Evasion of interferon-gamma responses by *Toxoplasma gondii* in murine and human fibroblasts

by

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ABSTRACT

Co-evolution of pathogen and host helps drive biological diversity. Unlike viral-host interactions, little is known about the co-evolution of eukaryotic pathogens with their hosts. The intracellular parasite *Toxoplasma gondii* is an excellent model organism for co-evolution studies because its hosts include all warm-blooded animals, and genetically diverse *Toxoplasma* strains may be adapted to specific hosts. *Toxoplasma* must evade host immunity without killing the host to establish a chronic infection and ensure transmission.

Interferon-gamma (IFNγ) activation of non-immune cells is crucial for host defense against *Toxoplasma.* In murine cells, interferon-inducible immune-related GTPases (IRGs) are essential to the IFNγ response because they disrupt the parasitophorous vacuole (PV). However, *Toxoplasma* secretes the contents of apical secretory organelles into the host cell during invasion, and some of these proteins strain-specifically promote mouse virulence by inactivating the IRGs. Here, we show that two secreted *Toxoplasma* factors, the protein kinase ROP18 and the pseudokinase ROP5, determine IRG evasion. We demonstrate that ROP5 binds to and inhibits the oligomerization of Irga6, allowing ROP18 to phosphorylate the IRGs to inhibit PV accumulation. However, humans lack interferon-inducible IRGs, and ROP5 and ROP18 do not affect *Toxoplasma* growth inhibition in human cells, suggesting these factors specifically evolved to battle the IRG system. Both ROP5 and the IRGs exhibit diversifying selection, and these proteins may provide a model for study of eukaryotic pathogen-host co-evolution.

We also uncover a novel mechanism of IFNγ-mediated *Toxoplasma* growth inhibition in human fibroblasts that correlates with host cell death that cannot be abrogated by inhibiting cell death pathways. Furthermore, we observed parasite egress from IFNγ-stimulated cells before replication, but inhibition of egress did not prevent cell death. Thus, the inhospitable intracellular environment of dying IFNγ-stimulated human fibroblasts triggers parasite egress. This disrupts the intracellular niche, prevents replication and could promote immune clearance or depletion of parasite secretory factors.

This work highlights the need for a parasite to balance immune evasion for increased parasite propagation with limiting parasite burden for host and parasite survival. Thus, host immune factors and parasite immune evasion factors have co-evolved, and strain differences may be due to adaptation to different hosts.

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Chapter One:

Introduction

Just as changes in the environment create purifying selection, co-evolution between species can generate diversifying selection when the fitness of one species antagonizes another. Co-evolution is not restricted to predator-prey relationships or species competing for resources, but also occurs in host-pathogen interactions. When a pathogen gains an advantage, hosts that alter the host-pathogen interaction site to restore host advantage are selected. This provides continual selective pressure on both host and pathogen to alter the interaction site between them for their own benefit. The arms race between host and pathogen leads to positive selection at host-pathogen interaction interfaces or even gene duplication, which can generate genetic diversity that affects the population structure of both species. There are several known examples of host-viral arms races between innate immunity genes and viral recognition or evasion factors, but few examples of eukaryotic pathogen-host counter-adaptations exist. The eukaryotic intracellular pathogen *Toxoplasma gondii* can parasitize many hosts, and genetically diverse strains may be adapted to survive specific host immune responses. Thus, *Toxoplasma* is a good model for the study of host-pathogen counter-adaptations.

Toxoplasma gondii

Toxoplasma gondii is an obligate intracellular protozoan parasite and the causative agent of toxoplasmosis. *Toxoplasma* can infect any nucleated cell of a warm-blooded animal, which is perhaps the widest host range of any parasite. It is estimated that a third of the world population is infected with *Toxoplasma,* and toxoplasmosis is a leading cause of death due to food-borne illness in the United States (Pappas and others 2009). While *Toxoplasma* infection is asymptomatic in most healthy individuals, it is a major opportunistic infection for the immunocompromised as well as a source of congenital complications. Furthermore, ocular

disease, neurologic symptoms, and severe toxoplasmosis have occurred in immunocompetent individuals (Bossi and others 2002; Grigg and others 2001).

Aside from having its own public health implications, *Toxoplasma* also serves as a model system for other apicomplexan pathogens such as *Plasmodium*, the causative agent of malaria, and *Cryptosporidium*, underlying the opportunistic diarrheal illness cryptosporidiosis. The ease of *in vitro* studies using various host cell types as well as the genetic tractability of *Toxoplasma* makes it a good model to study eukaryotic pathogens. Importantly, not all seropositive humans suffer symptoms of toxoplasmosis, and it is believed that strain differences may underlie different disease outcomes. *Toxoplasma* strain differences have been used to identify genetic loci associated with important phenotypes such as host transcriptional modulation and overall mouse virulence (Rosowski and others 2011; Saeij and others 2006; Saeij and others 2007). The identified genetic loci associated with virulence have helped confirm the immune pathways important for host defense, which may inform the study of host interactions with other eukaryotic pathogens.

Toxoplasma was first identified in 1908 in a rodent called the gundi in Tunis (Nicolle and Manceaux 1908), and simultaneously in a rabbit in Brazil (Splendore 1908). It was named for its arced morphology (toxo derives from bow or arc in Greek) and the rodent from which it was isolated. *Toxoplasma* was then isolated from other hosts, including birds, often by researchers attempting to study *Leishmania* or viruses. The first successful isolation of viable *Toxoplasma* used similar techniques to virus isolation and illustrated the obligate intracellular nature of the parasite (Sabin and Olitsky 1937). Not until *Toxoplasma* was isolated from a human infant with brain and eye lesions in 1939 was it recognized as a human pathogen capable of congenital transmission (Wolf and others 1939). Then in 1941, *Toxoplasma* was isolated from the brain of a

6 year-old child that died with neurological symptoms, illustrating that toxoplasmosis could be acquired after birth as well. When the immunocompromised population increased in the 1980s due to the AIDS epidemic and immunosuppressive cancer drugs, toxoplasmosis took on even greater clinical significance. *Toxoplasma* is now the third leading cause of death and economic losses due to food-borne illness in the United States (Hoffmann and others 2012).

Life Cycle and Transmission of Toxoplasma

Like many unicellular organisms, *Toxoplasma* has both sexual and asexual stages of its life cycle. The arc shaped parasites initially isolated are the fast growing form of its asexual cycle called tachyzoites. The acute stages of *Toxoplasma* infection are characterized by growth and dissemination of tachyzoites. Tachyzoites are able to cross the placenta, allowing for congenital transmission to the fetus if infection is acquired during pregnancy. In the chronic stages of infection, tachyzoites differentiate into slow-growing bradyzoites that form intracellular tissue cysts. Bradyzoites express different metabolic enzymes and surface antigens to slow growth and evade immune detection (Dzierszinski and others 2001; Kasper 1989; Yang and Parmley 1995). *In vitro*, tachyzoites can be induced to convert to bradyzoites by environmental stresses such as high pH or temperature, nutrient starvation, nitric oxide or the pro-inflammatory cytokine interferon-gamma (IFNγ) (Bohne and others 1993; 1994; Fox and others 2004; Jones and others 1986; Lyons and others 2002; Soête and others 1994). Survival of the host is crucial for survival of the parasite, so it is important for the immune response elicited by tachyzoites to initiate the conversion to encysted bradyzoites to ensure survival of the host and transmission of the parasite. Tissue cysts generally form in the nervous and muscular tissues and remain throughout the life of the host. Tissue cysts are an important part of the *Toxoplasma* life cycle because they allow for transmission through carnivorism without the occurrence of a sexual

cycle. The cyst wall is sensitive to digestion with pepsin or trypsin, but bradyzoites are resistant, allowing for infection by ingestion of tissue cysts (JACOBS and others 1960). Additionally, tissue cysts are not sensitive to current drug therapies and can reactivate in immunocompromised individuals leading to toxoplasmosis. The sexual cycle only occurs in the intestines of members of the Felidae family (Frenkel and others 1970). Infected cats can shed millions of highly infective oocysts that can survive in the environment for years. Oocysts can contaminate food and water, leading to outbreaks of toxoplasmosis, and indeed, *Toxoplasma* is absent from islands without cats, illustrating the importance of oocysts in the transmission of *Toxoplasma* infection (Benenson and others 1982; Wallace 1969).

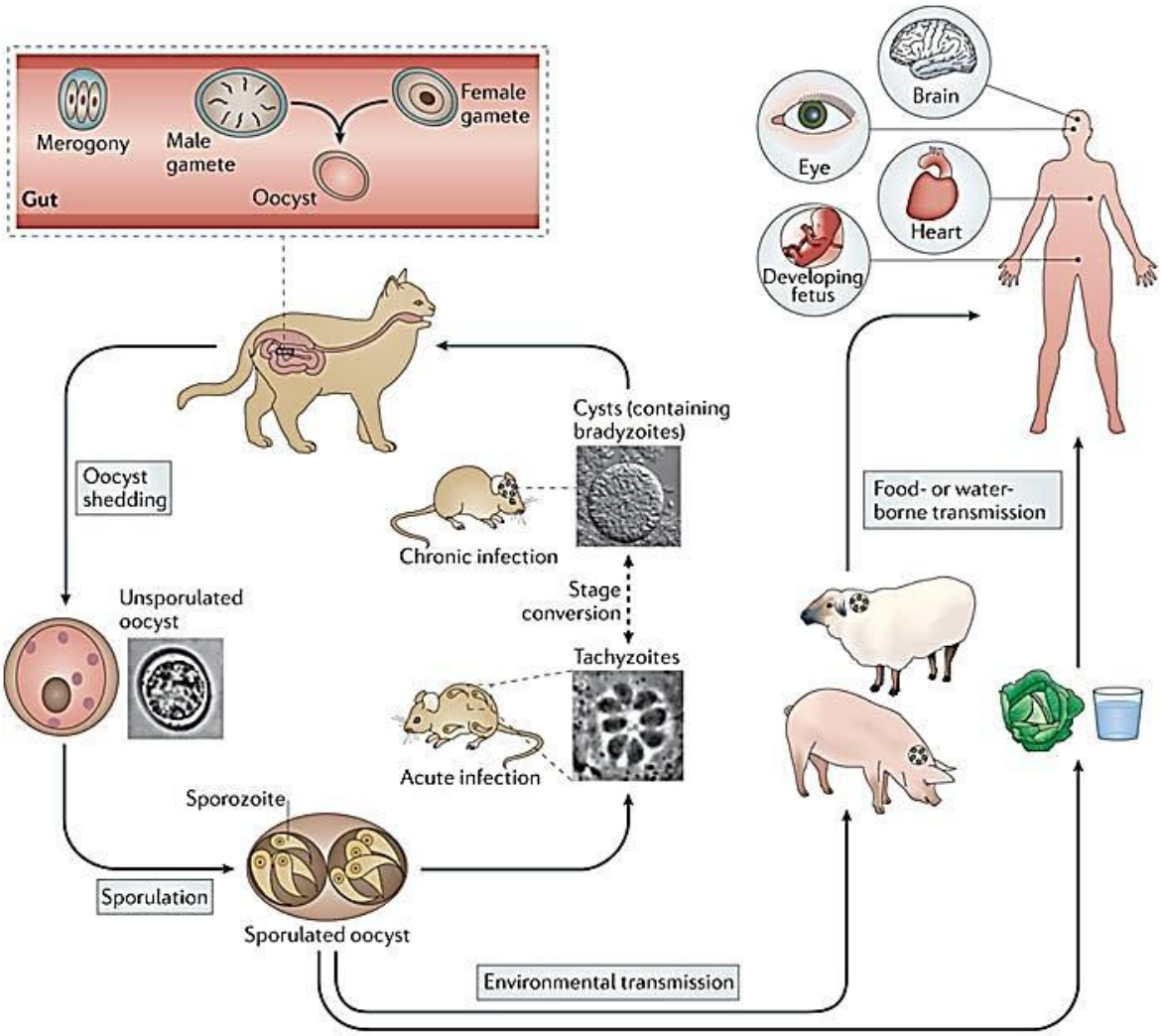

Figure 1: Life Cycle and Transmission of *Toxoplasma gondii*
(Reproduced from (Hunter and Sibley 2012) Copyright © 2012 with permission from Nature Publishing Group). *Toxoplasma* undergoes its sexual cycle in felines and has two life cycle stages in intermediate hosts: rapidly dividing tachyzoites, and dormant tissue cysts. Transmission occurs through ingestion of food or water contaminated with oocysts, ingestion of tissue cysts from other intermediate hosts, or congenitally.

Lytic Cycle of Toxoplasma

For *in vitro* studies of *Toxoplasma*, it is important to understand the lytic cycle of tachyzoites. The first step in the lytic cycle is active invasion of the host cell. Invasion begins with loose, non-specific attachment to the host cell surface, likely mediated by glycosylphosphatidyl inositol (GPI)-linked parasite surface proteins called surface antigens

(SAGs) (Mineo and Kasper 1994; Pollard and others 2008). Next, parasite cytosolic calcium release triggers the secretion of the contents of apical secretory organelles called micronemes and rhoptries (Carruthers and Sibley 1997). Microneme proteins are essential for motility and attachment to the host cell membrane (Carruthers and others 1999). Invasion is powered by the parasite actinomyosin motor and can be inhibited by prior treatment of the parasite with cytochalasin D, an actin polymerization inhibitor (Dobrowolski and Sibley 1996). Together with rhoptry neck proteins, microneme proteins form a moving junction between the parasite and host cell membranes that migrates from the apical end of the parasite to the posterior as the parasite actively invades forming a parasitophorous vacuole (PV) derived of host membrane (Alexander and others 2005; Hehl and others 2000). The moving junction helps exclude transmembrane proteins from the parasitophorous vacuole membrane (PVM) to prevent acidification of the vacuole or fusion with lysosomes (Mordue and others 1999).

After invasion, a third set of secretory organelles, the dense granules, are secreted, and several dense granule proteins, including GRA2, 4 and 6, help form a tubulovesicular network in the PVM (Labruyere and others 1999; Mercier and others 2002). It has been proposed that the tubulovesicular network is important for increasing the surface area of the PVM for host-parasite interactions. Additionally, the network creates negative curvature in the membrane, which may be important for localization of many proteins to the PVM, including secreted rhoptry and microneme proteins (Reese and Boothroyd 2009). Whatever the function of the tubulovesicular network, it appears to be important because GRA2 is one of only a few *Toxoplasma* genes for which deletion causes a reduction of *in vivo* mouse virulence (Mercier and others 1998).

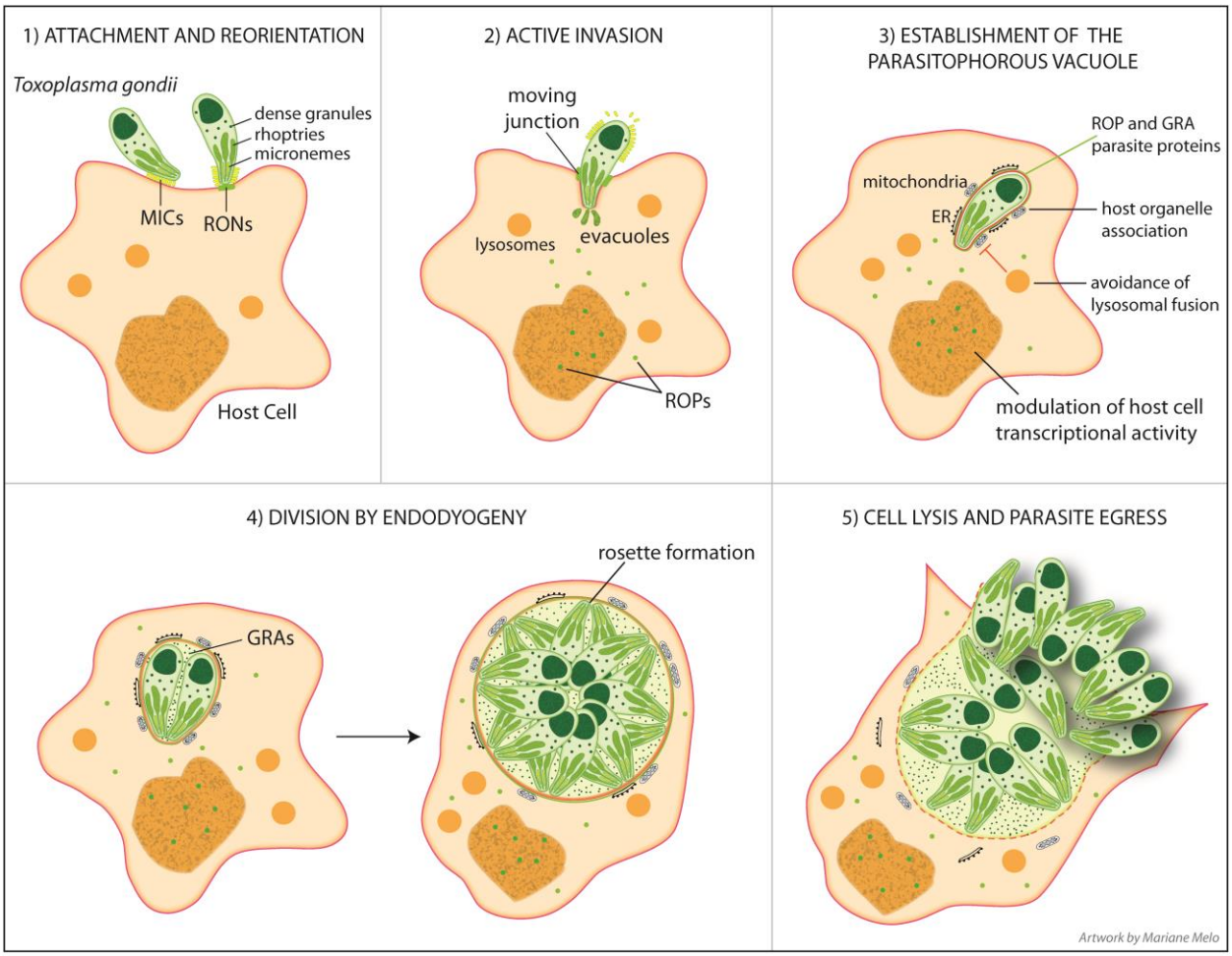

Figure 2: The Lytic Cycle of *Toxoplasma gondii*
(Illustration courtesy of Mariane Melo) *Toxoplasma* attaches and actively invades a host cell by secreting the contents of apical secretory organelles. Microneme and rhoptry proteins aid in the formation of a moving junction to exclude host membrane proteins from the parasitophorous vacuole membrane. Inside the cell, the parasite divides within this non-fusogenic vacuole by endodygeny. Finally, the parasites egress and lyse the host cell.

Parasites inside the vacuole divide asexually by endodygeny, a process in which the two daughter cells form inside the mother cell and organelles are partitioned by cytoskeletal scaffolding until the mother cell is consumed (GOLDMAN and others 1958). The nuclear envelope, cytoskeletal structures and organelles all remain intact throughout endodygeny, enabling immediate egress of viable parasites at any point in the process. After multiple rounds of replication, parasites actively egress from the host cell in an actinomyosin-dependent process

similar to invasion, resulting in host cell lysis. Egress requires parasite motility as well as PVM permeabilization by calcium-dependent secretion of the perforin-like protein TgPLP1 from micronemes (Kafsack and others 2009). Calcium ionophores, such as A23187, stimulate microneme secretion and motility, while calcium chelators, such as BAPTA-AM, inhibit it (Lovett and Sibley 2003). Indeed, *Toxoplasma* calcium-dependent protein kinases are essential for normal invasion, motility and egress (Garrison and others 2012; Lourido and others 2012; McCoy and others 2012). Cues for egress include a quorum sensing system based on accumulation of abscisic acid released by replicating parasites (Nagamune and others 2008) and activation of vacuolar NTPases by dithiols leading to depletion of host ATP and release of intra-host-cellular calcium (Stommel and others 2001). Furthermore, potassium efflux due to host cell membrane permeabilization can also lead to increased intracellular calcium levels within the parasite that activate motility and egress (Moudy and others 2001).

Population Structure of Toxoplasma

Genetic differences between infecting strains of *Toxoplasma* have been associated with different disease outcomes, making the population structure of *Toxoplasma* an important aspect of its epidemiology. In North America and Europe, *Toxoplasma* isolates derive largely from three main clonal lineages, types I, II and III (Howe and Sibley 1995). Strains from these haplotypes exhibit minimal within-type genetic diversity, and there is less than 1% genetic variation between these three lineages (Su and others 2003). It is not known what makes these three haplotypes so successful, but polymorphism between these clonal strains is generally limited to only two alleles, suggesting these strains could have risen from a recent cross between type II and two similar strains (Saeij and others 2005a). Despite the minimal genetic variation between lineages, major phenotypic differences exist between haplotypes including growth rate,

migratory capacity, oral infectivity of cysts, and activation or evasion of immune responses (Barragan and Sibley 2002; Fux and others 2007; Saeij and others 2005a; Saeij and others 2005b; Saeij and others 2007; Zhao and others 2009a). Importantly, these strains differ in the ability to promote virulence in mice with type I strains having an LD100 of 1 parasite and types II and III strains having LD50 of ~10^3 or ~10^5 parasites, respectively (Saeij and others 2006; Sibley and Boothroyd 1992). Crosses between these strains have enabled quantitative trait locus mapping of virulence in mice to identify genetic factors associated with mouse virulence (Behnke and others 2011; Saeij and others 2006; Taylor and others 2006).

Recently, a fourth clonal lineage prevalent in wild animals in North America was described and given the haplogroup number XII (Khan and others 2011). Several aspects of the *Toxoplasma* life cycle contribute to the clonality of these strains. The ability to transmit infection between intermediate hosts (all non-feline hosts) without a sexual cycle can reduce diversity. Furthermore, the parasite can differentiate into both macro- and micro-gametes, allowing self-fertilization to occur even in the sexual cycle. Cats shed millions of oocysts for a limited time after infection, so recombination requires infection with multiple strains in short succession, which is presumably a rare event in nature. In other regions of the world, particularly South America, the clonal lineages are rarely isolated, and more genetically diverse strains prevail (Lehmann and others 2006; Pena and others 2008). It has been proposed that the diversity of felines in South America compared to the relatively limited availability of the definitive host in North America has allowed for more recombination and diversity (Khan and others 2007). Likewise, in North America, strains have been selected for limited virulence and higher oral infectivity of tissue cysts to enhance transmission between intermediate hosts to compensate for reduced availability of definitive hosts. Interestingly, ocular toxoplasmosis and severe disease in

immunocompetent individuals is more frequent in South America, and South American isolates are also more virulent in mice (Bossi and others 2002; Gilbert and others 2008; Khan and others 2009). We recently used genome and RNA sequencing to produce a haplotype map for 26 strains representing global diversity (Minot and others 2012). This will allow for study of conserved haploblocks that may have a selective advantage as well as highly polymorphic regions undergoing positive selection to help make associations between genetic markers and virulence among the South American strains.

Immune Response to *Toxoplasma*

Toxoplasma must find a balance between activating the host immune response to allow the host to survive and continue supporting the parasite, and evading the immune response to allow for growth and dissemination of the parasite. Importantly, the immune response induces the encysted bradyzoite stage which not only limits parasite burden, but is necessary for oral transmission between hosts because it is resistant to digestion. However, a host can die not only from uncontrolled parasite burden, but also from excessive immunopathology. Thus, it is crucial to survival of both the host and the parasite that *Toxoplasma* elicits an appropriate immune response.

The majority of intermediate hosts, including humans, become infected with *Toxoplasma* through ingestion of tissue cysts or oocysts. After the cyst wall is digested, the parasite is released to infect intestinal epithelial cells which provide the first line of defense against infection. Infected intestinal epithelial cells produce cytotoxic molecules such as nitric oxide and secrete chemokines such as monocyte chemotactic protein (MCP-1) and macrophage inflammatory proteins (MIP-1α and MIP-1β) that attract macrophages, neutrophils, dendritic

cells (DCs) and lymphocytes (Mackay 2001; Mennechet and others 2002). MIP-1α and MIP-1β secreted by enterocytes also stimulate the expression of the chemokine receptor CCR5. Additionally, enterocytes produce interleukin-15 that activates natural killer (NK) cells. Despite initial immune responses in the intestine, parasites quickly transition to tachyzoites and migrate across the epithelium to disseminate throughout the body, possibly by infecting DCs and using them as Trojan horses (Bierly and others 2008).

Cell-mediated immunity plays an important role in surviving *Toxoplasma* infection (Frenkel 1967). Cell-mediated immunity is a non-specific immune response generated by activation of immune cells and release of cytokines to activate antimicrobial activity, whereas adaptive or humoral immunity relies on genome rearrangements for recognition of specific pathogens. The first immune cells to arrive at the site of infection are generally the neutrophils that circulate in the bloodstream and phagocytose and kill pathogens by releasing anti-microbial proteins from their granules. Additionally, neutrophils secrete chemokines to recruit other immune cells, and they directly interact with immature DCs to trigger maturation. Depletion of neutrophils in the early stages of infection or preventing neutrophil development by knocking out IL-17 makes mice more susceptible to *Toxoplasma* infection (Bliss and others 2001; Kelly and others 2005).

Macrophages are also phagocytes, and dendritic cells have high endocytic capacity to sample the environment for pathogens. These antigen-presenting cells use pattern recognition receptors such as toll-like receptors (TLRs) to identify pathogen associated molecular patterns (PAMPs). The importance of TLRs in recognizing *Toxoplasma* infection is illustrated by the inability of mice deficient for MyD88, the common adaptor molecule of TLR signaling, to survive infection with normally avirulent strains (Scanga and others 2002). Indeed, several

Toxoplasma TLR ligands have been identified including the GPI moieties that anchor parasite surface proteins which can activate TLR2 and TLR4 (Debierre-Grockiego and others 2007). Additionally, TLR7 and TLR9 recognize *Toxoplasma* RNA and DNA, respectively, and TLR11 and TLR12, which are expressed in mouse but not humans, recognize *Toxoplasma* profilin, a cytoplasmic molecule necessary for invasion (Andrade and others 2013; Koblansky and others 2013; Plattner and others 2008). Mice deficient in TLR7/9/11 or that have a mutation in UNC93B1, a protein that controls trafficking of TLR3/7/9/11/12/13 from the ER to endolysosomes, die in the acute stage of infection with avirulent *Toxoplasma* strains (Andrade and others 2013; Melo and others 2010; Pifer and others 2011). Aside from TLR activation, other pathways contribute to immune recognition, such as the chemokine receptor CCR5 which is activated by *Toxoplasma* cyclophilin-18 in DCs (Aliberti and others 2003). Mice deficient in CCR5 also have increased susceptibility to *Toxoplasma* infection (Aliberti and others 2000).

Downstream of TLR activation are the NF-KB and MAPK pathways leading to the secretion of the pro-inflammatory cytokine interleukin-12 (IL-12). While macrophages, neutrophils and DCs all secrete IL-12, DCs seem to be the most important source, and depletion of DCs suppresses IL-12 production and increases susceptibility to *Toxoplasma* infection (Liu and others 2006). In addition to secreting cytokines, macrophages and DCs migrate to the spleen and lymph nodes to present antigen to T cells to activate an adaptive Th1 response. The importance of T cell-mediated immunity in resistance to *Toxoplasma* infection is illustrated by the susceptibility of AIDS patients to toxoplasmosis. IL-12 activates $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells as well as NK cells that secrete the pro-inflammatory cytokine IFNγ (Chan and others 1991). Mice deficient in IFNγ or IL-12 die in the acute stage of infection by avirulent strains of *Toxoplasma* (Gazzinelli and others 1994; Scharton-Kersten and others 1996)

although humans with partial IFNγ receptor 1 deficiency can still control *Toxoplasma* infection (Janssen and others 2002). IFNγ promotes anti-parasitic activity and induces the conversion of tachyzoites to dormant tissue cysts.

