Modulation of innate immune signaling pathways by the intracellular pathogen *Toxoplasma gondii*

by

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Abstract

Toxoplasma gondii, an obligate intracellular protozoan parasite, is one of the most successful eukaryotic pathogens. It can infect virtually any warm-blooded animal, including humans, in whom it can cause serious disease. Its success is likely due to its ability to modulate host immune responses and host innate immune signaling pathways allowing it to establish a chronic infection with few symptoms in its hosts, which favors transmission to new hosts. Here, we report that *Toxoplasma* activates NF-κB and inhibits STAT1 signaling pathways to promote both its own survival and the survival of its host. We identified GRA15, a novel *Toxoplasma* secreted factor that activates the host cell NF-κB pathway. GRA15 is polymorphic between *Toxoplasma* strains and only active in the type II clonal lineage. GRA15 expression increases host pro-inflammatory cytokine production *in vivo*, thereby helping the host to control parasite growth. Conversely, *Toxoplasma* infection dampens the activation of other immune responses by inhibiting IFN-γ and STAT1 signaling. All of the *Toxoplasma* strains that we have tested directly inhibit the activity of STAT1, the transcription factor through which IFN-γ signals. We found that infection does not inhibit STAT1 phosphorylation, dimerization, nuclear translocation, or DNA binding. Instead, *Toxoplasma* must act even farther downstream, perhaps by inhibiting the recruitment of co-activators or RNA polymerase. Infection actually increased the association of STAT1 with DNA, which has been shown previously to be associated with decreased STAT1 transcriptional activity. The *Toxoplasma* effector that inhibits STAT1 remains unknown, but our results suggest that it is not secreted into the host cell upon invasion but must interface with its cellular target after the parasitophorous vacuole is formed. A deeper knowledge of how and why *Toxoplasma* modulates these processes will help us to understand more about the basic signaling pathways themselves and to discover clues on how to better treat *Toxoplasma* infections in humans.

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Chapter 1. Introduction

Intracellular pathogens

According to the World Health Organization, infectious disease is responsible for more than 15% of all world-wide deaths. This number is even higher in developing regions of the world such as Africa (> 43%) and Southeast Asia (> 17%) (Mathers et al., 2008). Of all of the causative agents of infectious diseases, the three that account for the most mortality—HIV/AIDS, Tuberculosis, and Malaria—are all intracellular pathogens.

Intracellular pathogens live within a specialized niche that allows them to evade certain mechanisms of the host immune system such as the complement system and antibodies, but this niche also presents its own set of problems with which the pathogens must deal. Every intracellular pathogen must get itself into a host cell, either by active invasion or by subverting host processes such as phagocytosis, and then get itself out of the host cell in order to disseminate to new cells and new hosts. Intracellular pathogens also must acquire nutrients and dispose of waste through the host cell. Hosts also possess immune mechanisms specifically designed to detect intracellular pathogens such as intracellular pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs), and to destroy such pathogens, such as T cell and NK cell mediated killing of infected cells, oxygen and nitrogen radicals, limitation of nutrients such as iron and tryptophan, and GTPases that can destroy the vacuoles within which these pathogens live and the membranes of the pathogens themselves.

Pathogens have in turn evolved virulence factors and mechanisms to evade multiple aspects of host-mediated killing. However, obligate intracellular pathogens also rely on their hosts for survival and it is not advantageous for the pathogen to kill its host before it is transmitted to a new one. Therefore, this interplay between the pathogen and the host is more of a balance than a battle, and successful pathogens might need to evade some host immune responses while actually inducing other immune responses. It is also likely that different pathogens and strains of pathogens are specifically adapted to different hosts and also that different hosts have adapted immune mechanisms against their own natural pathogens.

A major question that then arises is, why do different hosts have different responses to different intracellular infections and what host and pathogen factors account for these differences?

Toxoplasma gondii as a model intracellular eukaryotic pathogen

Much is known about how viruses and bacteria evade host immune responses and cause disease, but how eukaryotic pathogens modulate these responses, and what genetic factors lead to differences in how different strains of eukaryotic pathogens cause disease, is less well-studied.

Toxoplasma gondii is an obligate intracellular protozoan parasite that belongs to the same phylum Apicomplexa, *Plasmodium*, the causative agent of malaria, and it serves as an excellent model intracellular pathogen. The Apicomplexa are named for a complex at their apical end which is involved in the invasion of these parasites into host cells. Within its hosts, the parasite can infect any nucleated cell, and it can be grown easily in the lab within tissue culture cells and is therefore amenable to a variety of assays to study how it interact with host cells. With respect to questions regarding discovering host and pathogen factors that account for differences in the outcome of infection, it is an especially useful model. Mice are a natural host and mouse infection models have been widely studied, utilizing different infection routes (intraperitoneal injection or oral ingestion) as well as different mouse strains and mouse gene knockouts. The *Toxoplasma* genome has been sequenced and is ~65 Mb, consisting of 14 chromosomes and ~8000 genes (Gajria et al., 2008; Khan et al., 2005). Great genetic methods have been developed for *Toxoplasma* and it is relatively easy to ectopically express genes, knockout genes, and chemically mutagenize the parasite genome.

The natural life cycle of *Toxoplasma* is composed of a definitive host (the felines) and intermediate hosts (all warm-blooded animals, including mammals and birds) (Dubey, 2008; Wendte et al., 2011) (Fig. 1). After a cat eats an infectious stage of the parasite, within the gut of the cat haploid *Toxoplasma* parasites differentiate into micro- and macro-gametes and mate to form diploid oocysts. These oocysts are then shed into the environment where they undergo meiosis to form haploid sporozoites. Oocysts are orally infectious and lie dormant in the environment until they are consumed by an intermediate host or another cat. If ingested by an intermediate host, these sporozoites differentiate to a fast-growing life stage of the parasite, the tachyzoite, which can disseminate throughout the host. Intermediate hosts can pass this acute infection onto their progeny by congenital transmission. After the host mounts an immune response to combat this acute infection, these tachyzoites convert into slow-growing bradyzoites which are present during the chronic stage in tissue cysts. This encysted stage mainly forms in

the muscle and brain tissue of the host, and is crucial for its life cycle, as it is the other orally infectious stage of the parasite besides the oocyst. Both new intermediate hosts and cats can be infected by consumption of an intermediate host carrying these infectious tissue cysts. In order to form this chronic stage, *Toxoplasma* must establish a persistent infection, and therefore the balance between the pathogen and the host is especially important for this pathogen.

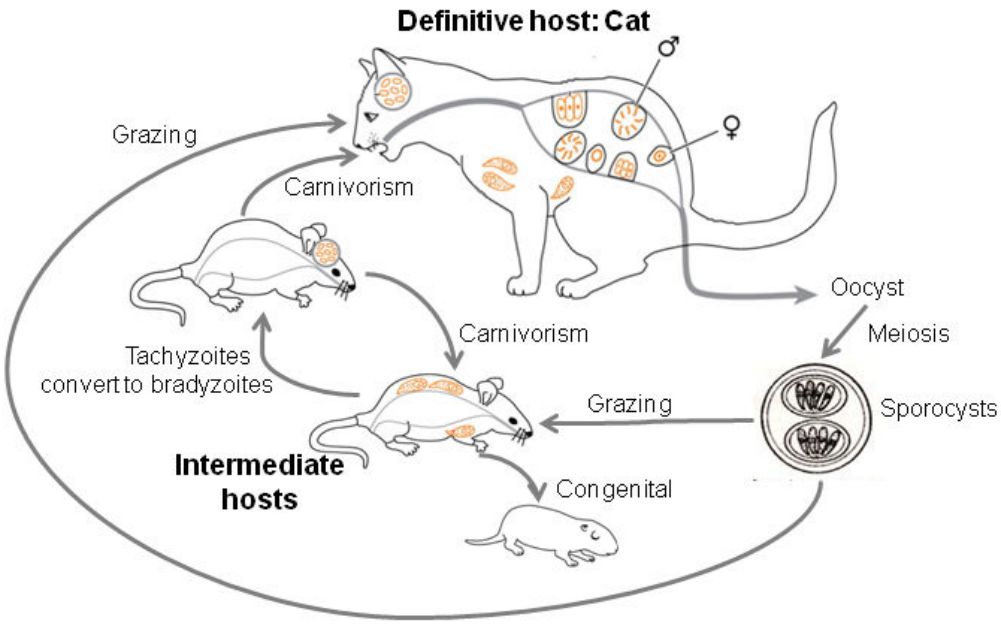

Figure 1. Life cycle of *Toxoplasma gondii*. Adapted from (Sibley and Ajioka, 2008).

Toxoplasma infection rates in humans vary greatly around the world, with ~60% seroprevalence in South America, ~20-30% in Europe and Asia, and ~10% in the United States (Pappas et al., 2009). Less data exists on infection rates in Africa, different studies have calculated seroprevalence rates between 20 and 80%, depending on the country (Pappas et al., 2009). Most human infections are asymptomatic chronic infections, however, *Toxoplasma* is an important opportunistic pathogen for immunosuppressed individuals and causes birth defects in developing fetuses upon congenital transmission (Montoya and Liesenfeld, 2004). Additionally, reactivation of dormant *Toxoplasma* infections in the brain can lead to retinal scarring and blindness and new highly virulent strains of *Toxoplasma* are arising in certain regions of the world such as South America that can cause disease even in immuno-competent individuals (Carme et al., 2009).

Many different *Toxoplasma* strains exist and the genomes and transcriptomes of many of these strains have been sequenced (Gajria et al., 2008; Minot et al., 2012). The population structure of *Toxoplasma* has been an area of active research. Originally, three major clonal lineages (types I, II, and III) were identified in North America and Europe on the basis of six restriction fragment length polymorphisms (RFLPs) (Howe and Sibley, 1995), and a fourth clonal lineage, haplogroup 12, was also later identified in North American wild animals (Khan et al., 2011). More recently, however, whole genome sequencing has shown that while clonal lineages do predominate in North America and Europe, large genetic diversity is the norm among strains in South America (Minot et al., 2012). These strains differ in many different phenotypes, for example, type I strains are categorically lethal in mice, with an $LD_{100} = 1$, while type II and type III infections are much less virulent ($LD_{50} \sim 10^2$ and $\sim 10^5$, respectively) (Saeij et al., 2006; Sibley and Boothroyd, 1992). Type II strains are also more commonly isolated from humans and associated with inflammatory disease while type III strains are more commonly isolated from wild animals (Boothroyd and Grigg, 2002; Dardé, 2004). These three types also differ in their ability to migrate *in vivo*, their attraction of different host cell types, and their induction of cytokine expression (Saeij et al., 2005a). Many of these strain differences are likely due to the adaptation of these different strains to different hosts with different immune systems, and parasite-induced pathology is likely caused by the infection of a host with a strain not adapted to that host's particular immune system.

Upon invasion of a host cell, *Toxoplasma* secretes an array of proteins into the cell from specialized secretory organelles: the micronemes, rhoptries, and dense granules (Fig. 2). Proteins from the micronemes and rhoptry neck are primarily involved in the formation of a moving junction through which *Toxoplasma* invades (Carruthers and Boothroyd, 2007). The parasite creates an invagination in the host plasma membrane, pulling it around itself to form a parasitophorous vacuole (PV) surrounded by a parasitophorous vacuole membrane (PVM) (Fig. 2) (Carruthers and Boothroyd, 2007). This vacuole does not enter endocytic or lysosomal pathways, as host proteins are excluded from the PVM during the invasion process (Mordue et al., 1999a, 1999b). During this process, rhoptry bulb contents are directly injected into the host cell, where they can traffic to cellular locations in the host cell such as the nucleus or back to the outside of the PVM (Boothroyd and Dubremetz, 2008), and some dense granule proteins are also

injected into the host cell at this time (Rosowski et al., 2011). However, there is evidence that dense granule proteins are also continually released into the PV (Dubremetz et al., 1993). Some of these dense granule proteins are then secreted into the host cell post-invasion through an unknown mechanism (Bougdour et al., 2013), likely involving protein export machinery similar to that used by malaria parasites upon infection of red blood cells (Haase and De Koning-Ward, 2010; Hsiao et al., 2013). *Toxoplasma* secreted rhoptry proteins affect a wide array of host cell processes, including JAK/STAT signaling (Denkers et al., 2012), MAPK signaling (Peixoto et al., 2010), and interfering with GTPase oligomerization and activity (Fleckenstein et al., 2012; Niedelman et al., 2012). Dense granule proteins can also be involved in remodeling the host cell, including recruiting endo-lysosomes to the vacuole (Coppens et al., 2006).

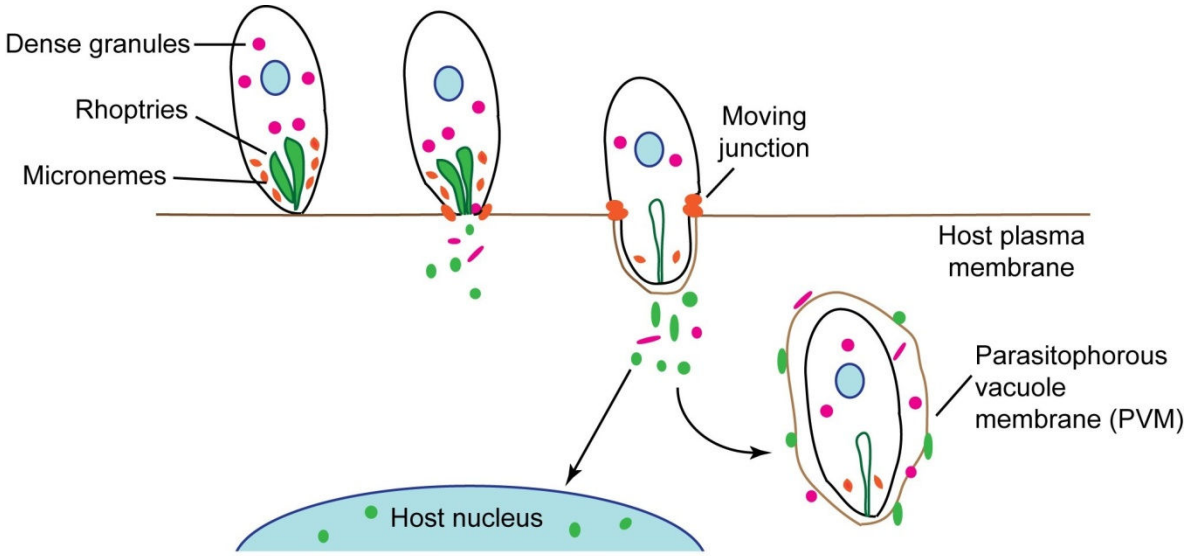

Figure 2. *Toxoplasma* invasion and protein secretion into the host cell.

The innate immune response to *Toxoplasma* infection in mice

The innate immune response to *Toxoplasma* infection in mice is comprised of two main phases (reviewed in (Denkers, 2003))(Fig. 3). First, the transcription factor nuclear factor-kappa B (NF-κB) is activated in cells such as macrophages and dendritic cells. Commonly, this activation is through the recognition of pathogen-associated molecular patterns (PAMPs) that activate Toll-like receptors (TLRs). The major TLR ligand in *Toxoplasma* is the protein profilin, which is recognized by both TLR11 and TLR 12 (Andrade et al., 2013; Koblansky et al., 2013;

Yarovinsky et al., 2005). *Toxoplasma* RNA and DNA can also activate TLR7 and TLR9 (Andrade et al., 2013) and glycosylphosphatidylinositols (GPIs) activate TLR2 and TLR4 (Debierre-Grockiego et al., 2007). The NF-κB family of transcription factors is comprised of five members which form hetero- and homo- dimers in the cell cytosol (Fig. 4). These factors are bound by inhibitor of kappa B (IκB) proteins, such as IκBα, which block nuclear localization signals of the NF-κB subunits. TLR activation, or activation of other receptors, leads to the phosphorylation of the inhibitor of kappa kinase (IKK) complex, which phosphorylates IκB proteins, marking them for ubiquitination and degradation. This frees NF-κB dimers to translocate to the nucleus and stimulate transcription of target genes (Fig. 4) (reviewed in (Vallabhapurapu and Karin, 2009)). The NF-κB dimer most commonly associated with transcriptional activation is the p65/p50 heterodimer (reviewed in (Vallabhapurapu and Karin, 2009)).

A major target gene of NF-κB p65/p50 dimers is the cytokine interleukin (IL)-12. This cytokine is produced in and secreted by macrophages and dendritic cells and it stimulates T cells and NK cells to produce the cytokine interferon-gamma (IFN-γ), beginning the second phase of the response (Fig. 3). IFN-γ receptors are expressed on the surface of many different cell types, and binding of IFN-γ leads to the phosphorylation of the transcription factor signal transducer and activator of transcription (STAT) 1 at tyrosine residue 701 (Fig. 5) (reviewed in (Platanias, 2005)). The tyrosine phosphorylated form of STAT1 can then dimerize and translocate to the nucleus, where STAT1 dimers bind gamma activated sequence (GAS) sites, and stimulate transcription of target genes. Initially STAT1 induces the expression of interferon regulatory factor (IRF) 1, another transcription factor. STAT1 and IRF1 together then induce both negative regulatory proteins, such as suppressor of cytokine signaling (SOCS) proteins, to downregulate the pathway and prevent excessive inflammation, and a gene expression program which restricts the growth of many pathogens, including *Toxoplasma* (reviewed in (Saha et al., 2010)). One set of genes upregulated by IFN-γ are genes encoding MHC molecules, which allow cells to present more antigen to and activate T cells. IFN-γ-inducible GTPases are also expressed, including the immunity-related p47 GTPases (IRGs) and p65 guanylate binding proteins (GBPs). The IRGs can coat *Toxoplasma* PVs and function as dynamins to strip both PV and parasite membranes (Ling et al., 2006; Martens et al., 2005), and the GBPs are required for the proper recruitment of

IRGs to the *Toxoplasma* PV (Yamamoto et al., 2012). Both the IFN-γ-induced production of nitric oxide (NO), through the upregulation of inducible nitric oxide synthase (iNOS/Nos2) (Khan et al., 1997), and degradation of tryptophan, through indoleamine 2,3-dioxygenase (IDO1) (Dai et al., 1994; Habara-Ohkubo et al., 1993; Pfefferkorn, 1984), also inhibit the growth/survival of intracellular pathogens such as *Toxoplasma*.

This innate response is crucial for mouse survival upon *Toxoplasma* infection. Mice lacking certain single NF-κB subunits are more susceptible to both the acute and chronic phase of *Toxoplasma* infection (Mason et al., 2004), and mice deficient in TLR11/12, IL-12, IFN-γ, STAT1, and IRF1 are all acutely susceptible to *Toxoplasma* infection (Gazzinelli et al., 1994; Koblansky et al., 2013; Lieberman et al., 2004; Silva et al., 2002; Suzuki et al., 1988).

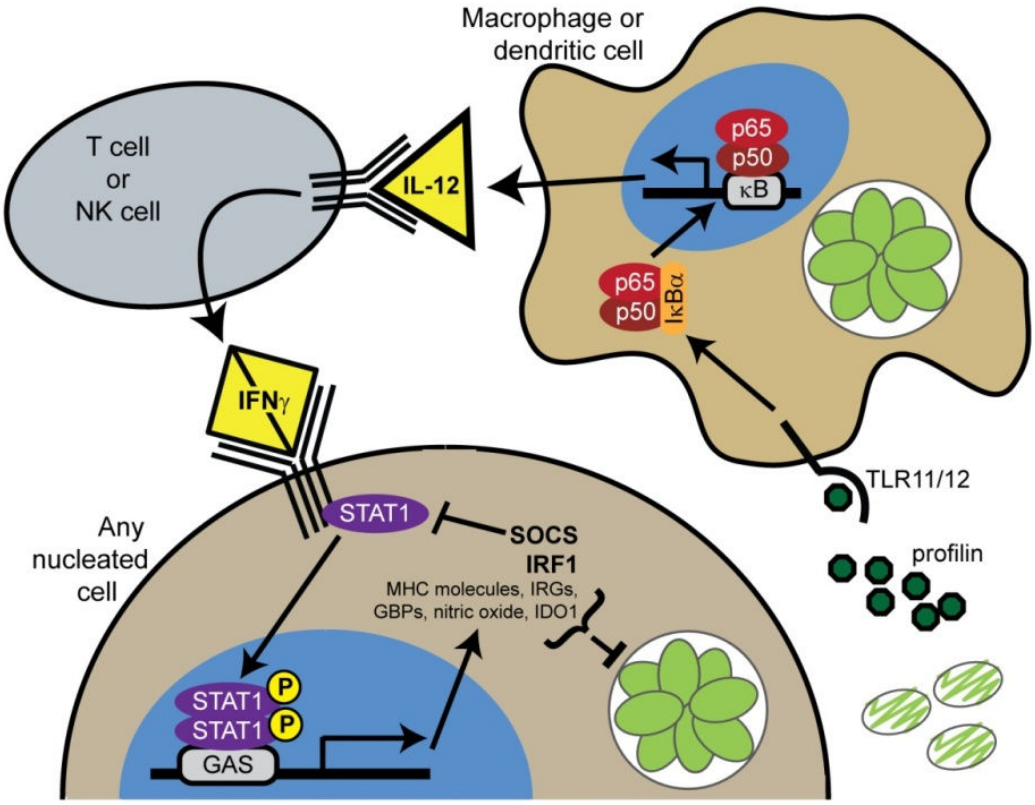

Figure 3. Innate immune response to *Toxoplasma*

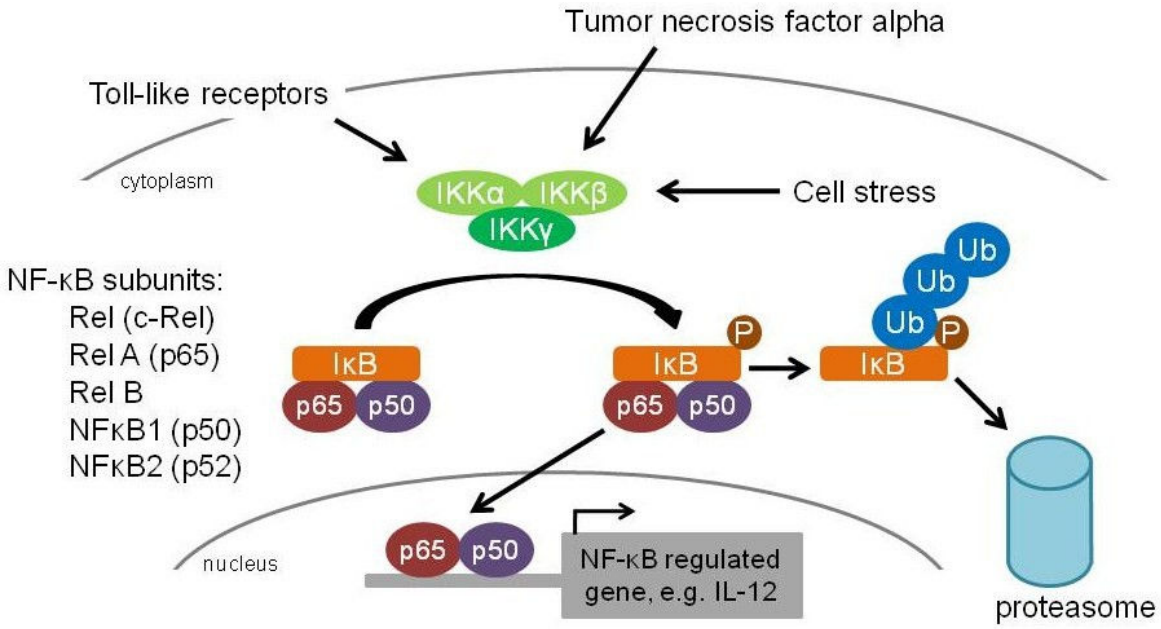

Figure 4. NF-κB signaling.

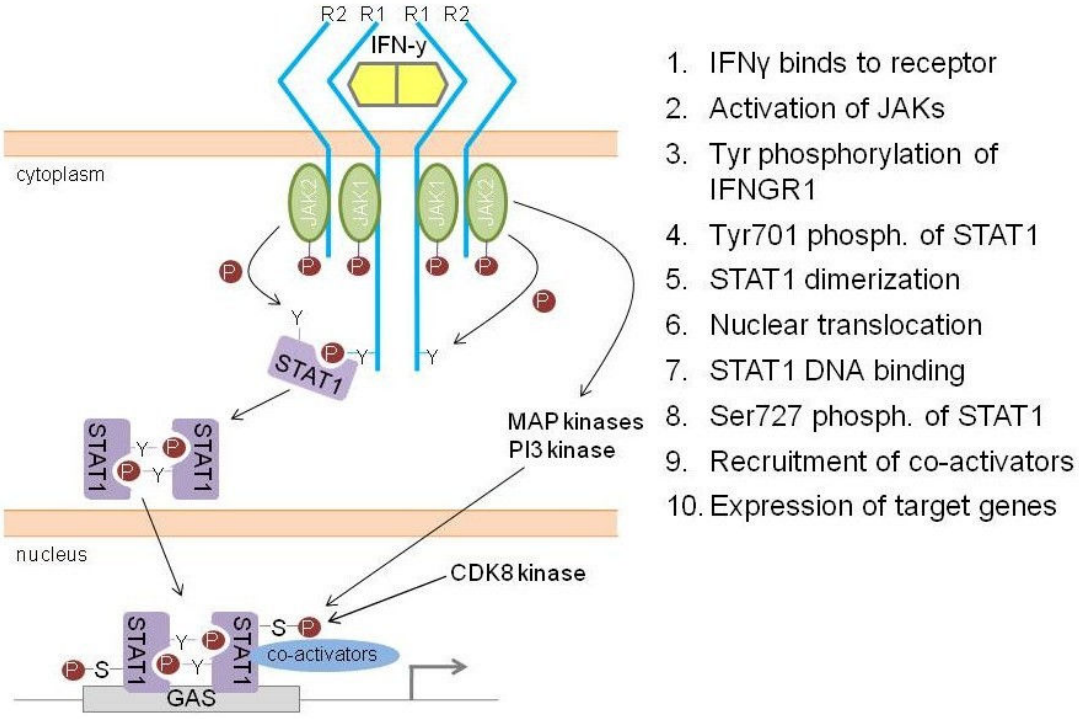

Figure 5. IFN-γ/STAT1 signaling

Strain differences in how *Toxoplasma* evades and modulates the immune response

The major hypothesis driving my thesis work is that the ability of *Toxoplasma* (and intracellular pathogens in general) to modulate host cell innate immune signaling pathways determines its ability to evade the host innate immune response and thus its success as a pathogen and its ability to live within and disseminate between hosts. A corollary of this hypothesis is that some differences between *Toxoplasma* strains in their ability to cause disease are due to differences in how these strains modulate host cell innate immune responses. At the beginning of this work this hypothesis had begun to be tested in experiments using an array of F1 progeny from crosses between the type I, II, and III clonal strains of *Toxoplasma*.

