N., Proell, M., Kufer, T.A., Schwarzenbacher, R., 2009. Evaluation of Nod-Like Receptor (NLR) Effector Domain Interactions. PLOS ONE 4, 1–11. https://doi.org/10.1371/journal.pone.0004931

Wan, K., Ajioka, J., 2004. Molecular Characterization of tgd057, a Novel Gene from Toxoplasma gondii. Journal of Biochemistry and Molecular Biology. https://doi.org/10.5483/BMBRep.2004.37.4.474

Wang, X., Kang, H., Kikuchi, T., Suzuki, Y., 2004. Gamma Interferon Production, but Not Perforin-Mediated Cytolytic Activity, of T Cells Is Required for Prevention of Toxoplasmic Encephalitis in BALB/c Mice Genetically Resistant to the Disease. Infection and Immunity 72, 4432–4438. https://doi.org/10.1128/IAI.72.8.4432-4438.2004

Wang, Y., Cirelli, K.M., Barros, P.D.C., Sangaré, L.O., Butty, V., Hassan, M.A., Pesavento, P., Mete, A., Saeij, J.P.J., 2019. Three Toxoplasma gondii Dense Granule Proteins Are Required for Induction of Lewis Rat Macrophage Pyroptosis. mBio 10, e02388-18. https://doi.org/10.1128/mBio.02388-18

Wang, Y., Sangaré, L.O., Paredes-Santos, T.C., Hassan, M.A., Krishnamurthy, S., Furuta, A.M., Markus, B.M., Lourido, S., Saeij, J.P.J., 2020. Genome-wide screens identify Toxoplasma gondii determinants of parasite fitness in IFNγ-activated murine macrophages. Nature Communications 11, 5258. https://doi.org/10.1038/s41467-020-18991-8

Wilson, D.C., Grotenbreg, G.M., Liu, K., Zhao, Y., Frickel, E.M., Gubbels, M.J., Ploegh, H.L., Yap, G.S., 2010. Differential regulation of effector- and central-memory responses to Toxoplasma gondii Infection by IL-12 revealed by tracking of Tgd057-specific CD8+ T cells. PLoS Pathogens 6, e1000815. https://doi.org/10.1371/journal.ppat.1000815

Wilson, D.C., Matthews, S., Yap, G.S., 2008. IL-12 Signaling Drives CD8+ T Cell IFN-γ Production and Differentiation of KLRG1+ Effector Subpopulations during Toxoplasma gondii Infection. The Journal of Immunology 180, 5935 LP – 5945. https://doi.org/10.4049/jimmunol.180.9.5935

Witola, W.H., Mui, E., Hargrave, A., Liu, S., Hypolite, M., Montpetit, A., Cavailles, P., Bisanz, C., Cesbron-Delauw, M.-F., Fournié, G.J., McLeod, R., 2011. NALP1 Influences Susceptibility to Human Congenital Toxoplasmosis, Proinflammatory Cytokine Response, and Fate of Toxoplasma gondii-Infected Monocytic Cells. Infection and Immunity 79, 756–766. https://doi.org/10.1128/IAI.00898-10

Xia, J., Venkat, A., Reese, M.L., Le Roch, K., Ay, F., Boyle, J.P., 2020. Third generation sequencing revises the molecular karyotype for Toxoplasma gondii and identifies emerging copy number variants in sexual recombinants. bioRxiv. https://doi.org/10.1101/2020.03.10.985549

Yamamoto, M., Ma, J.S., Mueller, C., Kamiyama, N., Saiga, H., Kubo, E., Kimura, T., Okamoto, T., Okuyama, M., Kayama, H., Nagamune, K., Takashima, S., Matsuura, Y., Soldati-Favre, D., Takeda, K., 2011. ATF6beta is a host cellular target of the Toxoplasma gondii virulence factor ROP18. The Journal of experimental medicine 208. https://doi.org/10.1084/jem.20101660

Yamamoto, M., Okuyama, M., Ma, J.S., Kimura, T., Kamiyama, N., Saiga, H., Ohshima, J., Sasai, M., Kayama, H., Okamoto, T., Huang, D.C., Soldati-Favre, D., Horie, K., Takeda, J., Takeda, K., 2012. A cluster of interferon-gamma-inducible p65 GTPases plays a critical role in host defense against Toxoplasma gondii. Immunity 37, 302–313. https://doi.org/S1074-7613(12)00250-6

Yamamoto, M., Standley, D.M., Takashima, S., Saiga, H., Okuyama, M., Kayama, H., Kubo, E., Ito, H., Takaura, M., Matsuda, T., Soldati-Favre, D., Takeda, K., 2009. A single polymorphic amino acid on Toxoplasma gondii kinase ROP16 determines the direct and strain-specific activation of Stat3. The Journal of experimental medicine 206, 2747–2760. https://doi.org/10.1084/jem.20091703

Yap, J.K.Y., Pickard, B.S., Chan, E.W.L., Gan, S.Y., 2019. The Role of Neuronal NLRP1 Inflammasome in Alzheimer's Disease: Bringing Neurons into the Neuroinflammation Game. Molecular Neurobiology 56, 7741–7753. https://doi.org/10.1007/s12035-019-1638-7

Yarovinsky, F., 2014. Innate immunity to Toxoplasma gondii infection. Nature Reviews Immunology 14, 109–121. https://doi.org/10.1038/nri3598

Zhao, Y., Ferguson, D.J., Wilson, D.C., Howard, J.C., Sibley, L.D., Yap, G.S., 2009. Virulent Toxoplasma gondii evade immunity-related GTPase-mediated parasite vacuole disruption within primed macrophages. Journal of immunology (Baltimore, Md.: 1950) 182, 3775–3781. https://doi.org/10.4049/jimmunol.0804190

Zhao, Y., Shao, F., 2012. NLRC5: a NOD-like receptor protein with many faces in immune regulation. Cell Research 22, 1099–1101. https://doi.org/10.1038/cr.2012.83

Zhou, R., Yazdi, A.S., Menu, P., Tschopp, J., 2011. A role for mitochondria in NLRP3 inflammasome activation. Nature 469, 221–225. https://doi.org/10.1038/nature09663