Aside from TLR activation, NOD-like receptors (NLR) are additional cytosolic sensors that mediate immunity to *Toxoplasma*. NLRs assemble into multi-protein complexes called inflammasomes that activate caspase-1 to cleave pro-IL-1β and pro-IL-18. The NLR NALP1 was identified as a susceptibility gene for congenital toxoplasmosis, and knockdown of NALP1 led to uncontrolled parasite growth and decreased secretion of the pro-inflammatory cytokines IL-1β, IL-12 and IL-18 in monocytes (Witola and others 2011). Inflammasomes are also closely linked to a pro-inflammatory cell death called pyroptosis (Fernandes-Alnemri and others 2007). Furthermore, ATP activation of the purinergic receptor P2X7R causes an efflux of intracellular K^+ that activates the inflammasome. Polymorphisms in P2X7R are associated with congenital toxoplasmosis, and loss of P2X7R in mouse and human macrophages compromises their ability to kill *Toxoplasma* (Jamieson and others 2010; Lees and others 2010). ATP activation of macrophages is associated with co-localization of parasitophorous vacuoles with lysosomes and apoptosis of the host cell (Corrêa and others 2010; Lees and others 2010).

Finally, as excess inflammation can cause pathology in the host, the anti-inflammatory cytokines IL-10, transforming growth factor-β, and IL-27 are also important for *Toxoplasma* immunity because they dampen the immune response to limit immunopathology. IL-10 deficient mice die in the acute stages of infection with *Toxoplasma* due to excessive inflammation, and TGF-β secreted by intestinal epithelial lymphocytes helps limit intestinal pathology (Gazzinelli and others 1996; Mennechet and others 2004). Successful parasites find a balance between activation and inhibition of the immune response to replicate without causing pathology. Thus, in

order to be successful in such a wide host range, it is believed that different strains may be adapted to survive the immune responses of a specific host and that virulence is a consequence of infecting the wrong host.

Interferon-gamma Induced Immunity to *Toxoplasma*

The pro-inflammatory cytokine IFNγ is a critical mediator of both innate and adaptive immunity to viruses and intracellular pathogens. IFNγ is secreted by natural killer and T lymphocytes, and while it is recognized as a major activator of macrophages, its receptor is expressed ubiquitously (Farrar and Schreiber 1993). Binding of IFNγ to its receptor causes dimerization of the receptor and activation of Janus kinases (JAKs). JAKs phosphorylate tyrosines on the receptor to create binding sites for the signal transducer and activator of transcription (STAT)-1, which is then also tyrosine phosphorylated by JAK. STAT1 then dimerizes and translocates to the nucleus to bind to Interferon-Gamma-Activated-Sequences (GAS) sites and initiate transcription of IFNγ-inducible genes.

The IFNγ receptor is necessary in both hematopoietic and non-hematopoietic cells to survive the acute stages of infection with *Toxoplasma* (Yap and Sher 1999). The ability to produce IFNγ remains critical for survival even in the chronic stages of infection to suppress reactivation of tissue cysts (Jones and others 1986). IFNγ induces expression of the major histocompatibility complex (MHC) class I in all nucleated cells and class II in professional antigen presenting cells to boost both cell-mediated immunity and humoral immunity. Additionally, IFNγ promotes cell-autonomous intracellular resistance mechanisms in both immune and non-immune cells that help control *Toxoplasma* infection including the production of nitric oxide (NO), nutrient deprivation, interferon-inducible GTPases, and autophagy.

Nitric Oxide

IFNγ induces the expression of inducible nitric oxide synthase (iNOS or NOS-2) which converts L-arginine to citrulline and NO. NO can inhibit essential mitochondrial enzymes and cause DNA damage to kill or inhibit growth of the parasite. However, NO can also cause increased pathology in the host. Treating mice with the iNOS inhibitor aminoguanidine or knocking out iNOS leads to uncontrolled parasite proliferation but reduced pathology with eventual mouse death in the chronic stages of infection (Khan and others 1997; Scharton-Kersten and others 1997). In chimeric mice that express iNOS only in hematopoietic or non-hematopoietic cells, it was shown that iNOS expression in only hematopoietic cells is sufficient for host resistance (Yap and Sher 1999). However, there is controversy over whether human macrophages produce NO to the same extent that mice do, and this effector mechanism may not be as important in humans.

Nutrient Deprivation

Another interferon-inducible enzyme important in *Toxoplasma* immunity is indoleamine-2,3-dioxygenase (IDO1) which degrades tryptophan. Because *Toxoplasma* scavenges this essential amino acid from its host, tryptophan degradation can limit parasite growth by starvation. Tryptophan supplementation or IDO1 inhibition has been shown to restore parasite growth in IFNγ-stimulated human lung cells and fibroblasts (Gupta and others 1994; Heseler and others 2008; Pfefferkorn 1984; Werner-Felmayer and others 1991). Furthermore, IFNγ was shown to inhibit *Toxoplasma* replication in rat enterocytes by limiting iron availability, and *Toxoplasma* growth was restored by addition of ferrous sulphate or holotransferrin (Dimier and Bout 1998). Prior to this work, tryptophan deprivation was the only characterized means for

IFNγ to inhibit parasite growth in non-immune human cells. Thus, nutrient deprivation is an important means of host defense against *Toxoplasma*.

Interferon-inducible GTPases

Among the most abundantly expressed proteins in response to IFNγ is a superfamily of IFN-inducible GTPases. This superfamily includes four subfamilies: the 21-47 kDa immunity-related GTPases (IRGs), the 65-73 kDa guanylate binding proteins (GBPs), the 72-82 kDa myxovirus resistant (Mx) proteins, and the 200-285 kDa very large inducible GTPases (VLIGs) (Boehm and others 1998; Haller and Kochs 2002; Klamp and others 2003). Crystal structures of IFN-inducible GTPases show globular G domains and helical domains of varying size that are important for determining protein-protein or protein-lipid interactions and subcellular localization (Ghosh and others 2004; Prakash and others 2000; Tiwari and others 2009). These proteins also generally share some biochemical properties including low affinity for GTP, GTP-dependent oligomerization and cooperativity, and the ability to interact with and modify lipid membranes. These structure and biochemical properties cause IFN-inducible GTPases to be grouped with dynamin-like or "large" GTPases that act as mechanoenzymes or scaffolds as opposed to the "small" monomeric GTPases or heterotrimeric G proteins that act as molecular switches. Mx proteins have antiviral qualities but are induced by other interferons, not IFNγ, and the role of VLIGs in immunity remains unknown, so these families will not be discussed further.

The IRG family, which is the most genetically diverse of these families both within and between species, is present to varying extents in fish, reptile and mammal genomes, but is absent in birds (Bekpen and others 2005). There are 21 protein-coding IRG genes in the mouse genome, most of which are IFNγ-inducible. Humans, on the other hand, have only two IRGs, neither of which are interferon-inducible; IRGC which is expressed only in the testis, and IRGM which has

a truncation in the nucleotide binding G-domain. Felines have only an IRGC-like gene (Premzl 2012). For mice, the IRGs play an important role in the defense against many intracellular pathogens, including *Toxoplasma.* Mice deficient in Irgm1 or Irgm3 die in the acute stages of toxoplasmosis, while Irgd and Irga6 deficient mice die in the chronic stages of infection (Collazo and others 2001; Liesenfeld and others 2011; Taylor and others 2000). Furthermore, expression of the IRGs is required even in non-hematopoietic cells, suggesting IRGs have non-redundant, crucial roles in cell-autonomous immunity to *Toxoplasma* (Collazo and others 2002; Collazo and others 2001; Taylor and others 2000).

In mice, two subfamilies of IRGs exist: the GKS class that has a canonical lysine in the conserved nucleotide binding site and the GMS class that has a methionine substitution for the key lysine and serves to negatively regulate the GTPase cycle of the GKS group by preferentially binding their GDP-bound state (Hunn and others 2008). The GMS proteins are membrane bound with Irgm2 localizing to the Golgi apparatus, Irgm3 localizing to the endoplasmic reticulum (ER) and Irgm1 localizing to the Golgi and endo-lysosomal membranes (Martens and others 2004). Irga6 and Irgb6 partition between membrane-bound and cytosolic fractions, and Irgd is largely soluble. Within minutes of infection, members of the GKS group accumulate on the PVM of *Toxoplasma* in a coordinated order led by Irgb6 and Irgb10 which stabilize the loading of the other members, perhaps due to the formation of mixed oligomers (Khaminets and others 2010). In the absence of GMS proteins, the GKS IRGs which are normally GDP-bound in the cytosol become activated and form cytotoxic aggregates, preventing localization to the PVM (Hunn and others 2008). Similarly, in the absence of autophagic proteins such as Atg5, cytotoxic aggregates also accumulate and prevent localization to the PVM (Khaminets and others 2010; Zhao and others 2008). Once IRGs reach the PVM, they promote disruption of the vacuole, possibly by

extracting lipid from the membrane similar to dynamin-mediated vesiculation, leading to tension and rupture of the membrane (Ling and others 2006; Martens and others 2005). Release of the parasite into the cytosol is followed by autophagosome elimination of the parasite in macrophages (Ling and others 2006) or necrotic death of the host cell in fibroblasts (Zhao and others 2009b).

Irgm1 is also necessary to prevent IFNγ-induced cell death of $CD4^+$ T lymphocytes, which are crucial for a Th1 response (Feng and others 2008). It has been suggested that localization to both Golgi and endo-lysosomal membranes allows Irgm1 to have a greater role in protecting the cell from lysosomal toxicity due to GKS aggregates than the other GMS proteins (Howard and others 2011). Human IRGM also plays an important immune role, although likely by a different mechanism than in mice. IRGM mediates autophagic destruction of *Mycobacterium tuberculosis* and *Salmonella typhimurium* in human cells, and some variants are associated with increased risk for Crohn's disease, an inflammatory bowel disease (McCarroll 2008; Singh and others 2010). However, a role in immunity to *Toxoplasma* for IRGM in humans has not been shown.

GBPs are well conserved in vertebrates and are some of the most abundantly expressed proteins in response to IFNγ (Boehm and others 1998). There are 11 mouse GBP genes distributed in 2 chromosomal clusters and 7 human genes on a single chromosome (Olszewski and others 2006). Most GBPs are predominantly cytosolic, but GBP-1, -2, and -5 of both mouse and human origin have CaaX sequences that promote isoprenylation which facilitates membrane targeting and protein-protein interactions (Nantais and others 1996). In mice, it was shown that Gbp-1,-2 and -5 localize to the parasitophorous vacuole alongside the IRGs (Virreira Winter and others 2011). Mice deficient in Gbp1, Gbp2 or a cluster of six GBPs on chromosome 3

including Gbp-1, -2, -3, -5 and -7 are susceptible to *Toxoplasma* and lack IRG localization to the parasitophorous vacuole (Degrandi and others 2013; Selleck and others 2013; Yamamoto and others 2012). mGbp1 and mGbp7 have also been shown to recruit oxidative and autophagic complexes for the elimination of listeria and mycobacterial infection (Kim and others 2011). mGbp7 binds the autophagic protein Atg4b and NADPH oxidase which generates superoxide, while mGbp1 binds p62/Sequestosome-1 to help deliver ubiquitinated proteins to the lysosome. Atg4b can be seen on the *Toxoplasma* PVM, but this is not affected in the absence of Gbp7, nor is oxidative burst (Yamamoto and others 2012). It is unknown if GBPs affect immunity to *Toxoplasma* in humans, but GBP5 from both mouse and humans was shown to be critical for assembling the NOD-like receptor protein 3 (NLRP3) inflammasome (Shenoy and others 2012). Inflammasome activation leads to release of pro-inflammatory cytokines IL-1β and IL-18, and is associated with pyroptosis. Indeed, GBP5 promotes pyroptosis in *Salmonella*-infected macrophages (Vestal and Jeyaratnam 2011). Additionally, hGBP1 and 2 localize to and limit the growth of chlamydia inclusions, suggesting the GBPs could play a similar role to the IRGs in humans (Tietzel and others 2009).

Autophagy

Autophagy is a homeostatic cellular process in which isolation membranes envelope a portion of cytoplasm or an organelle for delivery to the lysosome to degrade material too large for the proteasome. Autophagosomes can also deliver intracellular microbes to the lysosome, and autophagy is induced by several immune signals such as IFNγ, tumor-necrosis factor (TNF), and CD40 ligand. Indeed autophagosomes have been observed surrounding parasites released into the cytosol by IRG-mediated PVM disruption in IFNγ-activated macrophages, although this was not observed in fibroblasts (Ling and others 2006). Additionally, CD40 activation of mouse and

human macrophages induced fusion of the parasitophorous vacuole with lysosomes in a manner dependent on autophagic machinery (Andrade and others 2006). Furthermore, CD40 stimulation in mouse and human endothelial cells induced expression of autophagy proteins, increased autophagic flux and caused autophagosome recruitment to the parasitophorous vacuole (Van Grol and others 2013). As mentioned earlier, autophagy is also necessary for regulation of the IRGs, and IRGM regulates autophagy-dependent clearance of mycobacteria. More generally, autophagy can also facilitate MHC presentation of cytosolic antigens to promote adaptive immunity. Interestingly, in cells that have not been immune-activated, autophagy can actually promote parasite growth. In infected HeLa cells and fibroblasts, *Toxoplasma* induces host cell autophagy and recruits autophagosomes to the vacuole for nutrient capture, and parasite growth is limited in Atg5 deficient cells (Wang and others 2009).

Cell Death in Immunity

Another mechanism of host defense against intracellular pathogens is death of infected cells. There are three major modes of cell death in immunity: apoptosis, necroptosis and pyroptosis. Apoptosis is considered a non-inflammatory cell death characterized by cell shrinkage, membrane blebbing, DNA fragmentation and mitochondrial permeability. Apoptosis can be induced extrinsically by activation of death receptors such as the Fas or tumor-necrosis factor (TNF) receptors as well as intrinsically by mitochondrial release of cytochrome *c*, leading to caspase activation. Many intracellular pathogens manipulate cell death pathways, often preventing cell death initially until after replication occurs and then inducing cell death to efficiently exit the host cell. Indeed, there is evidence of both induction and inhibition of apoptosis by *Toxoplasma* (Gavrilescu and Denkers 2001; Goebel and others 1999; Khan and

others 1996; Liesenfeld and others 1997; Nash and others 1998; Orlofsky and others 1999). Necroptosis is programmed necrosis characterized by membrane rupture, nuclear swelling, reactive oxygen species and caspase-independent inflammation. TNF receptor activation in the absence of caspase-8 activation leads to necroptosis mediated by receptor-interacting protein kinase-1 (RIPK1) and RIPK3. Infected IFNγ-stimulated murine fibroblasts die by necrosis after IRG-mediated disruption of the parasitophorous vacuole, but it has not been shown that RIPK is necessary for this cell death (Zhao and others 2009b). Pyroptosis is an inflammatory cell death induced by the inflammasome and characterized by membrane rupture, DNA fragmentation, and release of pro-inflammatory cytokines. The NALP1 inflammasome and P2X7R-induced pyroptosis also contribute to *Toxoplasma* immunity (Lees and others 2010; Witola and others 2011).

Modulation of the Immune Response and Virulence

In order for *Toxoplasma* to be transmitted between hosts, it must establish a chronic infection which requires evading the host immune response, but not so much that it kills the host. The wide host range of *Toxoplasma* makes it necessary to counteract the different immune effectors of each host species. It is thought that different strains of *Toxoplasma* may be adapted for different host species, and that virulence observed in one species may be due to infection with a strain better suited for another host. Thus, *Toxoplasma* has strain-specific as well as more general abilities to modulate the host cell and avoid immune clearance. More generally, the parasite reorganizes host organelles and cytoskeleton around the PV to aid in scavenging nutrients (Coppens and others 2006; Sinai and Joiner 2001). *Toxoplasma* infection also alters host cell transcription of genes in metabolic, apoptosis, and inflammatory pathways (Blader and

others 2001). Many transcriptional differences may be to compensate for resources drained by the parasite, but there is also evidence that secreted parasite factors can directly mediate transcription. For instance, the early growth response 2 (EGR2) transcription factor is activated when *Toxoplasma* is allowed to attach and secrete rhoptry proteins into the host cell but is prevented from invading by cytochalasin D inhibition of parasite motility (Phelps and others 2008). Importantly, *Toxoplasma*-infected fibroblasts are unresponsive to IFNγ due to interference with STAT1 signaling and induced expression of suppressor of cytokine signaling (SOCS) proteins that inhibit the catalytic activity of JAKs (Lüder and others 2001; Zimmermann and others 2006). Inhibition of IFNγ-dependent signaling allows *Toxoplasma* to avoid the potent antimicrobial effector mechanisms downstream of IFNγ. However, later in infection, most cells will be activated prior to infection and other immune evasion mechanisms will be necessary.

Some host transcriptional responses to infection are strain specific. Fibroblasts infected with type I, II or III strains have different gene expression profiles (Saeij and others 2007). To identify the genetic basis of strain specific phenotypes such as host transcriptional modulation, the clonal strains have been crossed with each other in all pairwise combinations. The F1 progeny derived from these crosses were used in Quantitative Trait Locus (QTL) analyses of mouse virulence and expression QTL (eQTL) analyses of host transcriptional modulation to identify the genetic loci associated with these phenotypes. For instance, infection with type I and III strains leads to sustained phosphorylation and activation of STAT3/6, and eQTL analysis of F1 progeny from the II x III cross identified the rhoptry protein kinase ROP16 as the mediator of this phenotype (Saeij and others 2007). $ROP16_{I/III}$ directly phosphorylates STATs on the tyrosine residue required for activation leading to reduced IL-12 production (Ong and others

2010; Yamamoto and others 2009). Reduced IL-12 secretion dampens the immune response, which is beneficial for parasite survival, but also helps the host limit immunopathology.

There is also evidence for strain-specific activation of the transcription factor nuclear factor KB (NF-KB). NF-KB is activated by several immune signals important for *Toxoplasma* immunity including TLRs, TNFα and IL-1β, and importantly, NF- KB is necessary for IL-12 production (Trinchieri 2003). Mice deficient in NF-KB signaling have greater susceptibility to *Toxoplasma* infection (Caamaño and others 1999; Caamaño and others 2000; Franzoso and others 1998). Type II strains activate NF-KB and induce higher levels of IL-12 production, while type I strains can transiently block nuclear translocation of NF-KB, decreasing the production of inflammatory cytokines (Robben and others 2004; Shapira and others 2005). Again, eQTL mapping of F1 progeny from a II x III cross identified the dense granule protein GRA15 as the type II factor that activates NF-KB (Rosowski and others 2011).

QTL mapping was also used to identify *Toxoplasma* factors that mediate strain differences in mouse virulence. Five loci were shown to be associated with virulence in these QTL studies (Behnke and others 2011; Reese and others 2011; Saeij and others 2006; Taylor and others 2006). Not surprisingly, ROP16 and GRA15 were shown to underlie two of these loci, likely due to their abilities to modulate immune signaling (Rosowski and others 2011; Saeij and others 2006). Expression of $ROP16_{I/III}$ in a type II strain makes that strain less virulent because type II induction of IL-12 secretion promotes excessive inflammation that is curbed by STAT3/6 activity. However, deletion of ROP16 does not affect the ability of type I strains to induce mouse mortality (Butcher and others 2011). Another secreted polymorphic rhoptry protein kinase, ROP18, was also identified as a virulence locus in the II x III QTL study and the only virulence locus in the I x III cross (Saeij and others 2006; Taylor and others 2006). An insertion in the

promoter prevents expression of ROP18 in the avirulent type III strains, but expression of a type I or II allele of *ROP18* makes that strain virulent (Khan and others 2009; Saeij and others 2006). Furthermore, deletion of ROP18 partially attenuates the mouse virulence of type I strains (Fentress and others 2010). Recently, it was shown that $ROP18_I$ phosphorylates a conserved threonine in the G-domain of Irga6, Irgb6, and Irgb10 to inhibit their accumulation on the PVM (Fentress and others 2010; Steinfeldt and others 2010). Indeed, virulent type I strains are able to evade IRG accumulation and killing, while type II and III strains are not (Zhao and others 2009a). ROP18 was also shown to promote the degradation of the endoplasmic reticulum-associated transcription factor ATF6β, and DCs from ATF6β deficient mice have reduced ability to elicit IFNγ production from $CD8^+$ T cells (Yamamoto and others 2011). Thus, ROP18 could also lead to reduced IFNγ production.

Another important virulence locus identified in the QTL studies of virulence is the *ROP5* locus (Behnke and others 2011; Saeij and others 2006). This locus was the most significant virulence locus in the II x III QTL study and the only significant locus in the I x II study. ROP5 is a highly polymorphic rhoptry pseudokinase that localizes to the PVM, and deletion of *ROP5* in a type I strain significantly attenuates virulence (Behnke and others 2011; Reese and others 2011). The ROP5 locus contains 4-10 tandem duplications with at least 3 major isoforms called A, B and C that have differential abilities to complement the virulence of the ROP5 deleted strain (Reese and others 2011). Both type I and III strains have a virulent *ROP5* locus, but the mechanism by which ROP5 affects virulence and which ROP5 isoform is necessary to complement the virulence of type II were not known prior to this thesis.

Findings Presented in This Thesis

Because $ROP18_{II}$ can make a type III strain virulent, but type II strains are the most susceptible to the IRGs, we wondered if $ROP18_{II}$ can function in IRG evasion or if additional factors were necessary. Indeed, we found that $ROP18_{II}$ is functional in the type III background to reduce IRG accumulation, indicating other factors are involved in IRG evasion. The ability of ROP5 to promote virulence in avirulent strains and the fact that type II strains have the avirulent allele of *ROP5* made ROP5 a good candidate for this additional IRG evasion factor. Using an avirulent F1 progeny of a II x III cross that has the avirulent alleles of both *ROP18* and *ROP5*, we confirmed that $ROP5_{I}$ can reduce IRG accumulation on the PVM and that ROP18 can only inhibit accumulation of the IRGs on the PVM of strains that also express virulent *ROP5* alleles. We further show that the allelic combination of *ROP18* and *ROP5* also determines IRG evasion and virulence of non-canonical strains. This suggests evasion of the IRGs is crucial for *Toxoplasma*, and mice and other IRG-carrying hosts are important in the evolution of the parasite. Informed by genetic analysis of *ROP5* from these different strains, we then expressed ROP5-A_{III} or ROP5-C_{III} in a type II strain to find that ROP5-C but not A can promote IRG evasion and virulence in this background. Next, we demonstrated that ROP5 does not strongly interact with and is not necessary for the kinase activity of ROP18, but rather ROP5 directly interacts with one or more IRGs to inhibit oligomerization and allow ROP18 access to phosphorylate them. However, neither ROP18 nor ROP5 markedly affect survival in IFNγ-activated human cells which lack the multitude of IRGs present in murine cells. Along with the diversifying selection of ROP5 and the expansion of the IRG system in mice, this suggests these systems co-evolved and may provide a good model of eukaryotic pathogen-host co-evolution.

In Chapter Three, we uncovered a novel IFNγ-induced mechanism of resistance to *Toxoplasma* in human fibroblasts that does not depend on deprivation of tryptophan or iron. Additionally, we tested whether autophagy or GBPs control infection in IFNγ-stimulated human fibroblasts and found that infection is still controlled in HFFs deficient in GBP1 or ATG5. Then, we found that resistance is coincident with host cell death that is not dependent on caspases or the necroptosis mediators RIPK1 or RIPK3. Instead we show that host cell death is correlated with but not dependent on early egress of the parasite before replication. This IFNγ-induced cell death and early egress limits replication in HFFs and could promote clearance of the parasite by immune cells or depletion of secretory factors important for immune modulation.

Thus, we have assigned a role for the virulence factor that determines the majority of strain differences in mouse virulence, explained strain differences in IRG evasion, identified a model system of host-pathogen co-evolution and uncovered a novel mechanism of IFNγ-induced immunity in human fibroblasts. Finally, future directions will be discussed in Chapter Four.

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Chapter Two:

The Rhoptry Proteins ROP18 and ROP5 Mediate *Toxoplasma gondii* Evasion of the Murine, but not the Human, Interferon-gamma Response

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Daniel Gold performed mouse infection and the ROP18 kinase activity experiments. Emily Rosowski performed mouse infection experiments. Joris Sprokholt performed plaque assays in human fibroblasts. Daniel Lim generated the ROP18 kinase domains and performed kinase activity experiments. Ailan Farid helped amplify and sequence ROP5. Mariane Melo performed the RNASeq experiments. Eric Spooner performed the mass spectroscopy.

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Abstract

The obligate intracellular parasite *Toxoplasma gondii* secretes effector proteins into the host cell that manipulate the immune response allowing it to establish a chronic infection. Crosses between the type I, II and III strains, which are prevalent in North America and Europe, have identified several secreted effectors that determine strain differences in virulence. The polymorphic rhoptry protein kinase ROP18 was recently shown to determine the difference in virulence between type I and III strains by phosphorylating and inactivating the interferon-γ (IFNγ)-induced immunity-related GTPases (IRGs) that promote killing by disrupting the parasitophorous vacuole membrane (PVM) in murine cells. The pseudokinase ROP5 determines strain differences in virulence through an unknown mechanism. Here we report that ROP18 can only inhibit accumulation of the IRGs on the PVM of strains that also express virulent *ROP5* alleles. In contrast, specific *ROP5* alleles can reduce IRG coating even in the absence of *ROP18* expression and can directly interact with one or more IRGs. We further show that the allelic combination of *ROP18* and *ROP5* also determines IRG evasion and virulence of strains belonging to all other haplotypes. However, neither ROP18 nor ROP5 markedly affect survival in IFNγ-activated human cells which lack the multitude of IRGs present in murine cells. These findings suggest that ROP18 and ROP5 have specifically evolved to block the IRGs and are unlikely to have effects in species that do not have the IRG system, such as humans.

Author Summary

Toxoplasma gondii can infect any warm-blooded animal and is transmitted orally by consumption of tissue cysts. To facilitate transmission, the parasite must balance induction and evasion of host immune responses to allow parasite growth and persistence, while avoiding

excessive parasite burden which can kill the host before infectious cysts are formed. Different strains of *Toxoplasma* have likely evolved specific effector molecules to modulate the immune responses of different hosts. In many mammals, including mice but not humans, the cytokine interferon gamma (IFNγ) induces the immunity-related GTPases (IRGs) which are essential to the host immune response to *Toxoplasma.* They function by binding to and disrupting the parasite-containing vacuole. However, some *Toxoplasma* strains prevent the IRGs from disrupting the parasitophorous vacuole. It was previously shown that the secreted *Toxoplasma* kinase ROP18 promotes virulence in mice by phosphorylating the IRGs leading to their inactivation. We report that ROP18 requires another virulence factor, the secreted pseudokinase ROP5, to prevent IRG accumulation, and these two proteins determine the majority of strain differences in IRG evasion, even for divergent strains for which virulence determinants have not been studied. Additionally, we show that ROP18 and ROP5 do not affect *Toxoplasma* survival in IFNγ-stimulated human cells. Thus, ROP18 and ROP5 are strain and host-specific determinants of *Toxoplasma* immune evasion.