In two different studies, mice were infected with F1 progeny from either a I x III or a II x III cross, and mouse survival, among other phenotypes, was measured (Saeij et al., 2006; Taylor et al., 2006). Quantitative trait locus (QTL) mapping was then used to find regions of the *Toxoplasma* genome that were polymorphic between the strains and significantly associated with infection outcome. Both of these studies identified the polymorphic rhoptry kinase ROP18 as a significant predictor for the ability of a *Toxoplasma* strain to kill a mouse. Later, the rhoptry pseudokinase ROP5 was also identified from this same data (Reese et al., 2011). ROP18 and ROP5 cooperatively inhibit the activity of the IFN-γ induced IRGs (Behnke et al., 2012; Fleckenstein et al., 2012; Niedelman et al., 2012), demonstrating that the modulation of host cell processes and immune mechanisms by *Toxoplasma* does affect infection outcome and that these strains vary in how they modulate these processes.

However, these *Toxoplasma* virulence factors directly interact with specific host cell effector mechanisms, and it was still unclear whether strain-specific modulation of host cell transcription and transcription factors by *Toxoplasma* occurs and whether this modulation could affect disease outcome. Therefore, in another study, host gene expression was analyzed by microarrays upon infection of human fibroblasts with type I, II, or III strains *in vitro*, and indeed, subsets of host genes were found to be strain-specifically induced or inhibited by infection (Saeij et al., 2007). When these infections and microarray analyses were done with F1 progeny from a II x III cross, QTL mapping revealed that much of this strain-specific host cell gene expression is due to modulation of STAT3 and STAT6 transcription factor activity by another *Toxoplasma* rhoptry kinase, ROP16 (Saeij et al., 2007). ROP16 affects cytokine secretion and mouse survival

(Saeij et al., 2006, 2007), demonstrating that the modulation of signaling pathways by *Toxoplasma* can affect infection and disease outcome.

Summary

In the first third of my thesis work, I further analyzed these previously performed microarray analyses, focusing on host genes that were strain-specifically modulated by *Toxoplasma* infection, but whose expression was not dependent on the ROP16 effector. In the initial infection of HFFs with type I, II, and III parasites, we noticed that a subset of host genes was specifically induced by type II infection, and analysis of transcription factor binding sites in the promoters of these genes revealed that they were controlled by the NF-κB transcription factor. Therefore, we used F1 progeny from II x III crosses and QTL mapping to locate a genomic region responsible for NF-κB activation in type II strains. We then used a candidate gene approach to discover a novel dense granule protein, GRA15, responsible for this phenotype. A type II copy of GRA15 activates NF-κB p65 nuclear translocation, NF-κB-mediated transcription, and IL-12 secretion. While GRA15 affects parasite growth *in vivo* early after infection it does not affect overall mouse survival, probably because NF-κB also can be activated via many other pathways *in vivo*, including by the TLRs.

I then became interested in the second phase of the murine innate immune response to *Toxoplasma*, the IFN-γ response. Some strains of *Toxoplasma* can directly evade specific IFN-γ induced effector killing mechanisms such as the IRGs (Zhao et al., 2009), however, it has been known since 1998 that *Toxoplasma* infection can block the expression of IFN-γ induced MHC class II gene expression (Lüder et al., 1998), and this has been extended to the expression of other IFN-γ induced genes such as IRF1 and class II transactivator (CIITA) (Lüder et al., 2001). In fact, infection can dysregulate IFN-γ induced gene expression globally (Kim et al., 2007a). In studying the inhibition of this response, we had two major questions: 1) are there any differences in how type I, II, and III *Toxoplasma* strains modulate this response and 2) what is the mechanism of this inhibition? In my research I have found that while type I, II, and III strains do differentially modulate specific aspects of this pathway such as STAT1 phosphorylation and IRF1 expression through the polymorphic effectors ROP16 and GRA15, globally all of these strains equally inhibit STAT1 transcriptional activity and IFN-γ induced gene expression. I then

focused on the mechanism of this inhibition, measuring each phase of STAT1 activation to narrow down the exact step at which *Toxoplasma* acts. I found that this inhibition happens downstream of STAT1 binding to DNA and that it is not dependent on histone deacetylases. Additionally, this inhibition affects STAT1 in IFN-β induced interferon stimulated gene factor (ISGF) 3 complexes. The *Toxoplasma* effector responsible for this inhibition is still unknown, but I determined that it is not secreted into the host cell upon invasion.

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Chapter 2. Strain-specific activation of the NF-κB pathway by GRA15, a novel *Toxoplasma* gondii dense granule protein

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Abstract

NF-κB is an integral component of the immune response to *Toxoplasma.* While evidence exists that *Toxoplasma* can directly modulate the NF-κB pathway, the parasite-derived effector(s) involved are unknown. We determined that type II strains of *Toxoplasma* activate more NF-κB than type I or type III strains, and using forward genetics we found that this difference is due to the polymorphic protein GRA15, a novel dense granule protein that *Toxoplasma* secretes into the host cell upon invasion. A GRA15-deficient type II strain has a severe defect in both NF-κB nuclear translocation and NF-κB-mediated transcription. Further, human cells expressing type II GRA15 also activate NF-κB, demonstrating that GRA15 alone is sufficient for NF-κB activation. Along with the rhoptry protein ROP16, GRA15 is responsible for a large part of the strain differences in the induction of IL-12 secretion by infected mouse macrophages. *In vivo* bioluminescent imaging showed that a GRA15-deficient type II strain grows faster compared to wild-type, most likely through its reduced induction of interferon-gamma (IFN-γ). These results show for the first time that a dense granule protein can modulate host signaling pathways, and dense granule proteins can therefore join rhoptry proteins in *Toxoplasma*'s host cell-modifying arsenal.

Introduction

Toxoplasma gondii is an obligate intracellular parasite capable of infecting a wide range of warm-blooded hosts, including humans. *Toxoplasma* establishes a life-long chronic infection in the host by evading and subverting the immune system. *Toxoplasma* infection is usually asymptomatic in healthy humans, but can lead to flu-like and neurological symptoms in immunosuppressed patients and the fetuses of pregnant women infected for the first time. The vast majority of *Toxoplasma* strains isolated from Europe and North America belong to three clonal lineages, types I, II, and III, which differ in many phenotypes, including virulence (Saeij et al., 2005a). In mice, type I strains are categorically lethal, with an $LD_{100} = 1$, while type II or type III infections are not ($LD_{50} \sim 10^2$ and $\sim 10^5$, respectively) (Saeij et al., 2006; Sibley and Boothroyd, 1992). Strain differences in the modulation of host immune signaling pathways are one way by which this diversity arises. For example, the strain-specific modulation of the STAT3/6 signaling pathway by the secreted kinase ROP16 accounts for some of the strain

differences in virulence (Saeij et al., 2006). Evidence also exists for the strain-specific modulation of NF-κB (Robben et al., 2004), an important host signaling pathway in the regulation of inflammatory, immune, and anti-apoptotic responses.

The NF-κB family of transcription factors is composed of five members: p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RelB and c-Rel (Chap. 1 Fig. 4) (reviewed in (Vallabhapurapu and Karin, 2009)). In unstimulated cells, homo- or hetero-dimers of NF-κB are sequestered in the cytoplasm by members of the IκB (inhibitor of κB) family. Activation of NF-κB is initiated by the degradation of IκB proteins. This occurs via the activation of kinases called IκB kinases (IKKs), which phosphorylate two serine residues located in IκB regulatory domains, leading to their ubiquitination and subsequent proteasomal degradation. The NF-κB complex is then free to enter the nucleus where it can induce expression of specific genes that have NF-κB-binding sites in their promoters.

Many pathogens have developed strategies to modulate the host NF-κB pathway (reviewed in (Tato and Hunter, 2002)). Several bacteria and viruses inhibit NF-κB activation and its resultant recruitment and activation of immune cells, resulting in enhanced survival of the pathogen. Others pathogens induce NF-κB activation, which inhibits apoptosis, an important defense against intracellular pathogens, and increases cell migration, thereby recruiting new cells to infect. Further, NF-κB-mediated inflammation leads to tissue damage, allowing pathogens to cross tissue barriers. Thus, depending on the host, pathogen, and site of infection, an active NF-κB pathway can benefit either the host or the pathogen.

Mice deficient in some NF-κB family members have increased susceptibility to *Toxoplasma*, indicating the importance of this pathway in pathogen resistance (Mason et al., 2004). *C-Rel*$^{-/-}$ mice are highly susceptible to the acute stage of *Toxoplasma* intraperitoneal (i.p.) infection which can be rescued solely by treatment with IL-12, indicating that a major role of NF-κB in resistance to *Toxoplasma* is the induction of IL-12 secretion (Mason et al., 2004). IL-12 is a major mediator of the pro-inflammatory Th1 response development, and the major cause of chronic phase death in mice lacking RelB, p52, or the IκB protein Bcl-3 is also a deficient T cell response (Mason et al., 2004). While it is clear that the NF-κB pathway is important for an adequate response to *Toxoplasma* infection, the mice used in these studies all lack a particular

NF-κB subunit in every cell of their bodies, and it is currently unknown what the role of NF-κB is in specific cell types, such as those directly infected with *Toxoplasma*.

Evidence currently exists for both inhibition and activation of the NF-κB pathway in host cells by *Toxoplasma*. Less than six hours after infection, a type I strain was shown to block the nuclear translocation of p65 and the *in vitro* binding of NF-κB subunits to DNA (Butcher et al., 2001; Kim et al., 2004; Shapira et al., 2002). Induction of IL-12 in response to tumor necrosis factor-alpha (TNF-α) or lipopolysaccharide (LPS) stimulation was also reduced (Butcher et al., 2001; Kim et al., 2004). This inhibition of the NF-κB pathway was dependent on active invasion by live parasites (Butcher and Denkers, 2002). After more than six hours of infection, inhibition of p65 nuclear translocation and *in vitro* DNA binding was no longer observed (Kim et al., 2004; Leng et al., 2009). However, chromatin immunoprecipitation experiments showed that the *in vivo* binding of p65 to the TNF-α promoter was blocked even at late time points (Leng et al., 2009). Other groups, however, have shown that NF-κB is activated by a type I strain of *Toxoplasma*, and that this activation is necessary for the inhibition of apoptosis (Molestina and Sinai, 2005a; Molestina et al., 2003; Payne et al., 2003). A consistent observation has been the phosphorylation and ubiquitination of IκBα upon *Toxoplasma* infection, though it is unclear what effect this has on the nuclear translocation of NF-κB and transcription of downstream genes (Butcher et al., 2001; Molestina et al., 2003; Shapira et al., 2005). All of these studies used a type I strain of *Toxoplasma*, suggesting that different observations might be due to different cell types and/or host species. Strain differences have been observed in the manipulation of the host NF-κB pathway by *Toxoplasma*. Type II strains were shown to cause the translocation of NF-κB to the nucleus of mouse splenocytes and mouse bone marrow-derived macrophages (BMDM), while type I strains did not (Dobbin et al., 2002; Robben et al., 2004). This strain difference was also shown to have downstream effects, as infection of BMDM with type II parasites resulted in high levels of IL-12 secretion compared to infection with type I parasites (Robben et al., 2004). At present, the *Toxoplasma* factor(s) involved in the modulation of the NF-κB pathway is not known.

In our experiments, infection with type II strains induces a high level of NF-κB activation, while infection with type I or III strains does not. Using F1 progeny from a type II x type III cross we identify a type II gene responsible for NF-κB activation, *GRA15*. The protein

product of *GRA15* is a novel dense granule protein that is necessary and sufficient for p65 nuclear translocation and NF-κB mediated host cell transcription. We show that GRA15 activates the NF-κB pathway independent of MyD88 and TRIF but dependent on TRAF6 and the IKK complex. While GRA15 does not affect overall virulence of parasites, it does have more subtle phenotypes *in vivo*, affecting both parasite growth and cytokine levels.

Results

Host gene expression analysis shows strain-specific activation of the NF-κB pathway

We previously generated a large gene expression dataset from human foreskin fibroblasts (HFFs) infected with type I, II or III *Toxoplasma* strains (Saeij et al., 2007). In our dataset analysis, we focused on the human genes that were differentially regulated by type II strain infection because published data indicated that type II strains might induce more NF-κB nuclear translocation compared to type I strains (Dobbin et al., 2002; Robben et al., 2004). If *Toxoplasma* strains differ in the activation of the NF-κB pathway, this should lead to differences in expression of genes with NF-κB transcription factor binding sites (TFBS) in their promoters. 105 genes were found to be more than 2-fold upregulated in type II infections compared to type I and type III infections (Fig. S1A). Analysis of TFBS in the regulatory elements of these genes revealed enrichment of NF-κB TFBS in their promoters, and a network analysis of molecular relationships between the products of these 105 genes resulted in high scores for two networks whose central factors were the transcription factor NF-κB (network 1) and IL1β/PTGS2(COX-2) (network 2) (Fig. S1B). IL1β and COX-2 are also regulated by NF-κB (Newton et al., 1997; Vallabhapurapu and Karin, 2009). These data suggest that there is at least one polymorphic locus between type II and type I/III strains that modulates the NF-κB pathway.

We also looked for polymorphic loci between type II and type III strains that modulate host gene expression using quantitative trait locus (QTL) analysis of human gene expression levels of cells infected with 19 different F1 progeny from II x III crosses. We identified 3,188 human cDNAs that were regulated by a specific *Toxoplasma* genomic locus (Saeij et al., 2007). 1,176 of these human cDNAs were regulated by a locus on chromosome VIIb. The *Toxoplasma* polymorphic ROP16 kinase resides on this chromosome and, via its strain-specific activation of STAT3/6, is responsible for the differential expression of many of the genes that are regulated by

a chromosome VIIb locus (Saeij et al., 2007). Loci on chromosome X also influenced the expression of 563 human cDNAs. To discover if these cDNAs are regulated by a common transcription factor, we determined if any TFBS were enriched in the promoters of genes that are differentially modulated by F1 progeny with a type II allele versus a type III allele at each chromosome X marker. At many markers, the expression of genes with NF-κB TFBS in their promoters was enriched in F1 progeny with a type II genotype, suggesting that a *Toxoplasma* factor responsible for strain differences in NF-κB activation resides on chromosome X (Fig. S1C). Additionally, network analysis of molecular relationships between the 563 genes that were significantly influenced by a chromosome X locus and their gene products resulted in high scores for two networks, one of which had the transcription factor NF-κB as its central factor (Fig. S1C). We therefore hypothesized that a polymorphic *Toxoplasma* locus on chromosome X contributes to differential regulation of the host NF-κB pathway by type II and type I/III strains.

Toxoplasma type II parasites activate NF-κB

To investigate modulation of the NF-κB pathway by *Toxoplasma gondii*, we infected human foreskin fibroblasts (HFFs) with type I, II or III *Toxoplasma* strains for 1-24 hours, and measured nuclear translocation of the NF-κB p65 subunit by immunofluorescence (IF). Starting after four hours of infection and continuing until at least 24 hours of infection, many cells infected with a type II strain contained high levels of p65 in their nucleus, while a type I or a type III strain did not induce translocation of high levels of p65 to the nucleus (Fig. 1A and B, data not shown). We have observed the translocation of p65 by infection with various type II strains, including ME49, Pru, DAG, or Beverley, and the absence of high levels of p65 translocation after infection with both RH or GT1 type I strains and CEP or VEG type III strains. Uninfected HFFs surrounding infected HFFs did not contain increased p65 in the nucleus, indicating that activation is not caused by a secreted host factor or a contaminant in the medium. Activation of p65 translocation after infection with type II strains was not inhibited by previous infection with type I or type III strains, demonstrating that this translocation is due to specific activation by type II parasites rather than inhibition by type I/III parasites (Fig. 1C and D, data not shown). We have observed the activation of NF-κB p65 nuclear translocation by type II

parasites in 293T cells, HeLa cells, mouse BMDM, RAW264.7 murine macrophages, mouse embryonic fibroblasts (MEFs), and rat embryonic fibroblasts (REFs) (Fig. 3, data not shown).

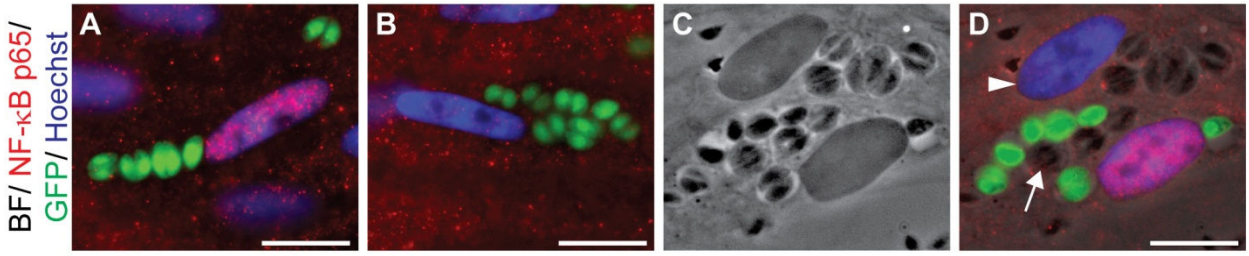


Figure 1. *Toxoplasma* strains differ in the activation of NF-κB. HFFs were infected with *Toxoplasma* strains for 18-24 hours, fixed, and stained with α-NF-κB p65 (red) and Hoechst dye (blue). Infection with **A.** a type II (GFP) strain; or **B.** a type III (GFP) strain; **C, D.** HFFs co-infected with a type I (non-GFP, arrow) and a type II (GFP) strain of *Toxoplasma* (C, brightfield; D, immunofluorescence). Cells infected with only type I parasites (arrowhead) do not contain nuclear NF-κB. This experiment has been repeated more than 10 times with similar results. Scale bar represents 5 μm.

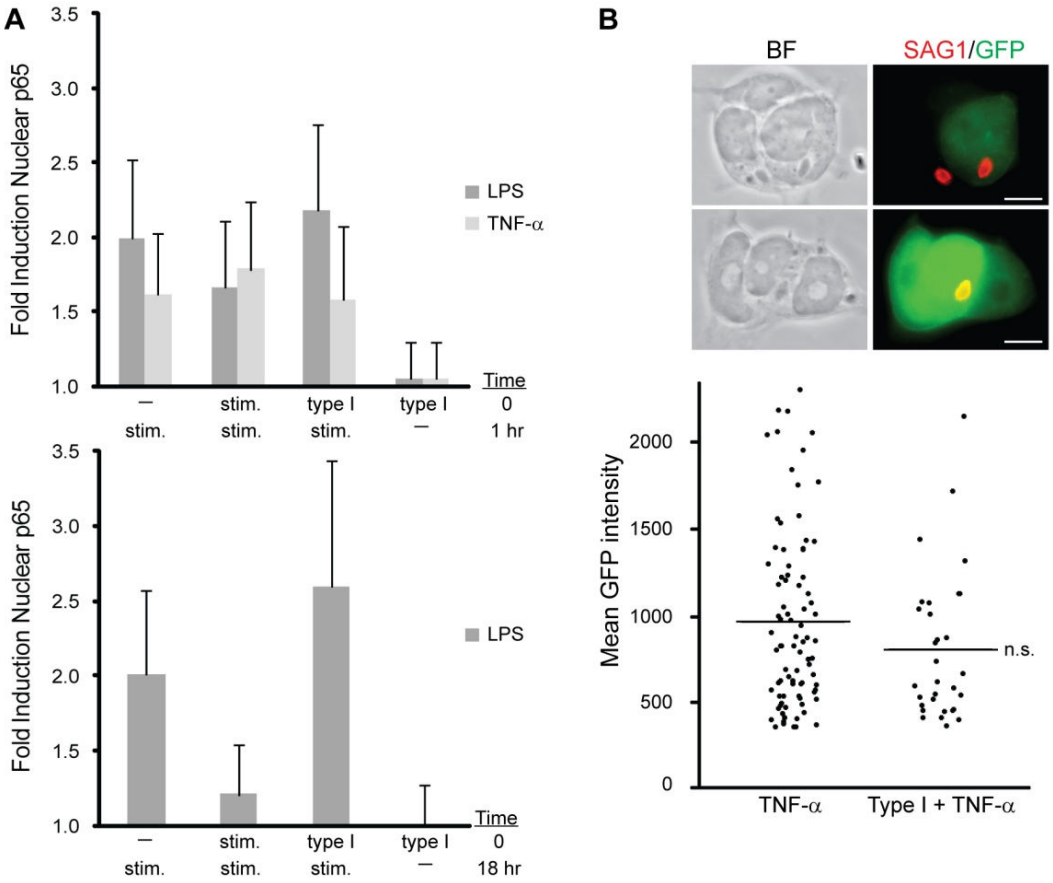
Type I parasites do not inhibit NF-κB activation

While we observed that a type I strain does not inhibit p65 nuclear translocation in a co-infection with a type II strain, previous reports have shown that in mouse macrophages infection with type I parasites can inhibit the activation of NF-κB in response to LPS or TNF-α (Butcher et al., 2001; Kim et al., 2004; Shapira et al., 2002). To further investigate if type I parasites can inhibit NF-κB translocation, we infected BMDM with type I parasites for 1 or 18 hours, stimulated the cells with LPS or TNF-α, and measured the translocation of p65 to the nucleus by IF (Fig. 2A). In uninfected cells, both LPS and TNF-α stimulation induced the translocation of p65 subunits to the nucleus. Pre-stimulation with LPS inhibited later LPS-induced translocation, as the activation of Toll-like receptor pathways induces negative feedback mechanisms to inhibit further signaling (Lang and Mansell, 2007; Wang et al., 2009). However, pre-infection with type I parasites did not inhibit LPS or TNF-α stimulated translocation, at early or late time points after infection. In fact, pre-infection with *Toxoplasma* led to higher levels of p65 translocation after LPS stimulation, perhaps due to increased TLR4 expression (Kim et al., 2004).

To test whether TNF-α stimulated NF-κB-mediated transcription can be inhibited by type I parasites, we used a HEK293 NF-κB reporter cell line which expresses GFP upon NF-κB activation. Infection of this reporter cell line with type II parasites results in high levels of GFP

in infected cells (data not shown). We added type I parasites to these cells for 45 minutes, stimulated the cells with TNF-α, and measured GFP levels of infected and uninfected cells by microscopy (Fig. 2B). After four hours of stimulation, both infected and uninfected cells had varying levels of GFP, with some cells containing almost no GFP and < 50% of cells having a high level of GFP. However, the distribution of GFP intensity in the populations of infected and uninfected cells was not significantly different. Unstimulated cells, either infected or uninfected, had negligible levels of GFP (data not shown). Type I parasites also did not inhibit NF-κB-mediated transcription of luciferase in a HEK293 NF-κB luciferase reporter cell line (data not shown).

To investigate if type I pre-infection might inhibit transcription of specific subsets of NF-κB regulated genes, we infected HFFs with a type I strain for 18 hours or left cells uninfected and subsequently stimulated the cells with TNF-α for six hours. We then performed gene expression analysis using Affymetrix microarrays. Comparing the expression data to uninfected HFF expression data, the genes regulated by TNF-α stimulation alone and type I pre-infection followed by TNF-α stimulation were not identical, however, genes with NF-κB TFBS in their promoters or belonging to an NF-κB related pathway were equally enriched in those two samples (Fig. 2C). We therefore conclude that type I parasites do not inhibit TNF-α or LPS stimulated NF-κB p65 translocation or TNF-α stimulated NF-κB-mediated transcription, but they may be able to modulate other pathways or host cell transcription factors which are important for expression of a small subset of TNF-α stimulated genes.



C

	FDR q-value	
Transcription Factor Binding Sites	**TNF-α**	**Type I + TNF-α**
GGGNNTTTCC_V$NFKB_Q6_01	0.0134	0.0033
NFKAPPAB_01	0.0174	0.0026
NFKB_Q6_01	0.0199	0.0152
CREL_01	0.0200	0.0907
NFKB_Q6	0.0213	0.0229
NFKAPPAB65_01	0.0236	0.1224
NFKB_C	0.0627	0.0041

Canonical Pathways	**TNF-α**	**Type I + TNF-α**
ST_TUMOR_NECROSIS_FACTOR_PATHWAY	0.0052	0.0025
TNFR2PATHWAY	0.0079	0.0473
TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY	0.0000	0.0056
CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	0.0000	0.0000
APOPTOSIS	0.0000	0.0060

Figure 2. Type I parasites do not inhibit NF-κB activation. A. C57BL/6 BMDM were untreated, stimulated with 100 ng/ml LPS or 20 ng/ml mouse TNF-α, or infected with type I parasites (MOI = 2) for one hour (top) or 18 hours (bottom). Cells were then re-stimulated for 30 min with LPS or 45 min with TNF-α, fixed, and stained with α-NF-κB p65 and Hoechst dye. The intensity of nuclear NF-κB p65 was quantitated in at least ten cells per treatment. Values represent the fold induction of nuclear p65 levels over uninfected, unstimulated cells. This experiment was done once in BMDM. A second experiment of HFFs infected with type I parasites and subsequently stimulated with TNF-α yielded similar results. **B.** A HEK293 NF-κB GFP reporter cell line was plated on coverslips and infected

with type I parasites (MOI = 1-2) for 45 min, then stimulated with 100 ng/ml human TNF-α for 4 hours, fixed, and stained with α-SAG1 (red). The GFP intensity was quantitated for at least 30 infected cells and 30 uninfected cells (bottom) (n.s. = not significant, two sample t-test). Scale bar represents 10 μm. This experiment was performed twice, with the same qualitative results. Similar results were also obtained with a HEK293 NF-κB luciferase reporter cell line. **C.** Microarray analysis was done on HFFs pre-infected with a type I strain (MOI = 7.5) for 18 hours, or left uninfected, and subsequently stimulated with 20 ng/ml human TNF-α for 6 hours. Genes were pre-ranked for both samples by the difference in expression as compared to uninfected, untreated HFFs, and gene set enrichment analysis (GSEA) was used to determine whether genes with NF-κB TFBS in their promoters or belonging to NF-κB related canonical pathways were enriched in either or both samples. FDR q-values less than 0.25 were considered significant. One array per strain and treatment was done.

A Toxoplasma genomic locus on chromosome X mediates strain-specific activation of NF-κB

To find the *Toxoplasma* genomic region(s) mediating the type II versus type I/III strain-specific difference in activation of NF-κB, we infected HFFs with 27 F1 progeny derived from crosses between type II and type III strains and measured NF-κB activation by IF. Only HFFs infected with F1 progeny having type II alleles for the genetic markers *ROP2* and *GRA6* at the right end of *Toxoplasma* chromosome X contained nuclear NF-κB p65 (Fig. S2). Thus, the genomic region in the vicinity of genetic markers *ROP2*-*GRA6* harbors one or more genes involved in the activation of NF-κB. We developed new RFLP markers to more accurately define the place of recombination in progeny that are recombinant for chromosome X around *ROP2* and *GRA6* and therefore limit the genomic region involved in the activation of NF-κB and the number of possible candidate genes. With these new markers, genotyping of STE7 refined the 3' boundary of the region and genotyping of S26 refined the 5' boundary of the region (Fig. S2). The refined region between the markers *SAG2E* and *RC4* contains 45 predicted type II genes (ToxoDB.org, v6.0).

To identify the *Toxoplasma* gene responsible for NF-κB activation, we employed a candidate gene approach. From the 45 predicted type II genes, the *SAG2CDXY* (*SRS49a/b/c/d*) locus was excluded, as a type II strain with this locus deleted still activates NF-κB (Saeij et al., 2008), data not shown). Our first criterion for a protein able to interface with the host cell and modulate host cell signaling was the presence of a signal sequence. Of the 41 remaining genes, 17 are predicted to have a signal sequence. Of these 17 genes, four (*ROP8*, *ROP2A*, *GRA6* and *63.m00001*) were consistently expressed in tachyzoites in infected macrophage cells, as

determined by *Toxoplasma* microarrays (data not shown). Our top candidate genes (*ROP2A*, *GRA6* and *63.m00001*) were then tested by adding a type II C-terminal HA-tagged copy of the candidate gene, including at least 1500 bp of the putative endogenous promoter, into a type I and/or III strain and we assayed whether these transgenic type I/III strains activate NF-κB. Type I and III strains stably expressing a copy of *63.m00001* activated NF-κB in HFFs, whereas strains expressing a copy of *GRA6* or *ROP2* did not, indicating that *63.m00001*, hereafter referred to as *GRA15*, is the locus on chromosome X which mediates the strain-specific activation of NF-κB (Fig. 3 and Fig. S3).

GRA15 mediates NF-κB p65 translocation

We quantified the nuclear p65 immunofluorescence signal of cells infected with type I, II, or III strains, confirming that a subset of type II infected cells had a high level of nuclear p65 (intensity > 800), resulting in a significant difference in the average nuclear p65 of type II infected cells compared to type I or type III infected cells (Fig. 3A, B). Type I (but not type III) infected cells did have significantly more nuclear NF-κB p65 than uninfected cells. Type I and type III strains engineered to stably express a type II, HA-tagged, copy of *GRA15* (type I $GRA15_{II}$ and type III $GRA15_{II}$) activated NF-κB (Fig. 3A, B). As with type II infected cells, a subset of type I $GRA15_{II}$ infected cells had very high levels of nuclear NF-κB, and the average nuclear p65 was significantly higher in these cells compared to type I infected cells. A time course experiment showed that this activation occurred starting four hours after infection (Fig. S4).

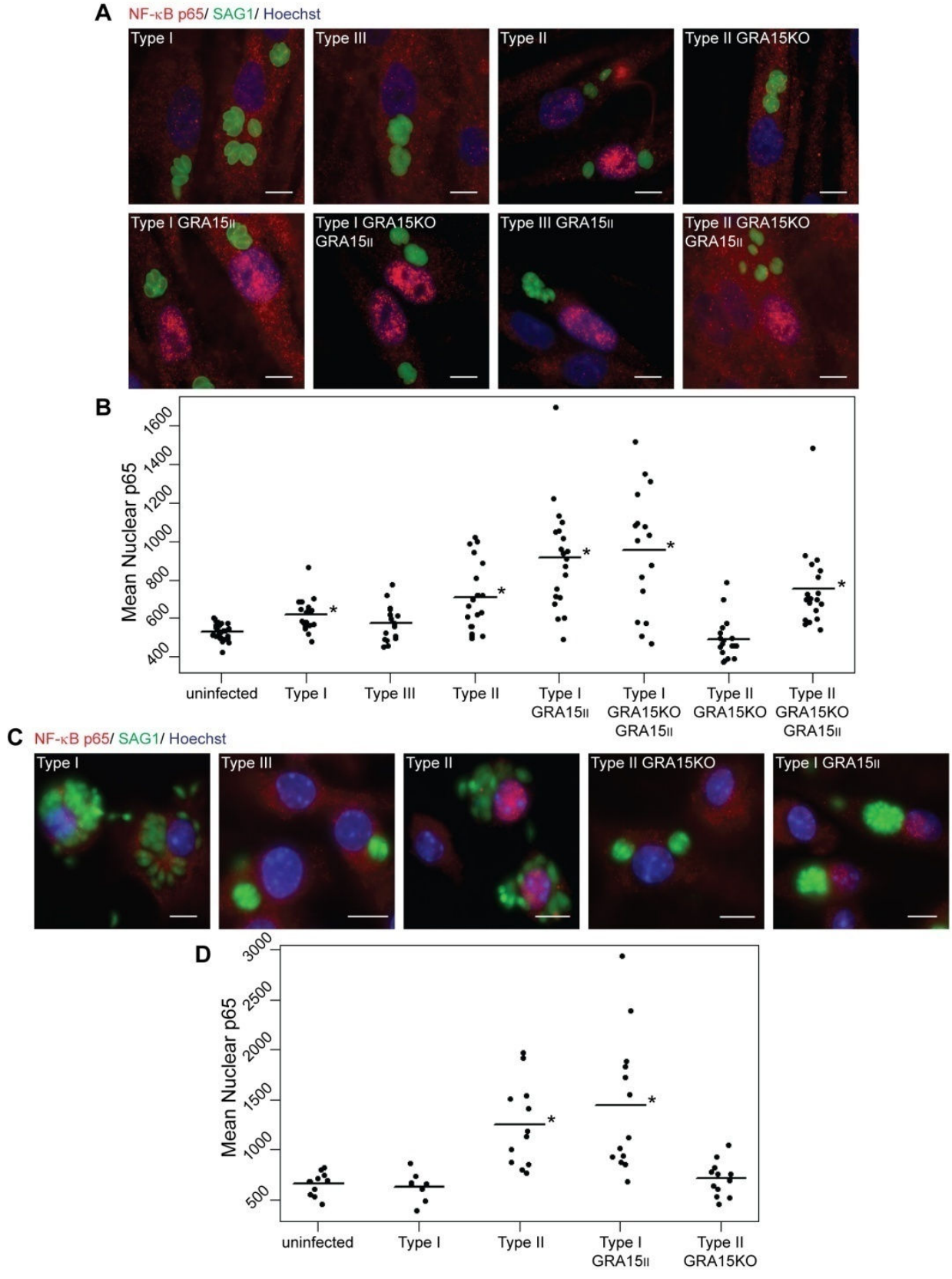

A
NF-κB p65/ SAG1/ Hoechst
Type I
Type III
Type II
Type II GRA15KO
Type I GRA15II
Type I GRA15KO GRA15II
Type III GRA15II
Type II GRA15KO GRA15II
B
Mean Nuclear p65
400
600
800
1000
1200
1400
1600
uninfected
Type I
Type III
Type II
Type I GRA15II
Type I GRA15KO GRA15II
Type II GRA15KO
Type II GRA15KO GRA15II
C
NF-κB p65/ SAG1/ Hoechst
Type I
Type III
Type II
Type II GRA15KO
Type I GRA15II
D
Mean Nuclear p65
500
1000
1500
2000
2500
3000
uninfected
Type I
Type II
Type I GRA15II
Type II GRA15KO

Figure 3. GRA15 mediates NF-κB p65 translocation. A. HFFs were infected with *Toxoplasma* strains for 18 hours, fixed, and stained with α-NF-κB p65 (red), α-SAG1 (green), and Hoechst dye (blue). Scale bar represents 10 μm. **B.** The amount of p65 in the nucleus was quantitated in at least 15 HFF cells for each strain. Asterix indicates significantly higher levels of nuclear p65 compared to uninfected cells (*$p < 0.0001$, two sample t-test). **C.** Mouse BMDM (BALB/c) were infected with *Toxoplasma* strains for 24 hours, fixed, and stained with α-NF-κB p65 (red), α-SAG1 (green), and Hoechst dye (blue). Scale bar represents 10 μm. **D.** The level of nuclear p65 in infected cells was quantitated in at least 12 infected cells per strain. Asterix indicates significantly higher levels of nuclear p65 compared to uninfected cells (*$p < 0.005$, two sample t-test). One replicate experiment was done in both HFFs and mouse BMDM with similar qualitative results.

To determine if type II *GRA15* was necessary for NF-κB p65 translocation in host cells, we generated type II *GRA15*KO strains (Fig. S5). Removal of the *GRA15* locus abolished p65 translocation by type II parasites in host cells, eliminating the subset of infected cells with a high level of nuclear p65 (Fig. 3A, B). The level of nuclear p65 in cells infected with type II *GRA15*KO parasites was not significantly different from the level in uninfected cells (Fig. 3B). To confirm that *GRA15* is responsible for the NF-κB activation phenotype, we transfected $GRA15_{II}$ back into a type II *GRA15*KO strain. NF-κB p65 nuclear translocation was rescued in this type II *GRA15*KO $GRA15_{II}$ strain (Fig. 3A, B). Additionally, a type I *GRA15*KO $GRA15_{II}$ strain activated translocation of p65, and infection with this strain or a type I $GRA15_{II}$ strain did not activate significantly different levels of nuclear p65, confirming that the type II copy of *GRA15* alone was sufficient for the nuclear translocation of p65 by type I strains of *Toxoplasma*. Similarly, in mouse BMDM, infection with type II strains, but not type I/III strains, activated a high level of p65 nuclear translocation. This activation was also due to the type II *GRA15* gene (Fig. 3C, D).

To examine if p65 was the only NF-κB family subunit activated by GRA15 we infected HFFs with type I $GRA15_{II}$ parasites for 24 hours, and examined p50, p52, RelB and c-Rel nuclear localization by IF (Fig. S6). Only p50 was specifically and significantly translocated to the nucleus by type I $GRA15_{II}$ strains (Fig. 6A). However, there was a significant increase in levels of nuclear c-Rel upon infection with both type I and type I $GRA15_{II}$ strains (Fig. 6D).

GRA15 activates NF-κB-mediated transcription

To determine if the nuclear NF-κB p65 triggered by $GRA15_{II}$ is transcriptionally active, we infected HFFs with type II, type II *GRA15*KO, type I, type I $GRA15_{II}$, type III, or type III $GRA15_{II}$ *Toxoplasma*, and hybridized RNA from the infections to Affymetrix human genome arrays to determine host cell gene expression. The expression of genes with NF-κB TFBS in their promoters and gene products belonging to an NF-κB related pathway was enriched in type II vs type II *GRA15*KO infections and type I/III $GRA15_{II}$ vs type I/III infections (Fig. 4A, Supp. data). 146 transcripts were strongly regulated by GRA15, regardless of the *Toxoplasma* strain genetic background, and we defined these genes as core, GRA15-regulated genes (Fig. 4B). Network analysis of molecular relationships between these 146 genes and their gene products also demonstrated an enrichment of genes involved in cytokine and NF-κB signaling (Fig. 4C).

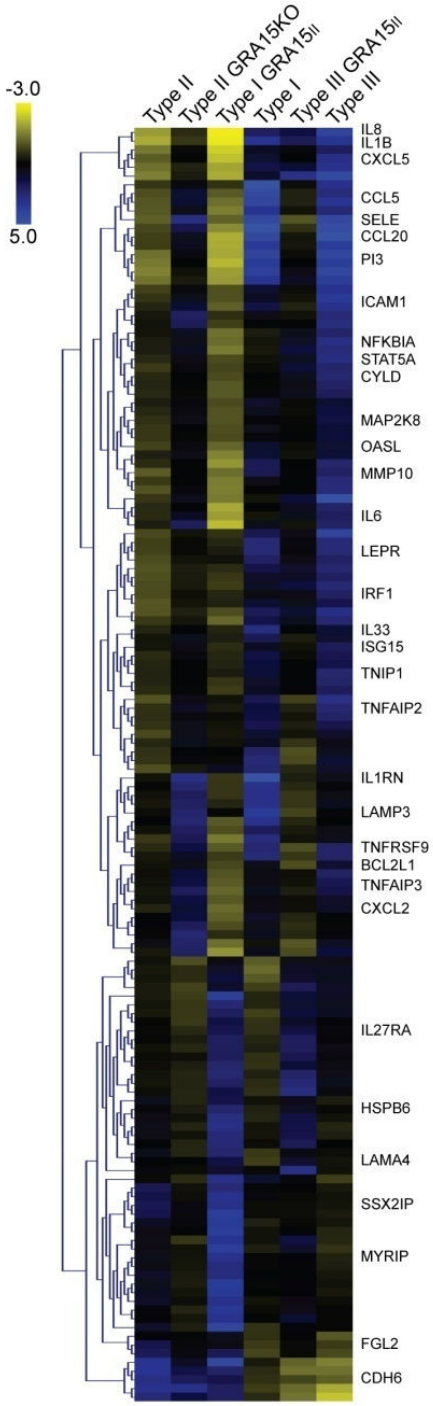
Type I *Toxoplasma* parasites also activate NF-κB p65 translocation in HFFs, although at a much lower level than type II strains (Fig. 3). Microarray analysis confirmed that a type I strain can cause the activation of NF-κB-mediated transcription. The expression of genes with NF-κB TFBS in their promoters and gene products belonging to an NF-κB related pathway was enriched in type I infected HFFs over uninfected HFFs (Supp. data). This activation is not dependent on *GRA15*; the expression of NF-κB regulated genes was enriched in a type I *GRA15*KO infection compared to uninfected cells, and not in a type I infection compared to a type I *GRA15*KO infection (data not shown).

We also infected wild-type and $p65^{-/-}$ MEFs with type II, type II *GRA15*KO, type I, or type I $GRA15_{II}$ *Toxoplasma* strains, and analyzed host cell gene expression by microarray (Fig. 4D). In WT MEFs, we found 18 genes to be core, GRA15-regulated genes (more than 2-fold different in type II vs type II *GRA15*KO and type I $GRA15_{II}$ vs type I infections). Of these 18 genes, only two are also GRA15-regulated in $p65^{-/-}$ host cells. This data indicates that the majority of host cell transcription induced by GRA15 was activated via the canonical p65/p50 NF-κB heterodimer, however it is possible that a small subset of genes was activated by other NF-κB subunits, such as c-Rel/p50 dimers, or other transcription factors.

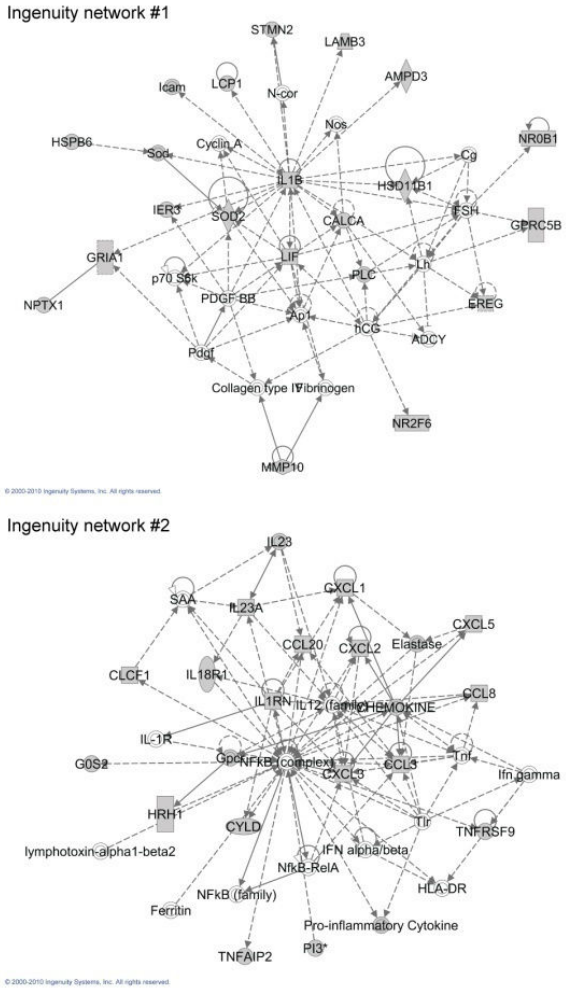
A

	Transcription Factor Binding Sites	FDR
Type II	NFKB_C	0.0000
vs	NFKB_Q6_01	0.0036
Type II GRA15KO	NFKB_Q6	0.0037
Type I GRA15$_{II}$	NFKB_Q6_01	0.0115
vs	NFKAPPAB_01	0.0115
Type I	NFKB_Q6	0.0117
Type III GRA15$_{II}$	NFKB_C	0.0532
vs	NFKB_Q6	0.0730
Type III	ICSBP_Q6	0.0740

B



C



D

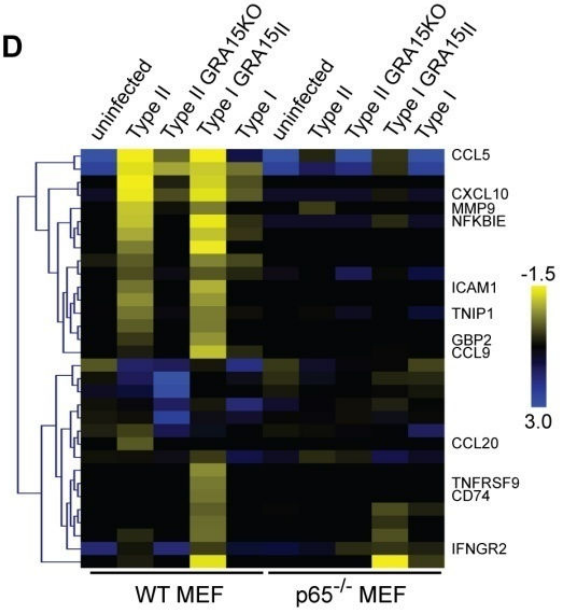


Figure 4. GRA15 activates NF-κB-mediated transcription. HFFs or MEFs were infected for 18-24 hours with type II, type II *GRA15*KO, type I, type III, type I $GRA15_{II}$ or type III $GRA15_{II}$ *Toxoplasma* strains, and host cell gene expression was analyzed by Affymetrix microarrays. At least two arrays were done per strain in HFFs and one array was done per strain in MEFs. **A.** The top three enriched, known TFBS from gene set enrichment analyses comparing type II versus type II *GRA15*KO and type I/III $GRA15_{II}$ versus type I/III infections are shown. **B.** For the 146 genes that are defined as core, GRA15-regulated genes, average $\log_2$ gene expression values were median-centered, genes were clustered by hierarchical clustering, and a heat map is presented. The complete set of genes is listed in Supp. data. **C.** Ingenuity pathway analysis was done for these 146 genes. The top two scoring networks are shown. **D.** In MEF arrays, 32 genes have the same expression level in WT and p65-/- cells and are regulated by GRA15. For these genes, $\log_2$ expression values were median-centered, genes were clustered by hierarchical clustering, and a heat map is shown. The complete set of genes is listed in Supp. data.

GRA15 is a polymorphic secreted dense granule protein

The *GRA15* coding region is predicted to be 1908 bp in type I and III strains, but only 1653 bp in type II strains, due to either an insertion or deletion (indel) (ToxoDB.org, v6.0). An intron is predicted in the type I and type III copies, very close to the indel. We sequenced the region around this indel of both type I and type II cDNA. Neither strain was found to have an intron in this region, and the indel was confirmed. To determine the full transcript of $GRA15_{II}$, we performed 5' and 3' RACE. Two $GRA15_{II}$ transcription start sites were found, one at -508 to -504 bp upstream of ATG and one at -277 bp upstream of ATG. Three polyadenylation sites were found, at +930, +992, and +1144 bp downstream of the stop codon.

ToxoDB.org currently contains sequences from one strain for each of the three North American/European clonal lineages of *Toxoplasma*: GT1 (type I), ME49 (type II), and VEG (type III). The genomic sequences of strains within the same lineage are thought to be very similar, however, we sequenced the *GRA15* locus from RH (type I), Pru (type II), and CEP (type III) strains as well, three other strains that we have used in our experiments. While Pru and CEP have an identical sequence to ME49 and VEG, respectively, the RH sequence contains a frameshift mutation at base 872. This frameshift leads to the mutation of a stretch of 22 amino acids (2 remain conserved), followed by a premature stop codon, truncating the protein to 312 amino acids, instead of 635 amino acids. The type I/III and II protein sequences differ most strikingly at the 84 amino acid indel near the C-terminus of the protein. Besides this indel, 5

other amino acids are polymorphic between types I/III and II and one other single amino acid is inserted or deleted (Fig. S7).

We next looked by IF at the localization of GRA15 in the parasite and infected host cells. To determine if GRA15 was secreted into the host cell, we performed evacuole staining on a five minute type I *GRA15*$_{II}$-HA infection of HFF cells using an antibody against the HA tag (Fig. 5A). HA staining is clearly present in evacuoles, partially co-localizing with evacuoles containing rhoptry proteins, indicating that GRA15 is a secreted protein. Evacuoles containing GRA15 can also be seen after attachment of cytochalasin D-treated parasites (data not shown). In parasitophorous vacuoles (PVs), co-staining of the GRA15$_{II}$-HA protein with either a rhoptry marker, ROP1, or a dense granule marker, GRA7, showed co-localization of GRA15$_{II}$-HA with GRA7, with almost no overlap between the HA tag and ROP1 (Fig. 5C). GRA15 staining overlaps GRA7 staining in the dense granules and within the PV. GRA15 also localizes to the outside of the parasitophorous vacuole membrane (PVM), and in a co-infection of type I (non-GFP) and type I *GRA15*$_{II}$-HA (GFP) parasites, HA staining can be seen localized in the PVM and on the outside of the PVM of a parasite expressing GRA15$_{II}$-HA (GFP), as well as on the outside of the PVM of a parasite not expressing GRA15$_{II}$-HA (Fig. 5B), which is consistent with dense granule localization. We therefore conclude that GRA15 is a dense granule protein.

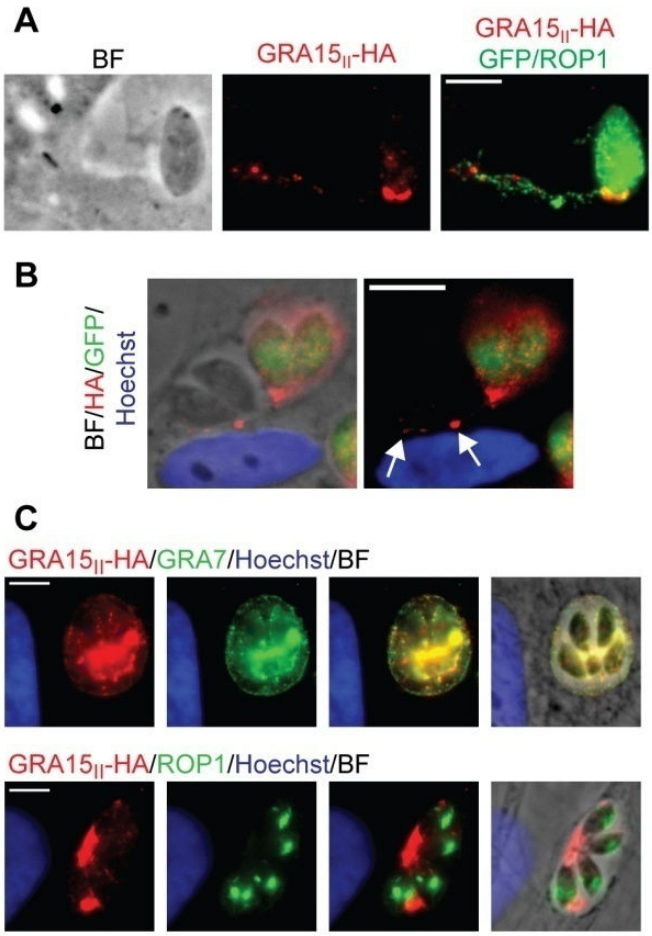


Figure 5. GRA15 is a secreted dense granule protein. A. Parasites expressing GFP and an HA-tagged copy of $GRA15_{II}$ were added to HFFs for five minutes to allow attachment and evacuole formation. Cells were then fixed and stained with α-HA (red), α-ROP1 (green) and Hoechst dye (blue). The HA tag is present with ROP1 in evacuoles, indicating that GRA15 can be secreted into the host cell. This experiment was repeated once with cytochalasin D-treated parasites with the same results. **B.** Co-infection of type I (non-GFP) and type I *GRA15*$_{II}$-HA (GFP) parasites, done once. Arrows indicate HA staining on the PVM of a non-GFP vacuole. **C.** Co-staining of $GRA15_{II}$-HA with a dense granule marker, GRA7, shows co-localization of HA-staining and GRA7 in both the dense granules and the parasitophorous vacuole. Conversely, co-staining of $GRA15_{II}$-HA with a rhoptry marker, ROP1, shows almost no overlap between the HA tag and the rhoptries. Co-staining was done once, but the same $GRA15_{II}$-HA staining pattern has been observed in more than five independent experiments. Scale bars represent 5 μm.