Introduction

Toxoplasma gondii is a widespread intracellular parasite capable of infecting most warm-blooded animals and is an important opportunistic pathogen for immunocompromised individuals and unborn fetuses. *Toxoplasma* divides within a non-fusogenic parasitophorous vacuole and has three apical secretory organelles, the micronemes, rhoptries and dense granules, which secrete proteins into the host cell during invasion that mediate crucial host-pathogen interactions (Melo and others 2011). In general, an asymptomatic but chronic infection is established in immunocompetent individuals. However, in rare cases *Toxoplasma* can cause

severe disease even in immunocompetent people. Diverse disease outcomes may be due to genetic differences between infecting strains (Boothroyd and Grigg 2002).

Toxoplasma has a partially clonal population structure of 12-15 (Khan and others 2011b; Su and others 2012) haplogroups with the majority of North American and European isolates belonging to the canonical type I, II and III strains (Howe and Sibley 1995; Khan and others 2011a), although haplogroup 12 has been recently shown to be prevalent in wild animals in North America (Khan and others 2011a). In mice, these strains differ in virulence, with type I strains having an LD_{100} of just one parasite, compared to the LD_{50} of ~10^3 or ~10^5 parasites for type II and III strains, respectively (Saeij and others 2006; Sibley and Boothroyd 1992). Type I strains may also be more virulent in humans, as they are more frequently isolated from cases of congenital or severe ocular toxoplasmosis than from animals (Grigg and others 2001; Howe and Sibley 1995). Interestingly, in South America, more genetically diverse strains are isolated, while the canonical strains are rarely found (Lehmann and others 2006). Some of these strains are associated with high mortality rates in mice. Additionally, there are high rates of ocular toxoplasmosis in humans in South America (Gilbert and others 2008; Pena and others 2008), and some strains isolated from French Guiana have been reported to be able to cause severe disseminated toxoplasmosis even in healthy individuals (Bossi and others 2002). The determinants of canonical strain-specific differences in murine virulence are well studied, but the same determinants for non-canonical strains or for human infection remain unknown.

Mice and humans diverge in the immune mechanisms used to resist *Toxoplasma.* Interferon-γ (IFNγ) is essential to murine *Toxoplasma* resistance, and IFNγ-deficient mice die after infection even with avirulent strains (Scharton-Kersten and others 1996). Some of the important downstream effectors of this immune activation are the IFNγ-inducible immunity-

related GTPases (IRGs), which belong to the dynamin family of GTPases and can cooperatively oligomerize to vesiculate membranes. Mice deficient in individual members of the IRG family die of toxoplasmosis, but at different stages of infection, and expression of the IRGs is required even in non-hematopoietic cells, suggesting IRGs have non-redundant, crucial roles in the innate immune response against *Toxoplasma* (Collazo and others 2002; Collazo and others 2001; Taylor and others 2000). Different IRGs are sequentially and cooperatively loaded onto the parasitophorous vacuole membrane (PVM) with Irgb6 and Irgb10 leading and stabilizing the loading of the other members (Khaminets and others 2010). The IRGs are able to disrupt the PVM and kill the parasite (Ling and others 2006; Martens and others 2005).

While mice have 23 IRG genes, humans have only two IRG genes: *IRGC* which is expressed only in the testis and *IRGM* which is expressed independently of IFNγ induction and has a truncation in the nucleotide binding G-domain (Bekpen and others 2005). Despite these differences, IRGM plays a role in autophagy-mediated destruction of *Mycobacterium tuberculosis* and *Salmonella typhimurium* in human cells, and some variants are associated with increased risk for Crohn's disease (McCarroll 2008; Singh and others 2010). Thus, IRGM may have an immune role, but its lack of GTPase activity suggests a distinct mechanism of action in humans. Humans do have other known IFNγ mediated mechanisms of resistance to *Toxoplasma*; for instance, IFNγ-induced indoleamine 2,3-dioxygenase (IDO1) degrades cellular tryptophan for which *Toxoplasma* is auxotrophic, thereby inhibiting *Toxoplasma* growth (Gupta and others 1994; Pfefferkorn 1984). The NALP1 inflammasome also mediates the innate immune response to *Toxoplasma*, and *NALP1* was recently identified as a susceptibility locus for human congenital toxoplasmosis (Witola and others 2011).

Toxoplasma strain differences in evasion of host-specific immune responses exist. For instance, type I strains are able to prevent the accumulation of IRGs on the PVM, while type II and III strains are susceptible to killing by the IRGs even when co-infecting the same cell as a type I parasite (Zhao and others 2009a). Because strain-specific evasion of the IRGs is correlated with increased virulence in the mouse, it is likely that the genetic determinants of IRG evasion will also be associated with virulence. Quantitative Trait Locus (QTL) analyses of the virulence of F1 progeny derived from type I x II, I x III and II x III crosses have identified the genetic loci associated with virulence, and subsequent experiments have identified the causative genes within these loci.

ROP18, a highly polymorphic rhoptry protein kinase, was identified as a virulence locus in the II x III QTL study and the only virulence locus in the I x III cross (Saeij and others 2006; Taylor and others 2006). ROP18 is highly expressed in type I and II strains, but an insertion in the promoter prevents expression in type III strains. Addition of a type I or II copy of *ROP18* into an avirulent type III strain makes that strain virulent (Khan and others 2009; Saeij and others 2006). Recently, it was shown that type I ROP18 can phosphorylate a conserved threonine in the G-domain of Irga6 and Irgb6, disrupting their accumulation on the PVM (Fentress and others 2010; Steinfeldt and others 2010). However, type II strains have the highest percent of vacuoles coated with IRGs (Khaminets and others 2010; Zhao and others 2009a) despite the fact that a type II copy of ROP18 is also able to make a type III strain virulent suggesting that other polymorphic proteins are involved in IRG evasion (Saeij and others 2006). ROP18 was also shown to promote the degradation of the endoplasmic reticulum-associated transcription factor ATF6β to compromise CD8 T cell-mediated adaptive immune responses (Yamamoto and others

2011). Importantly, ROP18-mediated ATF6β degradation occurs in human as well as murine cells.

The *ROP5* locus, which consists of a family of 4-10 tandem duplicates of highly polymorphic genes encoding for rhoptry pseudokinases that localize to the PVM, is another important virulence determinant in mice. Deletion of *ROP5* in a type I strain significantly attenuates virulence (Behnke and others 2011; Reese and others 2011). Furthermore, *ROP5* was the only significant virulence locus identified in the recent I x II QTL analysis and was the main virulence locus in the II x III QTL study (Behnke and others 2011; Saeij and others 2006). Both type I and III strains have a virulent *ROP5* locus, but the mechanism by which ROP5 affects virulence and which of the three major ROP5 isoforms, A, B or C, (Reese and others 2011) are necessary to complement the virulence of type II are not known.

A third virulence locus, identified in the II x III QTL study, contains the rhoptry protein kinase ROP16, which in type I and III strains leads to sustained phosphorylation and activation of STAT3/6 (Saeij and others 2007). It was recently shown that ROP16 and the dense granule protein GRA15, suggested to be the fourth virulence locus in the II x III QTL study (Rosowski and others 2011), affect the accumulation of p65 guanylate binding proteins (GBPs) on the PVM of mouse cells (Virreira Winter and others 2011). Because GBPs are also dynamin family members and were found on the same vacuoles as the IRGs, ROP16 and GRA15 might also affect the accumulation of the IRGs on the PVM. Furthermore, since the GBPs are present in humans, ROP16 and GRA15 could possibly affect survival in IFNγ-stimulated human cells.

Because the murine and human immune responses to *Toxoplasma* are so different, it cannot be assumed that ROP18, ROP5, ROP16 and GRA15, which determine *Toxoplasma* virulence in mice, similarly affect survival in human cells. Furthermore, it is currently unknown

for most of these proteins what effects they have outside the clonal lineages they were identified in. Many of the exotic strains are highly virulent in mice, but because they are so divergent from the canonical strains and no analyses have been done on crosses involving exotic strains, it is not known what factors drive virulence in these strains. For example, IRG evasion has not been measured for the exotic strains, and it may be that this is strictly a type I phenotype.

In this study, we find that ROP18 can only inhibit accumulation of the IRGs on the PVM of strains that also express virulent *ROP5* alleles. Expression of *ROP18* in strains that do not express virulent *ROP5* alleles does not affect IRG accumulation or *in vivo* virulence. In contrast, specific *ROP5* alleles can reduce IRG coating even in the absence of *ROP18* expression and directly interact with Irga6 to inhibit its oligomerization. Non-canonical strains exhibit differences in evasion of IRG mediated killing as well, and the allelic combination of *ROP18* and *ROP5* also explains strain differences in IRG evasion and virulence for these strains. However, neither ROP18 nor ROP5 markedly affect survival in IFNγ-activated human cells.

Results

Both ROP18$_{II}$ and ROP18$_{I}$ reduce IRG-mediated killing of type III

Type II strains have the highest percentage of IRG-coated vacuoles compared to types I and III strains (Khaminets and others 2010; Zhao and others 2009a) even though they possess a *ROP18* allele capable of conferring virulence to a type III strain (Saeij and others 2006). To determine if, like ROP18$_{I}$ (Fentress and others 2010; Steinfeldt and others 2010), ROP18$_{II}$ can reduce IRG coating in a type III background, we measured Irgb6 coating of the PVM in IFNγ-stimulated mouse embryonic fibroblasts (MEFs) infected with type I, II, III, III + ROP18$_{I}$, or III + ROP18$_{II}$ by immunofluorescence (Figure 1A). Indeed, transgenic expression in the type III

strain CEP of either *ROP18$_I$* or *ROP18$_{II}$* decreased the average number of vacuoles coated with Irgb6 from 45% to 23% (P = 0.001) for ROP18$_{\text{I}}$ or 29% (P = 0.003) for ROP18$_{\text{II}}$ (Figure 1B). Although it is generally assumed that once the PVM is coated, it will eventually lead to killing of the parasite inside, it has also been shown that *Toxoplasma* can escape a coated vacuole and invade a new cell (Virreira Winter and others 2011; Zhao and others 2009b). Therefore, to measure killing of *Toxoplasma*, 100 parasites were seeded on a monolayer of MEFs, either previously stimulated for 24 hours with IFNγ or left untreated, and the number of plaques that form after 4-7 days of growth was determined. Wild-type CEP (type III) had an average of 45% plaque loss when comparing plaques formed on IFNγ-stimulated to unstimulated MEFs. This percent plaque loss was similar to the percent vacuoles coated with Irgb6, suggesting that coated vacuoles are eventually destroyed. Furthermore, plaque loss is drastically reduced in Atg7 deficient MEFs (Supplemental Figure 1) in which the IRGs are mis-regulated as was shown for Atg5 deficient MEFs (Khaminets and others 2010; Zhao and others 2008), suggesting the killing observed is indeed due to the IRGs. Similar to the decrease in Irgb6 coating, the plaque loss of CEP + *ROP18$_I$* or *ROP18$_{II}$* was significantly decreased to 18% (P = 0.0002) and 21% (P = 0.0004), respectively (Figure 1B). The 23% PVM coating and 18% killing of CEP + *ROP18$_I$* is similar to the 25% coating and 35% plaque loss of the type I strain GT1. Thus, ROP18 expression can likely explain the majority of the difference in IRG coating and killing between type I and type III strains. However, despite the ability of ROP18$_{\text{II}}$ to reduce IRG coating of type III strain vacuoles and subsequent killing of the parasite, type II strains are still very susceptible to the IRGs, with 70% Irgb6 coating and 73% plaque loss for Pru (type II) (Figure 1B). Thus, there must be at least one other gene that is likely similar in type I and III but different in type II involved in IRG evasion.

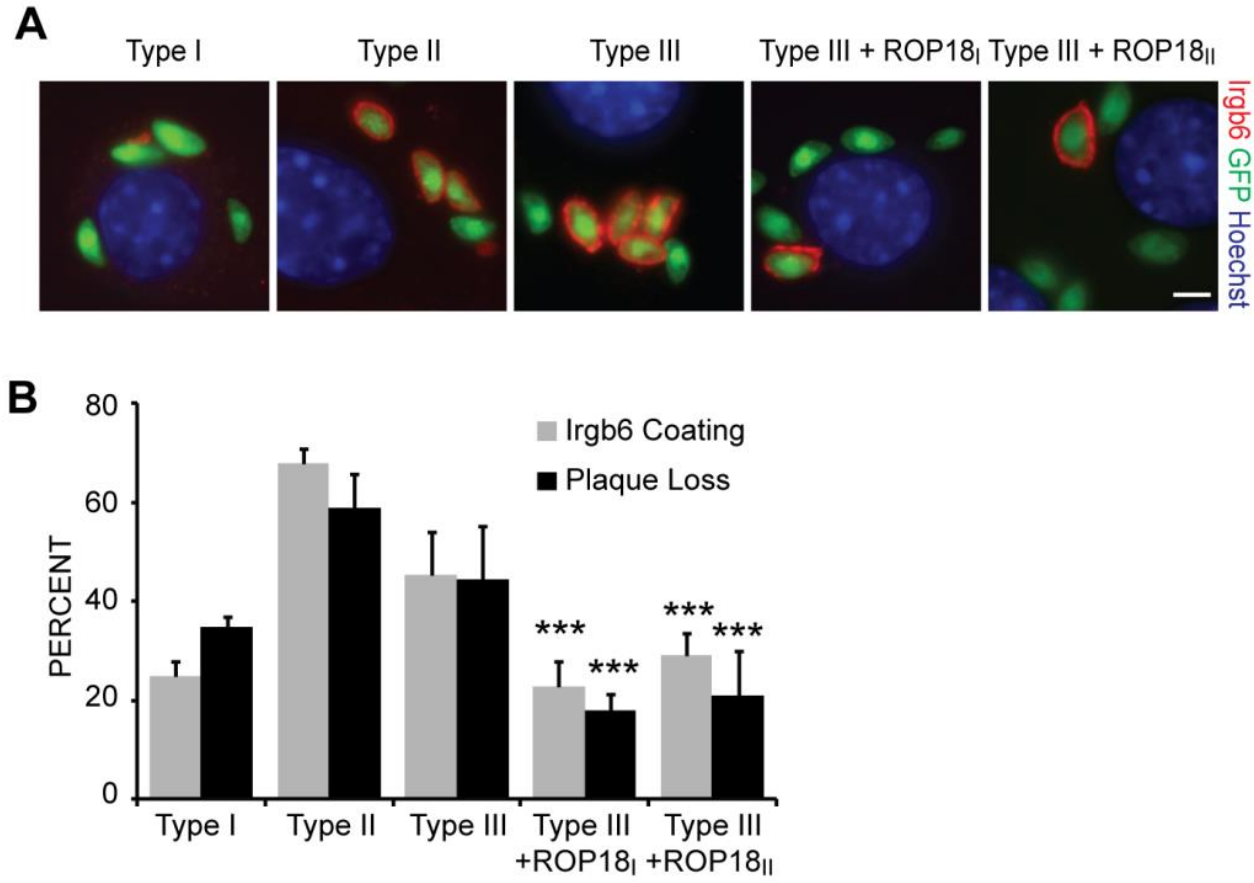


Figure 1: Type III strain expressing type I or type II ROP18 inhibits Irgb6 accumulation and killing
WT MEFs were stimulated for 24 hrs with IFNγ and infected with type I (GT1), type II (Pru), type III (CEP),CEP + $ROP18_I$ or CEP + $ROP18_{II}$ expressing GFP for 1 hr or allowed to form plaques for 4-7 days. **(A)** Immunofluorescence localization of Irgb6 (Red). Hoechst (blue). Scale bar represents 5 µm. **(B)** Quantification of Irgb6 localization on the parasite containing vacuole and percent plaque loss on stimulated MEFs compared to unstimulated MEFs. Mean ±SEM, n = 5 experiments, ***$p < 0.001$, Student's t test.

ROP5 reduces IRG-mediated killing

It was recently demonstrated that the *ROP5* cluster of pseudokinases accounts for the majority of variation in virulence between type I and II strains and between type II and III strains, with type I and III strains possessing a virulent *ROP5* locus (Behnke and others 2011; Reese and others 2011). Therefore, the *ROP5* locus is an excellent candidate for explaining strain differences in IRG evasion. We tested a potential role of ROP5 in mediating ROP18-independent

strain differences in IRG evasion by using the S22 strain, an avirulent F1 progeny from a II x III cross (Saeij and others 2005) which possesses the avirulent (non-expressed) $ROP18_{III}$ and $ROP5_{II}$ alleles. We compared the percent plaque loss and percent Irgb6 coated vacuoles between S22 and an S22 transgenic strain carrying the cosmid LC37, which contains the *ROP5* locus from the RH (type I) genome and was previously shown to have significantly increased virulence (Reese and others 2011). Expression of $ROP5_I$ significantly reduced the percent Irgb6 coating from 48% to 28% ($P < 0.001$, One-way ANOVA), and the percent plaque loss from 38% to 27% (n.s., One-way ANOVA) (Figure 2A). Thus, $ROP5_I$ can function independently of $ROP18_{I/II}$ to prevent IRG accumulation on the PVM and subsequent killing of the parasite.

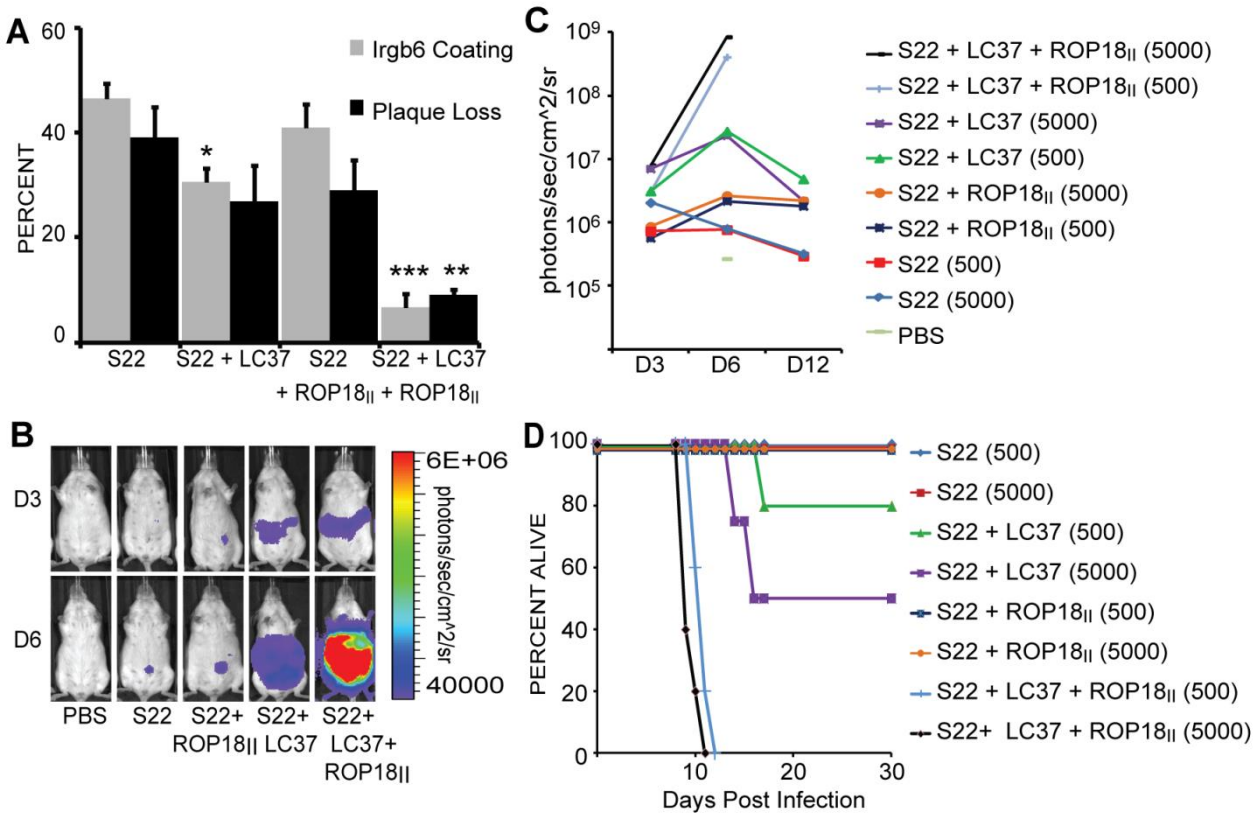


Figure 2: Virulent ROP5 promotes IRG evasion, independently of ROP18
(A) Quantification of Irgb6 localization on the parasitophorous vacuole (PV) and percent plaque loss on IFNγ-stimulated MEFs compared to unstimulated MEFs infected with S22, S22+$ROP18_{II}$, S22 LC37 and S22 LC37+$ROP18_{II}$. Mean ±SEM, n > 4 experiments, *p < 0.05, **p < 0.01, ***p < 0.001 One-way ANOVA with Bonferoni correction for all pairwise

comparisons. **(B)** *In vivo* imaging of mice infected with firefly luciferase expressing parasites of the indicated strains at days 3 and 6 post infection with 5000 parasites. One representative of 5 infected mice per strain is shown. **(C)** Quantification of *in vivo* imaging shown as average photons/sec/cm^2 for infected mice at days 3, 6 and 12. **(D)** Mouse survival of infection with indicated doses of each strain.

ROP18$_{II}$ requires ROP5 to reduce IRG coating

While ROP5 can function independently of ROP18 in reducing IRG accumulation on the PVM of S22 + LC37 vacuoles, type II strains which have a virulent allele of *ROP18* and an avirulent *ROP5* locus, have a high percentage of IRG-coated vacuoles. This suggests that either ROP18 cannot function independently of ROP5, or that ROP18 is inhibited in the type II background. We expressed *ROP18$_{II}$* in S22 and in S22 + LC37 to determine if ROP18$_{II}$ can function in the absence of virulent ROP5 alleles. ROP18$_{II}$ only slightly reduced Irgb6 coating in S22 from 47% to 41% (n.s., One-way ANOVA) and plaque loss from 39% to 24% (n.s., One-way ANOVA). However, ROP18$_{II}$ significantly reduced Irgb6 coating from 31% to 7% ($P < 0.001$) and plaque loss from 27% to 9% ($P < 0.01$) when expressed in S22 + LC37 (Figure 2A). Together, this suggests that ROP18 needs the virulent *ROP5* locus for its function. That the Irgb6 coating and plaque loss in S22 + LC37 + ROP18$_{II}$ are similar to those in RH (type I) signifies that these two genes are sufficient to complement IRG evasion and plaque loss in the S22 background.

To determine if the interactive effect of ROP18 and ROP5 on parasite survival also occurs *in vivo,* we infected outbred CD-1 mice by intraperitoneal injection with S22, S22 + ROP18$_{II}$, S22 + LC37 or S22 + LC37 + ROP18$_{II}$ tachyzoites expressing firefly luciferase and followed parasite growth and dissemination using *in vivo* imaging. On the third day after infection, the parasite burden in S22 + LC37 and S22 + LC37 + ROP18$_{II}$ infected mice was 10-fold higher than in S22 or S22 + ROP18$_{II}$ infected mice. By day six, both strains containing the

LC37 cosmid had disseminated throughout the peritoneal cavity, but S22 + LC37 + $ROP18_{II}$ infected mice had 35-fold higher luciferase activity than S22 + LC37 infected mice (P = 0.03), which in turn had 10-fold higher activity than S22 + $ROP18_{II}$ infected mice (P = 0.1) and 30-fold higher activity than S22 infected mice (P = 0.06). While S22 + $ROP18_{II}$ had a greater parasite burden than S22, this was not significant (P = 0.27). Indeed, S22 + LC37+ $ROP18_{II}$ killed 100% of the mice in the acute stages of infection at both a low and high dose (Figure 2B and C). Likewise, in keeping with the increased IRG evasion of S22 + LC37 but not S22 + $ROP18_{II}$, S22 + LC37 showed increased virulence compared to S22, but S22 + $ROP18_{II}$ infected mice survived the infection and did not show significant differences compared to S22 infected mice (Figure 2D). Thus, overall these results suggest that ROP18 only affects virulence in the context of a virulent *ROP5* locus.

IRG evasion differences in non-canonical strains

Although mouse virulence has been determined for many non-canonical strains (Khan and others 2009), it is unknown what factors determine virulence in these strains because they were not included in the previous QTL analyses. We wondered if virulent non-canonical strains could also evade IRG-mediated killing, or if IRG evasion is specific to type I strains. We measured percent plaque loss in IFNγ-stimulated MEFs as well as percent Irgb6-coated vacuoles for strains from haplogroups 1-11 (Khan and others 2011a; Khan and others 2007). In general, IRG evasion correlates with virulence as many of the most virulent strains have 25% or less Irgb6-coated vacuoles and plaque loss (Figure 3A). However, some exceptions are CASTELLS and COUGAR, which exhibit greater than 50% Irgb6 coating and plaque loss in stimulated MEFs, despite having a mortality rate of greater than 90% in CD-1 mice (Khan and others 2009).

These strains may have a different mechanism underlying their virulence in mice besides IRG evasion.

For most strains, the percent Irgb6 coating and plaque loss correlates with their *ROP18* allele (Figure 3A and Supplemental Figure 2) (Khan and others 2009). For example, CASTELLS and P89, as well as the type III strains CTG and VEG, have between 40 and 50% Irgb6 coating, and all these strains do not express ROP18 because they have a $ROP18_{III}$-like allele that contains an insertion in the promoter (Khan and others 2009). Strains expressing a type I-like allele of ROP18, with the exception of BOF, display 25% Irgb6 coating or less. Type II strains and COUGAR are highly susceptible to the IRGs with 70% and 53% Irgb6 coating respectively, despite having the virulent $ROP18_{II}$ allele. For type II strains, the avirulent *ROP5* locus likely explains the high degree of Irgb6 coating, but it is unknown what versions of *ROP5* are present in the non-canonical strains.

ROP5 sequence and expression explain strain differences in IRG evasion

It is currently unknown what determines the virulence and IRG evasion properties of the $ROP5_{I/III}$ locus because both copy number and amino acid sequence of the individual copies differ between the canonical strains (Reese and others 2011). For most of the strains mentioned above, Irgb6 coating correlates with their *ROP18* allele suggesting that they also have a virulent *ROP5* locus, as this is necessary for ROP18 to function (Figure 2). To identify differences that may be associated with virulence or IRG evasion, we sequenced the different *ROP5* isoforms from strains from haplogroups 1-11 (GenBank JQ743705-JQ743783). Based on the *Toxoplasma* genome sequence (www.ToxoDb.org) and our own genome sequencing of seven non-canonical *Toxoplasma* strains (Minot et al., submitted), we identified four distinct *ROP5* open reading frames, that we amplified and sequenced separately using isoform specific primers.