GRA15 affects total levels of phospho-IκBα, but does not affect PVM-associated phospho-IκBα

The nuclear translocation of NF-κB transcription factor subunits is dependent on the phosphorylation and degradation of an inhibitory protein, IκBα. We determined if GRA15 affected the overall levels of phospho-IκBα in infected cells by western blot, and quantified the fraction of phosphorylated IκBα compared to the total level of IκBα (Fig. 6A). Infection with a strain of *Toxoplasma* expressing $GRA15_{II}$ led to an increase in the fraction of total IκBα that was phosphorylated, although not to the extent of TNF-α-induced levels. This indicates that GRA15 activates NF-κB through the phosphorylation of IκBα.

Previously, a type I *Toxoplasma* protein extract was found to have IκBα-phosphorylating activity *in vitro* (Molestina et al., 2003). There is also evidence that this kinase activity can occur *in vivo*, as PVM-associated phospho-IκBα can still be observed in infected IKKα/β double knockout MEFs (Molestina and Sinai, 2005b). However, in these IKKα/β double knockout cells, after type I *Toxoplasma* infection NF-κB does not translocate to the nucleus or bind to DNA *in vitro*, and NF-κB mediated gene expression is severely decreased (Molestina and Sinai, 2005a). To determine if the accumulation of phospho-IκBα on the PVM correlated with NF-κB activation we infected HFFs with *Toxoplasma* strains and stained infected cells with a phospho-IκBα antibody. Although infection with type II parasites activated NF-κB to a much greater extent and lead to higher levels of total phospho-IκBα compared to type I parasite infection, in a mixed infection of type I (non-GFP) and type II (GFP) *Toxoplasma*, phospho-IκBα accumulated almost exclusively on type I vacuoles (Fig. 6B). We also found that the accumulation of phospho-IκBα at the PVM was independent of *GRA15*, as both type I and type I $GRA15_{II}$ PVMs accumulated phospho-IκBα, and neither type II nor type II *GRA15*KO PVMs accumulated visual levels of phospho-IκBα (Fig. 6C). Phospho-IκBα also did not accumulate on type III PVMs (Fig. 6C). We conclude that the accumulation of PVM-associated phospho-IκBα is specific to type I parasites and is not correlated with the overall level of NF-κB activation in the host cell.

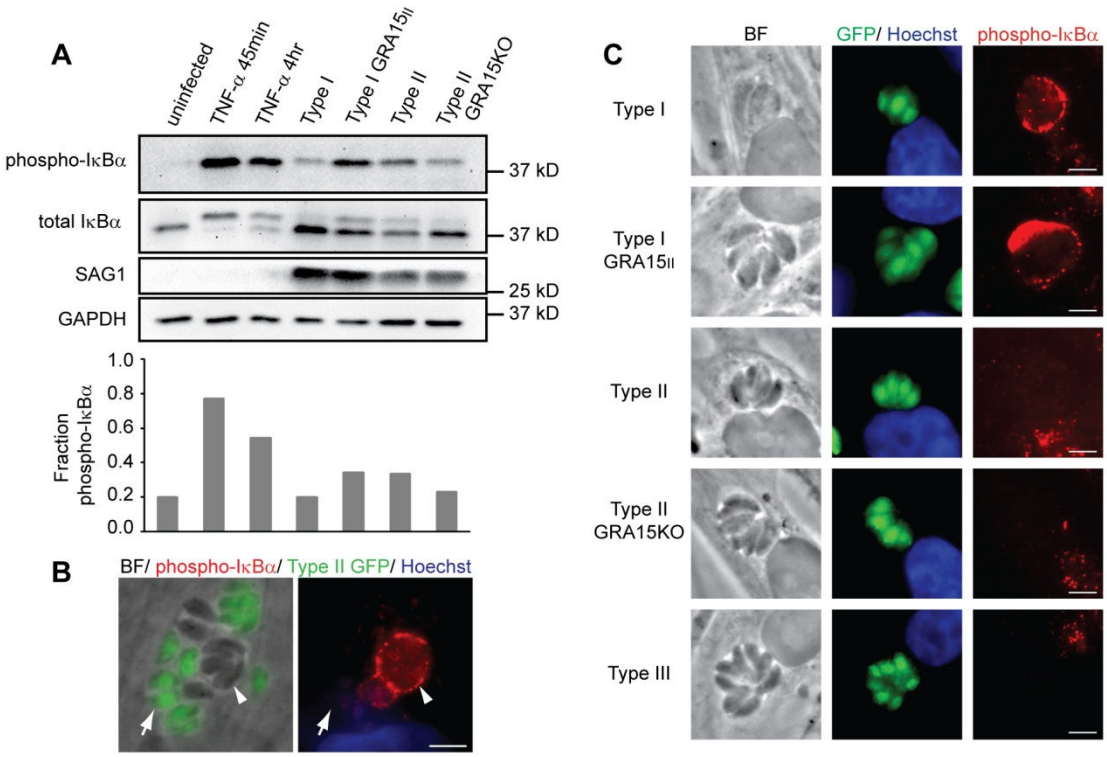


Figure 6. GRA15 activity affects total levels phospho-IκBα, but not PVM-associated phospho-IκBα. A. HFFs were infected with *Toxoplasma* strains for 24 hours or stimulated with TNF-α for indicated times, cell lysates were collected, run on an SDS-PAGE gel, and western blotted for phospho-IκBα, total IκBα, SAG1 (parasite loading control), and GAPDH (host cell loading control). From the total IκBα blot, the fraction of phosphorylated IκBα was determined by comparing the intensity of the upper band (phosphorylated form) to the total intensity of the lower and upper band. This experiment was repeated once with type I and type I $GRA15_{II}$ strains only with similar results. **B.** HFFs were co-infected with type I (non-GFP) and type II (GFP) parasites, fixed, and stained with α-phospho-IκBα (red) and Hoechst dye (blue). A type I PVM (non-GFP, arrowhead) and a type II PVM (GFP, arrow) are indicated. Scale bar represents 5 μm. **C.** HFFs were infected with *Toxoplasma* strains expressing GFP for 24 hours, fixed, and stained with α-phospho-IκBα (red) and Hoechst dye (blue). This experiment has been done three times with similar results. Scale bars represent 1 μm.

GRA15 is dependent on the IKK complex and TRAF6, but independent of MyD88 and TRIF

BLAST and Pfam searches for proteins with similar amino acid sequences or domains to $GRA15_{II}$ returned no significant results, providing no clues to the mechanism of GRA15 NF-κB activation (Altschul et al., 1990; Finn et al., 2008). To start to answer this question, we determined which components of the NF-κB signaling pathway were necessary for GRA15 activity. When NF-κB is activated, IκB proteins are phosphorylated and then degraded by the

proteasome. We previously determined that GRA15 leads to the phosphorylation of IκBα (Fig. 6), and to determine if GRA15 activity is dependent on the proteasome, we pre-treated cells with MG132, a proteasomal inhibitor. Our results show that activation of NF-κB by GRA15 required functional proteasomal degradation (Fig. 7A). IκB proteins are normally phosphorylated by the IKK complex, consisting of IKKα, IKKβ, and IKKγ (NEMO). IKKγ is a regulatory subunit in the complex, while the β and α subunits are active kinases. IKKβ has greater kinase activity than IKKα, and is the principal kinase responsible for the phosphorylation of IκBα (Ghosh and Karin, 2002; Li and Verma, 2002). In WT MEFs, a type I *GRA15*$_{II}$ strain induces a 3.4 fold increase in nuclear p65 compared to uninfected cells. However, in IKKβ$^{-/-}$ MEFs, this increase is only 1.9 fold (Fig. 7B). Many pathogens activate NF-κB via TLR agonists, and TLR signaling is mediated by the MyD88 and TRIF adaptor proteins. However, GRA15 is able to activate p65 nuclear translocation in MyD88/TRIF double knockout cells, indicating that it is not just a TLR ligand (Fig. 7D). GRA15-mediated p65 activation is also not dependent on the RIP1 adaptor protein, but is dependent on TRAF6 (Fig. 7C, data not shown). Thus GRA15 appears to modulate NF-κB at a specific step in the pathway downstream of MyD88 and TRIF, but upstream of, or in a complex with, TRAF6 and IKK proteins.

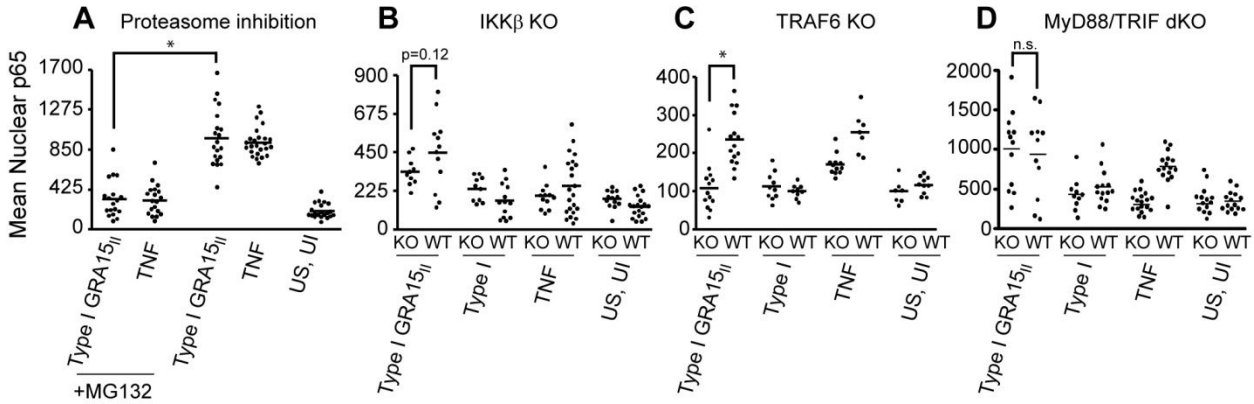


Figure 7. GRA15 activity is dependent on IKKβ and TRAF6 and independent of MyD88 and TRIF. Cells were infected with type I *GRA15*$_{II}$ or type I parasites for four hours, stimulated with 20 ng/ml TNF-α for one hour, or left unstimulated and uninfected (US, UI). Cells were fixed and probed with an α-NF-κB p65 antibody and mean nuclear staining was measured. **A**. HFF cells were pre-incubated with media containing 20 ng/ml MG132 proteasome inhibitor prior to infection and TNF-α stimulation. **B-D.** The activity of GRA15$_{II}$ in the absence of different components of the NF-κB pathway was assayed: **B.** IKKβ$^{-/-}$ MEFs, **C.** TRAF6$^{-/-}$ MEFs, **D.** MyD88$^{-/-}$/TRIF$^{-/-}$ BMDM host cells. These experiments were repeated at least two times and quantification was performed on a representative experiment for each factor assayed. Asterix (*) indicates data are significantly different, (p-value < 0.05, t-test), and n.s. indicates data are not significantly different.

GRA15 expressed in Hela cells is sufficient to activate NF-κB

We wanted to determine whether GRA15$_{II}$ alone is sufficient to activate p65 nuclear translocation, or if other *Toxoplasma* secreted factors that are common to all type I, II, and III strains are also needed for this process. When we transiently transfected HeLa cells with a vector expressing the type II copy of GRA15 N-terminally fused with GFP, the nuclei of transfected GFP-positive cells contained p65, while the nuclei of non-transfected, GFP-negative cells in the same culture did not (Fig. 8A). The level of this nuclear localization is equivalent with activation by intracellular type I GRA15$_{II}$ parasites (Fig. 8B). Expression of 55.m04955, an unrelated *Toxoplasma* protein, or Mob1A, a human protein present in the original vector, did not induce p65 translocation, indicating that this NF-κB activation is not due to cell stress from protein overexpression (Fig. 8A). GRA15$_{II}$ expression alone is therefore sufficient to recapitulate the induction of p65 nuclear translocation.

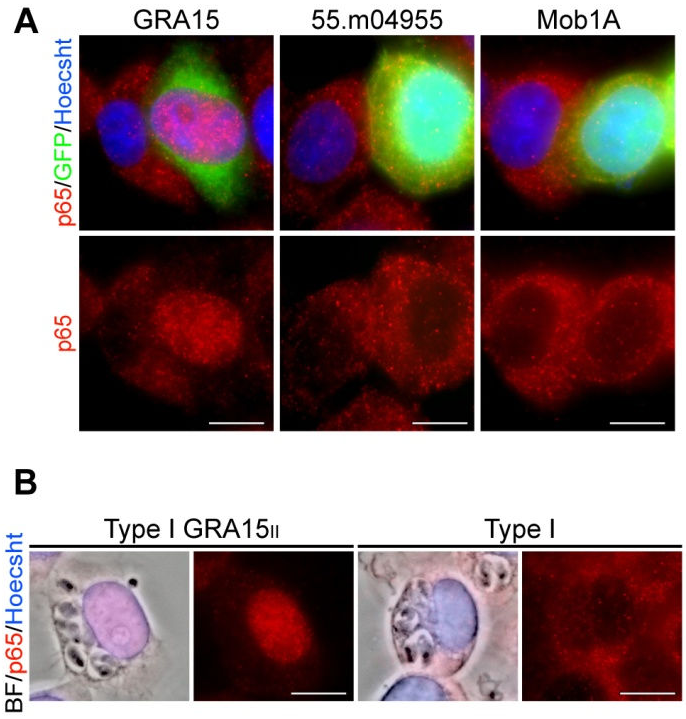


Figure 8. GRA15 expression alone is sufficient to activate NF-κB in HeLa cells. A. HeLa cells were transfected with *GRA15*$_{II}$, an unrelated *Toxoplasma* gene (*55.m04955*), or an unrelated human gene (*Mob1A*), fused to *GFP*. Cells were then fixed and stained with α-NF-κB p65 (red) and Hoechst dye (blue). All cells expressing GRA15$_{II}$-GFP contain activated NF-κB p65, whereas cells expressing 55.m04955-GFP or Mob1A-GFP do not. Non-transfected, non-GFP cells in the same culture also have no nuclear NF-κB p65. This experiment was repeated two more times with the same results. **B.** HeLa cells were infected with type I *GRA15*$_{II}$ or type I parasites for 24 hours, fixed, and stained with α-NF-κB p65 (red) and Hoechst dye (blue). Cells infected with a type I *GRA15*$_{II}$ strain contain comparable amounts of nuclear NF-κB p65 to transfected cells. This experiment was repeated a second time with similar results.

GRA15 affects in vitro parasite growth

To assay the effect of GRA15 on *in vitro* parasite growth, we infected monolayers of fibroblasts with type I, type I *GRA15*$_{II}$, type II or type II *GRA15*KO parasites, allowed the parasites to grow for 4-7 days, and then quantified the area of plaques formed on the monolayers. In HFF host cells, a type II GRA15KO strain formed significantly larger plaques than a type II strain ($p = 0.002$, t-test), and a type I strain formed significantly larger plaques than a type I *GRA15*$_{II}$ strain ($p = 0.024$, t-test) (Fig. S8A). However, in MEF host cells, type II and type II GRA15KO strains did not make significantly different size plaques ($p = 0.841$, t-test), and the

same was true of type I and type I *GRA15*$_{II}$ strains (p = 0.371, t-test) (Fig. S8A). This data indicates that GRA15 inhibits *in vitro* parasite growth in human fibroblasts, but not mouse fibroblasts.

GRA15 affects IL-12 production in vitro

In vitro infection of macrophages with different strains of *Toxoplasma* results in different levels of IL-12p40 secretion, with type II strains inducing much higher levels of IL-12p40, and some of this variation has been suggested to be due to strain differences in NF-κB activation (Kim et al., 2006; Robben et al., 2004; Saeij et al., 2007). To examine the role of GRA15-mediated NF-κB activation in the induction of IL-12p40 secretion, mouse BMDM were infected with type I, type I *GRA15*$_{II}$, type II, or type II *GRA15*KO *Toxoplasma*, and levels of IL-12p40 in the supernatant were determined by cytokine ELISA (Fig. 9A). As expected, type I infected BMDM secrete a low level of IL-12p40 which is not significantly higher than the level secreted by uninfected cells, while type II induces a high level of IL-12p40 secretion. When *GRA15* is removed from the type II strain, IL-12p40 secretion decreases more than 6-fold, implying a considerable role for this gene product in modulating host cell IL-12 signaling (p = 0.001, t-test). Similarly, the introduction of *GRA15*$_{II}$ in a type I strain leads to a significant increase in IL-12p40 secretion by BMDM (p = 0.008, t-test). IL-12p70 secretion was also higher after type II infection compared to a type I infection and partially dependent on the presence of a type II copy of *GRA15* (data not shown).

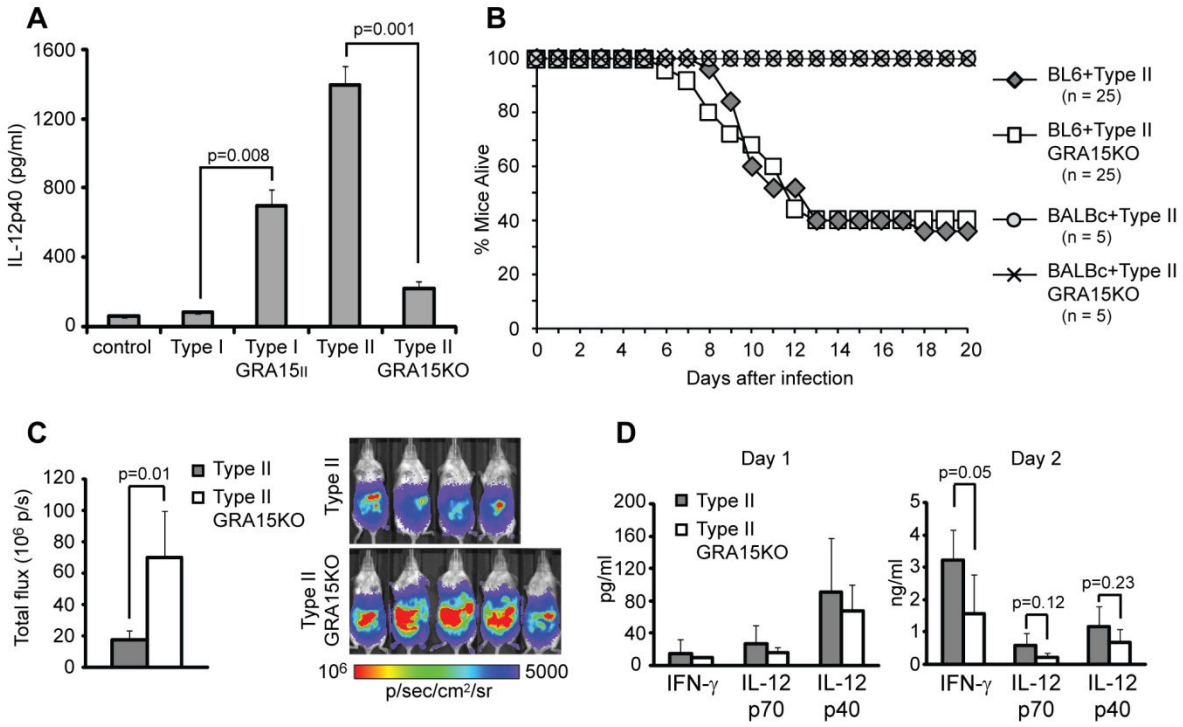


Figure 9. GRA15$_{II}$ promotes IL-12 secretion *in vitro* and affects parasite growth and host cytokine production *in vivo*. A. BALB/c BMDM were infected with *Toxoplasma* strains for 24 hours, supernatants were collected, and IL-12p40 levels were determined by cytokine ELISA. These experiments were performed at least three times in BMDM using triplicate samples, as well as in RAW264.7 macrophages, all with similar results. **B-D.** Mice were infected intraperitoneally with tachyzoites of either a type II or a type II GRA15KO strain. **B.** C57BL/6 or BALB/c mice were infected with 5,000 tachyzoites and survival of mice was monitored. In one experiment, 5 BALB/c mice were infected per strain, and in three separate experiments, a total of 25 C57BL/6 mice were infected per strain. **C.** BALB/c mice were infected with parasites which express the enzyme luciferase. Five days after infection, mice were intraperitoneally injected with luciferin, anesthetized, and the flux (photons/sec/cm^2/sr) was determined as a measure of parasite burden. Mice infected with a type II *GRA15*KO strain had significantly greater total flux (p/s) and therefore significantly greater parasite burden than mice infected with a type II strain. This burden difference five days after infection was observed in three independent experiments. **D.** One or two days after infection, infected BALB/c mice were euthanized and an i.p. cavity wash was collected for IFN-γ, IL-12p70, and IL-12p40 cytokine ELISA. On day 2 after infection, mice infected with a type II *GRA15*KO strain had significantly lower levels of IFN-γ in the intraperitoneal cavity than mice infected with a type II strain ($p = 0.05$, two-sample t-test). Five mice were infected per strain per day. Day 2 cytokine levels were measured in a separate experiment with similar results.

The *Toxoplasma* polymorphic rhoptry kinase ROP16 also affects IL-12 secretion, and previous microarray analyses determined that the expression of many GRA15-regulated genes are also strongly affected by a locus on chromosome VIIb, where *ROP16* resides (Saeij et al.,

2007). In these analyses, some genes, such as *SOCS2* and *SOCS3*, were regulated by loci on both chromosome VIIb and X (Fig. S9A). We wondered if the effect of ROP16 on these genes was through modulation of the NF-κB pathway. We infected a HEK293 NF-κB GFP reporter cell line with a type II strain or a type II transgenic strain expressing a type I copy of ROP16. A significantly greater percentage of type II infected cells than type II *ROP16*$_{I}$ infected cells are GFP-positive ($p = 1.5 \times 10^{-7}$, χ^2 test), indicating that in a type II background, a type I copy of ROP16 significantly inhibits NF-κB activation (Fig. S9B). In a HEK293 NF-κB luciferase reporter cell line, infection with a type II ROP16$_{I}$ strain also induced significantly less luciferase activity than infection with a type II strain (data not shown). Thus, both GRA15 and ROP16 affect NF-κB activity.

GRA15 affects in vivo parasite growth and cytokine production

To assay the effect of GRA15 on parasite virulence we infected C57BL/6 or BALB/c mice by i.p. injection with 5,000 tachyzoites of a type II or a type II *GRA15*KO strain and monitored mouse survival during the acute phase of infection (days 0-20) (Fig. 9B). C57BL/6 mice infected with either strain succumbed to infection at the same time, between days four and eighteen after injection. Additionally, the same percentage of mice in each group survived, ~40%. BALB/c mice infected with either a type II or a type II *GRA15*KO strain did not die after infection.

To determine the effect of GRA15 on *in vivo* parasite burden, BALB/c mice infected i.p. with tachyzoites of either a type II or a type II *GRA15*KO strain that express the enzyme luciferase were imaged throughout infection. At day five after infection, mice infected with a type II *GRA15*KO strain had a significantly higher parasite burden than mice infected with a type II strain (Fig. 9C) ($p = 0.01$, t-test). Similarly, expression of GRA15$_{II}$ in a type I strain inhibited *in vivo* parasite growth (Fig. S8B). Together, these experiments indicate that GRA15 inhibits *in vivo* parasite growth in both a type I and a type II background.

GRA15 affects IL-12 secretion by BMDM *in vitro*, and we also assessed whether it affects cytokine secretion *in vivo* at the site of infection. BALB/c mice were infected i.p. with tachyzoites of either a type II or a type II *GRA15*KO strain. One or two days after infection, mice were sacrificed, the intraperitoneal cavity was washed, and cytokine levels in the wash were

determined by ELISA. On day 2 after infection, mice infected with a type II *GRA15*KO strain had significantly less IFN-γ in their i.p. cavities than mice infected with a type II strain (Fig. 9D) ($p = 0.05$, t-test). While differences in IL-12p40 or p70 were not significant on either day 1 or day 2 due to large variations between mice, the average cytokine levels in type II *GRA15*KO infected mice were consistently lower than cytokine levels in type II infected mice. At these early time points, there was not a significant difference in parasite load between mice infected with either strain, as determined by imaging (data not shown).

Discussion

The modulation of the NF-κB pathway by *Toxoplasma* has long been an area of debate, with some reports stating that *Toxoplasma* activates NF-κB and others that *Toxoplasma* inhibits NF-κB activation. In this study, we have conclusively shown that the three North American clonal lineages of *Toxoplasma* differ in their activation of the host NF-κB pathway; type II strains activate a high level of NF-κB p65 translocation while type I and III strains do not (Fig. 3). Using F1 progeny from a type II x type III cross, we found that a locus on chromosome X is responsible for this polymorphic phenotype, and identified the novel *Toxoplasma* factor GRA15 at this locus (Fig. S2). The type II copy of *GRA15* (*63.m00001*) is necessary in type II strains and sufficient in type I and III strains for NF-κB nuclear translocation and transcriptional activity (Fig. 3, 4). We have observed this activation in a variety of human, mouse, and rat cell types, indicating that GRA15's activity is independent of host cell type and species. Additionally, when $GRA15_{II}$ is expressed in HeLa cells, it is sufficient to activate NF-κB.