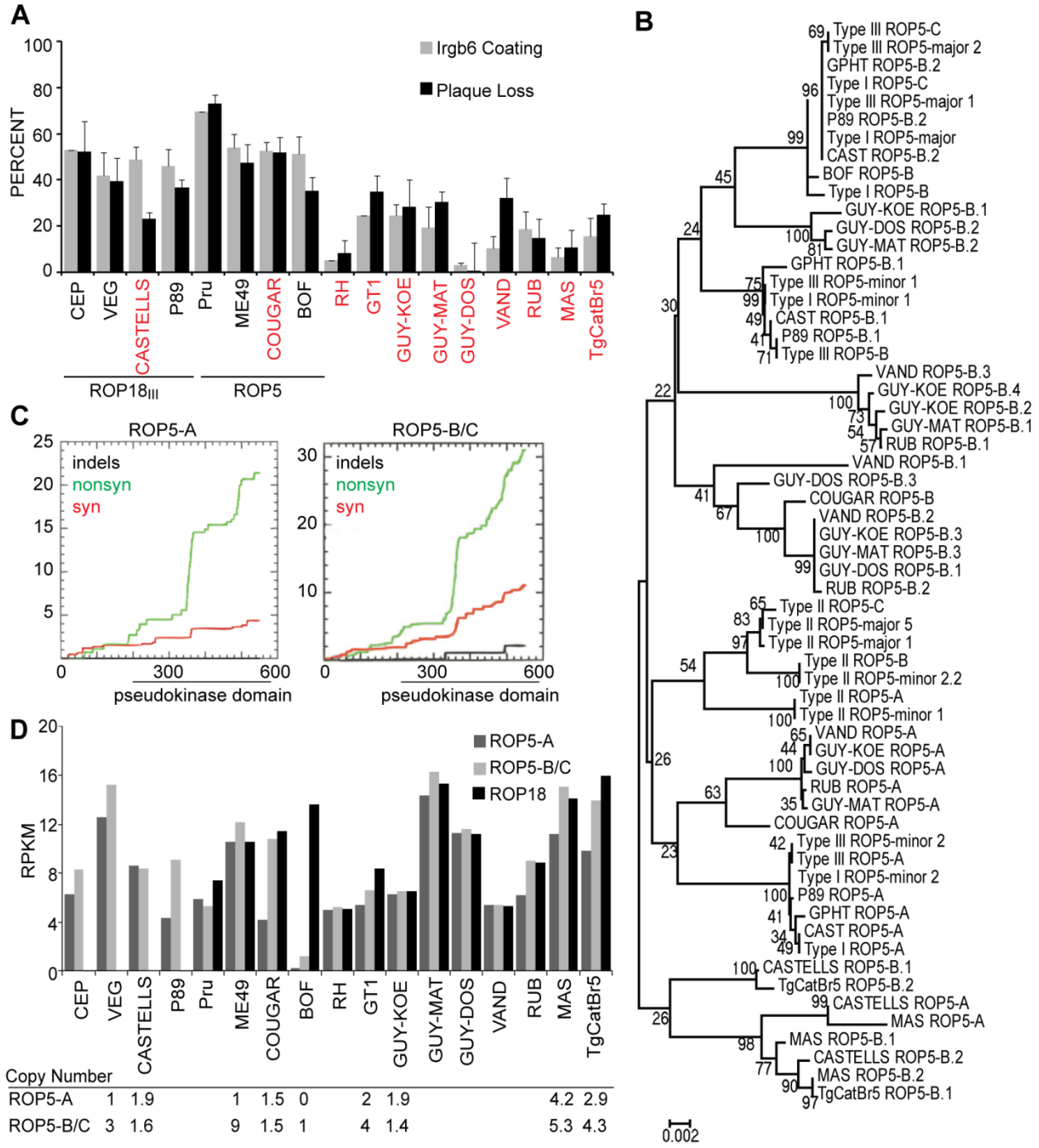


Figure 3: ROP5-A, ROP5-BC and ROP18 account for strain differences in IRG evasion in non-canonical strains

(A) Quantification of percent PVs with Irgb6 localization and percent plaque loss on IFNγ-stimulated MEFs compared to unstimulated MEFs infected with the indicated strains with strains having greater than 90% mortality in CD-1 outbred mice (Khan and others 2009) in red. The predicted reason for high IRG coating is indicated below the graph, which for CEP, VEG, CASTELLS and P89 is the unexpressed $ROP18_{III}$ allele, and for Pru, ME49, COUGAR and BOF is a divergent ROP5 allele. **(B)** Phylogenetic tree of *ROP5-A,B/C* and previously reported major and minor alleles (Behnke and others 2011) constructed from full-length coding nucleotide

sequences using Neighbor-Joining with 1000 bootstraps. **(C)** Cumulative behavior, codon by codon, of synonymous (red), nonsynonymous (green) and insertion/deletion (black) mutations in ROP5-A (left) and ROP5-B/C (right). **(D)** Relative expression of ROP5-A, ROP5-B/C and ROP18 determined by RNA-Seq of murine BMDM infected for 24 hrs with the indicated strains with similar levels of *Toxoplasma* RNA. ROP5-A and B/C copy number estimated by sequencing coverage of the *ROP5* locus versus the average genome coverage for strains for which the genome has been sequenced is indicated in the table below the graph.

Sequence chromatograms indicated that two or more alleles were present for the second *ROP5* gene. We therefore cloned the PCR product from this *ROP5* gene and sequenced multiple clones to obtain sequences from the different alleles, but some alleles may still be missing. Sequences from this expanded paralog matched what has previously been called both *ROP5-B* (minor) and *C* (major) genes (Figure 3B) (Behnke and others 2011; Reese and others 2011). We could not differentiate *B* and *C* alleles for all strains if they were not similar to the canonical strains, so we refer to them here as *B* copies. We determined that besides the three major *ROP5* copies that were previously described, 2 other highly divergent *ROP5* isoforms exist that we call *ROP5L-A* and *ROP5L-B* (Supplemental Figure 3). Interestingly, *ROP5L-A* and *ROP5L-B* are highly conserved between strains, but we find that these isoforms are not expressed in tachyzoites (Supplemental Figure 3) so they will not be discussed further. The previously described *ROP5* genes (*A, B and C*) (Reese and others 2011) are highly divergent with strong evidence for diversifying selection, especially in surface exposed residues (Reese and Boothroyd 2011) in the kinase domain (Figure 3C).

In general, for both *ROP5-A* and *BC*, alleles can be divided into groups with BOF, P89, CAST and GPHT grouping with the virulent I and III alleles (Figure 3B). A second allelic group consists of VAND, RUB, GUY-KOE, GUY-DOS and GUY-MAT. The ability to confer virulence of this allele group is unknown but because these strains are all highly virulent (Khan and others 2009) and able to evade the IRGs, these alleles are likely virulent. A third very

divergent group of alleles contains MAS, CASTELLS and TgCatBr5, but there is less diversity in the ROP5-A, B and C isoforms present in these strains. The COUGAR allele is most similar to but divergent from the second group, but interestingly, COUGAR has only one B/C allele. The avirulent *ROP5* locus from type II is also divergent, and a phylogenetic analysis of all *ROP5* alleles indicates that the type II *ROP5-B* and *C* genes are more closely related to *ROP5-A* than to *ROP5-B* or *C* of the other strains. These results suggest that ROP5-B and/or C could be important for IRG evasion and virulence since type II strains and COUGAR have high levels of IRG coating (Figure 3A) and seem to have either ROP5 alleles that are all ROP5-A-like (type II) or are missing ROP5-C (COUGAR) (Figure 3B).

We next tested whether differences in ROP5 expression or copy number could account for strain differences in IRG evasion. For example, BOF has virulent *ROP18* and *ROP5* alleles but is highly coated by Irgb6 (Figure 3A-B). To estimate copy number differences between the strains we have sequenced, we plotted the sequencing coverage of the *ROP5* locus versus the average genome coverage, as this was previously shown to be a good estimate for copy number (Yoon and others 2009). Most of the strains had about twice as many reads at *ROP5-A* and *B* as the rest of the genome, while MAS and TgCatBr5 have 3-5 copies of each gene (Figure 3D). However, coincident with our inability to amplify *ROP5-A*, we found that BOF is missing *ROP5-A* and has only one copy of *ROP5-B*.

We also looked at ROP5 expression levels determined using RNA-Seq data from 24 hour infections of murine bone marrow derived macrophages with each strain (Figure 3D). BOF has barely detectable expression of *ROP5-B* and no expression of *ROP5-A*, likely explaining its high Irgb6 coating despite virulent alleles of both *ROP18* and *ROP5*. Indeed BOF+LC37 has virtually no Irgb6 coating (0.33%) compared to BOF (40% Irgb6 coating, P= 0.001) (Figure 4A). *ROP5*

expression levels can also likely explain many intra-haplogroup strain differences where alleles of *ROP18* and *ROP5* are the same; for example, VEG has higher ROP5 expression levels compared to the other type III strain CEP, and VEG has slightly reduced IRG coating compared to CEP. Thus, higher ROP5 expression is correlated with reduced IRG coating, suggesting a non-enzymatic, dose-dependent role for ROP5 in IRG evasion.

ROP5-C complements IRG evasion in type II

Because the LC37 cosmid that reduced Irgb6 coating and plaque loss in S22 and BOF contains *ROP5-A, B* and *C* it is unknown which of these isoforms (or combination) is important for IRG evasion. However, the fact that type II *ROP5*s are less divergent and more similar to *ROP5-A* suggests type II is missing *ROP5-B* and *C*. Additionally, *ROP5-C* was previously described as the major allele with *A* and *B* as minor alleles when trace reads were assembled for the ROP5 coding region of types I, II and III (Behnke and others 2011). Therefore, we tested if *ROP5-*A_{III}*, ROP5-*C_{III} or LC37, which contains the entire *ROP5* locus, could complement IRG evasion in the type II background. Although some of the effects we see in the type II background will be due to an interaction with ROP18, because ROP18 is present in all backgrounds, we can still compare the effects of individual *ROP5* genes. We find, as expected, that expression of ROP5-A_{III} in the type II strain Pru led to only a slight but significant reduction in Irgb6 coating (51%, $P < 0.05$, One-way ANOVA), but expression of ROP5-C_{III} in Pru led to a significant reduction of IRG coating (36%, $P < 0.001$) similar to that of Pru + LC37 (38%, $P < 0.001$) compared to a heterologous control (62%) (Figure 4B). The 36% IRG coated vacuoles in Pru + ROP5-C_{III} is comparable to the 25% IRG coated vacuoles for GT1, suggesting that the lack of ROP5-C may account for the excessive IRG accumulation on type II vacuoles.

To see if ROP5-C_{III} can also increase the survival of type II parasites *in vivo*, we infected CD-1 mice with Pru, Pru + ROP5-A_{III}, Pru + ROP5-C_{III} or Pru + LC37. The growth and dissemination of Pru and Pru + LC37 was determined by *in vivo* imaging of luciferase activity. On the third day post infection, Pru + LC37-infected mice had twice the parasite burden of Pru-infected mice (Figure 4C and D). By day six, there was 50-fold higher luciferase activity in Pru + LC37 infected mice ($P < 0.0001$), and the parasites had disseminated throughout the peritoneal cavity. Indeed, 100% of Pru + LC37-infected mice died within 11 days of infection even at the lowest dose (Figure 4E). Mice infected with parasites expressing only ROP5-A_{III} or ROP5-C_{III} survived the infection (Figure 4E) but Pru + ROP5-C_{III}-infected mice had ruffled fur and lost significantly more weight (Figure 4F) than Pru-infected mice 15 days post infection ($P = 0.01$) while Pru + ROP5-A_{III}-infected mice continued to gain weight. Together, these results suggest that while expression of ROP5-C_{III} can reduce Irgb6 coating of type II parasites, ROP5-C_{III} only partially enhances the survival of type II parasites *in vivo* and the whole ROP5 locus is required to significantly increase virulence in mice.

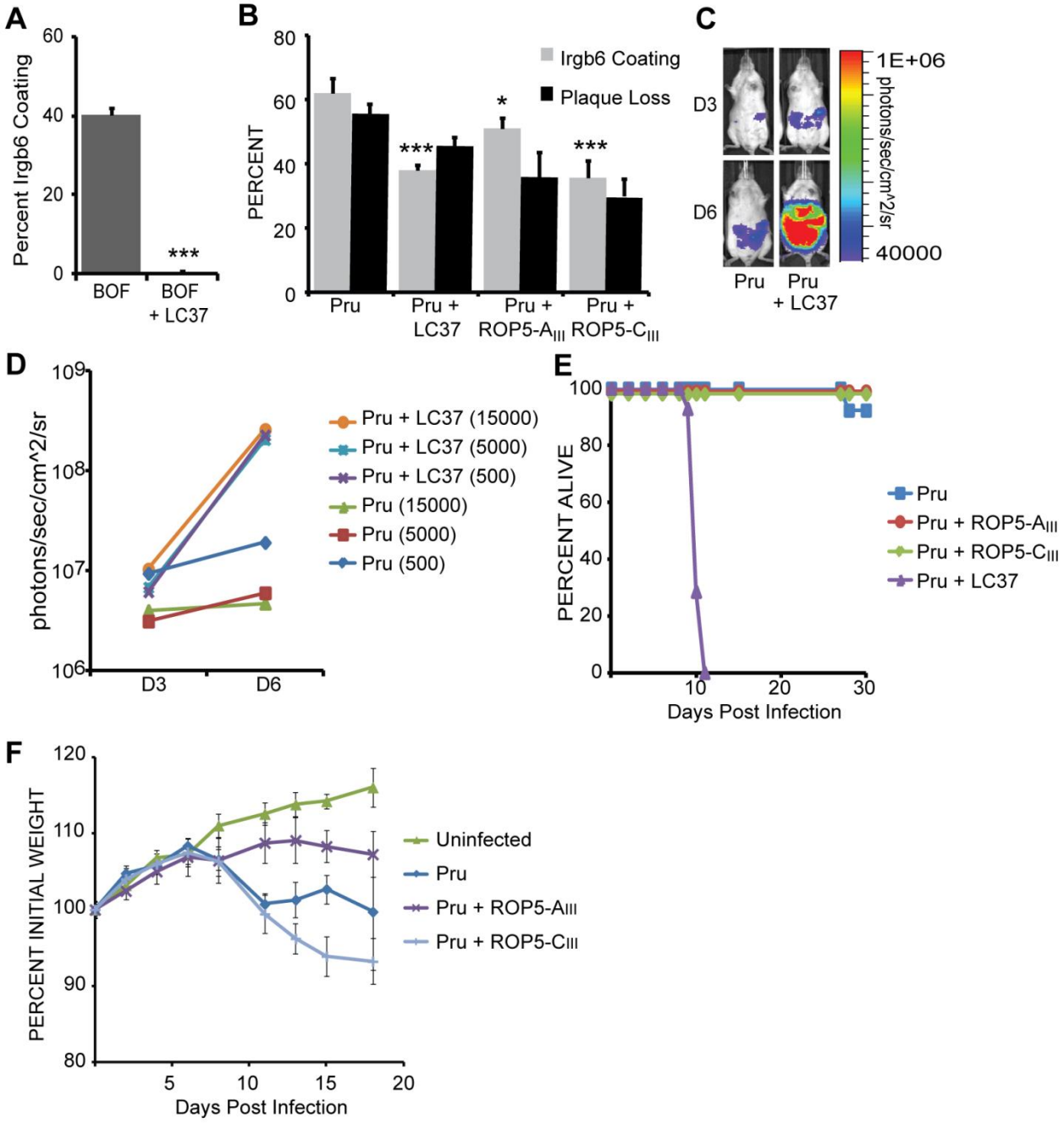


Figure 4: ROP5$_{III}$-C, but not ROP5$_{III}$A, inhibits IRG accumulation and increases mouse virulence

(A) Quantification of Irgb6 localization on the PV in IFNγ-stimulated MEFs infected with BOF and BOF + LC37. Mean ±SEM, n = 3 experiments, ***p < 0.001, Student's t-test. **(B)** Quantification of Irgb6 localization on the PV and percent plaque loss on IFNγ-stimulated MEFs compared to unstimulated MEFs infected with Pru, Pru + LC37, Pru+ROP5$_{III}$-A, and Pru+ROP5$_{III}$-C. Mean ±SEM, n > 4 experiments, *p < 0.05, ***p < 0.001 One-way ANOVA with Bonferoni correction for all pairwise comparisons. **(C)** *In vivo* imaging of mice infected with firefly luciferase expressing parasites of the indicated strains at days 3 and 6 post infection

with 5000 parasites. One representative of 5 infected mice per strain is shown. **(D)** Quantification of *in vivo* imaging shown as average photons/sec/cm^2 for infected mice at 3 doses on days 3 and 6. **(E)** Mouse survival of infection with indicated strains, n > 8 for each strain, combined results for doses 500, 5000 and 15000 (Pru and Pru + LC37 only). **(F)** Average percent change in weight over time for mice infected with the indicated strains, Mean +/- Std dev.

ROP5 does not interact with ROP18 and is not necessary for ROP18 kinase activity

It is not clear how ROP5 inhibits IRG accumulation at the PVM, but other pseudokinases have been shown to serve as protein scaffolds or to regulate the activity of kinases (Boudeau and others 2006). Since ROP18 requires ROP5 for fully efficient IRG evasion and there is an interactive effect of adding ROP18 and ROP5 to S22, it is possible that ROP5 and ROP18 interact directly. To test this hypothesis, we immunoprecipitated ROP5 and ROP18$_{II}$-HA from MEFs infected with CEP or CEP + ROP18$_{II}$-HA for one hour with or without previous IFNγ stimulation. We were unable to detect by western blot co-precipitation of ROP18 and ROP5 (Figure 5A). Furthermore, when recombinant tagged ROP18 kinase domain (Lim et al., submitted) is added to lysate from stimulated or unstimulated MEFs infected for one hour with Pru + ROP5-C$_{III}$HA prior to immunoprecipitation with anti-HA, we do not co-precipitate ROP18 (Supplemental Figure 5A) indicating that there is no direct interaction between ROP5-C$_{III}$ and the ROP18 kinase domain. Next we tested the hypothesis that ROP18 is only active in the presence of virulent *ROP5* alleles by immunoprecipitating ROP18$_{II}$-HA from MEFs infected with S22, S22 + ROP18$_{II}$HA, and S22 + LC37 + ROP18$_{II}$HA for one hour with or without previous IFNγ stimulation for use in an *in vitro* kinase assay. We found that there was no difference in the activity of ROP18, as measured by the phosphorylation of an optimized substrate (Lim et al., submitted) *in vitro*, immunoprecipitated from parasites with or without a virulent ROP5 (Figure 5B and Supplemental Figure 5B). This established that ROP18 was active

in all backgrounds and indicated that there are no irreversible effects of ROP5 on ROP18 kinase activity.

ROP5 directly interacts with and inhibits the oligomerization of Irga6

Because ROP5 does not directly interact with or irreversibly affect ROP18 kinase activity, we next tested the hypothesis that ROP5 directly interacts with one or more IRGs. We immunoprecipitated HA-tagged proteins from IFNγ stimulated or untreated MEFs infected with Pru, Pru + ROP5-A_{III}-HA, Pru + ROP5-C_{III}-HA, or RH + $GRA15_{II}$-HA for one hour lysed in the presence or absence of GTPγS. Co-precipitated proteins were separated by SDS-PAGE gel electrophoresis and identified by mass-spectrometry. We did not recover any ROP18 peptides, again indicating that ROP5 does not directly interact with ROP18. We did, however, recover 13 peptides (38% coverage) from Irga6 only in the Pru + ROP5-C_{III}-HA infected samples lysed in the presence of GTPγS (Figure 5C) suggesting a specific interaction between ROP5-C and Irga6 because the other HA-tagged, PVM associated proteins did not co-precipitate Irga6 under these conditions. Under different buffer conditions and in the absence of GTPγS, we also recovered 4 peptides of Irga6 and 2 peptides (9.8% coverage) of Irgb10 only in the Pru + ROP5-C_{III}-HA infected samples (data not shown). Because ROP5 lacks kinase activity (Reese and Boothroyd 2011) but reduces IRG localization to the PVM, we wondered if Irga6 binding by ROP5 could inhibit Irga6 oligomerization, which is necessary for its activity. To test this hypothesis, we measured the GTP-mediated oligomerization of recombinant Irga6 by dynamic light scattering in the presence of recombinant MBP-tagged ROP5 or MBP alone. We found the predicted hydrodynamic radius of Irga6 to be reduced in the presence of ROP5 but not MBP (Figure 5D). Thus, we find that ROP5-C_{III} binds and inhibits the oligomerization of at least one IRG.

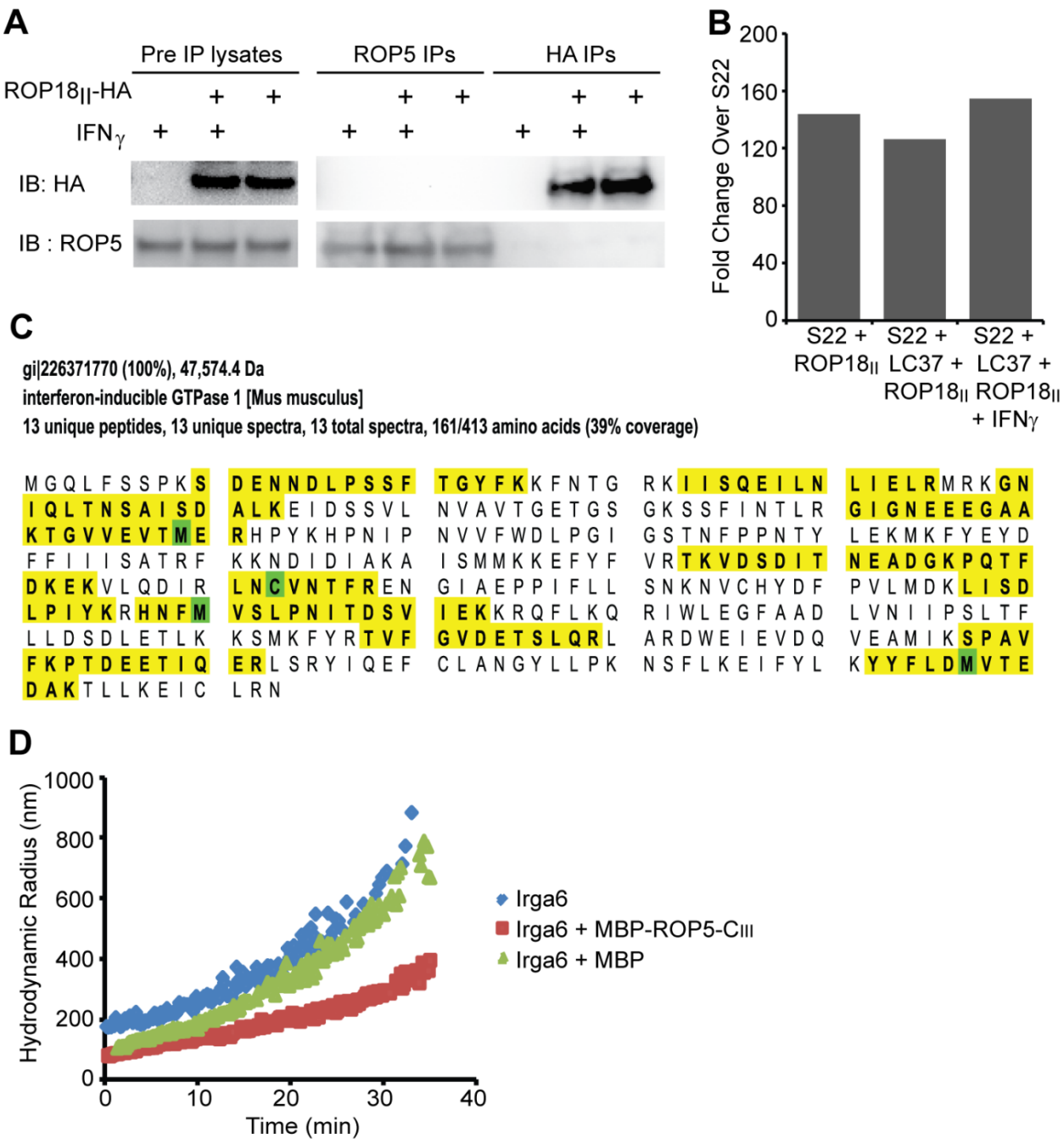


Figure 5: ROP5 interacts with Irga6, but not ROP18, and inhibits Irg6 oligomerization
(A) ROP5 and HA were immunoprecipitated from IFNγ-stimulated or unstimulated MEFs infected with CEP or CEP + ROP18$_{II}$-HA and inputs and precipitates were western blotted for ROP5 and HA. **(B)** Kinase activity of ROP18-HA immunoprecipitated from IFNγ-stimulated or unstimulated MEFs infected with S22, S22 + ROP18$_{I}$-HA or S22 + ROP18$_{I}$-HA + LC37 parasite strains. Half of the immunoprecipitated protein was western blotted with anti-HA antibodies and the relative amounts of ROP18-HA from each strain was quantified using the ImageQuant (GE Healthcare Life Sciences) software. The remaining immunoprecipitated proteins were incubated with ^{32}P-γ-ATP and a model peptide substrate (Lim, D., submitted) and spotted in quadruplicate

onto phospho-cellulose paper where the ^{32}P-γ-ATP incorporation was quantified by phosphorimage analysis. The kinase activity is expressed as a fold change over the S22 strain and normalized to the relative amounts of ROP18-HA that was immunoprecipitated. This experiment was performed twice and the graph represents the mean from those experiments. **(C)** Unique peptides and percent coverage of Irga6 recovered from mass spectrometry of proteins co-immunoprecipitated with ROP5-C_{III}-HA. Briefly, HA-immunoprecipitated proteins from IFNγ-stimulated or unstimulated MEFs infected with Pru, Pru + ROP5-A_{III}-HA, Pru + ROP5-C_{III}-HA or RH + $GRA15_{II}$-HA and lysed in the presence or absence of 0.5 mM GTPγS were separated by SDS-PAGE and analyzed by MS/MS. Irga6 peptides were recovered only in ROP5-C_{III}-HA samples lysed in the presence of GTPγS. **(D)** Oligomerization of 20 μM Irga6 with10 mM GTP at 37^{0}C in the presence of MBP-tagged ROP5-C or MBP shown as predicted mean hydrodynamic radius of the particle population determined by dynamic light scattering.

ROP16 and GRA15 do not affect IRG evasion

It was recently reported that p65 guanylate-binding proteins (GBPs), members of the dynamin superfamily that includes the IRGs, also accumulate on the *Toxoplasma* PVM alongside the IRGs (Virreira Winter and others 2011). Because ROP16 and GRA15 were shown to affect GBP coating, we were interested to see if ROP16 and GRA15 also affect IRG coating. We measured the effect on IRG coating and killing of ROP16 and GRA15 in types I, II and III genetic backgrounds. In a type I strain, the deletion of ROP16, the transgenic expression of $GRA15_{II}$, or both in combination did not significantly alter IRG coating or killing (Figure 6A and not shown). Likewise, type II Δ*gra15*, type II transgenically expressing $ROP16_I$, and type III transgenically expressing $GRA15_{II}$ showed no statistical differences in Irgb6 coating or plaque loss compared to their parental strains. Thus, while these genes may affect GBP coating, they do not significantly alter Irgb6 accumulation.

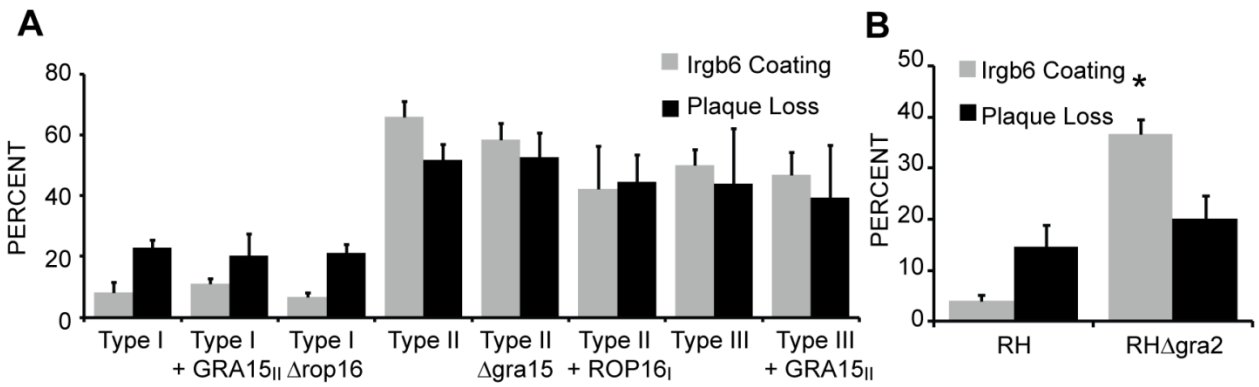


Figure 6: GRA2, but not ROP16 or GRA15, affects IRG evasion
WT MEFs stimulated for 24 hrs with IFNγ and infected with the indicated strains for 1 hr and fixed for immunofluorescence or allowed to form plaques for 4-7 days. **(A,B)** Quantification of Irgb6 localization on the parasite containing vacuole and percent plaque loss on stimulated MEFs compared to unstimulated MEFs infected with the indicated strains. Mean + SEM, n > 4 experiments. ***p < 0.001, Student's t-test.

PVM structure affects IRG accumulation

Not all of the F1 progeny in the I x II cross that have the type I ROP5 are as virulent as type I in mice (Behnke and others 2011) indicating that there are other genes besides *ROP5* and *ROP18* that affect virulence. While genetic location of the dense granule protein GRA2 has not been verified as a QTL affecting virulence, an RHΔ*gra2* strain is one of the few type I knockouts that have reduced mouse virulence (Mercier and others 1998). While the reason for this reduced virulence is unknown, it is known that GRA2 functions in the formation of the tubulovesicular network in the *Toxoplasma* PVM (Mercier and others 2002), which creates negative curvature in the PVM that might help to attract *Toxoplasma* proteins secreted into the host cell back to the PVM (Reese and Boothroyd 2009). Indeed it has been shown that the RHΔgra2 strain has reduced ROP18 localization to the tubulovesicular network in the *Toxoplasma* PVM (Reese and Boothroyd 2009). We therefore hypothesized that this GRA2-dependent ROP18 localization and/or the localization of other proteins, including ROP5, would be important for IRG evasion. Indeed, the RHΔgra2 strain has significantly increased IRG coating to 36% (P < 0.001) and

increased plaque loss on IFNγ-stimulated MEFs to 24% (P = 0.08) (Figure 6B). Therefore, we have shown that a protein required for the formation of the PVM structure also affects IRG accumulation.

Strain differences in survival in IFNγ-stimulated human foreskin fibroblasts

We wondered if there are strain differences in the survival of *Toxoplasma* in IFNγ-stimulated human cells since strain differences in virulence have been primarily studied in mice, and human cells lack the multitude of IRGs present in murine cells. We measured the percent plaque loss of different type I, II and III strains as well as of non-canonical strains in human foreskin fibroblasts (HFFs) stimulated for 24 hours with IFNγ (Figure 7A). In general, the percent plaque loss in IFNγ-stimulated HFFs is higher than in IFNγ-stimulated MEFs. The type I strains RH and GT1 have plaque losses of 54% and 63%, respectively, while the type II strains ME49 and Pru have plaque losses of 73% and 96%, respectively and the type III strains CEP and VEG have plaque losses of 90% and 67%, respectively. The non-canonical strains range in percent plaque loss from 47% (GUY-DOS) to 67% (CASTELLS). Thus, strain susceptibility to IFNγ-mediated killing in human cells does not correlate with that of murine cells.

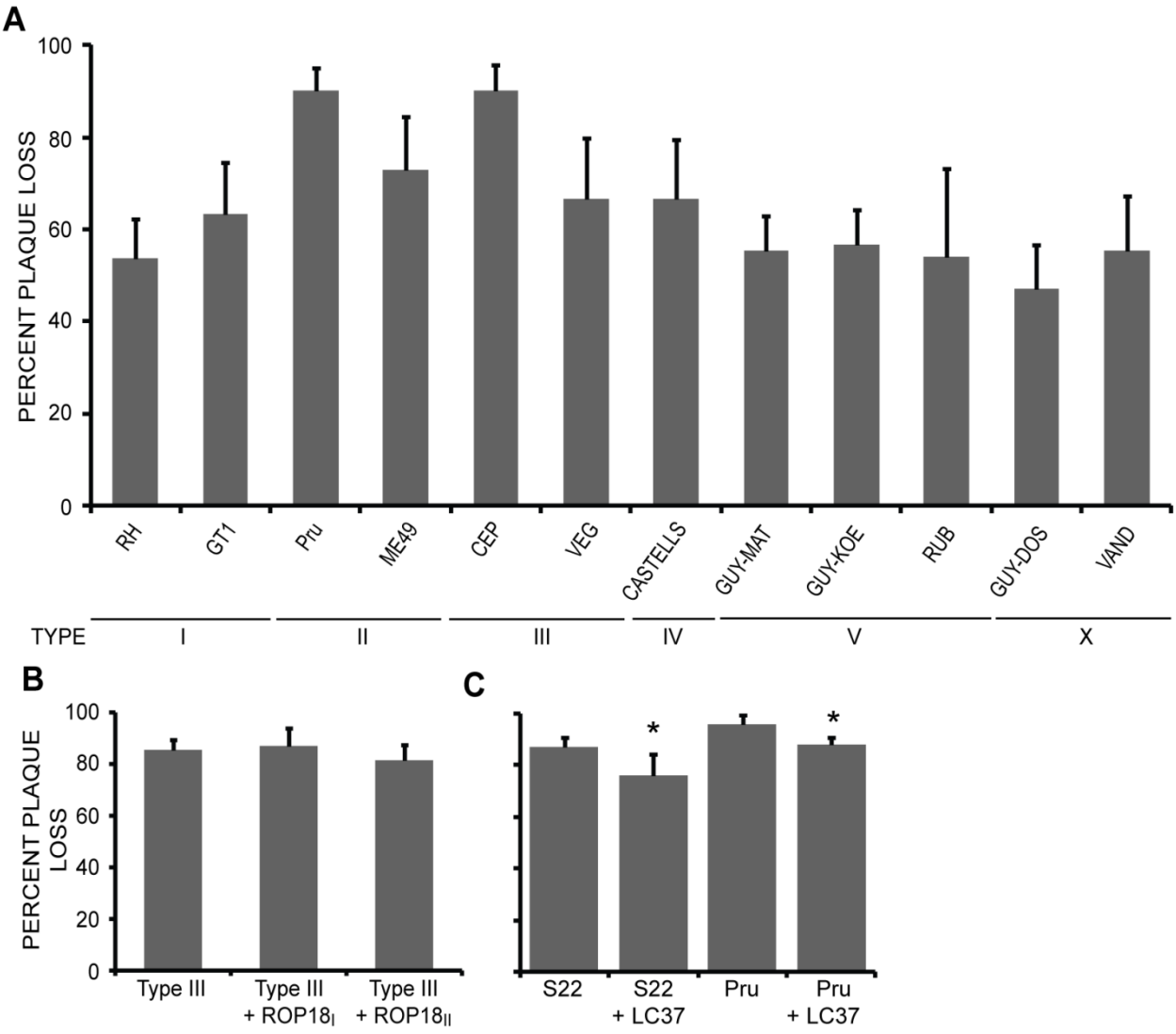


Figure 7: Strain differences in survival in IFNγ-stimulated human cells
Monolayers of HFFs, either previously stimulated for 24 hours with 100 U/ml of IFNγ or left untreated, were infected with 100-300 parasites. The number of plaques that form after 4-7 days of growth was determined. **(A)** Percent plaque loss of type I strains RH and GT1, type II strains ME49 and Pru, type III strains CEP and VEG, as well as the non-canonical strains CASTELLS, GUY MAT, GUY KOE, RUB, GUY DOS and VAND. Mean + Std. dev., $n \geq 3$ experiments. **(B)** Percent plaque loss for CEP, CEP + $ROP18_I$ and CEP + $ROP18_{II}$. Mean + Std. dev., $n \geq 5$ experiments. **(C)** Percent plaque loss of S22, S22 LC37, Pru and Pru + LC37. Mean + Std. dev, $n \geq 4$ experiments, * $p < 0.05$, Student's t-test.

ROP18 and ROP5 have a minimal effect on IFNγ-mediated killing in human foreskin fibroblasts

As we have shown, ROP18 and ROP5 are responsible for the majority of strain differences in IFNγ-susceptibility in murine cells, but *Toxoplasma* IFNγ-susceptibility in murine cells does not correlate with IFNγ-susceptibility in human cells. To test if ROP18 affects IFNγ-mediated killing in human cells, we first examined type III CEP strains transgenically expressing a virulent copy of ROP18. Neither $ROP18_I$ nor $ROP18_{II}$ expression in CEP decreases the percent plaque loss compared to the parental strain (Figure 7B), indicating that ROP18 is not responsible for strain differences in IFNγ-mediated killing in human cells. To see if ROP5 affects survival in IFNγ-activated human cells, we compared the percent plaque loss in IFNγ-stimulated HFFs between S22 and S22 + LC37 and between Pru and Pru + LC37. The percent plaque loss decreases from 87% for S22 to 76% for S22 + LC37 ($P = 0.03$) and from 96% in Pru to 88% for Pru + LC37 ($P = 0.01$) (Figure 7C). Although the differences in percent plaque loss due to expression of *ROP5* are significant, the differences are minimal (± 10%). Thus, virulent ROP18 and ROP5 are unable to decrease IFNγ-mediated killing in human cells.

Discussion

We report that the precise allelic combination of the *Toxoplasma* polymorphic *ROP18* and *ROP5* genes determines *Toxoplasma* strain differences in susceptibility to killing by IFNγ-stimulated MEFs, even for non-canonical strains. We also show that ROP18 and ROP5 function by inhibiting the accumulation of and subsequent killing by the IRGs. *Toxoplasma* strains also differ in their susceptibility to killing by IFNγ-stimulated HFFs, but this is not determined by ROP18 or ROP5.

Previous studies on the role of ROP18 in mediating strain differences in IRG accumulation on the PVM have produced inconsistent results. Initial studies of *in vivo* primed macrophages infected with the type III strain CTG expressing $ROP18_I$ and L929 cells expressing $ROP18_I$ infected with the type II strain ME49 showed minimal effects of ROP18 on Irgb6 and Irga6 coating (Khaminets and others 2010; Zhao and others 2009a). More recently, $ROP18_I$ was shown to phosphorylate a conserved threonine in the switch 1 loop of the GTPase domain of Irga6 and Irgb6 leading to their subsequent inactivation (Fentress and others 2010; Steinfeldt and others 2010). Here, we report that both $ROP18_I$ and $ROP18_{II}$ can prevent the accumulation of IRGs on the PVM but only when expressed in a genetic background that contains the virulent *ROP5* locus. The lack of virulent ROP5 in type II strains therefore likely explains why L929 expression of $ROP18_I$ did not affect IRG accumulation on type II vacuoles in those cells (Khaminets and others 2010).

Previously it was shown that the avirulent strain S22 transgenic for the cosmid LC37, containing *ROP5*, had slightly fewer Irgb6 coated vacuoles (~72%) than wild type S22 (87%) in IFNγ stimulated MEFs, but growth inhibition as measured by uracil uptake was not affected (Khaminets and others 2010). In contrast, we see a significant decrease in the percentage of vacuoles coated with Irgb6 and increased parasite survival when comparing S22 + LC37 with S22. This could be due to the concentration of IFNγ, the exact assay used or the genotype of the host cells used, as the IRGs are divergent between mouse strains. We find that LC37 also reduces Irgb6 coating and promotes parasite survival in Pru and BOF, and that ROP5-C can explain most of the reduction in IRG coating *in vitro*. However, Pru + LC37 was significantly more virulent in mice than Pru + ROP5-C_{III} suggesting the other *ROP5* genes may have additional roles in mouse virulence.

Currently, all *Toxoplasma* genes that determine strain differences in virulence were identified using pairwise crosses between type I, II and III. Because type I and III are progeny from a cross(es) between type II and a strain named alpha (similar to type VI) and beta (similar to type IX), respectively, these three strains are closely related to each other (Boyle and others 2006). In recent years it has become appreciated that in South America many other highly divergent strains exist and type I, II and III are rarely isolated. To date, no studies have been done to determine the virulence determinants of these strains. Here we report that for these strains the allelic combination and/or expression level of *ROP18* and *ROP5* also determine how well these strains evade the accumulation of the IRGs and their virulence in mice. Thus, surprisingly even though the North American/European and South American strains have diverged an estimated one million years ago (Khan and others 2007), they all use the same two genes to evade the murine IFNγ response. This suggests that evasion of host IRGs is crucial for *Toxoplasma*. However, it seems that the almost complete evasion of the IRGs of most strains would be an unsuccessful strategy to ensure transmission in mice as the host would be killed before infectious cysts are formed. This could mean that *ROP18/ROP5* allelic combinations of these highly virulent strains might have evolved to evade the IRGs of species that are more resistant to *Toxoplasma*, for example rats (Dubey 1996), and that mice are just an accidental host or it could be an artifact of the mouse lab strains commonly used. Strains such as the type II, type III, BOF (VI), P89 (IX) and CASTELLS (IV) that are either lacking (type II) or not expressing (BOF) virulent *ROP5* alleles or are not expressing *ROP18* (type III, P89 and CASTELLS) and are therefore less virulent in mice seem better adapted to cause chronic infections in mice. Indeed, the large majority of *Toxoplasma* isolates in North America and Europe belong to type II (Boothroyd and Grigg 2002).

ROP5 reduces IRG coating of the *Toxoplasma* PVM independently of ROP18 despite a lack of kinase activity (Reese and Boothroyd 2011). Many pseudokinases have been shown to act as scaffolds or regulators of active kinases (Boudeau and others 2006). We find that ROP5 is not necessary for ROP18 kinase activity *in vitro* nor did we find evidence for any direct interactions between ROP5 and ROP18 (Figure 5A). We find instead that ROP5 directly interacts with and inhibits the oligomerization of Irga6 (Figure 5C and D). Expression levels of ROP5 seem to correlate with the intra-haplotype differences in IRG coating between CTG and VEG, supporting a non-enzymatic, dose-dependent inhibition of the IRGs by ROP5. Importantly, both the IRGs and the ROP5 locus have expanded, perhaps due to an evolutionary arms race whereby each new host IRG gene required a new ROP5 gene so *Toxoplasma* could continue to evade IFNγ-mediated killing. Although ROP5 can inhibit IRG oligomerization, we see an interactive effect with ROP18. Perhaps the reduced oligomerization of IRGs in the presence of virulent *ROP5* alleles is reversible, but this de-oligomerization provides access for ROP18 to bind and phosphorylate the IRGs on the threonines in their switch I loop, to prevent re-activation. If this model is correct than the interaction of ROP18 with the IRGs (Fentress and others 2010; Steinfeldt and others 2010) should only occur in the presence of virulent ROP5 alleles.

To defend itself against the IRGs *Toxoplasma* must have evolved a mechanism to ensure appropriate trafficking of ROP18 and ROP5 to the PVM upon their secretion into the host cytoplasm. The N-terminal amphipathic helices (RAH domains) of both proteins were shown to be needed for efficient localization to the PVM, and it was speculated that their specificity for the PVM versus other membranes might be because of a preference for negative curvature (Reese and Boothroyd 2009). Indeed, we found that RHΔ*gra2* parasites that have a disrupted

tubulovesicular network (Mercier and others 2002) which provides much of the negative curvature of the PVM, have increased IRG accumulation. This indicates that the attraction of ROP5, ROP18 and possibly other secreted proteins to the PVM, which is attenuated in RHΔ*gra2* (Reese and Boothroyd 2009), outweighs the possible attraction the IRGs may have for the negative curvature of the PVM (Tiwari and others 2009). It is likely that the increased IRG accumulation on the PVM of RHΔ*gra2* accounts for its decrease in virulence (Mercier and others 1998).

Because all *Toxoplasma* strains seem to rely on ROP5 and ROP18 for evasion of the murine IFNγ response, these proteins could be an attractive drug target if they are also involved in evasion of the human IFNγ response. However, we find that although there are significant strain differences in susceptibility to IFNγ-mediated killing by HFFs, ROP5 and ROP18 do not markedly affect survival in those cells. This might not be surprising because humans do not possess the large variety of IRGs of murine cells (23 members) but only a single member (IRGM) that is not regulated by IFNγ (Bekpen and others 2005). The effector mechanisms induced by IFNγ in human cells that are effective against *Toxoplasma* include tryptophan degradation (Pfefferkorn 1984), $P2X_7$-mediated death of the host cell (Lees and others 2010) and activation of the NALP1 inflammasome (Witola and others 2011). While the IRGs do not mediate vacuolar destruction in human cells, we wondered if another group of dynamin-related large GTPase, the GBPs, could be involved in IFNγ-mediated killing by HFFs, but we failed to see GBP1 at the PVM in HFFs (data not shown).

The *Toxoplasma* strains that were most resistant to IFNγ-mediated killing by HFFs have also been shown to be able to cause severe disease even in immunocompetent humans. In future

studies, strain differences in survival in IFNγ-activated HFFs may provide insight into that mechanism.

Materials and Methods

Reagents

A rat monoclonal antibody against HA (3F10, Roche, 1:500 dilution), a goat polyclonal antibody against mouse TGTP (A-20, Santa Cruz Biotechnology, 1:100 dilution), a mouse monoclonal antibody against *Toxoplasma* surface antigen (SAG)-1 (DG52) (Burg and others 1988), a mouse monoclonal antibody against *Toxoplasma* dense granule protein (GRA) 7 (Rome and others 2008), and a mouse polyclonal antibody against the N-terminus of ROP5 (El Hajj and others 2007) were used in immunofluorescence assays or immunoprecipitations. Secondary antibodies were coupled with Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes). Mouse IFNγ from Peprotech and human IFNγ from AbD serotec were dissolved in DMEM with 10% FBS.

Parasites and Cells

Parasites were maintained *in vitro* by serial passage on monolayers of human foreskin fibroblasts (HFFs) at 37°C in 5% CO2. The following representatives for each haplotype were used: RH and GT1 for type I, ME49 and Pru for type II, CEP and VEG for type III, MAS and CASTELLS for type IV, GUY-KOE and GUY-MAT for type V, GPHT and BOF for type VI, CAST for type VII, TgCatBr5 for type VIII, P89 for type IX, GUY-DOS and VAND for type X and COUGAR for type XI. A Pru strain engineered to express firefly luciferase and GFP (PruΔHXGPRT A7) (Kim and others 2007), a CEP and RH strain engineered to express clickbeetle luciferase and GFP (CEPΔHXGPRT$^-$ C22 and RH 1-1) (Boyle and others 2007), CEP + $ROP18_{II}$, Pru + $ROP16_I$ (Saeij and others 2006), RHΔGRA2 (Mercier and others 1998), RHΔROP16 (Ong and

others 2010), RH + $GRA15_{II}$ and CEP + $GRA15_{II}$ (Rosowski and others 2011) have been described previously. HFFs were grown as described previously (Rosowski and others 2011). WT C57BL6/J MEFs were a gift from A. Sinai (University of Kentucky College of Medicine, Lexington, KY), Atg7+/- and Atg7-/- MEFs (Komatsu and others 2005) from Masaaki Komatsu (The Tokyo Metropolitan Institute Medical Science) and all MEFs were grown in HFF media supplemented with 10 mM Hepes. All parasite strains and cell lines were routinely checked for Mycoplasma contamination and it was never detected.

Immunofluorescence assays

Parasites were allowed to invade monolayers of MEF cells grown on coverslips and incubated for 24 hours with or without 1000 U IFNγ. After 20 minutes, non-invading parasites were washed away with PBS 3 times, and the infection proceeded for 1 hour. The cells were then fixed with 3% (v/v) formaldehyde in PBS for 20 minutes at room temperature, permeabilized with 0.2% saponin and blocked in PBS with 3% (w/v) BSA and 5% (v/v) FBS. Percent Irgb6 coating was determined in a blind fashion by finding intracellular parasites and then scoring Irgb6 coating as positive or negative.

Characterization of ROP5 sequences

The coding sequence for ROP5 from types I (GT1), II (ME49), and III (VEG) was predicted from ToxoDB genomic sequence using ORF Finder (NCBI). ROP5 genomic DNA from additional strains was amplified by PCR with isoform specific primers confirmed by sequence chromatograms. *ROP5* was amplified with the following primers:

forward 5'CGATTCACGCTTTCCATGT'3, reverse 5'TCCTTCAGCGGAAAACAGAT'3 for ROP5-A, forward 5'CATTTCATGCCTTCCCAGTT'3, reverse 5'GCGCTCGAGTACTTGTCCTG'3 for ROP5-B/C, forward

5’GTCCCTGGAAAACTGTTTCG’3, reverse 5’GTGAACAGAGAGCGTCCAA’3 for ROP5-D, forward 5’ATTCTGCAATGCCCAAAAGA’3, reverse 5’TTCATGTTGGATACGGCAAC’3 for ROP5-E and 5’AAAAGGCGCGGCGAGCTAGCGTC’3 as an alternate forward primer for ROP5-A for MAS and CASTELLS.

The ROP5-B/C PCR products produced mixed sequence and therefore the PCR product was cloned and multiple clones were sequenced.

The following primers were used to sequence ROP5-A and ROP5-B

5’ATAGGTAACCGGGACGCTTG’3, 5’CCACTTCGGAAGAGACTTGC’3, 5’GGACAGACGCAGGCTTTTAC’3

The following primers were used to sequence ROP5-D and ROP5-E

5’TGAGCTGAAAACCGACTTCAC’3, 5’GGTGACTGGAACACTCGACA’3, 5’TTTTCCGGACCTTGTCTTTG’3, 5’TTCGGGAGAGACTTGCTCAG’3, 5’GCTGTGACAGTTCCGACTCA’3

Sequences were aligned using ClustalX and Neighbor-Joining phylogenetic trees were made with Molecular Evolutionary Genetic Analysis (MEGA) software version 4.1 with 1000 bootstraps and default settings (Kumar and others 2001). The Non-synonymous Analysis Program (SNAP) was used to calculate the proportion of synonymous and non-synonymous changes in coding regions (Korber 2000).

Generation of transgenic parasites

The coding region and putative promoter *(766* bp upstream of the start codon for *ROP5-A* and 681 bp upstream for *ROP5-B)* of ROP5-A and ROP5-B was amplified from type III *Toxoplasma* genomic DNA by PCR *(A forward, 5′*-CCACGCATTCTTCCACTCAGTACCG-*3′; B forward, 5′*-CCACAATGGCTACCAGGTCCTGCG-*3′; A/B reverse, 5′-*

CTA*CGCGTAGTCCGGGACGTCGTA*CGGGTAAGCGACTGAGGGCGC-*3')*. The coding region of ROP18, along with putative promoter (742 bp upstream of the ATG start codon), from type I *Toxoplasma* genomic DNA was amplified by PCR. (Forward 5'-CACCAGATTCGAAACGCGGAAGTA-3`; Reverse 5`-TTACGCGTAGTCCGGGACGTCGTACGGGTATTCTGTGTGGAGATGTTCCTGCTGTTC -3`). These primers amplified these genes specifically as confirmed by sequencing and the sequence matched the previously published data (Reese and others 2011; Behnke and others 2011). Sequence coding for an HA tag was included in the reverse primer (denoted with italics) to C-terminally tag the protein. ROP5-A_{III}HA, ROP5-C_{III}HA and $ROP18_{I}$ were then cloned into pENTR/D-TOPO (Invitrogen), and then cloned into pTKO-att (described in (Rosowski and others 2011) by LR recombination (Invitrogen). The pTKO-att-$ROP5_{III}$HA vectors were then linearized by digestion with HindIII (NEB) which does not cut either gene. Linearized vector was transfected into PruΔHXGPRT parasites by electroporation as described previously (Rosowksi and others 2011). The pTKO-att-ROP18 vector was linearized by digestion with NdeI (NEB) and transfected into CEPΔHXGPRT^{-} C22 parasites by electroporation. Stable integrants were selected in media with 25 mg/ml mycophenolic acid (Axxora) and 25 mg/ml xanthine (Alfa Aesar) and cloned by limiting dilution. To express $ROP18_{II}$ in the S22 and S22 LC37 parasite strains, 35 µg of pTKO-att-$ROP18_{II}$HA (Saeij and others 2006) was linearized by HindIII (NEB) and 1 µg of pTUB-CAT were co-transfected by electroporation. Stable integrants were selected by passage of 10^6 parasites every 2 days in 2 µM chloramphenicol. Expression of ROP18 and $ROP5_{III}$ was confirmed by IF and Western blot for HA staining (Supplemental Figure 6A-D). The LC37 cosmid was expressed in PruΔHXGPRT A7 and BOF by transfecting 50 µg and selecting twice extracellularly for 1.5 hours with 5 µg/ml phleomycin. Insertion was

confirmed by PCR with the Type I ROP5 specific forward primer (5'-TTTTCCGCAGGCCGTGGC-3') and ROP5A/B reverse for Pru and amplification of ROP5-A for BOF.

Plaque Assay

For the plaque assay, 100-300 parasites per well were added to monolayers of MEFs seeded the day before or HFFs seeded two days before and either previously stimulated with 1000 U mouse IFNγ, 100 U human IFNγ or left unstimulated for 24 hours before infection in a 24 well plate in either MEF media or DMEM with 1% FBS for HFFs. Infections were then incubated for 4-7 days at 37°C and the number of plaques was counted using a microscope.

In vivo *imaging analysis.*

CD-1 mice were intraperitoneally (i.p.) infected with 500 or 5000 syringe-lysed tachyzoites in 300 µl PBS using a 28 gauge needle. On days 3, 6 and 12 post infection, parasite burden and dissemination was measured by bioluminescence emission imaging. Mice were injected i.p. with 3 mg firefly D-luciferin (Gold Biotechnology) dissolved in PBS, anesthetized with isofluorane, and imaged with an IVIS Spectrum-bioluminescent and fluorescent imaging system (Xenogen Corporation). Images were processed and analyzed with Living Image software. The MIT Committee on Animal Care approved all protocols. All mice were maintained in specific pathogen-free conditions, in accordance with institutional and federal regulations.

High-throughput genomic and RNA sequencing:

For genomic sequencing, DNA was isolated from freshly lysed *Toxoplasma* parasites using a Trizol-based extraction (Invitrogen). This DNA was subsequently prepared for high-throughput sequencing according to the Illumina single-end genomic DNA kit protocol (COUGAR, CASTELLS and MAS) and 36 nucleotides of each library was sequenced on an Illumina GAII

and processed using the standard Illumina pipeline. Paired-end sequencing Illumina libraries were constructed for the genomic DNA of P89, GUY-KOE, TgCatBr5, BOF using the Nextera Illumina compatible DNA sample prep kit (Epicenter) and amplified with the modified PCR protocols described previously (Aird and others 2011). Sequence reads were aligned to the *Toxoplasma* and human genomes using the Maq software package (Li and others 2008). Reference *Toxoplasma* genomes from a type II (Me49) a type I (GT1) and a type III (VEG) strain were obtained from http://toxodb.org (release 6.3). For RNA sequencing, murine bone-marrow derived macrophages (BMDM) were seeded in 6 well plates at 70% confluency and infected with different strains of *Toxoplasma* at three multiplicity of infections (MOIs): 15, 10 and 7.5. After 24 hrs total RNA was extracted from all infected cells using the Qiagen RNeasy Plus kit. Integrity, size and concentration of RNA was then checked using the Agilent 2100 Bioanalyser. The RNA was processed for high-throughput sequencing according to standard Illumina protocols. Briefly, after mRNA pull down from total RNA using Dynabeads mRNA Purification Kit (Invitrogen), mRNA was fragmented into 200-400 base pair-long fragments and reverse transcribed to into cDNA, before Illumina sequencing adapters were added to each end. Libraries were barcoded and subject to paired end sequencing on the Illumina HiSeq2000 (40+40 nucleotides) and processed using the standard Illumina pipeline. All libraries were spiked with trace amounts of the phiX bacteriophage for quality control purposes. After sequencing, the samples were de-barcoded to separate reads from the multiplexed samples using a custom Perl script. Reads were assembled into full sequences by mapping to exons and across exon junctions using the organism's genomes as a template. Maq was used to estimate *Toxoplasma* transcript abundance for ROP5 and ROP18 based on our sequenced alleles. A more detailed analysis of the genome and RNA-seq data will be described elsewhere.