It had been previously reported that infection with type I strains of *Toxoplasma* activates NF-κB in MEF and Henle 407 intestinal epithelial host cells (Ju et al., 2009; Molestina et al., 2003). Careful quantification of IF experiments showed that type I strains do slightly activate NF-κB p65 translocation, but the level of nuclear p65 is much higher in type II infected cells than type I infected cells (Fig. 3). It is possible that other groups have concluded that type I strains do not activate NF-κB because this activation is so low compared to that of LPS or TNF-α stimulation (Butcher et al., 2001; Shapira et al., 2002, 2005). Our IF experiments also showed that a type I strain activates c-Rel nuclear translocation, which might also affect host cell transcription (Fig. S6). By microarray analysis, some NF-κB regulated genes are induced by type

I infection, but a much greater number are induced by type II infection, and infection with a type I strain does not activate detectable GFP or luciferase transcription from an NF-κB reporter cell line (data not shown), in concordance with another published report (Shapira et al., 2005). This low-level activation is also not dependent on GRA15, as there is no enrichment in NF-κB activation in a type I infection over a type I *GRA15*KO infection. Type III strains do not activate any p65 nuclear translocation or NF-κB-mediated transcription.

Our results agree with previous observations that phosphorylated IκBα accumulates on the PVM upon *Toxoplasma* type I infection (Butcher et al., 2001; Molestina et al., 2003; Shapira et al., 2005). However, levels of PVM-associated phospho-IκBα are not correlated with total levels of phospho-IκBα or the level of NF-κB transcriptional activity in the host cell. Phospho-IκBα is not observed on type II PVMs, and, while infection with *Toxoplasma* strains expressing $GRA15_{II}$ leads to higher total levels of phospho-IκBα, the presence of GRA15 does not affect PVM-associated phospho-IκBα (Fig. 6). A type I *Toxoplasma* protein capable of phosphorylating IκBα may play a role in low-level activation of NF-κB in type I strains (Molestina and Sinai, 2005b). However, it is clear that the GRA15 protein in type II strains activates NF-κB to a much greater extent.

On the other hand, some groups have reported that infection with type I *Toxoplasma* strains inhibits NF-κB activation after stimulation with the cytokine TNF-α or the TLR ligand LPS (Butcher et al., 2001; Kim et al., 2004; Shapira et al., 2002). This inhibition has been observed after less than 6 hours of infection in HFFs (Shapira et al., 2005), mouse BMDM (Kim et al., 2004; Shapira et al., 2002), and thioglycolate-elicited cells (Butcher et al., 2001), and was not observed after six or 12 hours of infection in mouse BMDM (Kim et al., 2004; Leng et al., 2009). Our experiments confirm that type I *Toxoplasma* cannot inhibit LPS stimulated NF-κB nuclear translocation or TNF-α stimulated NF-κB transcriptional activity at a late time point in infection (Fig. 2A, C). But, after a short infection (< 5 hr), we also do not observe inhibition of NF-κB p65 nuclear translocation or NF-κB mediated-transcription, contradicting these reports (Fig. 2A, B). It is true that some infected cells do not respond to LPS or TNF-α stimulation, and this observation may have lead to the conclusion that type I strains can inhibit NF-κB signaling. However, quantification of NF-κB p65 translocation and NF-κB reporter transcription in many

uninfected and infected cells shows that pre-infection with *Toxoplasma* does not alter the response of populations of cells to these stimuli.

The *GRA15* gene product is a novel *Toxoplasma* dense granule protein which is secreted into the host cell upon invasion (Fig. 5), representing the first identified Apicomplexan dense granule protein that can modulate host cell signaling pathways. Rhoptry proteins have already been identified as parasite factors that can alter host cell behavior, but our findings indicate that dense granule proteins should be viewed as candidate factors as well. It is still unclear why only the type II copy of *GRA15* activates NF-κB. Amino acid polymorphisms between the type II and type I/III copies of GRA15, including an insertion/deletion and several single amino acid changes, or the expression level of *GRA15* may be responsible for this polymorphic phenotype.

Although the GRA15 protein is secreted into the host cell upon parasite invasion, cells infected with type I $GRA15_{II}$ parasites did not have substantial p65 in their nuclei until approximately four hours post-infection (Fig. S4), and in our experiments NF-κB activation was usually assayed 18-25 hours post-infection. These slow kinetics are not unprecedented, Rac GTPase has been shown to initiate NF-κB nuclear translocation with a time course similar to that of GRA15 (Boyer et al., 2004). Additionally, the amount of GRA15 interacting with host cell proteins likely increases after PV formation as dense granule proteins are made and continuously secreted from intracellular parasites, and higher levels of GRA15 may be necessary to initiate NF-κB activation.

The precise mechanism by which GRA15 activates NF-κB has yet to be discovered. Our data suggests that GRA15 initiates canonical NF-κB activation, which preferentially induces the p65/p50 heterodimer (Hayden and Ghosh, 2004). A simple hypothesis is that GRA15 acts as a TLR ligand to activate the canonical NF-κB pathway, however the activation of p65 translocation by GRA15 is not dependent on either MyD88 or TRIF, two proteins that are essential for TLR signaling. We did find that the activity of GRA15 is dependent on both IKKβ and TRAF6, suggesting that GRA15 acts either upstream of or in a complex with these proteins. A type I $GRA15_{II}$ strain activated more nuclear p65 translocation in WT MEFs than in $IKK\beta^{-/-}$ or $TRAF6^{-/-}$ MEFs, although in $IKK\beta^{-/-}$ cells this difference was not significant ($p = 0.12$). This is probably because IKKα also has phosphorylating activity. Our microarray data further defines the placement of GRA15 in the NF-κB signaling pathway. GRA15 is able to constitutively

activate NF-κB, but this activation leads to the expression of negative feedback regulators, such as the deubiquitinating enzymes (DUBs) A20 (*TNFAIP3*) and CYLD, which normally act to quickly downregulate NF-κB signaling. Since TRAF6 and the IKK complex are both targets of these DUBs, it is likely that GRA15 acts in concert with these proteins, rather than upstream (Sun, 2008). We are currently looking for direct binding partners of GRA15 by co-immunoprecipitation.

We found that a type I copy of *ROP16* can inhibit NF-κB activation in a type II strain (Fig. S9B). Why this inhibition occurs in a type II strain but not a type I strain is unknown, but the genetic backgrounds of type I and type II strains are very different and other polymorphic factors likely exist that affect STAT and/or NF-κB signaling pathways. How ROP16 inhibits NF-κB activation is also unclear, but it is likely to be through its activation of STAT6 and/or STAT3 (Butcher et al., 2005a; Hoentjen et al., 2005; Nelson et al., 2003; Ohmori and Hamilton, 2000). This inhibition has significant consequences, for example, GRA15 and ROP16 have opposing effects on the expression levels of many genes, including IL-12, a particularly important cytokine in *Toxoplasma* infection (Fig. 9A) (Gazzinelli et al., 1994; Saeij et al., 2007). In fact, the single amino acid difference in ROP16 which causes it to be less active in type II strains (Yamamoto et al., 2009) may have been selected for in type II strains specifically to increase NF-κB activation by $GRA15_{II}$. However, GRA15 and ROP16 are expected to have additive or synergistic effects on the expression of other genes, such as the *SOCS* genes (Supp Fig. 9A). In any case, the modulation of host cell gene expression will depend upon the exact allelic combination of a variety of factors that *Toxoplasma* possesses, including *GRA15* and *ROP16*.

Early in infection (days 1-5), GRA15 affected both cytokine production and parasite growth *in vivo*. When a host is first infected by live parasites, type II strains expressing GRA15 activate NF-κB in host cells and induce IL-12 secretion, whereas infection with type II *GRA15*KO parasites, or type I/III strains, does not cause this early activation. IL-12 stimulates NK cells and T cells to secrete IFN-γ, and the observed effect of GRA15 on IFN-γ levels was likely via an effect on IL-12. While levels of IL-12 in the intraperitoneal cavity were not significantly different between mice infected with a type II or type II *GRA15*KO strain, the levels were consistently lower in type II *GRA15*KO infected mice, and this difference may have been enough to lead to a significant difference in IFN-γ levels (Fig. 9D). IFN-γ is the main mediator

of host resistance to *Toxoplasma*, and differences in IFN-γ levels probably also explain the growth difference that we observed *in vivo* between a type II and type II *GRA15*KO strain at a slightly later time point, five days after infection (Fig. 9C). However, as the infection progresses and parasites lyse out of host cells, pathogen associated molecular pattern (PAMP) proteins within the PV such as profilin and cyclophilin are released, and NF-κB will be activated via TLR signaling and CCR5 signaling by all strains. At this stage, IL-12 production, IFN-γ production, and parasite growth are then independent of the GRA15 locus, which might explain why a type II strain does not differ in overall virulence from a type II *GRA15*KO strain (Fig. 9B). The location of GRA15 on chromosome X does indicate that it could represent a previously identified chromosome X virulence locus (Saeij et al., 2006).

Plaque assays showed that GRA15 also affects parasite growth *in vitro* (Fig. S8A). However, this *in vitro* effect occurred specifically in human cells and not in mouse cells. The cause of this difference is currently unknown, but one possibility is that genes affecting amino acid levels, lipid levels, or levels of other nutrients are partially NF-κB regulated in human cells but not in mouse cells.

$GRA15_{II}$ may also have other effects *in vivo* that remain untested. As an intracellular pathogen, *Toxoplasma* must use host cells to traffic through the body of the host animal. NF-κB activation by $GRA15_{II}$ increases expression of many chemokines and adhesion molecules (our microarray data), and strain differences in NF-κB activation may therefore lead to differences in the ability to induce migration of host cells, as previously reported (Lambert et al., 2006, 2009). Infection of hosts with other pathogens is also highly relevant to disease outcome, and the activation of NF-κB by *Toxoplasma* might increase transcription of HIV retroviral sequences with NF-κB binding sites in their promoters (Gazzinelli et al., 1996). Lastly, NF-κB activation leads to a pro-inflammatory Th1-type immune response which may promote inflammatory disease manifestations such as encephalitis and colitis, both of which have been observed mainly after type II strain infections (Hunter and Remington, 1994; Liesenfeld, 1999).

Materials and Methods

Parasites and Cells

Parasites were maintained *in vitro* by serial passage on monolayers of human foreskin fibroblasts (HFFs) at 37°C in 5% CO2. RH or GT1 were used as representative type I strains, ME49 or Pru as representative type II strains and CEP or VEG as representative type III strains. A Pru strain engineered to express firefly luciferase and GFP (Pru Δ*HXGPRT* A7) (Kim et al., 2007b), and CEP and RH strains engineered to express clickbeetle luciferase and GFP (CEP *HXGPRT*$^-$ C22 and RH 1-1) (Boyle et al., 2007) have been described previously. Pru and RH strains expressing HXGPRT and generated from unsuccessful knockout transfections were used in assays comparing parasite growth. F1 progeny from type II x type III crosses were described previously (Khan et al., 2005; Sibley et al., 1992). A Pru strain expressing a type I copy of ROP16 was also described previously (Saeij et al., 2007). HFFs were grown in DMEM (GIBCO BRL) supplemented with 10% heat inactivated fetal bovine serum (FBS) (PAA), 2 mM L-glutamine, 50 μg/mL each of penicillin and streptomycin, and 20 μg/ml gentamycin. Bone-marrow derived macrophages (BMDM) were obtained from female BALB/c, C57BL/6, or MyD88/TRIF double knockout (a gift from Hidde Ploegh) mice. Bone marrow was isolated by flushing hind tibias and femurs using a 27 gauge needle and/or by crushing the bones using a mortar and pestle, followed by passage over a cell strainer. Cells were suspended in DMEM supplemented with 20% L929 cell-conditioned medium, 10% heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1x MEM non-essential amino acids, and 50 μg/mL each of penicillin and streptomycin. 3-6 x 10^6 cells were plated in 10 cm non-tissue culture treated dishes (VWR) and incubated at 37°C, 5% CO_2 in humidified air. After 6-7 days cells were washed with PBS to remove non-adherent cells, harvested by dislodging with a cell-scraper in PBS, and replated for the assay. HEK293 stable cell lines with four copies of the NF-κB consensus transcriptional response element driving the expression of GFP or GFP and luciferase (System Biosciences) were grown in DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1x MEM non-essential amino acids, 10 mM HEPES, 50 μg/mL each of penicillin and streptomycin, and 20 μg/ml gentamycin. HEK293 cells were passed every 2-4 days using 0.05% Trypsin-EDTA. Wild-type mouse embryonic fibroblasts (MEFs) were gifts from Michael Karin and Anthony Sinai, IKKβ$^{-/-}$ MEFs were a gift from

Michael Karin (Li et al., 1999), NF-κBp65$^{-/-}$ MEFs were a gift from Anthony Sinai, and TRAF6$^{-/-}$ MEFs were kindly provided by Katherine Fitzgerald. MEFs were grown in DMEM (GIBCO BRL) supplemented with 10% non-heat inactivated fetal bovine serum (FBS) (PAA), 2 mM L-glutamine, 1 mM sodium pyruvate, 1x MEM non-essential amino acids, 10 mM HEPES, and 50 μg/mL each of penicillin and streptomycin. MEFs were passed using 0.05% Trypsin-EDTA. All parasite strains and cell lines were routinely checked for Mycoplasma contamination and it was never detected.

Reagents

Antibodies against HA (3F10, Roche), mouse NF-κB p65 (sc-8008), human NF-κB p65 (sc-109), NF-κB p50 (sc-8414), NF-κB p52 (sc-7386), NF-κB RelB (sc-28689), NF-κB c-Rel (sc-71), *Toxoplasma* surface antigen (SAG)-1 (DG52) (Burg et al., 1988), *Toxoplasma* dense granule protein GRA7 (Dunn et al., 2008), *Toxoplasma* rhoptry protein ROP1 (Tg49) (Ossorio et al., 1992), phospho-IκBα (sc-8404), total IκBα (sc-847), and GAPDH (sc-32233), were used in the immunofluorescence assay or in western blotting. Immunofluorescence secondary antibodies were coupled with Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes). Secondary antibodies used in western blotting were conjugated to peroxidase (Kirkegaard & Perry Laboratories). Purified LPS (Calbiochem), recombinant mouse TNF-α (AbD Serotec), and recombinant human TNF-α (Invitrogen) were used to stimulate cells. MG132 (Calbiochem) was used in proteasomal inhibition.

Immunofluorescence (IF)

Parasites were allowed to invade cells on coverslips and incubated for different time points. The cells were then fixed with 3% (v/v) formaldehyde in PBS for 20 minutes at room temperature, permeabilized with 100% ethanol and/or 0.2% (v/v) Triton-X 100, and blocked in PBS with 3% (w/v) BSA and 5% (v/v) goat serum. Coverslips were incubated with primary antibody for one hour at room temperature or overnight at 4°C, and fluorescent secondary antibodies and Hoechst dye were used for antigen and DNA visualization, respectively. Coverslips were mounted on a glass slide with Vectashield (Vector Laboratories), and photographs were taken using NIS-Elements software (Nikon) and a digital camera (Coolsnap

EZ; Roper Scientific) connected to an inverted fluorescence microscope (model eclipse Ti-S; Nikon). Quantification of nuclear signal was performed by randomly selecting at least 10 infected cells per *Toxoplasma* strain and measuring the average signal intensity per nucleus using the NIS-Elements software and Hoechst dye to define nuclei. For evacuole staining, this standard immunofluorescence protocol was modified slightly. Parasites were added to HFFs on coverslips, spun down to bring them into contact with host cells, and allowed to attach to and invade host cells for five minutes at 37°C. Unattached parasites were washed off with PBS, and cells were fixed as above, blocked in PBS with 5% (v/v) fetal bovine serum and 5% (v/v) normal goat serum for one to two hours at room temperature, and permeabilized by incubation in PBS with 0.2% (w/v) saponin at 37°C for 20 min. For proteasomal inhibition HFF monolayers were pre-treated with 20 ng/mL of MG132 for one hour at 37°C. Cells were infected with parasites and spun down. Four hours following MG132 addition, cells were washed with PBS and fresh media containing no inhibitor was added. The monolayer was incubated for one additional hour prior to fixation. To synchronize infection during time course assays, HFF monolayers were incubated on ice with cold media for ten minutes prior to infection. For infection, supernatant from fully lysed parasite flasks were pelleted and washed three times with PBS and resuspended in cold media. Following infection, monolayers were incubated on ice for 30 minutes, then unattached parasites were washed off with cold PBS. Fresh pre-warmed media was added and cells were incubated at 37°C to allow invasion and infection for the determined length of time.

Generation of transgenic parasites

The GRA15 coding region and putative promoter (1940 bp upstream of the start codon) was amplified from type II *Toxoplasma* genomic DNA by PCR (F: 5'-CCCAAGCTTGACTGCCACG TGTAGTATCC -3'; R: 5'-TTA*CGCGTAGTCCGGGACGTCGTACGGGTA*TGGAGTTACCGCTGATTGTG T -3'). Sequence coding for an HA-tag was included in the reverse primer (denoted with italics) to C-terminally tag the protein. $GRA15_{II}HA$ was then inserted into pCR8/GW (Invitrogen) by TOPO-TA cloning. Gateway cassette A was ligated into pTKO at the EcoRV site, creating a Gateway destination vector (Invitrogen), pTKO-att (Fig. S12), and $GRA15_{II}HA$ was cloned into pTKO-att by LR recombination (Invitrogen). The pTKO-att-$GRA15_{II}HA$ vector was then linearized by

digestion with XhoI (NEB). XhoI cuts off 244 bp of the putative promoter, leaving 1696 bp intact upstream of the start codon. Linearized vector was transfected into RH Δ*HXGPRT* and CEP *HXGPRT*⁻ C22 parasites by electroporation. Electroporation was done in a 2 mm cuvette (BioRad) with 2 mM ATP (MP Biomedicals), 5 mM GSH (EMD), in a GenePulser Xcell (BioRad), with the following settings: 25 μFD, 1.25 kV, ∞ Ω. Stable integrants were selected in media with 50 μg/ml mycophenolic acid (Axxora) and 50 μg/ml xanthine (Alfa Aesar) and cloned by limiting dilution. Expression of $GRA15_{II}$ was confirmed by IF for HA staining. Parasite strains already containing the *HXGPRT* gene (RH *GRA15*KO and Pru A7 *GRA15*KO) were co-transfected with 35 μg pTKO-att-$GRA15_{II}$ and 1 μg pTUB5-BLE (Soldati et al., 1995), containing the ble selectable marker. Stable integrants were selected extracellularly with 50 μg/ml Phleomycin (InvivoGen), and HA staining was confirmed by IF.

The ROP2 and GRA6 coding regions and putative promoters (at least 1500 bp upstream of the start codon) were amplified from type II *Toxoplasma* genomic DNA by PCR (ROP2F: 5'-CACCGAGGTTGGAACTGTG -3'; ROP2R: 5'-C*TTACGCGTAGTCCGGGACGTCGTACGGGTA*GATTGCCGTAACCGCCT -3'; GRA6FW: 5'- CCCAAGCTTGAAGGACTGCGTTGAGTGTTTT -3'; GRA6RV: 5'-GGAATTC*TTACGCGTAGTCCGGGACGTCGTACGGGTA*AAAATCAAACTCATTCACACT TC -3'). Sequence coding for an HA-tag was included in the reverse primers (denoted with italics) to C-terminally tag the proteins. $ROP2_{II}$HA was then inserted into pENTR/D (Invitrogen) and $GRA6_{II}$HA was inserted into pCR8/GW (Invitrogen) by TOPO cloning, and then cloned into pTKO-att by LR recombination (Invitrogen). The pTKO-att-$ROP2_{II}$HA or $GRA6_{II}$HA vectors were then linearized by digestion with NotI (NEB). Linearized vector was transfected into parasites by electroporation. Electroporation and selection was done as above, and HA staining was confirmed by IF.

Generation of GRA15 knockout

A targeting construct (Fig. S5A) was engineered using a modified pTKO-att vector, pTKO2, and MultiSite Gateway Pro 3-Fragment Recombination (Invitrogen). The hypoxanthine-xanthine-guanine ribosyl transferase (*HXGPRT*) selectable marker was removed from pTKO-att by Cre recombinase (NEB) to form pTKO2 (Fig. S12). 5' and 3' flanking regions of GRA15

were cloned from type I and type II genomic DNA. Primers contained att recombination sites (denoted in primer sequence with italics), and amplified 2083 bp, 100 bp upstream of the GRA15 start codon, and 2071 bp, 34 bp downstream of the GRA15 stop codon (5'F: 5'-*GGGGACAAGTTTGTACAAAAAAGCAGGCTTA*AGGGTCTGAACGTGTGCA-3'; 5'R: 5'-*GGGGACAACTTTGTATAGAAAAGTTGGGTG*ACCCGGCTTAAGTTGGTG-3'; 3'F: 5'-*GGGGACAACTTTGTATAATAAAGTTGC*ATGACCAAAAACCGATAA-3'; 3'R: 5'-*GGGGACCACTTTGTACAAGAAAGCTGGGTA*CAAGTCGGCACATGCTTAGA-3'). These flanking regions were then cloned around the *HXGPRT* selectable marker flanked by 5' and 3' UTRs from DHFR, amplified from pTKO with primers containing att recombination sites (denoted in primer sequence with italics) (DHFR::HPT F: *GGGGACAACTTTTCTATACAAAGTTGC*TCAGCACGAAACCTTGCAT; DHFR::HPT R: *GGGGACAACTTTATTATACAAAGTTGT*GTGTCACTGTAGCCTGCC). Prior to transfection, the knockout vector was linearized with the restriction enzyme NotI (NEB). RH Δ*HXGPRT*, Pru Δ*HXGPRT*, and Pru Δ*HXGPRT* A7 parasites were transfected with the knockout construct by electroporation, as described above. Stable integrants were selected as above and cloned by limiting dilution. PCR with a forward primer upstream of the 5' flanking region (P1: 5'-CATGGATGCTAATCGGCTTT-3') and a reverse primer within the *HXGPRT* cassette (P2: 5'-GATCCAGACGTCTTCAATGC-3'; P3: 5'-GGGGACAACTTTATTATACAAAGTTGTGTGTCACTGTAGCCTGCC-3') confirmed a disruption in the GRA15 locus. Additionally, PCR was done to confirm the inability to amplify GRA15 (P4: 5'-GATGATGGATCCATAATTCGGTGGCTTGGG-3'; P5: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTATCGGCACATGCTTAGAAG -3') (Fig. S5B).

Microarray

For human arrays, Human Foreskin Fibroblasts (HFFs) were grown in a T25 to confluency. Parasite strains were syringe lysed and washed once with PBS. HFFs were infected with Pru Δ*HXGPRT* A7, Pru Δ*HXGPRT*, Pru A7 *GRA15*KO, Pru *GRA15*KO, RH 1-1, RH Δ*HXGPRT*, RH $GRA15_{II}$ (a transgenic RH strain expressing a type II copy of GRA15), RH *GRA15*KO, CEP *HXGPRT*- C22, or CEP C22 $GRA15_{II}$ (a transgenic CEP strain expressing a

type II copy of GRA15) at varying MOIs. For mouse arrays, wild-type or $p65^{-/-}$ MEFs were grown in a 12-well plate to confluency. Parasite strains were syringe lysed and washed twice with PBS. MEFs were infected with Pru Δ*HXGPRT* A7, Pru A7 *GRA15*KO, RH 1-1, or RH $GRA15_{II}$ at varying MOIs.

Plaque assays were done to assess viability of parasites and infections with similar MOIs were chosen. Some samples were also stimulated with TNF-α. At least two biological replicates were done for every sample, except RH *GRA15*KO infection, TNF-α stimulation, RH Δ*HXGPRT* pre-infection followed by TNF-α stimulation, and all MEF samples. 18-24 hours after infection or six hours after stimulation, total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol and cleaned up using RNeasy Mini or MinElute Kit (Qiagen). RNA was labeled and hybridized to a human or mouse Affymetrix array (Human U133A 2.0 or Mouse 430A 2.0) according to the manufacturer's protocol. Probe intensities were measured with the Affymetrix GeneChip Scanner 7G and were processed into image analysis (.CEL) files with either Affymetrix GeneChip Operating Software or Affymetrix Expression Console Software. Intensity values were normalized using the MAS5 algorithm such that the median intensity on the array was 500 using Expression Console software. The MAS5 algorithm gives a signal intensity value for every probe as well as a present, marginal, or absent call, based on mismatch probes. For all probes called present, signal intensity values less than 50 were increased to a minimum value of 50. For all probes called marginal or absent, the signal intensity value was set to 50. Expression data was clustered using MultiExperiment Viewer (Saeed et al., 2003, 2006). Microarray data has been uploaded to GEO Datasets under accession number GSE25476.

Gene set enrichment analysis (GSEA) was used to find candidate transcription factors and canonical pathways that are modulated differently between *Toxoplasma* infections (Mootha et al., 2003; Subramanian et al., 2005). This program uses *a priori* defined sets of genes and determines whether the members of these sets of genes are randomly distributed throughout a ranked list or primarily found at the top or bottom. As GSEA is generally used to generate hypotheses, gene sets enriched with a false discovery rate (FDR) < 0.25 were considered significant. Both transcription factor and canonical pathway gene sets from the Molecular Signatures Database were evaluated for enrichment (c2.cp.v2.5.symbols,

c3.tft.v2.5.symbols.gmt) (Subramanian et al., 2005). The gsea2 java release was run using all default settings. For analyses on RH *GRA15*KO, TNF-α, and RH Δ*HXGPRT* + TNF-α, and all MEF infections, for which we only did one array, MOI-matched arrays and the GSEA preranked function were used.