Immunoprecipitations, western blotting and kinase assays

Immunoprecipitations were each performed with 5 μg of rat anti-HA antibodies (3F10, Roche) or mouse anti-ROP5 (El Hajj and others 2007) conjugated to 25 μl of protein G dynabead slurry (Life Technologies). The HA antibodies were crosslinked at room temperature with 5mM Bis(Sulfosuccinimidyl) suberate (*BS3*) (Pierce) prepared in conjugation buffer (20 mM sodium phosophate, 150 mM sodium chloride, pH 7.5) for 30 minutes and quenched by adding 50 mM Tris-Cl, pH 7.5 for 15 minutes and finally washed with conjugation buffer. For each immunoprecipitation (IP) condition, 4.2 x 10^6 MEFs were infected at an MOI of ~5-10, with the strain and condition indicated. After 1 hpi, uninvaded parasites were washed away with PBS and the infected cells were treated with 0.25% trypsin for 5 minutes at 37 °C. The cells were quenched and harvested with growth media and subsequently washed with PBS + 1 mM PMSF and lysed for 15 minutes at 4 °C with light agitation in 1 ml of IP lysis buffer [50 mM HEPES-KOH (pH 7.5), 300 mM NaCl, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM $NaVO_4$, 1 mM PMSF, 1% NP-40, and protease inhibitor cocktail (Roche)]. The lysate was then centrifuged at 16,000g for 30 minutes at 4 °C and the supernatant was collected. For ROP18 binding assays, 1 μg of ROP18 recombinant kinase domain[residues 187-554, fused to a series of N-terminal fusion tags consisting of: (His_6)-(glutathione S-transferase)-(maltose binding protein)-(*Streptococcus* protein B1 domain)-(TEV cleavage site), (Lim, D et al., submitted)] were incubated for 30 minutes before adding conjugated and crosslinked antibody beads described above and agitating them for 3 hours at 4 °C. The beads were washed 3x with IP wash buffer [10 mM HEPES-KOH (pH 7.5), 150 mM NaCl, 20 mM β-glycerophosphate, and 0.5% IGEPAL], washed 3x with Hepes-buffered saline (HBS) and boiled in sample buffer. The samples were

western blotted with anti-GST HRP conjugate (GE Healthcare Life Sciences) and anti-HA (3F10, Roche) antibodies.

Immunoprecipitations for kinase assays were performed as above but with several changes. The cleared lysates were incubated with 10 μg of rat anti-HA antibodies (3F10, Roche) per IP reaction and incubated for 90 minutes, washed 5x with the IP lysis buffer and 3x with IP wash buffers and HBS (all buffers contained 300 mM NaCl). Half of the beads were boiled in sample buffer for western blotting with ant-HA antibodies and the other half used for the kinase assay. Kinase assays using a ROP18 model peptide substrate (NH_3-KKKKKWISEHTRYFF-$CONH_2$) (Lim, D. et al., submitted) were conducted at room temperature with a reaction buffer consisting of 50 mM HEPES pH 7.5, 300 mM NaCl, 10 mM DTT, 10 mM $MgSO_4$ and 60 μM cold ATP. Each reaction contained 0.5 mM of peptide substrate and 2 to 10 μCi of ^{32}P-γ-ATP. Reactions were stopped after 30 minutes by spotting on Whatman P81 phospho-cellulose paper, which were then dried and washed with 0.425% phosphoric acid until no significant radioactivity remained in the washes. Radioactivity captured on P81 filters was then quantified by phosphorimage analysis with ImageQuant 5.2 software (Molecular Dynamics).

Mass Spectrometry

Immunoprecipitations were performed as above with a monolayer of confluent MEFs in a T175 lysed in the presence or absence of 0.5 mM GTPγS (Sigma) and precipitated using 30 μg of HA antibody. The washed beads were boiled in sample buffer and samples were subjected to SDS–PAGE and colloidal coomassie staining. For mass spectrometry analysis, proteins were excised from each lane of a coomassie stained SDS-PAGE gel encompassing the entire molecular weight range. Trypsin digested extracts were analyzed by reversed phase HPLC and a ThermoFisher LTQ linear ion trap mass spectrometer. Peptides were identified from the MS data using

SEQUEST algorithms44 that searched a species-specific database generated from NCBI's non-redundant (nr.fasta) database.

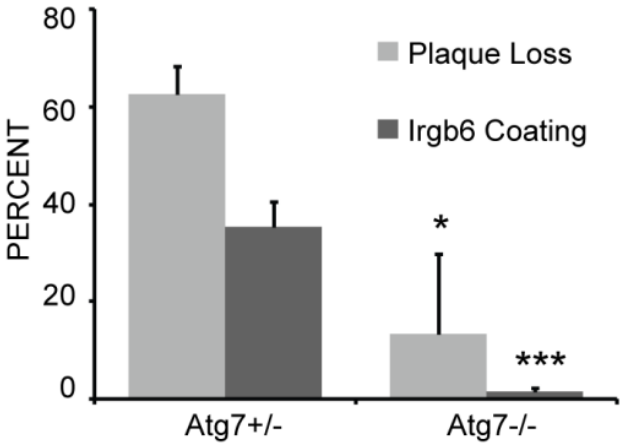
Dynamic Light Scattering

Recombinant Irga6 [residues 1-413, fused to a series of N-terminal fusion tags consisting of: (His_6)-(glutathione S-transferase)-(maltose binding protein)-(*Streptococcus* protein B1 domain)-(TEV cleavage site), (Lim et al, submitted)] oligomerization was monitored in 50 mM Tris/ 5 mM $MgCl_2$/ 2 mM DTT by dynamic light scattering (DLS). Oligomerization was initiated by the addition of 10 mM GTP (Sigma) to 20 μM Irga6 in the presence or absence of 40 μM recombinant MBP-tagged ROP5-C_{III} or MBP. The reaction was mixed by pipetting and immediately transferred to a quartz cuvette and equilibrated to 37^0C. DLS was performed using a DynaPro NanoStar Light Scatterer (Wyatt Technologies) with an acquisition time of 10 sec over 35 minutes and analyzed using the DYNAMICS software version 7.1.4. The mean hydrodynamic radius of the population was estimated using the standard curve of molecular weight for globular proteins and is not equal to the actual size of the oligomer.

Ethics Statement

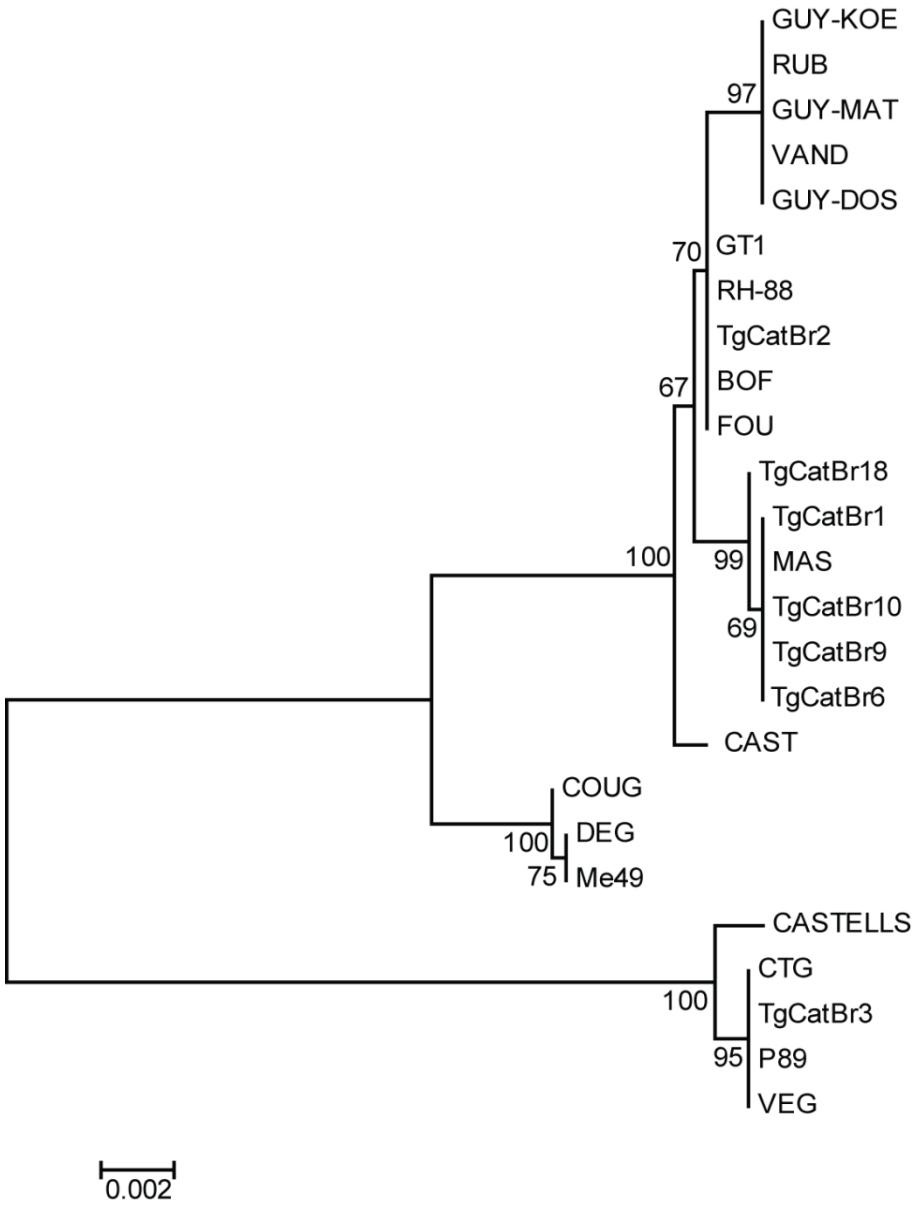
This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The MIT Committee on Animal Care (assurance number A-3125-01) approved all protocols. All mice were maintained in specific pathogen-free conditions, and all efforts were made to minimize suffering.

Supplemental Figures

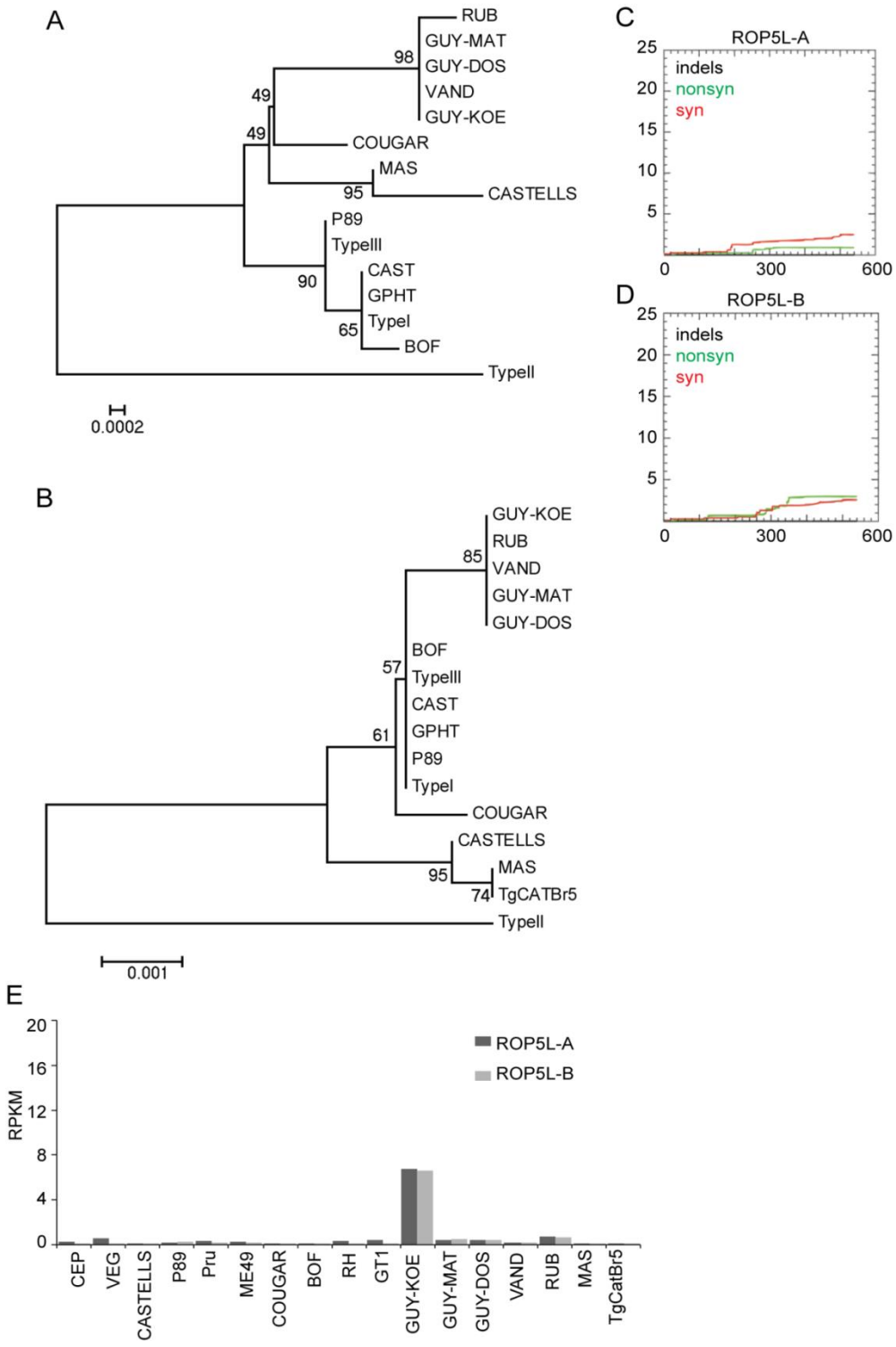


Supplemental Figure 1: IFNγ-induced plaque loss is reduced when the IRGs are mis-regulated

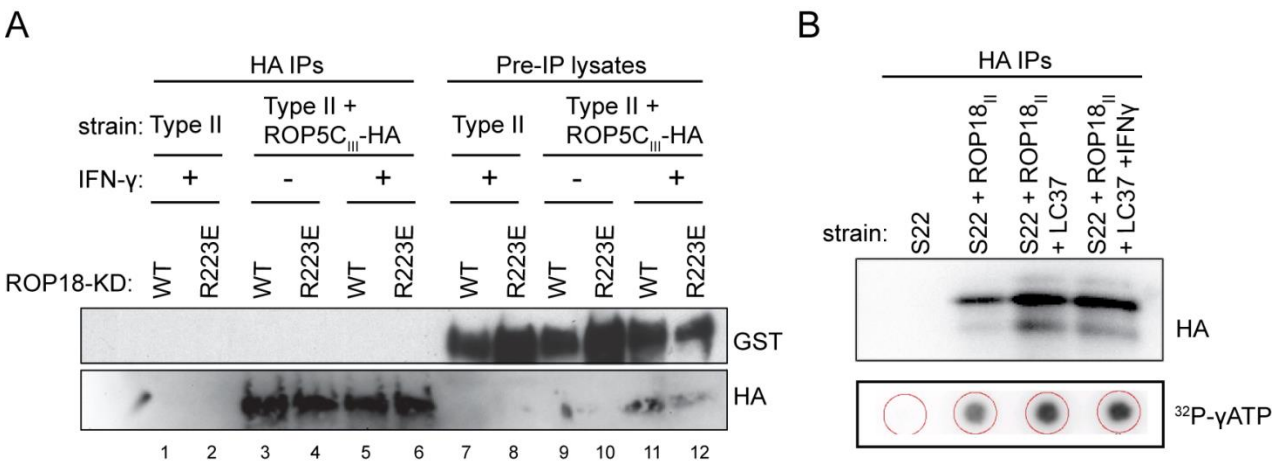
Monolayers of Atg7+/- and Atg7-/- MEFs were stimulated for 24 hrs with IFNγ and infected with type II (Pru) for 1 hr or allowed to form plaques for 7 days. Immunofluorescence of Irgb6 PV coating and percent plaque loss on stimulated compared to unstimulated MEFs. Mean + Std Error, n = 3 experiments, *P < 0.05, ***P < 0.001, Student's t-test.



Supplemental Figure 2: Phylogenetic analysis of ROP18
Phylogenetic analysis of coding nucleotide sequences by Neighbor Joining with 1000 bootstraps of ROP18 alleles (Khan and others 2009).

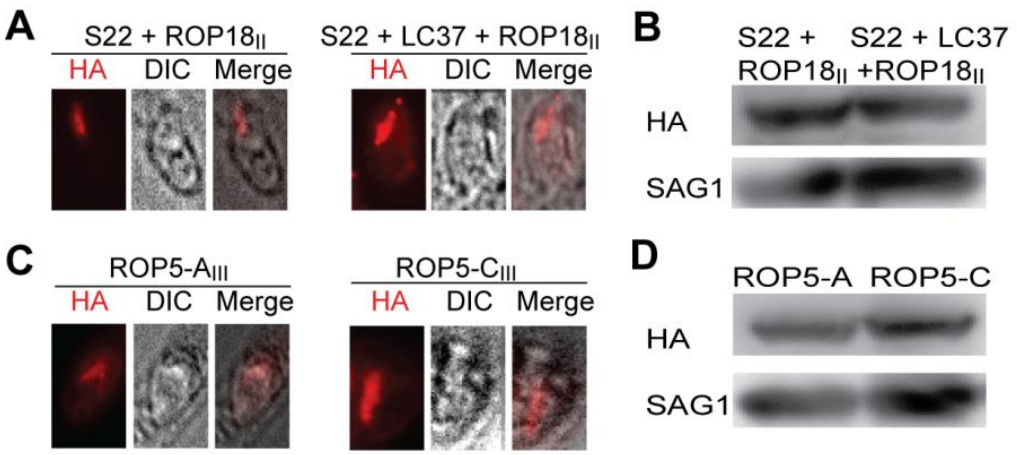


Supplemental Figure 3: Phylogenetic analysis and expression of ROP5L-A and B
(A) ROP5L-A and B **(B)** phylogenetic analysis of coding nucleotide sequences by Neighbor Joining and cumulative mutations codon by codon by type **(C and D** respectively**). (E)** Expression by RNA-Seq analysis of 24 hour infection with indicated strains in bone marrow derived macrophages.



Supplemental Figure 5: ROP5 does not directly interact with ROP18 and is not necessary for ROP18 kinase activity

(A) Wild-type (wt) or mutant R223E recombinant proteins comprising the kinase domain (KD) of ROP18$_I$ fused to MBP-GST were added to lysates prepared from IFNγ-stimulated and unstimulated MEFs infected with type II or type II + ROP5C$_{III}$-HA parasites and incubated for 30 minutes before immunoprecipitating the reactions with anti-HA antibodies. Both the immunoprecipitates (lanes 1-6) and pre-IP lysates (lanes 7-12) were Western blotted with anti-GST and anti-HA antibodies. **(B)** Kinase activity of ROP18-HA immunoprecipitated from IFNγ-stimulated or unstimulated MEFs infected with S22, S22 + ROP18$_I$-HA or S22 + ROP18$_I$-HA + LC37 parasite strains. Half of the immunoprecipitated protein was Western blotted with anti-HA antibodies (top). The remaining immunoprecipitated proteins were incubated with ^{32}P-γ-ATP and a model peptide substrate (Lim, D., submitted) and spotted in quadruplicate onto phospho-cellulose paper where the ^{32}P-γ-ATP incorporation was quantified by phosphorimage analysis (bottom). This experiment was performed twice and the graph represents the mean from those experiments.



Supplemental Figure 6: Expression and localization of transgenic ROP18 and ROP5
(A) Immunofluorescence of HA (red) in S22 + $ROP18_{II}$-HA and S22 + LC37 + $ROP18_{II}$-HA parasites as well as DIC and merged images **(B)** Western blot for HA (top) and SAG1 (bottom) comparing expression of S22 + $ROP18_{II}$-HA and S22 + LC37 + $ROP18_{II}$-HA strains used for mouse infections. **(C)** Immunofluorescence of HA (red) in Pru + ROP5-A_{III}-HA and Pru + ROP5-C_{III}-HA parasites as well as DIC and merged images. **(D)** Western blot for HA (top) and SAG1 (bottom) comparing expression of Pru + ROP5-A_{III}-HA and Pru + ROP5-C_{III}-HA strains used for mouse infections.

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Chapter Three:

Cell death of interferon-gamma stimulated human fibroblasts upon *Toxoplasma gondii* infection induces early parasite egress and limits parasite replication

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Under review in Infection and Immunity

Joris helped design and implement preliminary experiments that led to this research, but all data presented here was produced and analyzed by W. Niedelman.

Abstract

The intracellular protozoan parasite *Toxoplasma gondii* is a major foodborne illness and opportunistic infection for the immunosuppressed. Resistance to *Toxoplasma* is dependent on interferon-γ (IFNγ) activation of both hematopoietic and non-hematopoietic cells. While IFNγ-induced innate immunity in non-hematopoietic cells has been extensively studied in mice, it remains unclear what resistance mechanisms are relied on in non-hematopoietic human cells. Here, we report an IFNγ-induced mechanism of resistance to *Toxoplasma* in primary human foreskin fibroblasts (HFFs) that does not depend on deprivation of tryptophan or iron. Additionally, infection is still controlled in HFFs deficient in the p65 guanylate binding protein GBP1 or the autophagic protein ATG5. Resistance is coincident with host cell death that is not dependent on the necroptosis mediator RIPK3 or caspases, and is correlated with early egress of the parasite before replication. This IFNγ-induced cell death and early egress limits replication in HFFs and could promote clearance of the parasite by immune cells.

Introduction

Innate immunity, in which immune cells recognize pathogen-associated molecular patterns and secrete pro-inflammatory cytokines to activate anti-microbial responses, is crucial in host defense against intracellular pathogens. For instance, the cytokine interferon-gamma (IFNγ) activates macrophages and many non-immune cells to cell-autonomously fight infections of many intracellular organisms, including the protozoan parasite *Toxoplasma gondii* (Suzuki and others 1988). *Toxoplasma* actively invades host cells, divides within a non-fusogenic parasitophorous vacuole, and then destroys the cell upon active egress, making intracellular resistance mechanisms important for host defense (Melo and others 2011).

Toxoplasma can infect all warm-blooded animals, including humans (Hill and Dubey 2002). It is estimated that a third of the global population is infected with *Toxoplasma.* Most infections in humans are asymptomatic, but *Toxoplasma* establishes a lifelong chronic infection by forming dormant cysts in brain and muscle tissue. However, *Toxoplasma* can cause severe disease and death in immunosuppressed individuals and in developing fetuses of pregnant women. It is also an important cause of ocular disease in both immunocompetent and immunosuppressed individuals (Gilbert and others 2008; Grigg and others 2001). In a recent study, *Toxoplasma* was among the top 5 pathogens responsible for the majority of economic losses and quality of life impairment due to foodborne illness in the United States (Hoffmann and others 2012).

Many resistance mechanisms effective against *Toxoplasma* have been identified in macrophages. For instance, in mouse and human macrophages, CD40 stimulation induces autophagic killing of the parasite by fusion of parasitophorous vacuoles with lysosomes (Andrade and others 2006). Additionally, activation of the purinergic receptor P2X7R leads to killing of the parasite by fusion with lysosomes followed by apoptotic death in human macrophages (Corrêa and others 2010; Lees and others 2010). The NALP1 inflammasome receptor was also identified as a susceptibility locus for human congenital toxoplasmosis, and silencing NALP1 leads to uncontrolled parasite growth in human monocytes (Witola and others 2011). While IFNγ-induced expression of nitric oxide synthase (Nos2) in macrophages is important for controlling the chronic stages of infection in mice (Scharton-Kersten and others 1997), nitric oxide production does not appear to play a role in controlling *Toxoplasma* infection in human macrophages (Murray and Teitelbaum 1992). However, IFNγ not only activates macrophages, but also induces anti-*Toxoplasma* activity in non-immune cells (Suzuki and others

1988). Indeed, in chimeric mice, IFNγ receptors were shown to be necessary in both hematopoietic and non-hematopoietic cells to survive *Toxoplasma* infection (Yap and Sher 1999). While in mice the main IFNγ-inducible effector mechanism against the acute phase of *Toxoplasma* infection is the p47 immunity-related GTPases (IRGs) that localize to and disrupt parasitophorous vacuoles (Martens and others 2005), humans lack the multitude of IRGs present in mice (Bekpen and others 2005). Indeed, ROP5 and ROP18, the virulence factors that allow *Toxoplasma* to evade the IRGs in mice, do not affect the ability of the parasite to survive in IFNγ-activated human foreskin fibroblasts (HFFs) (Niedelman and others 2012). Much less is known about the effector mechanisms of non-immune cells in humans compared to mice.

The main characterized mechanism of resistance to *Toxoplasma* in non-immune human cells is nutrient deprivation. For instance, *Toxoplasma* is auxotrophic for tryptophan, and the IFNγ-inducible enzyme indoleamine 2,3-dioxygenase (IDO1) degrades tryptophan. Tryptophan supplementation has been shown to restore parasite growth in IFNγ-stimulated human lung cells and fibroblasts (Gupta and others 1994; Heseler and others 2008; Pfefferkorn 1984; Werner-Felmayer and others 1991). However, in one study in human endothelial cells, neither tryptophan supplementation nor oxygen scavengers reduced IFNγ-induced inhibition of *Toxoplasma* growth (Woodman and others 1991). Furthermore, IFNγ was shown to inhibit *Toxoplasma* replication in rat enterocytes by limiting iron availability, and *Toxoplasma* growth was restored by addition of ferrous sulphate or holotransferrin (Dimier and Bout 1998). Although IFNγ-activated human monocytes were shown to down-regulate transferrin receptor expression to limit the growth of other microbes (Byrd and Horwitz 1989), iron supplement did not restore growth of *Toxoplasma* in IFNγ-activated human macrophages (Murray and others 1991). However, macrophages might have other mechanisms for resisting *Toxoplasma* growth that could make iron depletion

unnecessary, and it is unknown if iron deprivation plays a role in non-immune cell resistance. Together, these studies suggest that methods of resistance vary by cell type and that other resistance mechanisms remain to be uncovered.

There are other anti-microbial effectors induced by IFNγ that non-immune cells can utilize in *Toxoplasma* resistance. For example, though humans do not possess the large family of IRGs present in mice and some other mammals, humans do have another family of large IFNγ-induced GTPases called the p65 guanylate binding proteins (GBPs). In mice, it was shown that GBPs localize to the parasitophorous vacuole alongside the IRGs (Virreira Winter and others 2011), and mice deficient in a cluster of six GBPs are susceptible to *Toxoplasma* and lack IRG localization to the parasitophorous vacuole (Yamamoto and others 2012). Humans have 5 interferon-inducible GBPs, and it is possible that they could play a similar role in *Toxoplasma* resistance in human cells.

Another resistance mechanism induced by IFNγ is autophagy via PI3K activation (Matsuzawa and others 2012). Autophagosomes not only sequester organelles and cytoplasmic protein aggregates, but intracellular microbes as well, to deliver their contents to the lysosome for degradation. Autophagosome sequestration of *Toxoplasma* in human non-immune cells has not been reported, but autophagy is also important for the regulation of some proteins, such as the IRGs and GBPs in murine cells (Traver and others 2011; Zhao and others 2008). Furthermore, some instances of excessive autophagy have been reported to correlate with cell death (Levine and Yuan 2005), and cell death, autophagic or otherwise, can also prevent parasite proliferation. Several cell death pathways have been implicated in immunity: caspase-dependent apoptosis, RIP kinase-dependent necroptosis and caspase-1 and IL-1β-dependent pyroptosis

which occurs only in inflammatory cells (Han and others 2011). It remains to be seen if autophagy or host cell death plays a role in *Toxoplasma* resistance in non-immune cells.

Here we report that in IFNγ-stimulated HFFs, neither tryptophan supplementation nor IDO1 inhibition can restore parasite growth. Furthermore, iron supplementation does not relieve IFNγ-induced growth inhibition. Additionally, *Toxoplasma* resistance is not significantly altered in cells deficient for GBP1 or ATG5. Instead, we find that IFNγ stimulation and *Toxoplasma* infection leads to increased host cell death that is unaffected by chemical inhibition of necroptosis or caspases or knockdown of the necroptosis mediator RIPK3. Interestingly, we find that IFNγ and infection induced host cell death is correlated with but not dependent on early egress of the parasite. Parasite proliferation is inhibited even through multiple rounds of re-invasion and egress without replication. Importantly, early egress of the parasite not only limits parasite burden by preventing growth, but disrupts the intracellular niche, which could promote parasite clearance by immune cells *in vivo*.

Results

Tryptophan supplementation does not rescue Toxoplasma proliferation in IFNγ-stimulated human foreskin fibroblasts

To study intracellular resistance to *Toxoplasma* infection in primary non-immune cells, we sought to measure *Toxoplasma* growth inhibition by IFNγ in HFFs. Plaque formation includes all parts of the lytic cycle rather than measuring simply division, so the number and size of plaques can more accurately reflect *in vivo* parasite burden than other measures of growth such as parasite per vacuole counts. Therefore, we infected monolayers of cells either previously stimulated with IFNγ for 24 hours or left unstimulated and compared the number of plaques

formed after 4 days of growth to determine the percent plaque loss due to IFNγ stimulation. In HFFs, IFNγ stimulation causes 82% plaque loss and 81% reduction in plaque area (Figure 1 A and B). Previous studies have shown that in some human cell types, IFNγ-stimulation inhibits *Toxoplasma* growth by depletion of tryptophan (Heseler and others 2008; Pfefferkorn 1984; Schmidt and others 2009), while in other cell types tryptophan supplementation cannot restore parasite growth (Woodman and others 1991). To test the role of tryptophan deprivation in the control of *Toxoplasma* proliferation in IFNγ-stimulated primary HFFs, we measured the percent plaque loss due to IFNγ when we supplemented the media with L-tryptophan (L-Trp) simultaneously with infection or when we inhibited IDO1 by addition of 1-methyl-L-tryptophan (1-MT) (Cady and Sono 1991) at the time of IFNγ stimulation. As a positive control, we used HeLa cells that were shown to limit *Toxoplasma* growth by tryptophan depletion (Schmidt and others 2009). Indeed, the percent plaque loss on IFNγ-activated HFFs is only minimally reduced, from 82% to 64%, in the presence of tryptophan supplement (*p*-value = 0.028) and is unaffected by 1-MT (88% plaque loss) (Figure 1A). Furthermore, while tryptophan supplementation results in larger plaques than control (mean 125 mm^2 with tryptophan compared to 99.8 mm^2 without), it does not restore plaque size in IFNγ-stimulated cells (mean 24 mm^2 IFNγ with tryptophan) (Figure 1B). However, parasite survival is almost completely restored when IFNγ-activated HeLa cells are supplemented with tryptophan or 1-MT (plaque loss of 12 and 13%, respectively, *p*-value = 0.006) (Figure 1A), suggesting that the compounds are functional and that some cells do indeed solely rely on tryptophan degradation for *Toxoplasma* resistance. The inability of tryptophan to restore *Toxoplasma* growth in IFNγ-stimulated HFFs indicates a different mechanism of resistance in these cells.

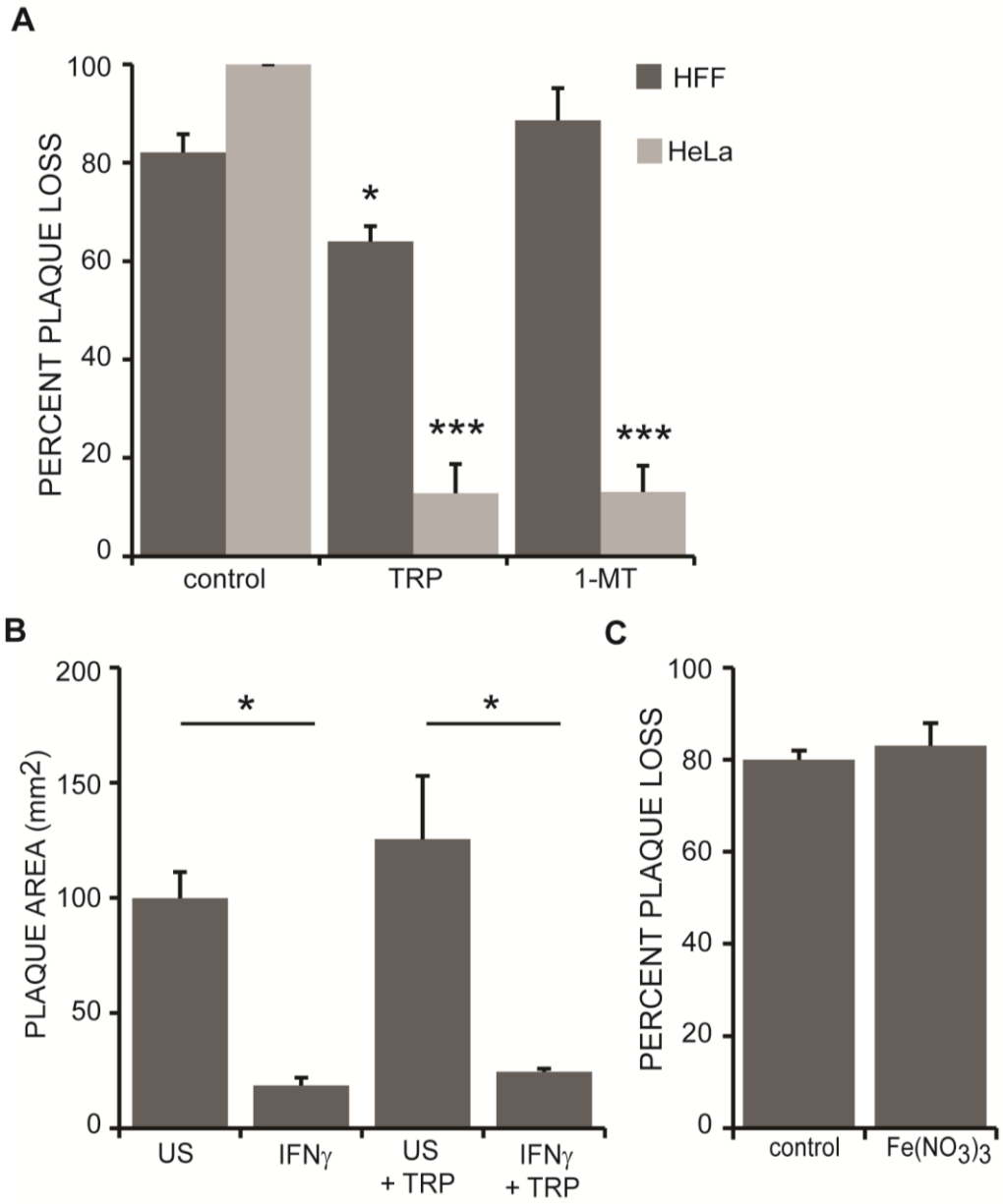


Figure 1: IFNγ-mediated resistance in HFFs is not dependent on tryptophan or iron depletion

(A) Percent plaque loss on HFFs or HeLa cells previously stimulated with 100 U/mL IFNγ for 24 hours was determined in the presence of 1 mM tryptophan (TRP) supplement added upon infection, 1 mM IDO1 inhibitor (1-MT) added at the time of IFNγ stimulation, or as a control, the same volume of 0.1N NaOH, the solvent used to dissolve both compounds. Mean + SE, n > 3 experiments. * represents p-value < 0.05, *** represents p-value < 0.001 (Student's t-test). **(B)** The area of the plaques formed on IFNγ-stimulated or unstimulated (US) HFFs in the presence or absence of tryptophan added upon infection. Mean + SE, n = 3 experiments, * represents p-value < 0.05 (Student's t-test). **(C)** Percent plaque loss on IFNγ-stimulated HFFs was determined in the presence of 25 µM ferric nitrate ($Fe(NO_3)_3$) added upon infection . Mean + SE, n = 3 experiments.

IFNγ-induced Toxoplasma resistance in human foreskin fibroblasts is not dependent on iron depletion

Because *Toxoplasma* is also auxotrophic for iron (Dimier and Bout 1998; Gail and others 2004), we wondered if HFFs could use iron depletion to curb parasite growth. To test this hypothesis, we performed the plaque assay in media supplemented with 25 μM ferric nitrate or 250 μM deferoxamine, an iron chelator. As expected, no plaques form in the presence of deferoxamine because *Toxoplasma* requires iron to grow. However, iron supplementation did not restore growth in IFNγ-induced HFFs (83% plaque loss compared to 80% without iron) (Figure 1C), and indeed, we found that higher concentrations of iron inhibited parasite growth on unstimulated HFFs (data not shown). Thus, the observed inhibition of *Toxoplasma* growth in IFNγ-stimulated HFFs is not dependent on iron depletion.

Autophagy is not necessary for IFNγ-induced inhibition of Toxoplasma proliferation in human foreskin fibroblasts

IFNγ stimulation also induces autophagy (Matsuzawa and others 2012), which could be important for inhibiting *Toxoplasma* replication in HFFs either by sequestration in autophagosomes or regulation of other effectors, as is the case for IRGs and GBPs in murine cells (Zhao and others 2008). To determine if autophagosomes do sequester parasitophorous vacuoles, we used immunofluorescence to stain for LC3, a marker of autophagosomes, in IFNγ-stimulated HFFs. We rarely observed (less than 1%) LC3 localized around the parasitophorous vacuole, making it unlikely that sequestration of PVs by autophagosomes is responsible for the inhibition of *Toxoplasma* growth in IFNγ-stimulated HFFs. However, autophagy could regulate other factors necessary for growth inhibition. Because autophagy inhibitors also affect the parasite ((Ghosh and others 2012; Wang and others 2010) and data not shown), we inhibited host

autophagosome formation by creating stable *ATG5* knockdown HFF cell lines to test if autophagy is necessary for IFNγ-induced resistance to *Toxoplasma*. Knockdown was confirmed by RT-qPCR and Western blot, and a limited amount of LC3 conversion to the lipidated form associated with autophagosomes was observed (Supplementary Figure 1A and B). IFNγ-stimulated ATG5 deficient HFFs are not less able to resist *Toxoplasma* proliferation than LacZ shRNA control HFFs (52% plaque loss compared to 43% plaque loss for LacZ shRNA control, *p*-value = 0.39) (Figure 2A). Additionally, ATG5 deficient HFFs still limit plaque size on IFNγ-stimulated monolayers (Figure 2B). Because we do not see co-localization of parasitophorous vacuoles with autophagosomes or altered plaque loss in autophagy deficient cells, it seems autophagy is not necessary for IFNγ-induced inhibition of parasite replication in HFFs.

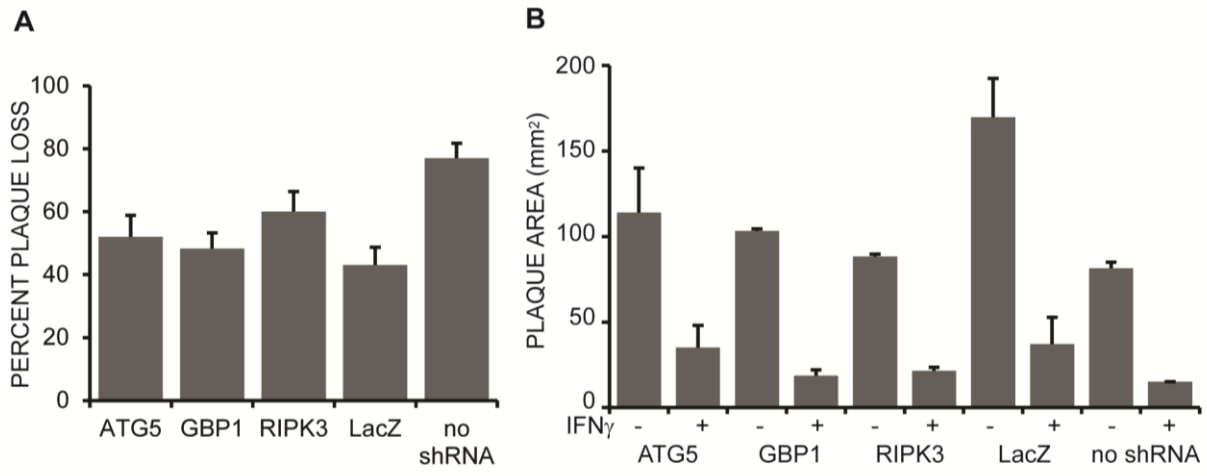


Figure 2: GBP1, ATG5 and RIPK3 are not necessary for IFNγ-mediated resistance in HFFs
Lentiviral shRNA was used to knockdown *GBP1*, *ATG5* and *RIPK3* or LacZ as a control. **(A)** Percent plaque loss on IFNγ-stimulated HFFs for the indicated gene knockdown as compared to no shRNA control. Mean + SE, n > 3 experiments. **(B)** The area of the plaques formed on IFNγ-stimulated or unstimulated HFFs with the indicated gene knocked down. Mean + SE, n = 3 experiments.

GBP-1 is not necessary for IFNγ-induced Toxoplasma resistance in human foreskin fibroblasts

Another possible cause for the inhibition of *Toxoplasma* growth in IFNγ-stimulated HFFs is that host GBP proteins could localize to the parasitophorous vacuole and promote membrane remodeling or vacuolar destruction, as is observed in murine cells. To determine if human GBPs colocalize with the PV, we stained IFNγ-stimulated HFFs with an antibody that recognizes GBP1-5. At a very low frequency (less than 1%), we do observe vacuolar localization of GBPs, but it is unlikely that this low level of localization could explain the significantly decreased parasite survival in IFNγ-stimulated HFFs. However, the GBPs were also shown to promote pyroptosis in *Salmonella* infected macrophages and associate with autophagic machinery and components of the NADPH oxidase, so they could still play a role in resistance without localizing to the parasitophorous vacuole (Kim and others 2011; Vestal and Jeyaratnam 2011). To test if the GBPs are necessary for the observed IFNγ-induced resistance in HFFs, we created stable *GBP1* knockdown HFF cell lines, as GBP1 has been shown to localize to chlamydial inclusions to inhibit their growth (Tietzel and others 2009). After confirming knockdown by RT-qPCR (Supplemental Figure 1A), we performed the plaque assay with IFNγ-stimulated HFFs in which GBP1 had been knocked down. IFNγ-stimulated GBP1 deficient HFFs are not less able to resist *Toxoplasma* than a LacZ shRNA control as measured by IFNγ-induced plaque loss (48% plaque loss GBP1 knockdown compared to 43% LacZ shRNA controls, p-value = 0.43), and plaque size in IFNγ-stimulated GBP1 knockdown cells is also reduced compared to unstimulated GBP1 knockdown cells (Figure 2A and B). Because GBP1 deficient HFFs are still able to reduce plaque size and plaque number upon IFNγ stimulation, GBP1 is not likely the main resistance mechanism induced by IFNγ in primary HFFs.

Infected, IFNγ-stimulated human fibroblasts undergo cell death independently of caspases, RIP kinases, autophagy or purinergic receptor activation

In infected human macrophages, P2X7R activation induces cell death to prevent parasite replication (Lees and others 2010). Additionally, in IFNγ-activated MEFs, infected host cells undergo necrotic cell death after IRG-mediated disruption of the parasitophorous vacuole (Zhao and others 2009). To investigate if HFFs also undergo cell death during infection as a means to prevent parasite replication, we stained infected and IFNγ-stimulated cells with propidium iodide (PI), a DNA dye that is excluded from viable cells but able to permeate dying cells (Fink and Cookson 2005). We compared the number of cells that were positive for PI to total number of cells, as measured by staining with the cell-permeable nuclear stain Hoechst 33342 which stains both viable and nonviable cells. We found that as early as 8 hours post infection, there is a significant increase in PI positive nuclei in infected, stimulated cells (27% PI positive) compared to uninfected, stimulated HFFs (0.1%, *p*-value = 0.04) or unstimulated, infected (1%, *p*-value = 0.004) HFFs (Figure 3A). As expected, this cell death in IFNγ stimulated, infected cells is MOI dependent, but independent of tryptophan (Figure 3B). After 24 hours, the number of PI positive nuclei reached 43% in infected, IFNγ-stimulated HFFs, but only 4.5% in unstimulated, infected HFFs (*p*-value = 0.001). Thus, cell death is associated with IFNγ-mediated resistance to *Toxoplasma* in HFFs, but it is unclear if the observed cell death is related to parasite clearance.

Next, we wondered if chemical inhibitors of cell death pathways could reduce IFNγ-induced death of infected cells. We measured the percent of PI positive nuclei in IFNγ-stimulated, infected HFFs in the presence of the caspase inhibitor Z-VAD-fmk to block apoptosis or the necroptosis inhibitor necrostatin-1 (Nec-1). We found no difference in PI positive nuclei in the presence of either inhibitor (39% PI positive for Z-VAD-fmk and 28% for Nec-1 compared

to 31% control, *p*-value = 0.30 and 0.61 respectively) (Figure 3B). Accordingly, HFFs with the necroptosis signal transducer RIPK3 knocked down by lentiviral shRNA infection did not have less plaque loss due to IFNγ (60% plaque loss compared to 43% LacZ shRNA control, *p*-value = 0.12) (Figure 2) or reduced PI staining (20% PI positive compared to 19% for LacZ control, *p*-value = 0.57) (Figure 3C). This suggests that IFNγ is not activating a programmed cell death pathway in infected cells, but because cell death pathways intersect it remains possible that chemical inhibition cannot prevent previously initiated cell death from proceeding down another pathway.

High levels of autophagy often accompany cell death, so we also tested if inhibition of autophagy with the PI3K inhibitor 3-methyladenine (3-MA) or ATG5 knockdown could prevent the observed cell death in IFNγ-stimulated, infected HFFs. ATG5 deficient HFFs show similar cell death (24% PI positive) in infected stimulated cells to cells targeted with LacZ control shRNA (19% PI positive, *p*-value = 0.53) (Figure 3C) and we found no difference in PI positive nuclei in the presence of 3-MA (26% compared to 31% control, *p*-value = 0.56) (Figure 3B). Additionally, cell death in IFNγ-stimulated infected GBP1-deficient HFFs is not significantly different than in lacZ control cells (18% PI positive compared to 19% PI positive LacZ control, *p*-value = 0.91) (Figure 4C), indicating GBP1 is not required to promote IFNγ and infection induced cell death.

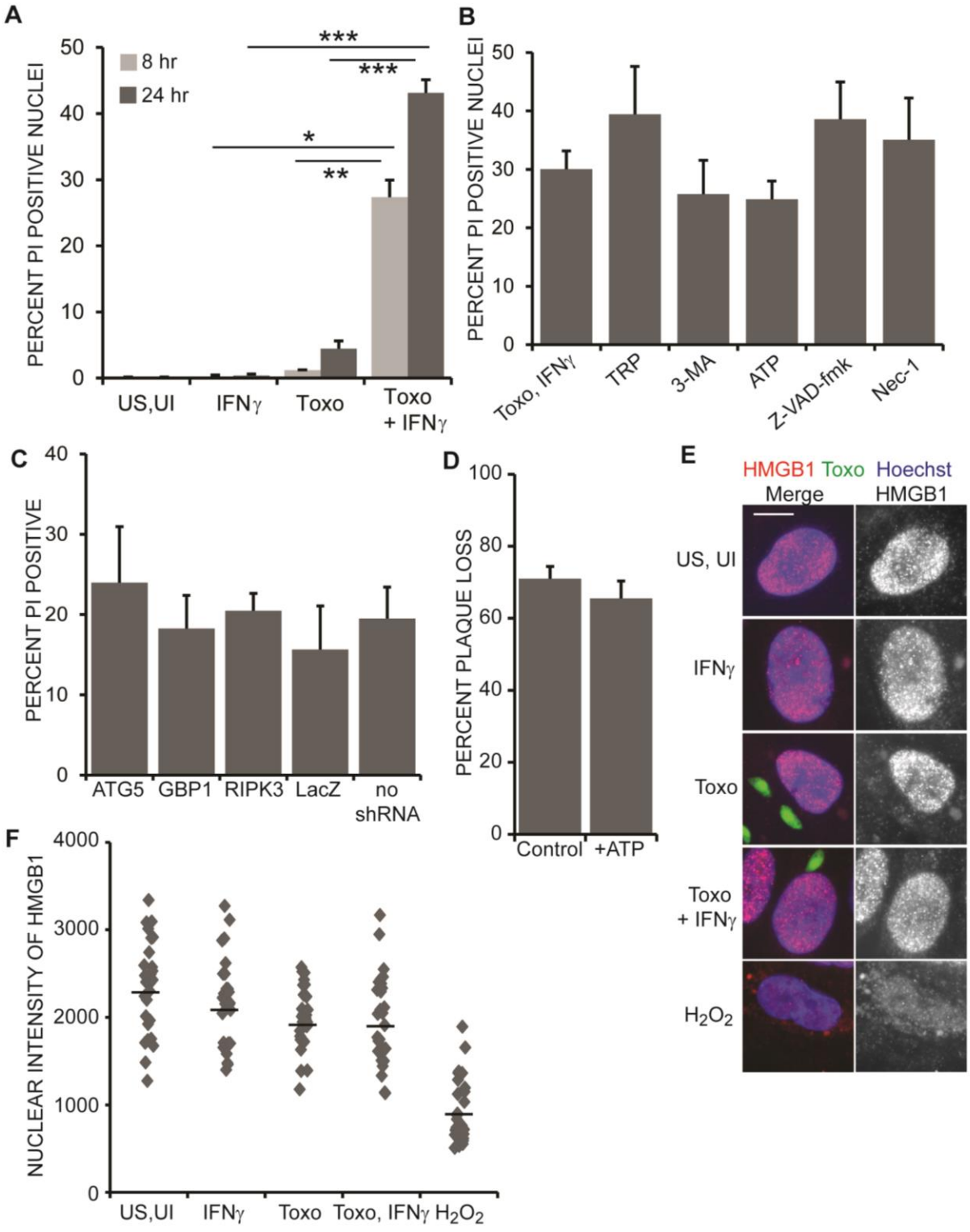


Figure 3: IFNγ-stimulated, infected HFFs undergo cell death independently of apoptosis, necroptosis or autophagy

(A) IFNγ-stimulated or unstimulated (US) HFFs were infected for 8 or 24 hours at an MOI of 3 or left uninfected (UI). Propidium iodide (PI) and membrane-permeable Hoechst were added for

15 minutes before visualization, and the percent PI positive nuclei was determined. Mean + SE, n > 3 experiments. * represents p-value < 0.05, ** represents p-value < 0.01, *** represents p-value < 0.001 (Student's t-test). **(B)** Percent PI positive nuclei in infected, IFNγ-stimulated HFFs in the presence of 1 mM tryptophan (TRP), 3mM ATP or the indicated autophagy (10 mM 3-MA) or cell death inhibitors (50 μM Nec-1 or 100 μM Z-VAD-fmk). Mean + SE, n = 3 experiments. **(C)** Percent PI positive cells at 8 hours post infection of IFNγ-stimulated HFFs deficient in the indicated gene. Mean + SE, n = 3 experiments. **(D)** Percent plaque loss on IFNγ-stimulated HFFs was determined in the presence or absence of 3 mM ATP. Mean + SE, n = 3 experiments. **(E)** IFNγ-stimulated or unstimulated (US) HFFs were infected with parasites expressing GFP (green) for 8 hours or left uninfected (UI) or necrosis was induced by 45 minutes of 1 mM hydrogen peroxide, fixed and stained for HMGB1 (red), Hoechst (blue). Merged image left, HMGB1 image right. Scale bar represents 10 μm. **(F)** Quantification of mean nuclear HMGB1 from (C). Dots represent individual nuclei, lines represent the mean, representative of 3 experiments.

In macrophages, purinergic receptor activation leads to fusion of parasitophorous vacuoles with lysosomes and host cell death (Corrêa and others 2010). Because human skin fibroblasts were shown to express P2X7R (Solini and others 1999), we tested if purinergic receptor activation contributes to host cell death and/or parasite control in HFFs by measuring PI positive nuclei and IFNγ induced plaque loss in the presence of 3 mM ATP. We found no significant differences in PI positive infected, IFNγ-stimulated cells in the presence of ATP (25% PI positive compared to 30% control, *p*-value = 0.37) (Figure 3B) or percent plaque loss in the presence of ATP (66% loss compared to 71% control, *p*-value = 0.48) (Figure 3D). This suggests that purinergic receptor activation does not induce host cell death or parasite clearance in HFFs.

Infected, IFNγ-stimulated MEFs undergo necrosis after disrupting the parasitophorous vacuole (Zhao and others 2009). High-mobility group protein B1 (HMGB1) normally resides in the nucleus, but is released into the supernatant by necrotic cells (Scaffidi and others 2002). To test if infected HFFs die via necrosis, we analyzed the nuclear intensity of HMGB1 after 8 hours of infection compared to 45 minutes of hydrogen peroxide induced necrosis. Quantification of mean nuclear fluorescence of HMGB1 in infected and uninfected HFFs indicated that HMGB1 levels are 15% lower in the nuclei of infected cells (p = 0.004) compared to uninfected, but in a

manner independent of IFNγ stimulation (16% lower in Toxo + IFNγ than uninfected, unstimulated) (Figure 3E and F). However, hydrogen peroxide treated cells had 62% lower mean nuclear HMGB1 than uninfected, unstimulated cells. This indicates that the observed cell death after infection and IFNγ stimulation is not likely to be necrotic.