Distant Regulatory Elements of co-regulated genes (DiRE) (Gotea and Ovcharenko, 2008) and Ingenuity pathway analysis (IPA) were also used. For every gene in a list, DiRE detects regulatory elements throughout the entire gene locus, and looks for enrichment of TFBSs. IPA takes a list of genes and overlays them onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these genes are then algorithmically generated based on their connectivity. A network is a graphical representation of the molecular relationships between genes/gene products. Genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge. All edges are supported by at least 1 reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base. The two networks with the highest score are indicated.

5' and 3' Rapid amplification of cDNA ends (RACE)

Total RNA was isolated from HFFs infected with Pru parasites using TRIzol reagent (Invitrogen) and cleaned up using RNeasy Mini Kit (Qiagen). RACE-ready cDNA was synthesized using a GeneRacer Kit with SuperScript III RT (Invitrogen). Nested PCR was done on the RACE-ready cDNA to determine both 5' and 3' transcript ends using gene-specific primers (5' GRA15R: 5'-AGTCCTCCCCGTTTTCGGTCTGTT-3'; 5' GRA15 nestedR: 5'-GACTCTGAACGGGGACGGGTAGTC-3'; 3' GRA15F: 5'-CTGTCCACTCAATAGACCCCGTTGT-3'; 3' GRA15 nestedF: 5'-AAGATGCCGTGCAAAGCCAACTTC-3'), provided GeneRacer 5' and 3' primers, and Phusion enzyme (Finnzymes). PCR products were cloned into the pCR4Blunt-TOPO vector (Invitrogen) and sequenced. Sequences were analyzed with Sequencher software (Gene Codes).

Characterization of GRA15 sequence

The coding sequence for GRA15 from types I (GT1), II (ME49), and III (VEG) was predicted from ToxoDB genomic sequence using ORF Finder (NCBI). GRA15 genomic DNA

from additional strains (RH, Pru, CEP) was amplified by PCR and sequenced (F: 5'-TCCGACTCAGTGCGGGAAA -3'; R: 5'- ATCCAGGTCCCCAAAGG -3'). To check for the presence of a predicted intron in the type I/III ORF, type I cDNA was amplified by PCR and sequenced (F: 5'- CACGTACACAACCCATCTCG -3'; R: 5'-CGAATTCTCATGGAGTTACCGCTGATT -3'). 5' and 3' UTRs were determined by RACE, as described above. Amino acid alignments were done with ClustalW2 (EMBL-EBI). Similarity to known sequences was queried using BLAST (NCBI, (Altschul et al., 1990)) and PfamA (Finn et al., 2008).

Western blot

HFFs in a 6-well plate were infected with parasites (MOI = 5) for 24 hours. Infected cells were washed with ice-cold PBS, lysed by addition of lysis buffer, boiled for five minutes and subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, which was blocked in PBS/0.1% Tween-20/5% nonfat dry milk and incubated with primary and secondary antibodies. The blot was incubated with a luminal-based substrate (Immun-Star WesternC, BioRad) and chemiluminescence was detected using a CCD camera (Bio-Rad Chemidoc XRS). The bands were visualized using Quantity One 1-D analysis software and analyzed using ImageJ.

GRA15 expression in HeLa cells

A type II copy of GRA15 or a type II copy of 55.m04955 was inserted into pIC242 (a gift from Ian Cheeseman), a Moloney Murine Leukemia Virus (MMLV) retroviral vector containing an N-terminal GFP protein fusion, by restriction/ligation. Amino acids 51-551 of $GRA15_{II}$ were included, as amino acids 1-50 were predicted to be a signal sequence by the signal peptide cleavage prediction server, SignalP (Bendtsen et al., 2004; Nielsen and Krogh, 1998). These insertions replaced the original gene insert, Mob1A. Expression of GFP fusion proteins was promoted by the endogenous retroviral long terminal repeats. HeLa cells were then transiently transfected with expression vectors by lipofection using Fugene 6 Plus Transfection Reagent (Roche). Confluent cell cultures were split 1:10 into a 24-well plate (BD Falcon) containing glass coverslips. The cells were allowed to incubate at 37°C and 5% CO_2 for four hours.

Following incubation, the medium was replaced with 1 ml of fresh supplemented DMEM, and liposomes were added dropwise to the cells. Liposomes were generated according to manufacturer protocol. Briefly, 3 µl of Fugene reagent was mixed into 20 µl of unsupplemented DMEM and allowed to stand at room temperature for five minutes. Next, 0.5 µg of appropriate plasmid DNA for each transfection was added, mixed and incorporated into liposomes for 20 minutes prior to addition to cells. Cells were left in contact with liposome for 24 hours until the cells were fixed and stained for NF-κB p65. This experiment was performed two times.

In vitro cytokine ELISA

BALB/c BMDMs were seeded (10^5 per well) in 96-well plates and left to adhere overnight at 37°C 5% CO_2. Cells were infected with freshly lysed *Toxoplasma* tachyzoites at multiplicity of infection 20, 10 and 5, supernatants (200 µL) were collected 24-48 h after infection, and stored at -20°C if necessary. IL-12p40 levels were determined, for the cells infected with equal numbers of viable parasites as determined by plaque assay, using a commercial available ELISA kit (BD Biosciences OptEIA™ Mouse IL-12 (p40) ELISA Set) following the manufacturer's instructions.

Infection of mice

Female BALB/c or C57BL/6 mice that were 6 to 10 weeks old (Jackson Laboratories, Bar Harbor, Maine) were used in all experiments. For intraperitoneal (i.p.) infection, tachyzoites were grown *in vitro* and extracted from host cells by passage through a 27-gauge needle, washed three times in PBS and quantified with a hemocytometer. Parasites were diluted in PBS, and mice were inoculated i.p. with tachyzoites of each strain (in 300 µl) by using a 28-gauge needle. To image mice infected with a parasite strain which expressed the enzyme luciferase, mice were injected i.p. with 3 mg firefly D-luciferin dissolved in PBS, anesthetized with isoflurane, 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.

Intraperitoneal wash and in vivo cytokine ELISA

One or two days after i.p. infection, mice were sacrificed and the i.p. cavity was washed with 5 ml PBS. The i.p. wash was spun at 450 xg for 5 minutes to pellet cells. Supernatant was collected and stored at -80°C if necessary. IFN-γ, IL-12p40, and IL-12p70 levels were determined using commercially available ELISA kits (eBioscience, ELISA Ready-SET-Go!) following the manufacturer's instructions.

Plaque Assay

For all assays comparing the effect of *Toxoplasma* on the host cell, cells were infected with different MOIs and a plaque assay was done to determine the viability of each strain. The infections with the closest MOIs were then used. For the plaque assay, one hundred parasites per well were added to confluent HFFs in a 24 well plate and were incubated for 5-7 days at 37°C. The number of plaques was counted using a microscope. Plaque assays were also performed to assess the viability of parasites used to infect mice. To assay *in vitro* parasite growth, plaque size was measured using NIS-Elements software (Nikon) and a digital camera (Coolsnap EZ; Roper Scientific) connected to an inverted fluorescence microscope (model eclipse Ti-S; Nikon).

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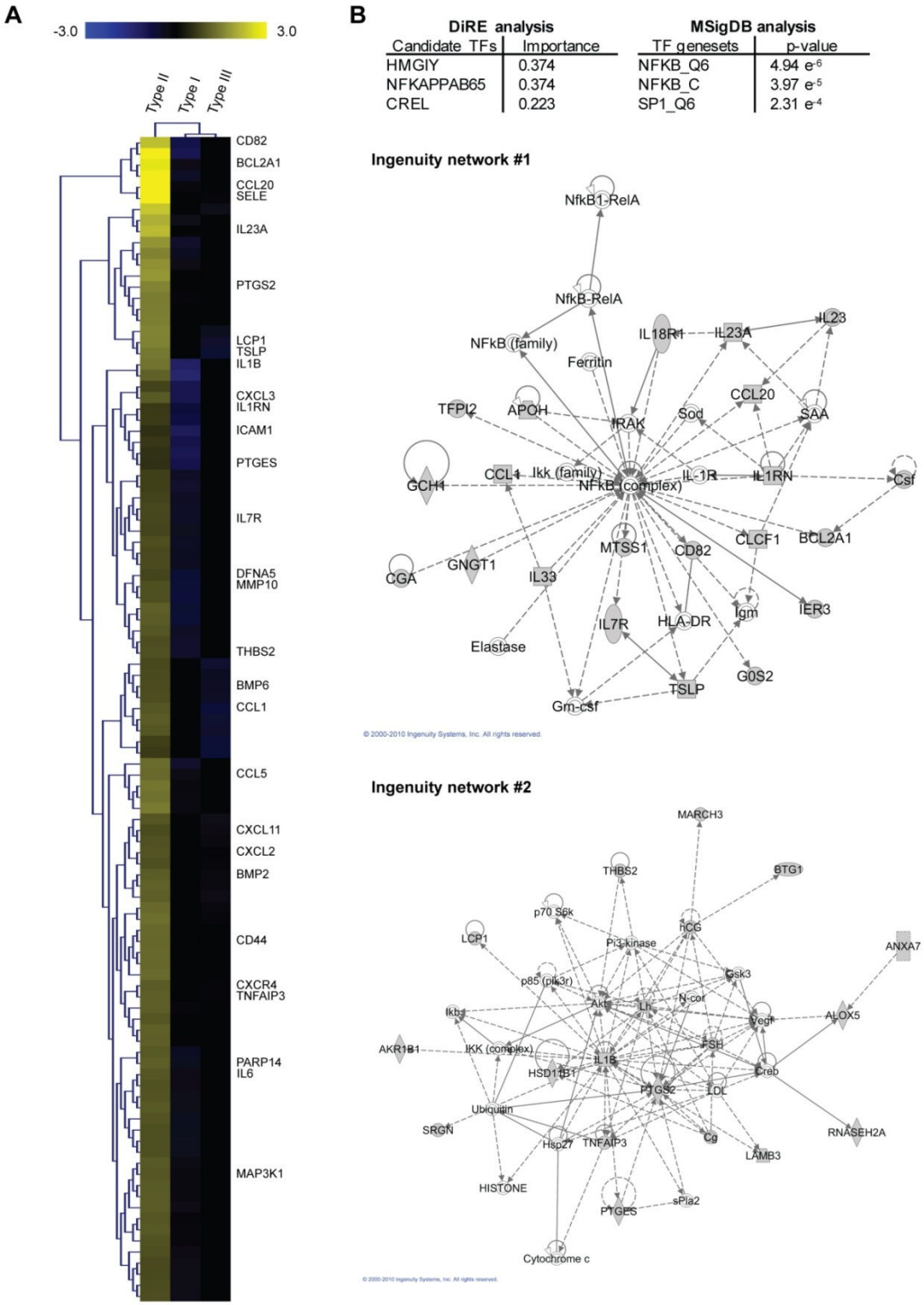
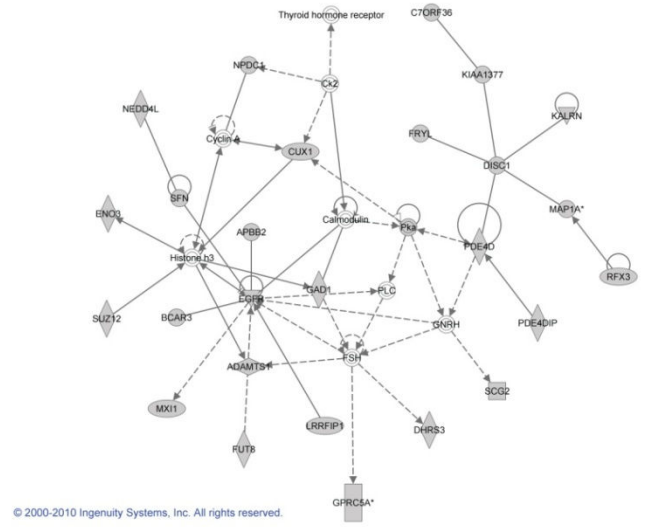
Supplementary Figures



C

Chromosome X marker	NF-κB enriched gene set	FDR q-value
AK129	NFKB_C	0.0001
GRA2	NFKB_C	0.0023
AK63	NFKB_C	0.0055
AK65	NFKAPPAB65_01	0.0293
SRS4		
AK66	NFKB_C	0.1782
AK131		
AK34	NFKB_C	0.1823
L366	NFKB_C	0.1090
M2AP	NFKB_C	0.1858
AK154	NFKB_C	0.0005
AK157	NFKB_C	0.0037
GRA6	NFKB_C	0.2231
RC4	NFKB_C	0.2354

Ingenuity network #1



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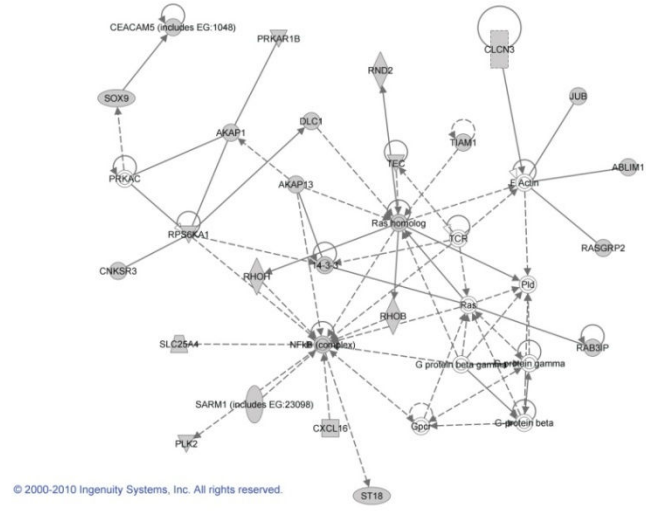


Figure S1. A type II locus on chromosome X strain-specifically induces expression of NF-κB regulated genes. **A, B.** HFFs were infected with type I, II, or III *Toxoplasma* and host gene expression was determined by microarray analysis (three arrays per strain). A. 105 genes are more than 2-fold upregulated in type II strains compared to type I/III strains. Average $\log_2$ gene expression values were median-centered, genes and strains were clustered by hierarchical clustering, and a heat map is presented made. The complete set of genes is listed in Supp. data. B. For this list of genes, the top three DiRE enriched TFBS and Molecular signatures database enriched TFBS are shown and the top two Ingenuity networks are shown. **C.** HFFs were infected with F1 progeny from a II x III cross and host gene expression was determined by microarray analysis (one array per strain). Top: For each chromosome X marker, F1 progeny were split into two groups based on their genotype. TFBS enriched in genes differentially expressed by infection with progeny with a type II genotype were determined. NF-κB gene sets, among others, were found to be significant. Bottom: Ingenuity pathway analysis was done using genes that had a sign LOD score for any chromosome X marker. The top two networks are shown.

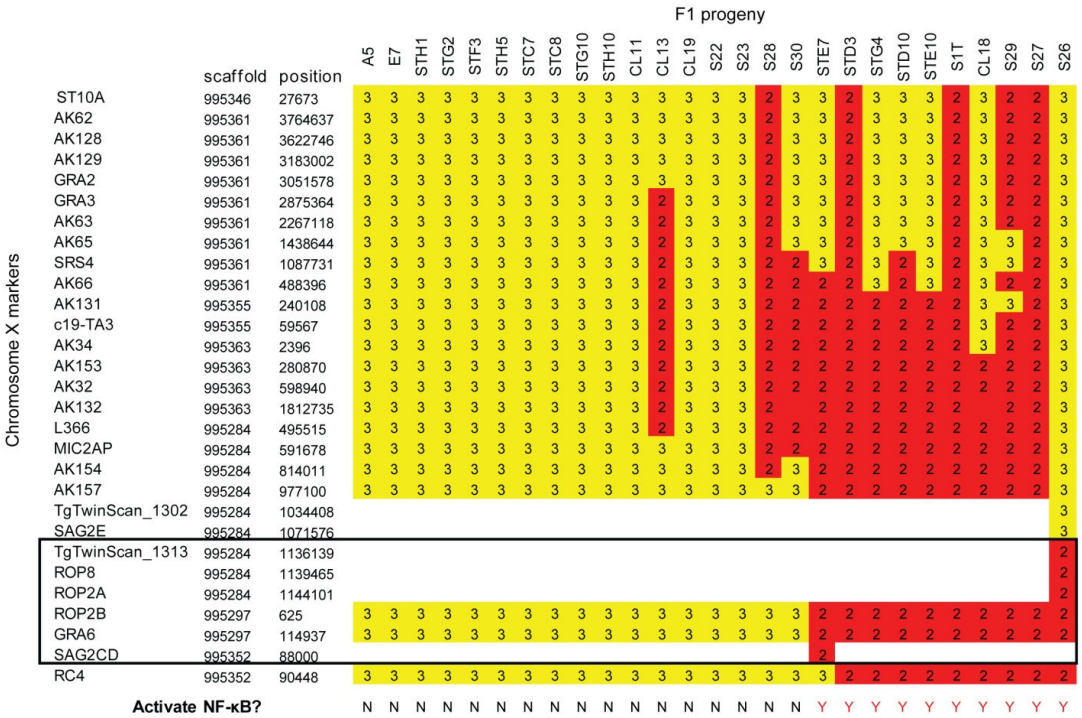


Figure S2. Mapping the strain-specific activation of NF-κB. The NF-κB activation phenotype of 27 F1 progeny derived from crosses between type II (NF-κB activating) and type III (NF-κB non-activating) strains was determined by IF. These progeny have also been genotyped at markers throughout the genome as either type II (red) or type III (yellow) (Toxomap.wustl.edu). New RFLP markers were developed and used to further genotype progeny STE7 and S26. A black box outlines the minimal region in which the locus responsible for strain-specific NF- κB activation must reside.

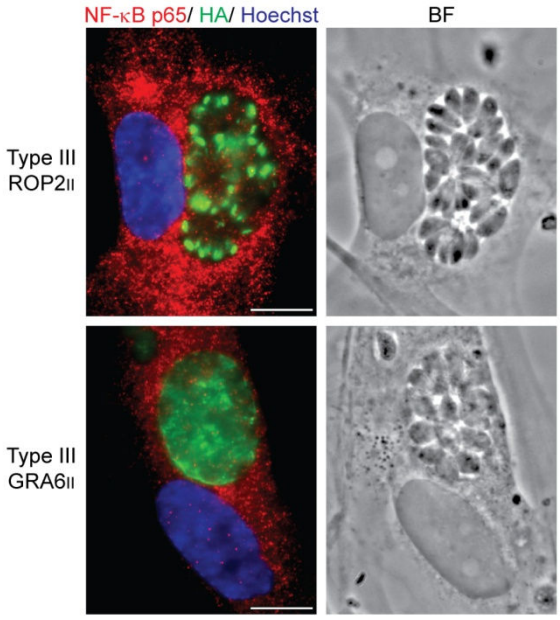


Figure S3. Type III strains complemented with *GRA6* or *ROP2* do not activate NF-κB. HFFs were infected with transgenic type III strains stably expressing an HA-tagged type II copy of either *ROP2* or *GRA6*. Cells were fixed 18-24 hours later and stained with α-NF-κB p65 (red), α-HA (green), and Hoechst dye (blue). Scale bar represents 10 μm. This experiment was done once.

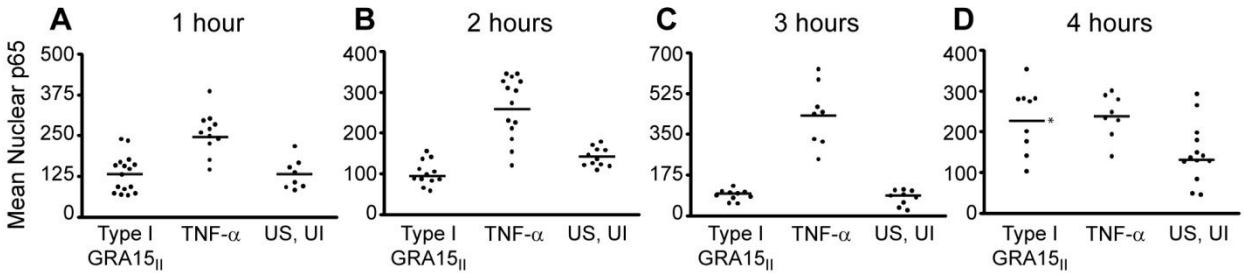


Figure S4. Time course of NF-κB activation by GRA15$_{II}$. HFF cells were infected with a type I *GRA15*$_{II}$ *Toxoplasma* strain, stimulated with TNF-α, or left unstimulated and uninfected (US, UI). For infections, cells were infected on ice with fully lysed parasites. Cells and parasites were kept on ice for 30 minutes, then allowed to infect host cells at 37°C. Coverslips were fixed every hour for four hours. **A.**, **B.**, **C.**, and **D.** correspond to 1, 2, 3, and 4 hour time points, respectively. The time course was repeated two times and quantification was performed on a representative experiment. Asterix (*) indicates mean nuclear p65 in infected cells was significantly different from in US, UI cells ($p < 0.05$, t-test).

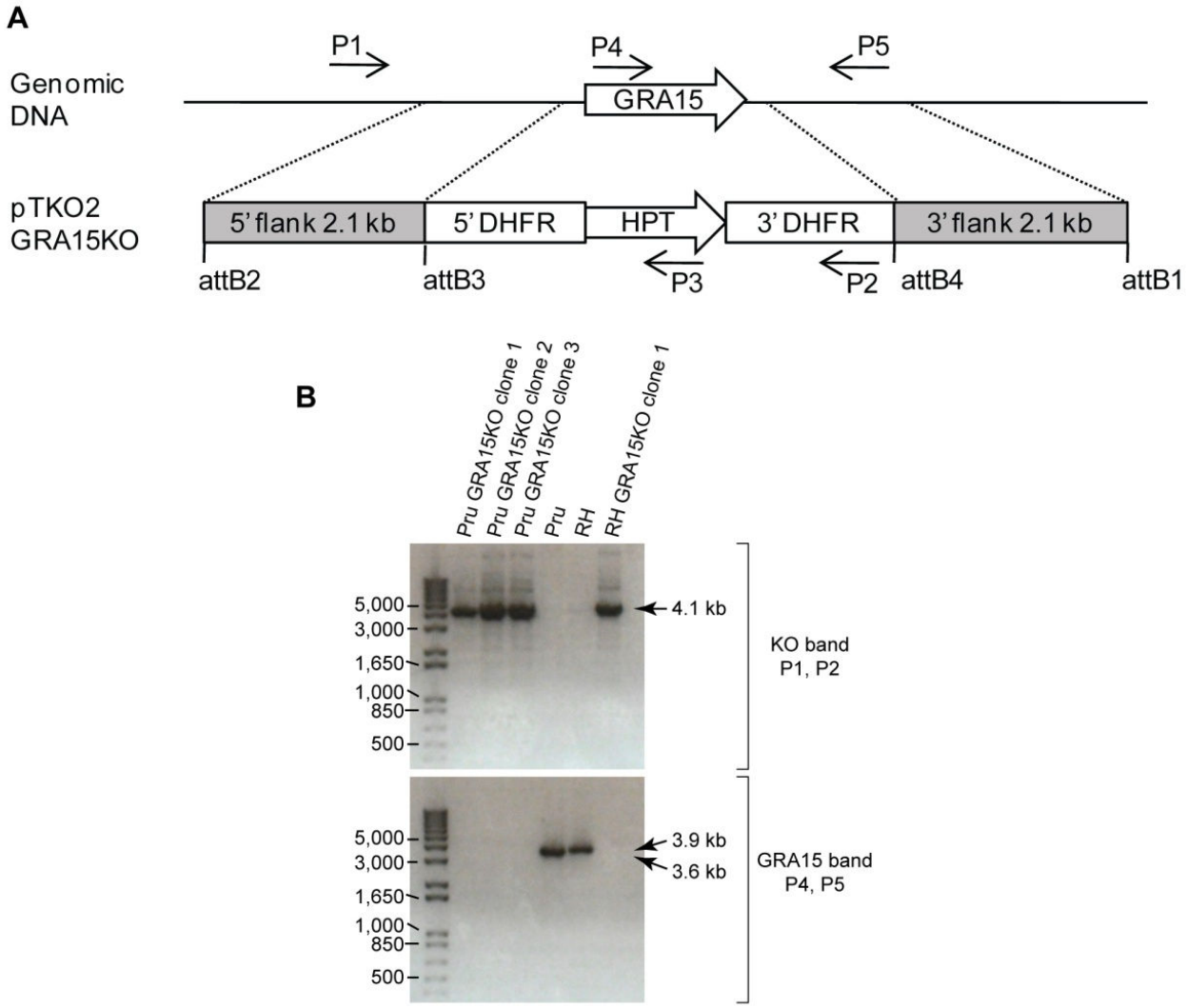


Figure S5. Generation and confirmation of *GRA15*KO. A. Schematic of the GRA15 locus, not drawn to scale. Double homologous recombination between the knockout construct (pTKO2 GRA15KO) and genomic DNA replaces *GRA15* with the *HXGPRT* (*HPT*) gene, which was used for positive selection. Primers used in PCRs to confirm a knockout are shown (P1-P5). **B.** After transfection of *Toxoplasma* strains with the GRA15KO vector, parasites were cloned by limiting dilution, and genomic DNA was isolated. This genomic DNA was then used as template in a PCR reaction to amplify a band that is only present in a successful knockout (top, P1 and P2 from Fig. 3, amplifies a 4.1 kb fragment). The intact *GRA15* locus was also amplified as a negative control (bottom, P4 and P5, amplifies a 3.9 kb fragment in type I/III strains and a 3.6 kb fragment in type II strains).

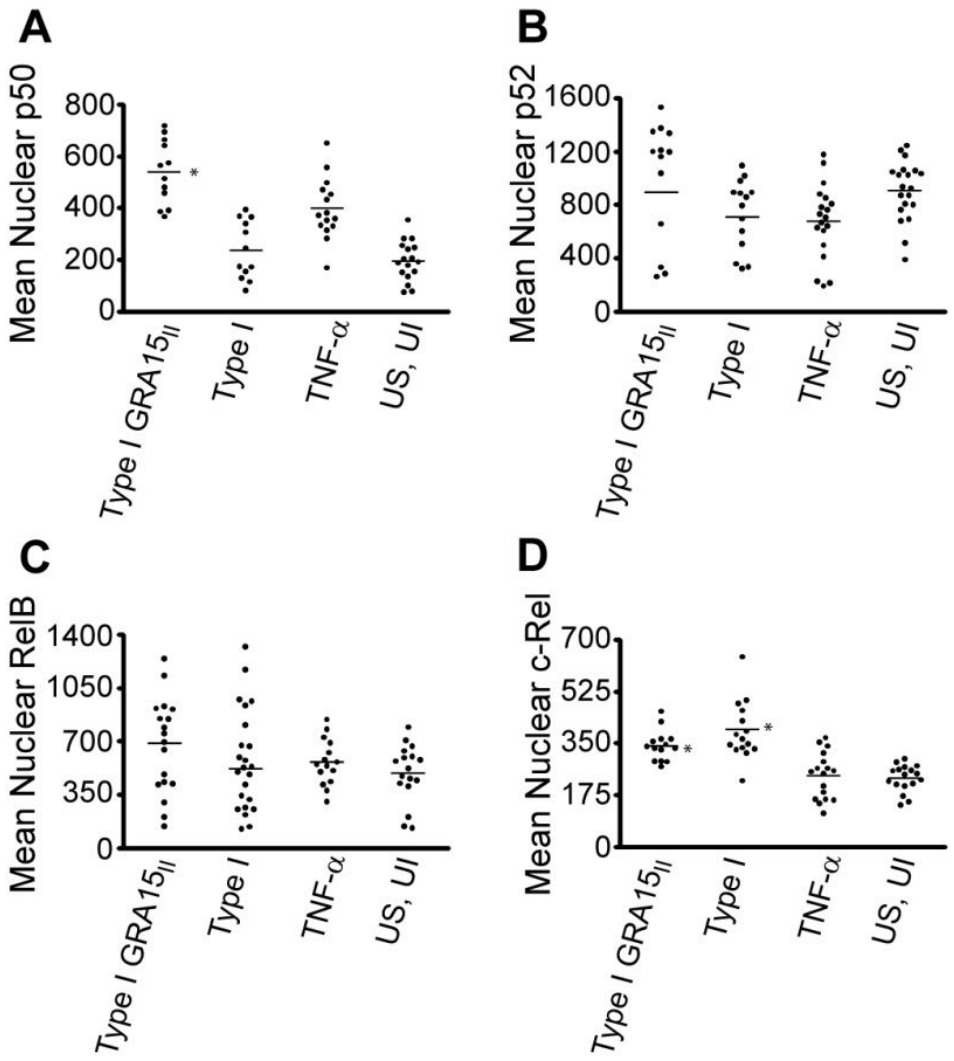


Figure S6. GRA15 activation of NF-κB family subunits. HFF cells were infected for 24 hours with type I or type I *GRA15* II parasites, stimulated with TNF-α for one hour, or left unstimulated and uninfected (US, UI). Cells were fixed and probed with antibodies to different NF-κB subunits: **A.** p50, **B.** p52, **C.** RelB, **D.** c-Rel, and mean nuclear staining was quantified. These experiments were repeated three times and quantification was performed on a representative experiment for each factor assayed. Asterix (*) indicates mean nuclear levels of the subunit are significantly different from levels in US, UI cells ($p < 0.05$).

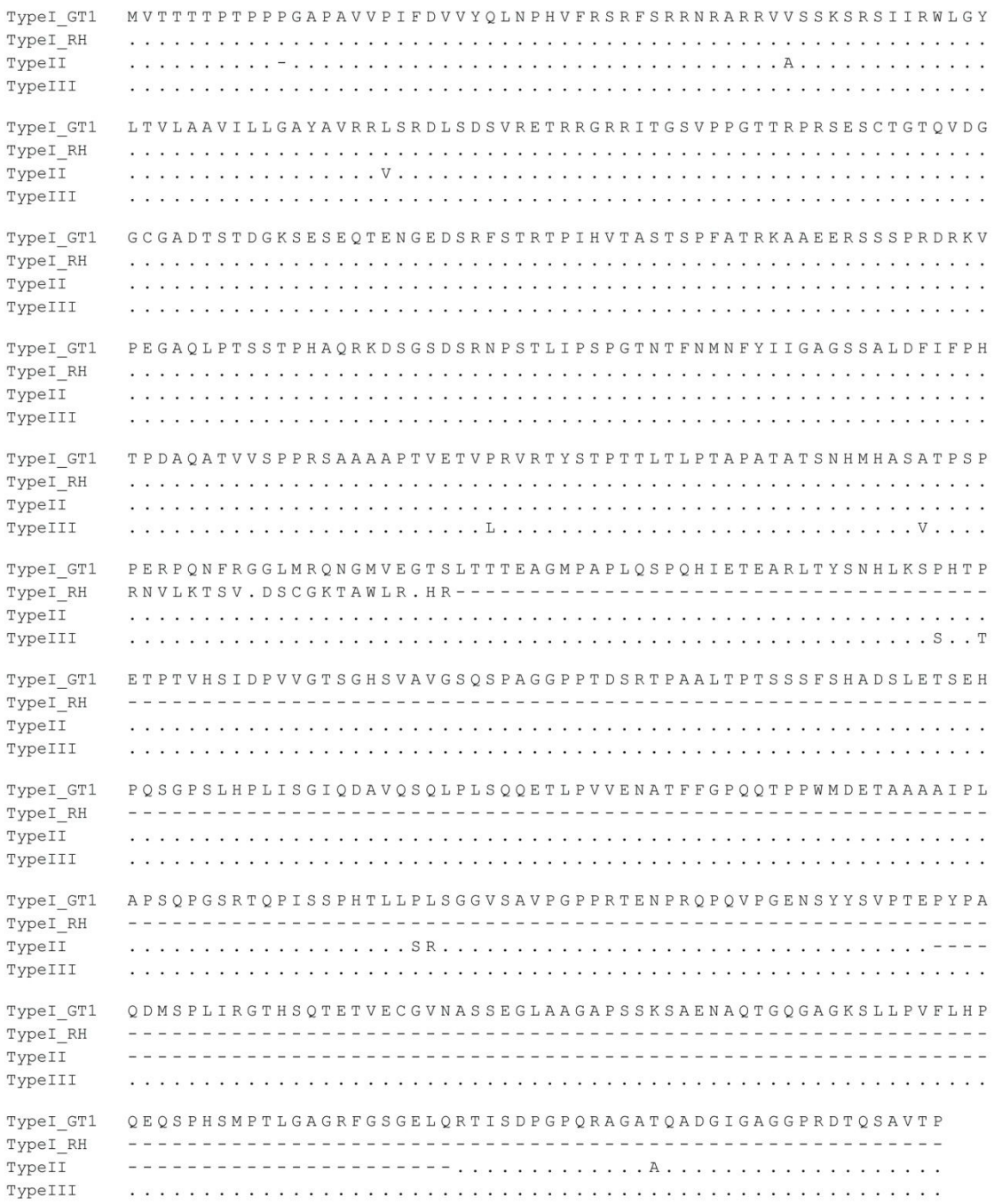
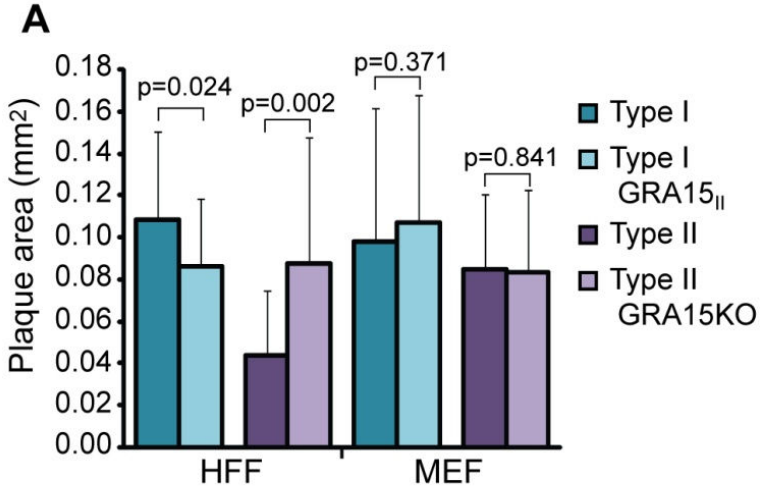


Figure S7. GRA15 amino acid alignment. The coding sequence of *GRA15* from types I (GT1), II (ME49), and III (VEG) was predicted by ToxoDB genomic sequence using ORF Finder (NCBI). *GRA15* was also sequenced in RH (I), Pru (II), and CEP (III). RH had a frameshift mutation at amino acid 290, resulting in the mutation of 20 amino acids and a premature stop codon at amino acid position 312. The type II sequence contains an 84 amino acid deletion of amino acids 519-602, as well as a single amino acid deletion and 5 single amino acid polymorphisms. Conserved (.) and missing (-) residues are indicated. The alignment was done by ClustalW2 (EMBL-EBI).



B

Organ	Competitive Index Type I $GRA15_{II}$ vs. Type I
I.P. cavity	0.22 ± 0.06*
lung	0.096 ± 0.070*
spleen	0.19 ± 0.02*

Figure S8. GRA15 affects *in vitro* parasite growth and inhibits *in vivo* parasite growth in a type I background. **A.** One hundred parasites of a type I, type I *GRA15*$_{II}$, type II, or type II *GRA15*KO strain were added to confluent fibroblast monolayers (either HFFs or MEFs) in a 24-well plate and incubated at 37°C (four days for type I infections, six days for type II infections in MEF host cells, and seven days for type II infections in HFF host cells). The size of at least 15 plaques per strain per host cell was measured. This experiment was done three times in HFF host cells and twice in MEF host cells with similar results. P-values are from a two-sample t-test. **B.** One BALB/c mouse and one C57BL/6 mouse were co-infected with 500 type I GFP^+ parasites and 500 type I $GRA15_{II}$ GFP^- parasites by i.p. injection. A plaque assay was done to confirm the ratio of GFP^+ : GFP^- parasites at injection. Five days later, mice were sacrificed, an i.p. wash was done, and the spleen and a lung were isolated. The spleen and lung were ground through a cell strainer (70 μm and 100 μm, respectively), and then spun along with the i.p. wash to pellet parasites. Pellets were resuspended in fresh media, added to HFF monolayers in T175 flasks, and incubated at 37°C. 4-7 days later, T175s were scraped, parasites were pelleted, counted, and 2,000 parasites were added to HFF monolayers in T25 flasks. After 3-4 days at 37°C, GFP^+ and GFP^- plaques were counted. Competitive index values were calculated and the averages from two mice are presented. Asterix indicates the CI is significantly different from 1 ($p < 0.05$, one sample t-test). This experiment was done once.

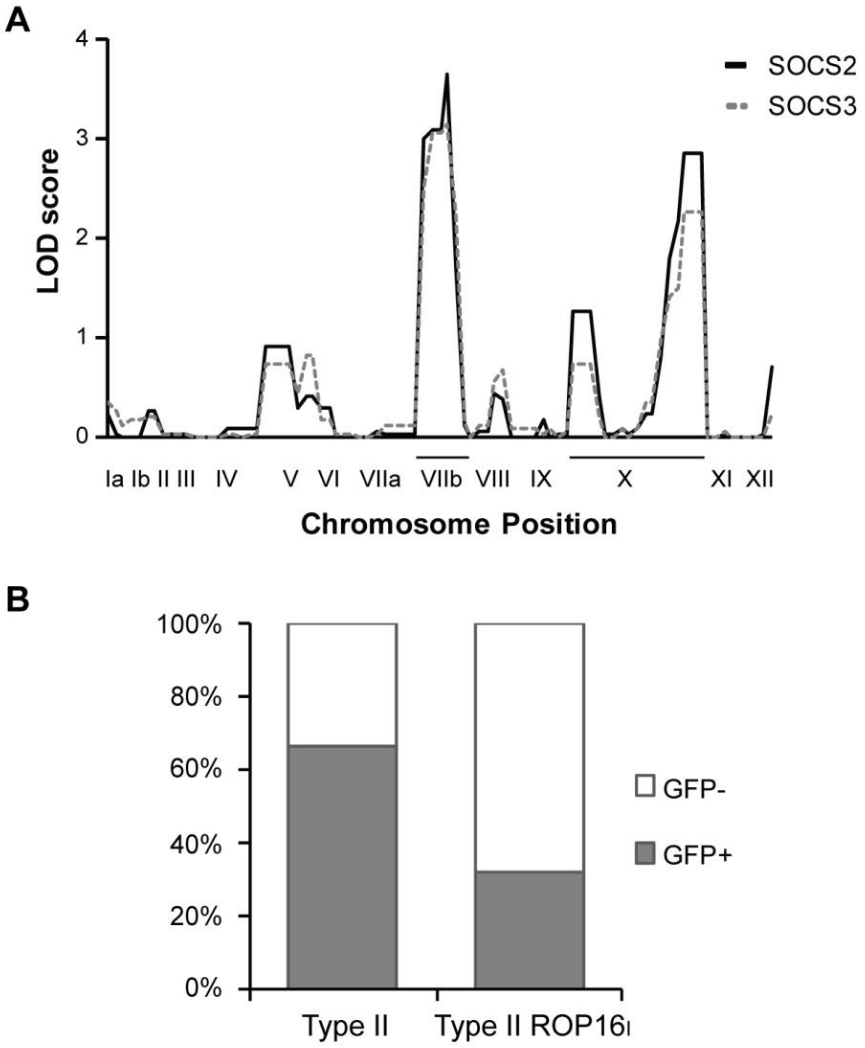


Figure S9. **ROP16 can affect the expression of GRA15-regulated genes. A.** SOCS2 and SOCS3 map to loci on chromosome VIIb and X. Previously, microarrays and QTL analysis were done on F1 progeny from a type II x type III cross to map the differential expression of host genes (Saeij et al., 2007). LOD scores for the SOCS2 and SOCS3 human genes are plotted for markers across the *Toxoplasma* genome. **B.** ROP16 inhibits GRA15-activated NF-κB in a reporter cell line. A HEK293 NF-κB GFP reporter cell line was infected with type II or type II $ROP16_I$ parasites. For each strain, the GFP level of at least 50 cells infected with only one vacuole containing at least two parasites was determined. The percent of GFP positive and GFP negative host cells for each strain is shown. The relative percentages of GFP positive and GFP negative cells after infection with these two strains is significantly different ($p = 1.5 \times 10^{-7}$, χ^2 test). This experiment has been done twice, and in two additional experiments similar results were obtained after infection of a HEK293 NF-κB luciferase reporter cell line.

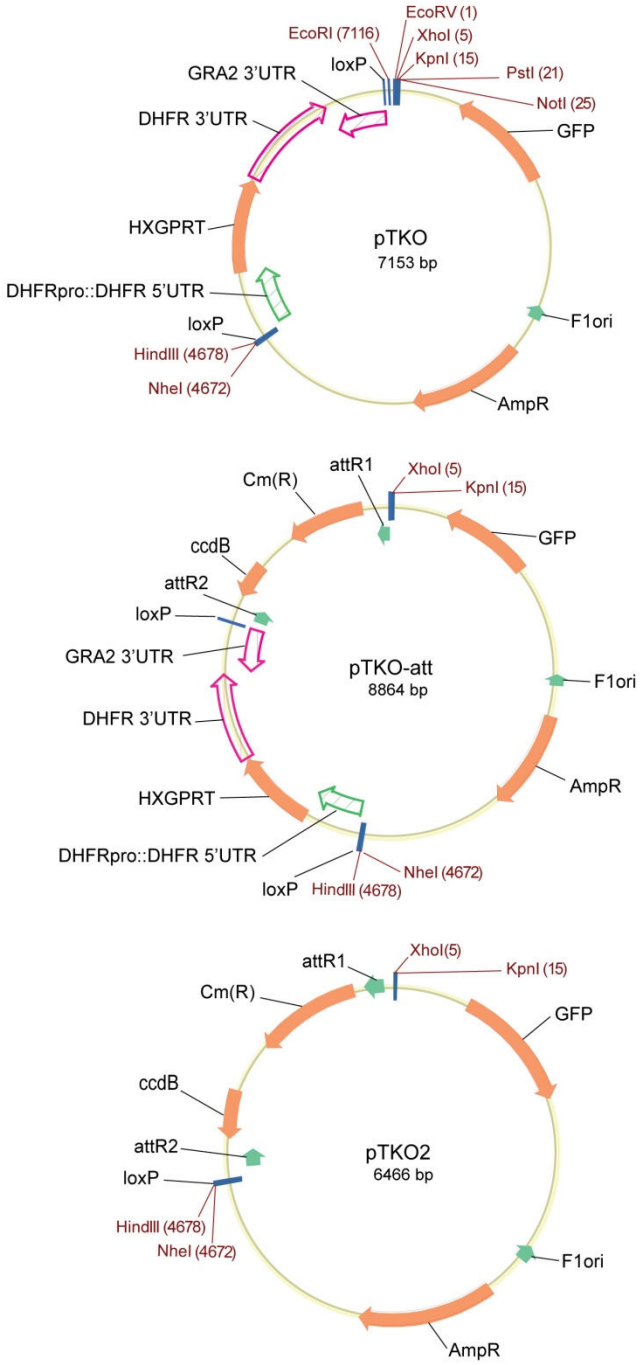


Figure S10. pTKO, pTKO-att, and pTKO2 vectors. pTKO contains the *HXGPRT* selectable marker, flanked by the *DHFR* promoter and UTRs. Gateway cassette A, containing the *ccdB* and *Cm(R)* genes flanked by attR1 and attR2 recombination sites, was ligated into pTKO at the EcoRV site, creating a Gateway destination vector, pTKO-att. The *HXGPRT* cassette was also removed from the pTKO-att vector by Cre recombination at loxP sites to form pTKO2.

Chapter 3. *Toxoplasma gondii* Clonal Strains All Inhibit STAT1 Transcriptional Activity but Polymorphic Effectors Differentially Modulate IFN-γ-Induced Gene Expression and STAT1 Phosphorylation

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Abstract

Host defense against the parasite *Toxoplasma gondii* requires the cytokine interferon-gamma (IFN-γ). However, *Toxoplasma* inhibits the host cell transcriptional response to IFN-γ, which is thought to allow the parasite to establish a chronic infection. It is not known whether all strains of *Toxoplasma* block IFN-γ responsive transcription equally and whether this inhibition occurs solely through the modulation of STAT1 activity or whether other transcription factors are involved. We find that strains from three North American/European clonal lineages of *Toxoplasma*, types I, II, and III, can differentially modulate specific aspects of IFN-γ signaling through the polymorphic effector proteins ROP16 and GRA15. STAT1 tyrosine phosphorylation is activated in the absence of IFN-γ by the *Toxoplasma* kinase ROP16, but this ROP16-activated STAT1 is not transcriptionally active. Many genes induced by STAT1 can also be controlled by other transcription factors and therefore using these genes as specific readouts to determine *Toxoplasma* inhibition of STAT1 activity might be inappropriate. Indeed, GRA15 and ROP16 modulate the expression of subsets of IFN-γ responsive genes through activation of the NF-κB/IRF1 and STAT3/6 transcription factors, respectively. However, using a stable STAT1-specific reporter cell line we show that strains from the type I, II, and III clonal lineages equally inhibit STAT1 transcriptional activity. Furthermore, all three of the clonal lineages significantly inhibit global IFN-γ-induced gene expression.

Introduction

The cytokine interferon-gamma (IFN-γ) and the transcription factor it activates, signal transducer and activator of transcription (STAT) 1, are critical to host defense against the obligate intracellular parasitic pathogen *Toxoplasma gondii*; mice deficient in elements of this pathway are acutely susceptible to *Toxoplasma* infection (Lieberman et al., 2004; Scharton-Kersten et al., 1996; Yap and Sher, 1999). Activated STAT1 induces the expression of genes with gamma activated sequence (GAS) elements in their promoters, including the interferon regulatory factor (IRF) 1 transcription factor. STAT1 and IRF1 together induce a broad transcriptional program including effector mechanisms that mediate pathogen destruction or inhibition of pathogen growth (Saha et al., 2010).

However, *Toxoplasma* infection can inhibit IFN-γ-induced gene expression in host cells, and was first shown to inhibit the basal and IFN-γ-induced expression of MHC class II molecules, in a variety of cell types (Lüder et al., 1998, 2003; Yang et al., 1996). Since then, *Toxoplasma* has also been shown to inhibit the expression of IRF1 (Lang et al., 2006; Lüder et al., 2001), class II transactivator (CIITA) (Lang et al., 2006; Lüder et al., 2001, 2003), inducible nitric oxide synthase (iNOS/NOS2) (Rozenfeld et al., 2005; Seabra et al., 2002), interferon inducible GTPase 1 (IIGP1) (Zimmermann et al., 2006), and chemokine (C-X-C motif) ligand 9 (MIG/CXCL9) (Zimmermann et al., 2006). This inhibition occurs in a variety of cell types, including human foreskin fibroblasts (HFFs), human glioblastoma cells, murine bone marrow-derived macrophages (BMDMs), RAW264.7 murine macrophages, murine dendritic cells, and murine microglial cells. Microarray analyses showed that *Toxoplasma* infection can dysregulate the entire IFN-γ-induced gene expression program in both HFFs (Kim et al., 2007a) and BMDMs (Lang et al., 2012).

Toxoplasma infects virtually all warm-blooded animals, including ~30% of the worldwide human population (Montoya and Liesenfeld, 2004). Many different strains of *Toxoplasma* have been isolated from various hosts, and in North America and Europe the majority of *Toxoplasma* isolates from humans and livestock belong to three main clonal lineages: types I, II, and III (Howe and Sibley, 1995). These strains differ in the modulation of multiple host cell signaling pathways through polymorphic effectors secreted into the host cell from rhoptry and dense granule organelles (Melo et al., 2011). While all of these strains can inhibit the expression of at least certain IFN-γ-induced genes, it is unknown whether all of the strains can inhibit global IFN-γ-induced gene expression and STAT1 transcriptional activity, or whether the degree of inhibition varies between *Toxoplasma* strains.

Many STAT1 regulated genes can be induced or repressed by other transcription factors, for example NF-κB and STAT3/6, and such genes might not be the best readouts to determine if *Toxoplasma* specifically inhibits STAT1 activity. Another question that is still unanswered is whether the activation of other transcription factors by *Toxoplasma* affects the IFN-γ response. Specifically, the modulation of STAT3/6 and NF-κB transcription factors through the effector proteins ROP16 (Saeij et al., 2007) and GRA15 (Rosowski et al., 2011), respectively, might affect this response.

The polymorphic rhoptry kinase ROP16 from type I and III strains activates the transcription factors STAT3 and STAT6 (Ong et al., 2010; Saeij et al., 2007; Yamamoto et al., 2009). In STAT3 deficient cells (Costa-Pereira et al., 2002) or cells with STAT6 knocked down (Baus et al., 2009), increased transcription of STAT1 target genes has been found, suggesting that STAT3 and STAT6 can antagonize STAT1 activity. STAT6 can also compete for promoter sites with STAT1 (Ohmori and Hamilton, 1997). It is therefore possible that the activation of STAT3/6 by ROP16 helps to suppress IFN-γ-induced signaling.

SOCS family proteins are important negative regulators of the IFN-γ response and in *Socs1*$^{-/-}$ BMDM, *Toxoplasma* could not inhibit the IFN-γ response as well as in wild-type BMDM (Zimmermann et al., 2006). ROP16 is a strong activator of SOCS family gene expression; in murine BMDM, *Socs1*, *2*, and *3* are more than 10-fold induced by ROP16 expression (Jensen et al., 2011). It is therefore possible that ROP16 plays a role in the inhibition of the IFN-γ response through the induction of *Socs* genes. Furthermore, the expression of genes that are co-regulated by both STAT1 and STAT3/6 transcription factors could also be affected by ROP16. If the expression level of such a gene was chosen to measure STAT1 activity, incorrect conclusions might be drawn.

The type II version of the dense granule protein GRA15 activates the host cell NF-κB pathway (Rosowski et al., 2011). NF-κB also co-regulates many of the same genes as STAT1, and NF-κB activation combined with STAT1 activation synergistically induces IRF1 expression and activity (Robinson et al., 2006). It is therefore possible that strains possessing an active copy of GRA15 do not inhibit IFN-γ-induced gene expression as well as other strains, or differentially inhibit subsets of IFN-γ responsive genes. In fact, a type II Δ*gra15* strain grows faster *in vivo* than a type II strain (Rosowski et al., 2011), and *GRA15* corresponds to a *Toxoplasma* virulence locus (Rosowski et al., 2011; Saeij et al., 2006).

In this report we show that the polymorphic effectors GRA15 and ROP16 do contribute to strain differences in the modulation of IFN-γ/STAT1 signaling. Type II GRA15 induces the expression of IRF1, which can induce a subset of IFN-γ responsive genes. ROP16 induces the tyrosine phosphorylation and nuclear translocation of STAT1 but this STAT1 is not transcriptionally active. In spite of these differences, type I, II, and III parasites can all inhibit global IFN-γ-induced transcription as determined by microarray analysis. Because many

STAT1-regulated genes can be controlled by other transcription factors we directly measured STAT1 activity with a stable STAT1 specific reporter cell line and find that neither GRA15 nor ROP16 affects the ability of *Toxoplasma* to inhibit STAT1 transcriptional activity.