Parasites infecting IFNγ-stimulated HFFs egress without replication

It is unclear if cell death leads to parasite clearance or occurs as a result of it, so to clarify the order of events, we performed live imaging of infected IFNγ-stimulated or unstimulated cells over the course of 20 hours. Interestingly, while parasites divide normally in unstimulated HFFs, in IFNγ-stimulated HFFs we observed early egress of non-replicated parasites accompanied by host cell death in 85% of infected cells as early as 5 hours after infection. The remainder of infected cells died, but whether or not the parasite egressed could not be determined due to the timing of the image intervals. Many egressed parasites then infected neighboring cells, only to egress again several hours later (Figure 4/Supplemental Video). Although the IFNγ-stimulated HFFs appear not to kill infecting *Toxoplasma* parasites, the intracellular niche is disrupted and replication is prevented by early egress, explaining the reduced plaque size and number on stimulated monolayers.

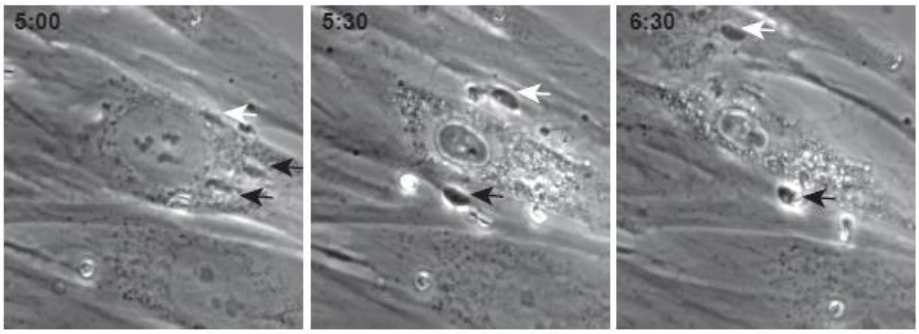


Figure 4: Parasites infecting IFNγ-stimulated HFFs egress without replication
Live imaging of infected, IFNγ-stimulated HFFs at the indicated time point after infection. Arrows point to parasites.

Inhibition of egress does not reduce cell death in IFNγ-stimulated, infected fibroblasts

Cell membrane permeabilization leads to a loss of intracellular potassium which can activate parasite motility (Moudy and others 2001). However, it is also possible that the parasite senses another signal that leads to egress, causing the observed host cell membrane permeabilization and death. To determine if egress leads to cell death, we measured the percent PI positive nuclei in IFNγ-stimulated HFFs infected with parasites that were unable to egress. We used parasites that express TgCDPK3, which was shown to be necessary for egress, with either a glycine (G) or methionine (M) at the gatekeeper position which determines the sensitivity to the inhibitor 3-methyl-benzyl pyrazolo [3,4-*d*] pyrimidine (3-MB-PP1). The $TgCDPK3^{G}$ strain cannot egress in the presence of the inhibitor, while $TgCDPK3^{M}$ is uninhibited. As a positive control, we measured percent PI positive nuclei for these strains in the presence of 3-MB-PP1 and a calcium ionophore A23187, which induces egress. The inhibitor is able to prevent calcium ionophore induced egress of $TgCDPK3^{G}$ but not $TgCDPK3^{M}$. However, there is no difference in PI positive nuclei in IFNγ-stimulated HFFs infected with either strain in the presence or absence of the inhibitor (Figure 5). This indicates that cell death is not caused by egress, but rather that the parasite egresses to escape a dying cell.

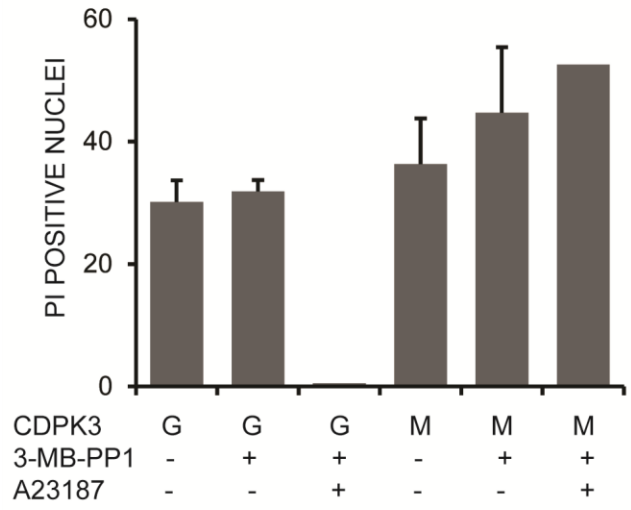


Figure 5: Inhibition of egress does not prevent cell death in infected IFNγ-stimulated HFFs Percent PI positive nuclei in IFNγ-stimulated HFFs infected with parasites expressing either TgCDPK3^{G} (sensitive to 3-MB-PP1) or TgCDPK3^{M} (insensitive to 3-MB-PP1) for 8 hours, and 5μM of the inhibitor 3-MB-PP1 or DMSO was added for the last 4 hours. Mean + SE, n = 3. As a positive control, unstimulated HFFs were infected with the indicated strains for 8 hours, with 3-MB-PP1 for the last 4 hours and then 2 μM of the calcium ionophore A23187 was added for 20 minutes.

Discussion

Toxoplasma establishes a lifelong infection in hosts by forming cysts in brain and muscle tissue, and therefore cell-autonomous immunity in non-immune cells is important for limiting parasite burden and cyst formation. Previously, the main characterized mechanism for controlling parasite growth in non-immune human cells was IFNγ-induced deprivation of tryptophan. Here we report that tryptophan supplementation does not restore parasite growth in IFNγ-stimulated primary HFFs. We do not find evidence of other reported anti-*Toxoplasma* mechanisms such as iron deprivation or vacuolar destruction by p65 guanylate binding proteins (GBPs) or autophagy being involved in the observed resistance in HFFs. Instead, we observe that IFNγ- stimulated HFFs undergo cell death upon *Toxoplasma* infection that induces parasites to

egress as early as 5hr after infection, before replication occurs, leading to limited parasite proliferation and potentially promoting clearance by immune cells *in vivo*.

Previous studies showed that some non-immune human cells such as HeLa cells (Schmidt and others 2009) and human fibroblasts (Gupta and others 1994; Pfefferkorn 1984) controlled *Toxoplasma* infection by tryptophan degradation via induction of the IFNγ-induced enzyme IDO1, while other cell types such as endothelial cells (Dimier and Bout 1997; Woodman and others 1991) used a tryptophan-independent resistance mechanism. Our results confirm that HeLa cells do rely on tryptophan degradation to inhibit *Toxoplasma* replication, but in contrast to previous work with human fibroblasts, we find that tryptophan supplementation does not restore parasite growth in primary foreskin fibroblasts. The origin of the human fibroblasts from previous studies was not reported (Gupta and others 1994; Pfefferkorn 1984), but it may be that differences in the specific tissue from which the fibroblasts were derived or the use of transformed rather than primary human fibroblasts could explain these differences in IFNγ-mediated parasite clearance mechanisms.

Similarly, we find that iron supplementation does not abrogate resistance, but excess iron can inhibit parasite growth, even in unstimulated cells. Many cellular functions and immune mechanisms are sensitive to iron concentration in the cell (Weiss 2002). It is possible that the observed resistance mechanism in HFFs is in some way regulated by iron, but it would be difficult to differentiate that effect from other effects iron has on the cell. It is at least clear that iron deprivation is not responsible for limiting *Toxoplasma* growth in stimulated HFFs because iron supplementation does not restore growth.

We report that IFNγ stimulated HFFs limit *Toxoplasma* growth by dying before parasite replication can occur. It is not clear how cell death is induced, but it is unaffected in HFFs

deficient for RIPK3, GBP1 or ATG5 or in the presence of autophagy or cell death inhibitors. It is possible that, due to incomplete knockdown, the remaining protein expressed in these knockdowns is enough to function. It also remains possible that other GBPs aside from GBP1 promote resistance without vacuolar localization or that GBP1 itself is also involved, but that its function is redundant. However, the fact that chemical inhibition of autophagy and cell death pathways is also unable to prevent IFNγ and infection induced cell death suggests that these pathways are either dispensable or redundant. Cell death pathways are so intertwined that inhibition of one pathway can cause cell death to proceed down another pathway. For instance, TNF activation can lead to apoptosis or necroptosis depending on caspase-8 activation (Han and others 2011). Thus, cell death is difficult to inhibit and therefore determining the mediators involved will be a challenge. It will also be interesting to determine what factors of *Toxoplasma* infection contribute to this cell death.

Early egress and reinvasion have also been observed in murine peritoneal exudate cells (Tomita and others 2009). The observed egress was reported to be triggered externally by activated macrophages in a manner dependent on intracellular calcium and sensitive to a p38 MAPK inhibitor. Natural egress from a host cell is triggered by a reduction in cytoplasmic potassium concentration due to host membrane permeabilization (Moudy and others 2001). Egress can also be induced *in vitro* by calcium ionophores, DTT and cell death inducers such as *fas* ligand or perforin (Black and others 2000; Moudy and others 2001; Persson and others 2007; Stommel and others 1997). The fact that egress can be triggered externally by environmental cues suggests that the parasite may have adapted to be able to evacuate inhospitable cells. In the previous report, parasites that had egressed and reinvaded were preferentially restricted *in vivo* (Tomita and others 2009). It was suggested that the early egress triggered externally could

reshuffle parasites to previously stimulated cells that are better able to restrict growth. Early egress from stimulated non-immune cells may be similarly beneficial by promoting infection of immune cells with other clearance mechanisms or by depleting the contents of secretory organelles used for invasion and host cell manipulation. At the very least, even if egressed parasites are not able to invade an immune cell, parasite burden is limited by the lack of or delay in replication. Thus, even if human fibroblasts do not possess the vacuole destroying abilities of immune cells or murine fibroblasts, they can still play an important role in limiting the course of *Toxoplasma* infection.

Materials and Methods

Reagents

A mouse monoclonal antibody against GBP1-5 (G-12, Santa Cruz, 1:100 dilution), a rabbit polyclonal antibody against LC3B (2775, Cell Signaling 1:700 dilution), a rabbit polyclonal antibody against ATG5 (2630, Cell Signaling 1:1000 dilution), a rabbit polyclonal antibody against human HMGB1 (ab18256, Abcam 1:900 dilution), a rat polyclonal antibody against GBP1 (1B1, Santa Cruz 1:500 dilution), a rabbit polyclonal antibody against RIPK3 (M-2, Santa Cruz 1:500 dilution) and a mouse monoclonal antibody against β-actin (ab8226, Abcam 1:10000 dilution) were used in immunofluorescence assays or Western blotting. Secondary antibodies were coupled with Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes) or HRP (Kirkegaard and Perry Laboratories). L-tryptophan (MP Biochemicals) and 1-methyl-L-tryptophan (Sigma-Aldrich) were dissolved in 0.1 N NaOH before use. Indole (Sigma-Aldrich), ferric nitrate (MP Biomedicals), deferoxamine (CalBiochem) and dextran sulfate (Sigma-Aldrich) were dissolved in water before use. Necrostatin-1 (Sigma-Aldrich), 3-methyladenine (Sigma-Aldrich), z-VAD-

FMK (Axxora), A23187 (Sigma-Aldrich), and 3-MB-PP1 (CalBiochem) were initially dissolved in DMSO and further dissolved in DMEM before use. Hoechst 33342 (Invitrogen) was dissolved in DMSO. Human IFNγ from AbD serotec was dissolved in DMEM with 10% FBS.

Parasites and Cells

Parasites were maintained *in vitro* by serial passage on monolayers of human foreskin fibroblasts (HFFs) at 37°C in 5% CO2. HFFs were grown as described previously (Rosowski and others 2011) and HeLa cells were grown in HFF media supplemented with 1mM sodium pyruvate. An RH strain engineered to express clickbeetle luciferase and GFP (RH 1-1) (Boyle and others 2007) was described previously. RH strains engineered to express TgCDPK1^{M} and either TgCDPK3^{G} or TgCDPK3^{M} were gifts from S. Lourido and were described previously (Lourido and others 2012).

Immunofluorescence assays

Parasites were allowed to invade monolayers of HFF cells grown on coverslips previously incubated for 24 hours with or without 100 U/mL IFNγ, and infection proceeded for 8 hours. The cells were then fixed and prepared for immunofluorescence as described previously (Rosowski and others 2011).

Plaque Assay

For the plaque assay, 100-300 parasites per well were added to monolayers of HFFs seeded two days before and either previously stimulated with 100 U/mL human IFNγ or left unstimulated for 24 hours before infection in a 24 well plate. Infections were then incubated for 4 days at 37°C and the number of plaques was counted using a microscope.

PI staining

HFFs were seeded into a 24 well plate just as for the plaque assay. The media was changed the next day and cells were stimulated with 100U/mL IFNγ. Syringe lysed parasites were passed through a Millipore 5 µm filter to remove lysed nuclei before infection. A "parasite only" well was used to ensure no host nuclei were added to the HFFs. After 8 or 24 hours of infection, propidium iodide (PI) and Hoechst 33342 (Invitrogen) were added and staining was imaged 15 minutes later using a fluorescence microscope.

Live Microscopy

HFFs were plated on 24-well glass bottom plates, the next day the media was changed and the cells were stimulated with IFNγ for 24 hours before infection. Infection was synchronized by spinning at 900 rpm for 3 minutes, and washing with PBS 5 times after an hour infection. Infected cells were then imaged every 30 minutes over a 20 hour period using a 40X objective (NA = 0.95) on a Nikon TE2000 inverted microscope equipped with an environmental chamber, Hamamatsu ORCA-ER digital camera and NIS Elements Imaging Software.

shRNA Knockdowns

HFFs were infected with lentivirally packaged shRNA vectors (Broad RNAi Consortium) in the presence of 8 µg/mL polybrene (Sigma Aldrich) for 24 hours (ATG5 target sequence: 5'-CCTTTCATTCAGAAGCTGTTT-3'; GBP1 target sequence: 5'-CCAGATGAGTACCTGACATAC-3'; RIPK3 target sequence: 5'-GGCGACCGCTCGTTAACATAT-3'; LacZ target sequence: 5'-GTCGGCTTACGGCGGTGATTT-3'). Infection media was removed and the following day cells were switched to and maintained in media containing 2 µg/mL puromycin (Invitrogen). All experiments were performed in media without puromycin and knockdown was re-confirmed at

the end of the experiment. Knockdown was confirmed by RT-qPCR by comparison to β-actin and no shRNA control cells. Briefly, RNA was isolated with TRIZOL (Invitrogen) and cleaned up with the Qiagen RNeasy kit. Reverse transcription was done using the Superscript III Reverse Transcriptase system (Invitrogen) using oligo dT. Quantitative real time PCR was performed with the following primers: β-Actin FW: 5'-CATGTACGTTGCTATCCAGGC-3', RV: 5'-CTCCTTAATGTCACGCACGAT-3'; ATG5 FW: 5'-AGAAGCTGTTTCGTCCTGTGG-3', RV: 5'-AGGTGTTTCCAACATTGGCTC-3'; GBP1 FW: 5'-CTCTTAAACTTCAGGAACAGGAGC-3', RV: 5'-CATGATCATTGTACCACATGCC-3'; RIPK3 FW: 5'-AATCCAGTAACAGGGCGACC-3', RV: 5'-GCCTCAGGATCTTTAGGGCC-3'.

Statistical Analysis

All comparisons were analyzed for statistical significance using two-tailed Student's t-tests.

Supplemental Figures

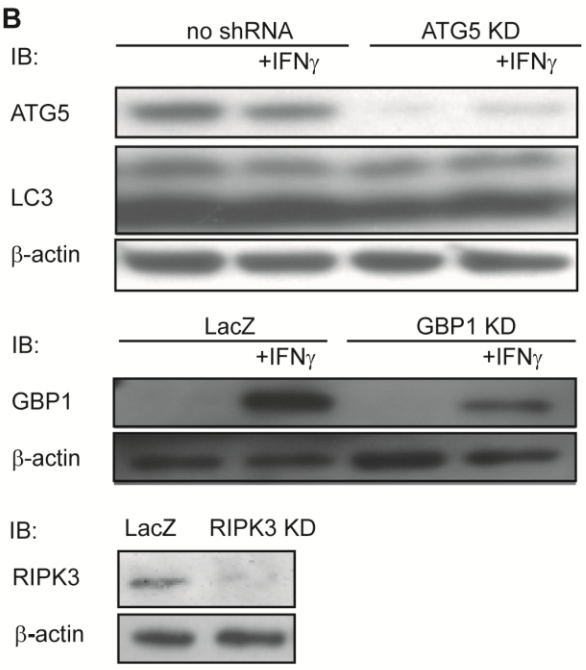
A

	Actin C_T	ATG5 C_T	$2^{-\Delta\Delta C}{}_T$
no shRNA	19.16	27.08	1
ATG5 KD	24.28	35	0.14

	Actin C_T	GBP1 C_T	$2^{-\Delta\Delta C}{}_T$
no shRNA	19.16	25.59	1
GBP1 KD	16.19	23.21	0.01

	Actin C_T	RIPK3 C_T	$2^{-\Delta\Delta C}{}_T$
no shRNA	19.16	29.8	1
RIPK3 KD	20.83	35	0.09

B



Supplemental Figure 1
(A) Results of qPCR presented as C_T values for actin and the indicated gene. Relative expression was determined by comparing C_T for the knocked down gene to actin and to a no shRNA control using $2^{-\Delta\Delta Ct}$ **(B)** Western blot of ATG5 knock down cells and no shRNA control at the end of a plaque assay performed in media without puromycin (Top), GBP1 knockdown cells and LacZ control (Center) or RIPK3 knockdown and LacZ control stimulated with IFNγ (Bottom).

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Chapter Four:

Conclusions and Future Directions

Summary

The work in this thesis demonstrated that the allelic combination of *ROP18* and *ROP5* determines IRG evasion and virulence of all strains tested. Additionally, the *ROP5-C* but not *A* isoform reduced IRG coating in type II by directly interacting with the IRGs to inhibit their oligomerization and allow access for ROP18 to phosphorylate and permanently inhibit them. Thus, type II strains are the most susceptible to the IRGs despite a virulent *ROP18* because ROP18 requires virulent *ROP5* alleles to inhibit the IRGs. We further showed that ROP18 and ROP5 do not affect parasite survival outside the context of the IRGs, such as in human cells. These findings suggest that mouse models of *Toxoplasma* virulence are limited in predicting *Toxoplasma* virulence factors that are important in humans. However, the fact that the two factors that explain the majority of strain differences in virulence promote IRG evasion indicates the importance of IRGs in the evolution of the parasite. The expansion and diversifying selection of ROP5 and IRGs in mice suggests they will provide a good host-pathogen co-evolution model.

We then identified a new mechanism of resistance to *Toxoplasma* in non-immune human cells in which parasite proliferation is prevented by host cell death. This cell death is not dependent on caspases, the necroptosis pathway, autophagy or GBP1. Furthermore, the parasite is adapted to escape the inhospitable environment of a dying cell, so we observed early egress of non-replicated parasites from IFNγ-stimulated human fibroblasts. Thus, IFNγ-induced cell death does not kill the parasite, but it does limit replication and disrupts the intracellular niche. This could promote clearance of the parasite by immune cells or depletion of secretory factors important for immune modulation.

Future Directions

While we did explain the strain differences in evasion of mouse IRGs, assign a function to the virulence factor ROP5 and uncover a novel mechanism of IFNγ-induced resistance in human fibroblasts, many questions remain to be answered.

Why is type II ROP5 less effective in inactivating the IRGs?

In our expression and sequence analysis of *ROP5*, we found that type II ROP5-B and C are more closely related to ROP5-A from the other strains. Additionally, we showed that ROP5-A_{III} could not reduce IRG coating in type II to the extent that ROP5-C_{III} can. Furthermore, Irga6 was only immunoprecipitated with ROP5-C not ROP5-A. Another group also found that two copies of ROP5-A_{III} were not able to complement the IRG evasion of type I *Δrop5* as well as one copy each of ROP5-A_{III} and B_{III} (Fleckenstein and others 2012). This suggests that ROP5-A is not able to inhibit the oligomerization of the IRGs as well as ROP5-B or C. Because IRG inhibition occurs through binding, one hypothesis for this result is that ROP5-A, and therefore possibly type II ROP5 by extension, is less able to bind IRGs. However, it was also demonstrated that several IRGs immunoprecipitated type I ROP5 peptides unique to all three isoforms, indicating that ROP5-A is able to bind the IRGs (Fleckenstein and others 2012). Furthermore, HSQC spectra of labeled ROP5-B_I in the presence or absence of Irga6 indicate that the region of ROP5 that binds Irga6 is on the side of the protein opposite from the pseudoactive site, which is one of the least divergent regions of protein surface among isoforms. Indeed, most polymorphisms on the opposite face from the pseudoactive site are between isoforms rather than between strains, with the exception T211M shared by ROP5-A_{II} and C_{II} (Reese and Boothroyd 2011). While these results are not inconsistent with the hypothesis that type II ROP5 has reduced IRG-binding because type II ROP5 alleles are divergent even from ROP5-A from all other

strains, it remains to be demonstrated that type II ROP5 has lower binding affinity for IRG proteins than type I ROP5. Perhaps it is not just binding affinity but binding confirmation that mediates the inhibition of IRG oligomerization. Indeed, ROP5 binding to IRGs is increased in the presence of GDP, suggesting ROP5 preferentially binds IRGs in their inactive state to inhibit their oligomerization (Fleckenstein and others 2012). It may be that type II ROP5 does not bind inactive IRGs preferentially. Thus, binding affinity of the different ROP5 isoforms from types I and II to Irga6 in the presence of different nucleotides should be determined to distinguish these two possible explanations for the ineffectiveness of type II ROP5. Furthermore, it would be helpful to identify which ROP5 residues specifically interact with the IRGs to study the evolution of these proteins and their interactions.

Can GRA2 explain the remaining within-type strain differences in IRG evasion?

Although both type I strains tested, GT1 and RH, are virulent in mice, they differ in percent Irgb6 coating (25% IRG-coated vacuoles for GT1 compared to 5% for RH). These differences cannot be explained by polymorphism or expression levels of *ROP5* or *ROP18* because these genes have equal or higher expression in GT1 compared to RH. Additionally, Pru, a type II strain, expressing $ROP5_I$ has 38% Irgb6 coating while S22, an avirulent F1 progeny with $ROP18_{III}$ and $ROP5_{II,}$ transgenically expressing $ROP18_{II}$ and $ROP5_I$ has 9% Irgb6 coating. We found that GRA2 also contributes to IRG evasion by creating negative curvature in the PVM to attract rhoptry proteins, such as ROP18 and ROP5 (Reese and Boothroyd 2009). Both GT1 and Pru have non-synonymous single nucleotide polymorphisms (SNPs) in GRA2 compared to RH. To see if polymorphism in GRA2 can explain the remaining strain differences in IRG coating, we have complemented RHΔ*gra2* with GRA2 from RH, GT1 and Pru. Both Pru and GT1 GRA2 can reduce IRG coating to almost the same extent as RH GRA2 but interestingly,

they do not reduce plaque loss (data not shown). These data are inconclusive as to whether GRA2 polymorphism explains the remaining strain differences in IRG coating. However, it would be interesting if one or two SNPs in GRA2 affected the formation of the tubulovesicular network that creates the negative curvature of the PVM, which appears to be important for many interactions with the host cell and is likely conserved between strains (Mercier and others 2002). Thus, if GRA2 polymorphism does affect IRG evasion, it might indicate the role of GRA2 in IRG evasion is not related to PVM structure but perhaps a direct protein-protein interaction. Indeed, deletion of GRA6, which also disrupts tubulovesicular network formation but to a lesser extent than GRA2 (Mercier and others 2002), does not affect IRG coating (data not shown). However, the effect of GRA2 deletion on IRG coating is not large (36% coating in RHΔ*gra2* compared to 5% coating in RH) so if GRA6 has even less effect on PVM structure, the effect on IRG coating may be negligible. Thus, it remains to be determined whether polymorphism in GRA2 explains the remaining differences in IRG evasion and if GRA2's role is determined by PVM structure or protein interactions.

What Toxoplasma genes are important for human disease and immune evasion?

The known factors associated with virulence were identified in mice using QTL studies of the F1 progeny of crosses between canonical strains. While some of these factors, such as ROP16 and GRA15 do modulate immune signaling pathways even in humans, we demonstrated the two factors that account for the majority of strain differences in virulence ROP5 and ROP18 do not affect survival in IFNγ-stimulated human fibroblasts. The prominence of the IRGs in the murine immune response and the absence of the IRGs in humans portends that mouse models of *Toxoplasma* virulence cannot fully reflect human disease. Additionally, the use of only canonical strains in crosses does not reflect the genetic diversity of *Toxoplasma*, further limiting the

representativeness of the known virulence factors. QTL studies of virulence in humans are not feasible, although *in vitro* phenotypes associated with disease in humans could be studied by QTL if strain differences exist. Perhaps the recent sequencing of 26 strains reflecting global diversity and the identification of highly conserved or polymorphic genes will be useful in associating genotypes with phenotypes (Minot and others 2012). Forward genetic screens can also identify mutants with differential survival in IFNγ-stimulated human cells to identify other *Toxoplasma* factors important for survival and immune evasion. This method has been used to identify a *Toxoplasma* patatin-like protein that suppresses nitric oxide production for survival in murine macrophages (Mordue and others 2007). Because *Toxoplasma* evades death in IFNγ-stimulated HFFs by egress, an essential process for parasite survival, there will not likely be strain differences in this phenotype, and the *Toxoplasma* factors responsible are likely the ones already identified in studies of egress. However, there are likely additional *Toxoplasma* factors that affect disease outcome in humans that are yet to be uncovered.

What factors promote cell death in infected, IFNγ-stimulated human fibroblasts?

IFNγ-stimulated, infected HFFs die in a manner independent of caspases, precluding both apoptosis and pyroptosis, and the necroptosis mediators RIPK1 (illustrated by necrostatin-1 inhibition) and RIPK3 (shown by knockdown) (Fink and Cookson 2005). Furthermore, when the parasite is prevented from egress, cell death is still observed, suggesting it is not caused by egress-induced lysis. Because cell death pathways are interconnected, inhibition of one pathway can cause cell death to proceed down another, making it difficult to prevent cell death by removing only one mediator (Han and others 2011). We have also attempted to block cell death by combining necrostatin-1 and Z-VAD-fmk inhibition, to no avail. Perhaps, a more comprehensive shRNA screen, possibly with pooled shRNAs, could be informative, but it may

be impossible to inhibit cell death once it is triggered. Alternatively, overexpression studies could identify factors sufficient for host cell death. It may also be helpful to identify what aspect of *Toxoplasma* infection contributes to host cell death, as this is not observed in uninfected but stimulated HFFs. How is the parasite sensed? Does the parasite need to infect the cell or can secreted parasite factors induce cell death in a stimulated cell? If there is indeed an IFNγ induced parasite sensor that causes cell death in infected cells, such as the inflammasome that is thought to be only expressed in immune cells, it may be possible to use this sensor to kill infected cells without immune activation or excessive inflammation.

Concluding Remarks

The work presented in this thesis demonstrates the importance of the IRGs in the murine immune response to *Toxoplasma* because the two factors that control the majority of strain differences in virulence interact to disable the IRGs. The diversifying selection of ROP5 and the IRGs suggests they have co-evolved, and these proteins will provide a model of eukaryotic pathogen-host co-evolution. Additionally, we uncovered a novel mechanism of resistance in IFNγ-stimulated human fibroblasts that disrupts the intracellular niche by cell death-induced egress without replication. While the mechanisms of ROP5 and GRA2 inhibition of IRG accumulation and the means of promoting cell death in IFNγ-stimulated infected cells are not fully understood yet, this work has advanced our understanding of mouse virulence and the human immune response to *Toxoplasma gondii*.

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