Results

A type II strain activates IRF1 via GRA15 and NF-κB

Infection of HFFs with a type II Pru strain of *Toxoplasma* was previously shown to induce the expression of 46 genes that were also defined as IFN-γ regulated (Kim et al., 2007a), raising the possibility that type II strains are not as good at inhibiting IFN-γ-induced gene expression as other clonal lineages. To compare the ability of type I, II, and III strains to inhibit the IFN-γ response we pre-infected HFFs with RH(I), Pru(II), or CEP(III) strains, or left cells uninfected, and subsequently stimulated the cells with IFN-γ or left them unstimulated. We then visualized and quantified the amount of IRF1 in the nucleus by immunofluorescence. IRF1 is a primary response gene induced directly by STAT1 upon IFN-γ stimulation. After three hours of infection, with IFN-γ stimulation for the last two hours, cells pre-infected with either RH(I), Pru(II), or CEP(III) all had significantly lower levels of IRF1 in their nuclei than uninfected cells (Fig. 1A, B), as was previously seen for type I, II, and III strains (Kim et al., 2007a; Lang et al., 2006; Lüder et al., 2001). However, after 24 hours of infection, with IFN-γ stimulation for the last six hours, while RH(I), Pru(II), and CEP(III) infection all significantly inhibited IRF1 expression compared to uninfected cells, cells pre-infected with a Pru(II) strain had significantly higher IRF1 in their nuclei than cells pre-infected with a RH(I) strain (Fig 1A, B). Cells pre-infected with a Pru(II) strain also had higher levels of IRF1 than cells pre-infected with a CEP(III) strain but this difference was not statistically significant. These data suggest that a Pru(II) strain does not inhibit IRF1 expression as well as RH(I) or CEP(III) strains.

We next determined IRF1 levels after infection in the absence of IFN-γ. In unstimulated cells infected with Pru(II) for 24 hours, we find ~2.5 fold higher nuclear IRF1 levels than in uninfected cells or cells infected with either RH(I) or CEP(III) (Fig 1A, B). These data suggest that the different IRF1 protein levels observed in Pru(II) and RH(I) infected cells after IFN-γ treatment may not be due to differences in the ability of these strains to inhibit IFN-γ-induced

gene expression but instead due to the induction of IRF1 by Pru(II) infection, independently of IFN-γ.

Although IRF1 is primarily induced by interferons it also has three NF-κB binding sites in its promoter (Saha et al., 2010) and these are important for the synergistic induction of genes by IFN-γ and TNF-α (Robinson et al., 2006). Type II parasites activate NF-κB-mediated transcription via the dense granule protein GRA15 (Rosowski et al., 2011) and we hypothesized that GRA15-mediated NF-κB activation could drive the expression of IRF1. To test this hypothesis, we also infected HFFs with a Pru Δ*gra15* strain (Fig. 1A, B). After 24 hours of infection, this strain induced significantly less IRF1 protein than a Pru strain ($p < 0.001$) and Pru Δ*gra15* infected cells have similar IRF1 levels as cells infected with RH(I) and CEP(III) strains which possess inactive copies of GRA15 (Rosowski et al., 2011). An RH(I) strain ectopically expressing a type II copy of GRA15 also induced IRF1 expression in HFF host cells (Fig. 1C).

To determine whether this GRA15-mediated activation of IRF1 is dependent on STAT1, we infected U3A STAT1-deficient cells (McKendry et al., 1991; Müller et al., 1993) with either Pru(II) or Pru Δ*gra15* parasites, or stimulated the cells with IFN-γ. While IFN-γ treatment, which relies on STAT1 signaling, does not activate IRF1 expression in these cells, infection with Pru(II) parasites does, and this activation is again dependent on the presence of GRA15 (Fig. 1D), demonstrating that the GRA15-mediated induction of IRF1 is through a different transcription factor. GRA15 is known to activate the NF-κB p65 transcription factor (Rosowski et al., 2011), and since it is also known that NF-κB can activate the expression of IRF1 (Robinson et al., 2006; Saha et al., 2010), we hypothesized that GRA15 was inducing IRF1 through the activation of NF-κB p65. Indeed, in a previous microarray analysis (Rosowski et al., 2011), while *Irf1* transcript was induced by infection of wild-type MEFs with GRA15-expressing Pru(II) parasites, infection with this strain did not induce *Irf1* transcript in $p65^{-/-}$ MEFs, strongly suggesting that induction of IRF1 expression by GRA15 is through the NF-κB p65 transcription factor.

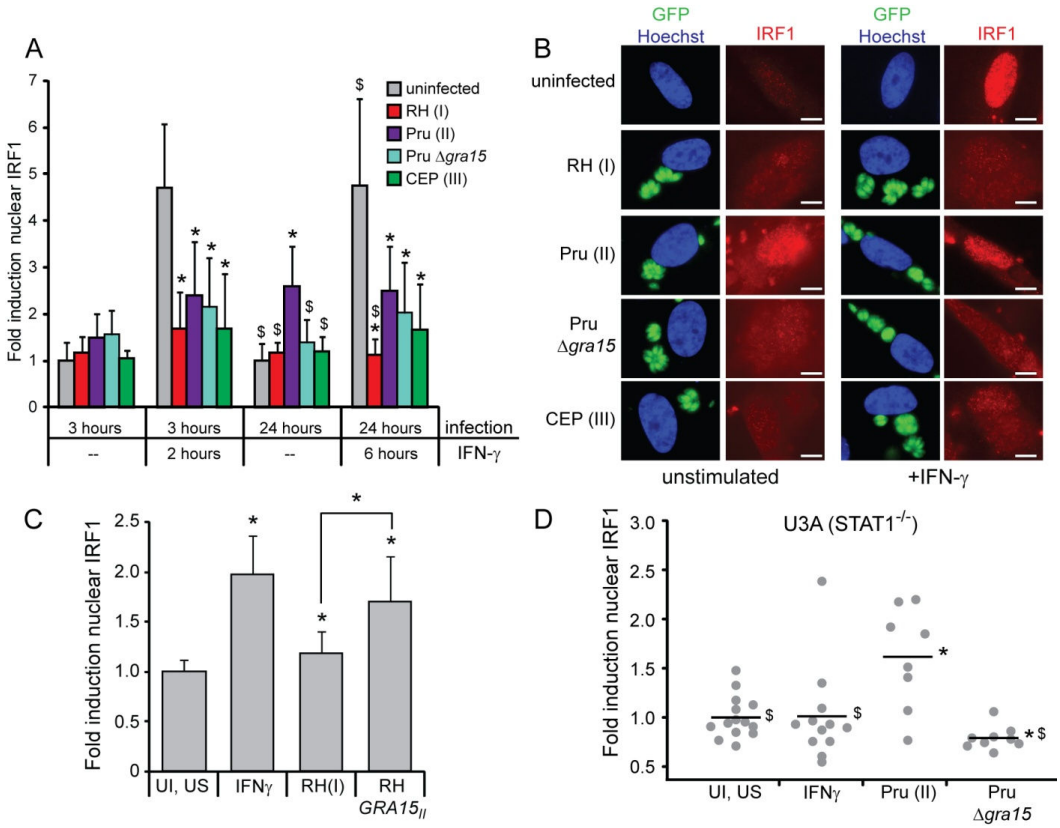


Figure 1. Type II GRA15 affects the expression of IRF1. HFFs were infected with RH(I), RH *GRA15$_{II}$*, Pru(II), Pru Δ*gra15*, or CEP(III) parasites and/or stimulated with 100 U/ml IFN-γ, subsequently fixed and permeabilized and stained with an antibody against IRF1 (red) and with Hoechst dye (blue, nucleus). **A, B.** HFFs were infected for three or 24 hours with GFP-expressing parasites (green), or left uninfected, and stimulated with IFN-γ for the last two or six hours, or left unstimulated. Nuclear localization of IRF1 was quantified in at least 12 randomly selected cells per condition and normalized to uninfected, unstimulated cells (A) and a representative cell for each condition is shown (B). Scale bar represents 10 µm. This experiment was performed three times with similar results. Data and standard deviation from one representative experiment are shown. Asterisk (*) indicates $p < 0.05$ compared to uninfected cells, dollar sign ($) indicates $p < 0.05$ compared to type II infected cells. **C.** HFFs were infected with an RH(I) or RH *GRA15$_{II}$* strain, left uninfected, or left uninfected and stimulated with IFN-γ for 24 hours. Nuclear localization of IRF1 was quantified in at least 30 randomly selected cells and normalized to uninfected, unstimulated cells. Data and standard deviation from one experiment are shown. Asterisk (*) indicates $p < 0.05$ compared to uninfected cells or as represented by brackets. **D.** U3A STAT1-deficient cells were infected with Pru(II) or Pru Δ*gra15* parasites for 20 hours, left uninfected, or stimulated with IFN-γ for one hour. Nuclear localization of IRF1 was quantified in at least 8 randomly selected cells per condition, and normalized to uninfected, unstimulated cells. This experiment was performed twice with similar results, data from one representative experiment are shown. Asterisk (*) indicates $p < 0.05$ compared to uninfected cells, dollar sign ($) indicates $p < 0.05$ compared to type II infected cells.

IRF1 is itself a transcription factor and to test whether GRA15 might be responsible for more than just the expression of IRF1, but also the expression of other IFN-γ regulated genes that were found to be induced by Pru infection (Kim et al., 2007a), we re-analyzed the microarray data from which this observation was made. We found 775 oligonucleotide probes that were at least two-fold induced in HFFs by IFN-γ treatment and by Pru infection. These 775 probes correspond to 374 genes also present in a microarray analysis of HFFs infected with GRA15-deficient and GRA15-overexpressing *Toxoplasma* strains (Rosowski et al., 2011). Of these 374 genes, 43 were previously found to be at least two-fold GRA15-regulated in at least one parasite genetic background (Rosowski et al., 2011), a significant enrichment ($p = 0.03$, hypergeometric test), indicating that GRA15 does induce the expression of a subset of IFN-γ responsive genes (Supp. data). Therefore, while type I, II, and III *Toxoplasma* strains can all inhibit the IFN-γ-induced expression of IRF1, type II strains also induce IRF1 expression, independently of STAT1, most likely through GRA15-mediated activation of NF-κB p65. This IRF1 induction also leads to the expression of a small subset of other IFN-γ responsive genes.

Toxoplasma infection affects STAT1 phosphorylation

After IFN-γ treatment, STAT1 is tyrosine phosphorylated in the cytoplasm which allows it to traffic to the nucleus. Most recently, it was shown that infection of cells with type II Pru (Kim et al., 2007a) or NTE parasites (Lang et al., 2006, 2012) does not inhibit IFN-γ-induced STAT1 trafficking into the nucleus. Previously however, the nuclear translocation of STAT1 was reported to be inhibited by type II (NTE) *Toxoplasma* infection (Lüder et al., 2001); the tyrosine phosphorylation of STAT1 was reported to be inhibited by type I (BK) infection (Zimmermann et al., 2006), and type I (RH), type II (Pru), and type III (CL14) *Toxoplasma* strains were suggested to cause dephosphorylation of STAT1 in the nucleus (Kim et al., 2007a).

To determine if there are strain differences in the effect of infection on IFN-γ-induced STAT1 phosphorylation and localization, we infected HFFs for one hour with either RH(I), Pru(II), or CEP(III) parasites, subsequently stimulated the cells for two hours with IFN-γ, and quantified STAT1 tyrosine phosphorylation and nuclear translocation by immunofluorescence (Fig. 2A, B). Quantification of the immunofluorescence signal revealed that levels of IFN-γ-

induced nuclear phospho-STAT1^{Tyr} were actually higher in infected cells compared to uninfected cells (Fig. 2A). We therefore find that none of the tested *Toxoplasma* strains inhibit the tyrosine phosphorylation or nuclear accumulation of phospho-STAT1^{Tyr} after IFN-γ treatment, which agrees with the majority of previous results.

Since we observed higher levels of phospho-STAT1^{Tyr} in infected cells as compared to uninfected cells after IFN-γ stimulation, we wondered whether infection with type I, II, or III parasites induces nuclear phospho-STAT1^{Tyr} in the absence of IFN-γ. We infected HFFs for three hours with either RH(I), Pru(II), or CEP(III) parasites, and quantified STAT1 tyrosine phosphorylation and nuclear translocation by immunofluorescence. Indeed, we observed nuclear phospho-STAT1^{Tyr} in unstimulated cells infected with three or more RH(I) or CEP(III) parasites, and to a lower level in cells infected with three or more Pru(II) parasites (Fig. 2B). We quantified this signal in cells infected with three or more parasites and find that infection results in a significant increase in nuclear phospho-STAT1^{Tyr} levels in RH(I) and CEP(III) infected cells (Fig. 2A). Pru(II) infection also significantly induces phospho-STAT1^{Tyr}, although not as highly as RH(I) or CEP(III) parasites (Fig. 2A).

We next sought to determine what parasite factor induces phospho-STAT1^{Tyr} after host cell infection. It is known that the secreted rhoptry kinase ROP16 from type I and III strains can directly tyrosine phosphorylate STAT3 and STAT6 (Ong et al., 2010; Yamamoto et al., 2009). The first 700 amino acid residues of STATs 1-6 share up to 40% identity (Schindler and Darnell, 1995), raising the possibility that ROP16 also induces the tyrosine phosphorylation of STAT1. To determine if ROP16 is required for the tyrosine phosphorylation of STAT1 by RH(I) parasites in non-IFN-γ-stimulated conditions, we also infected HFFs with RH Δ*rop16* parasites and again visualized phospho-STAT1^{Tyr} nuclear accumulation by immunofluorescence. As compared to cells infected with RH(I) parasites, cells infected with RH Δ*rop16* parasites had significantly less phospho-STAT1^{Tyr} in their nuclei, with levels almost as low as in uninfected cells (Fig. 2A, B). We next infected HFFs with a Pru strain that overexpresses the type I copy of *ROP16*. The ectopic expression of ROP16$_I$ in a type II background led to an increase in phospho-STAT1^{Tyr} after infection (Fig. 2A, B). However, deletion of *ROP16* from a type I parasite background or overexpression of *ROP16$_I$* in a type II parasite background did not affect the level of phospho-STAT1^{Tyr} in infected cells after IFN-γ treatment, indicating that the increase in phospho-

STAT1^{Tyr} in infected cells after IFN-γ stimulation occurs independently of ROP16 (Fig. 2A, B). Together, these results demonstrate that in the absence of IFN-γ, ROP16 can induce the tyrosine phosphorylation of STAT1.

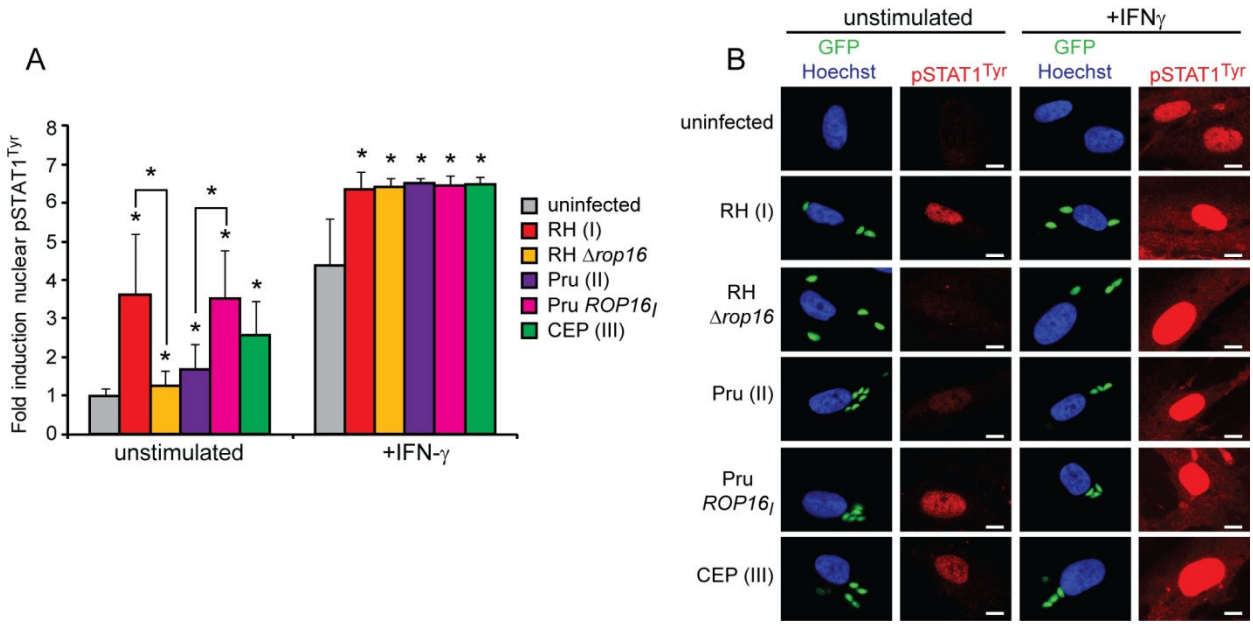


Figure 2. ROP16 activates STAT1 tyrosine phosphorylation and nuclear translocation

A, B. HFFs were infected with a GFP (green) expressing RH(I), RH Δ*rop16*, Pru(II), Pru *ROP16$_I$*, or CEP(III) strain, or left uninfected, for three hours, and 100 U/ml IFN-γ was added for the last two hours of infection, or cells were left unstimulated. Cells were fixed, permeabilized, and stained with α-phospho-STAT1^{Tyr} (red) and Hoechst dye (nucleus, blue). Nuclear localization of phospho-STAT1^{Tyr} was quantified in at least 30 randomly selected cells infected with at least three parasites (A) and a representative cell for each condition is shown (B). Scale bar represents 10 μm. This experiment was performed for each condition at least two times with similar results. Data and standard deviation from one representative experiment are shown. Asterisk (*) indicates $p < 0.05$ compared to uninfected cells or as indicated by brackets.

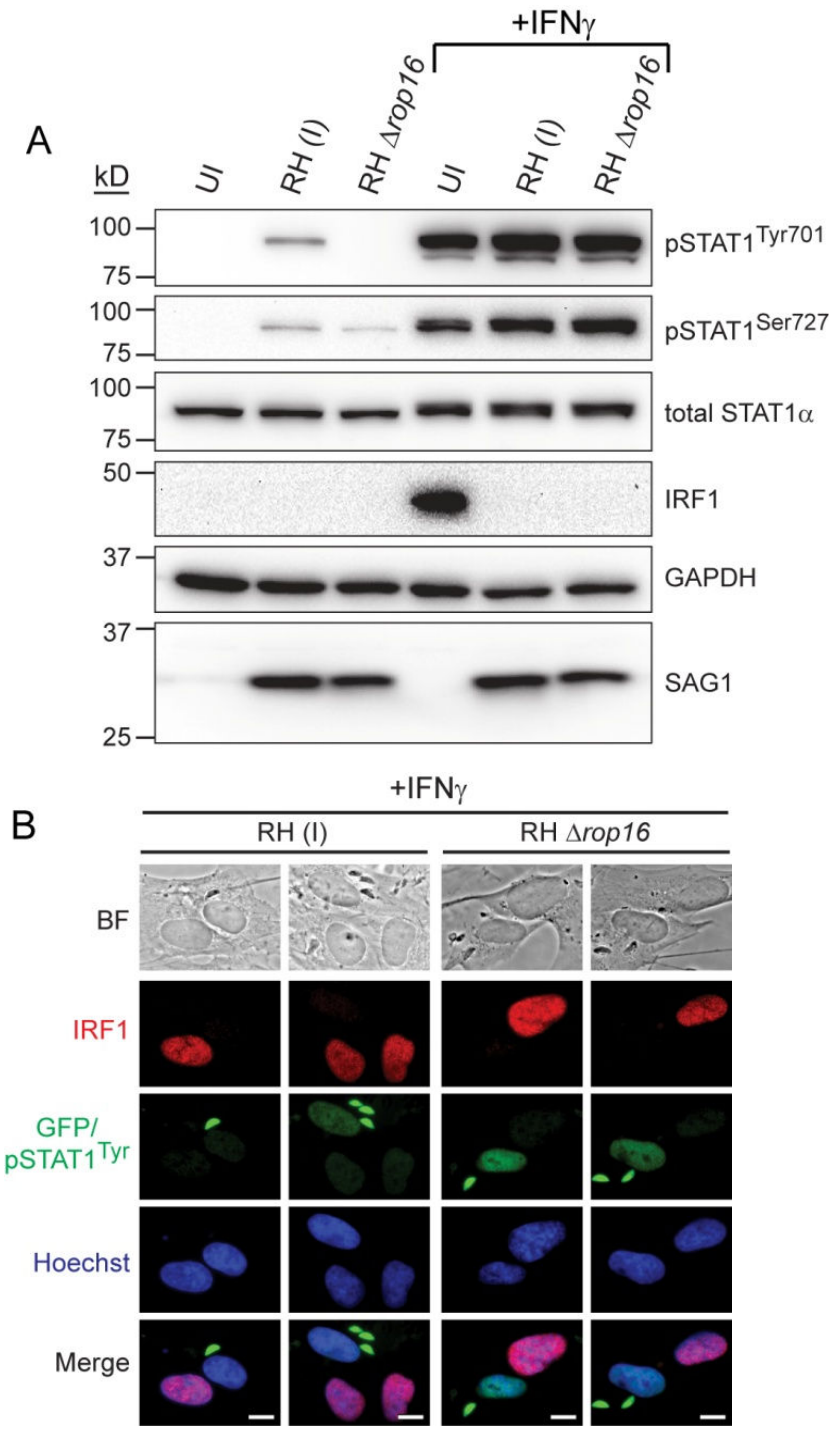
ROP16 activated STAT1 is not transcriptionally active

Our results indicate that ROP16 can directly activate STAT1 and it is therefore possible that strains with an active ROP16 (I and III) might be less efficient in inhibiting IFN-γ mediated STAT1 activation. On the other hand, ROP16 also activates STAT3 and STAT6, both of which can induce *SOCS* gene expression, which might inhibit IFN-γ/STAT1 signaling (Hebenstreit et al., 2003; Naka et al., 1997). Indeed, we previously showed that *Socs1*, a potent inhibitor of the

IFN-γ/STAT1 signaling pathway, is one of the host genes most highly induced by ROP16 expression (Jensen et al., 2011). To determine if ROP16 might play a role in the modulation of the IFN-γ response, we infected HFFs with an RH(I) or RHΔ*rop16* strain for three hours, or left cells uninfected, and subsequently stimulated with IFN-γ for one hour, or left cells unstimulated, and analyzed IRF1 protein levels by Western blot. While only RH(I) infection induced the tyrosine phosphorylation of STAT1 in the absence of IFN-γ (Fig. 3A), RH(I) and RH Δ*rop16* parasites both completely inhibited IFN-γ-induced IRF1 expression (Fig. 3A), indicating that ROP16-induced phospho-STAT1^{Tyr} is not transcriptionally active and that ROP16 is not required for the ability of *Toxoplasma* infection to block IFN-γ-induced STAT1-mediated gene expression. These results were confirmed in an immunofluorescence assay. After two hours of infection with either RH or RH Δ*rop16* parasites, HFFs were subsequently stimulated with IFN-γ for two hours, cells were fixed and permeabilized, and IRF1 expression and STAT1 tyrosine phosphorylation were visualized. As seen previously (Fig. 2A, B), after IFN-γ treatment, cells infected with either RH or RH Δ*rop16* had higher nuclear phospho-STAT1^{Tyr} than uninfected cells (Fig. 3B). But, as in the Western blot results (Fig. 3A), both strains clearly inhibited IFN-γ-induced IRF1 expression (Fig. 3B).

In addition to STAT1 tyrosine phosphorylation at residue 701, which is required for dimerization and nuclear translocation, STAT1 also must be serine phosphorylated at residue 727 for full transcriptional activity (Platanias, 2005). We wondered whether ROP16 or type I, II, or III strains of *Toxoplasma* affect this serine phosphorylation. It was previously shown that infection with a Pru(II) strain of *Toxoplasma* does not interfere with IFN-γ-induced serine phosphorylation of STAT1 in HFFs (Kim et al., 2007a), but this has not been shown for any type I or III strains. We infected HFFs with an RH(I), RH Δ*rop16*, Pru(II), or CEP(III) strain, or left cells uninfected, for three hours, subsequently stimulated cells with IFN-γ for one hour, or left cells unstimulated, and analyzed lysates by Western blot. We first blotted for IRF1 as a control to confirm that infection with any of these strains inhibited the IFN-γ-induced accumulation of IRF1 (Fig. 3C), as we have shown by immunofluorescence (Fig. 1A, B, Fig. 3B). IRF1 was not inhibited as strongly in this infection as compared to the previous Western blot (Fig. 3A) due to a lower MOI. Additionally, Pru(II) infection alone led to the induction of IRF1 protein, in concordance with previous immunofluorescence experiments (Fig. 1A,B). We next analyzed

STAT1 phosphorylation in these lysates. Consistent with our immunofluorescence data (Fig. 2A,B), infection with RH(I) or CEP(III) led to a high level of phospho-STAT1^{Tyr} as compared to uninfected cells while a Pru(II) strain also induced phospho-STAT1^{Tyr} but to a lesser extent (Fig. 3C). Deletion of *ROP16* from RH almost completely abolished this tyrosine phosphorylation (Fig. 3C). In addition, none of these strains inhibited the IFN-γ-induced accumulation of phospho-STAT1^{Tyr}. Looking next at STAT1 serine phosphorylation, we found that infection with any of the *Toxoplasma* strains that we tested induced the serine phosphorylation of STAT1 slightly, but none of these strains strongly inhibited IFN-γ induced serine phosphorylation of STAT1 (Fig. 3C). These results indicate that ROP16 does not play a role in serine phosphorylation of STAT1 and that type I, II, and III strains do not differentially modulate STAT1 serine phosphorylation. Thus, *Toxoplasma* infection alone can induce low levels of STAT1 serine phosphorylation independently of ROP16 and ROP16 mediates the tyrosine phosphorylation and subsequent nuclear translocation of STAT1, but this nuclear phospho-STAT1$^{Tyr701/Ser727}$ does not seem to be transcriptionally active.


A
+IFNγ
UI
RH (I)
RH Δrop16
UI
RH (I)
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75
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50
IRF1
37
GAPDH
SAG1
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B
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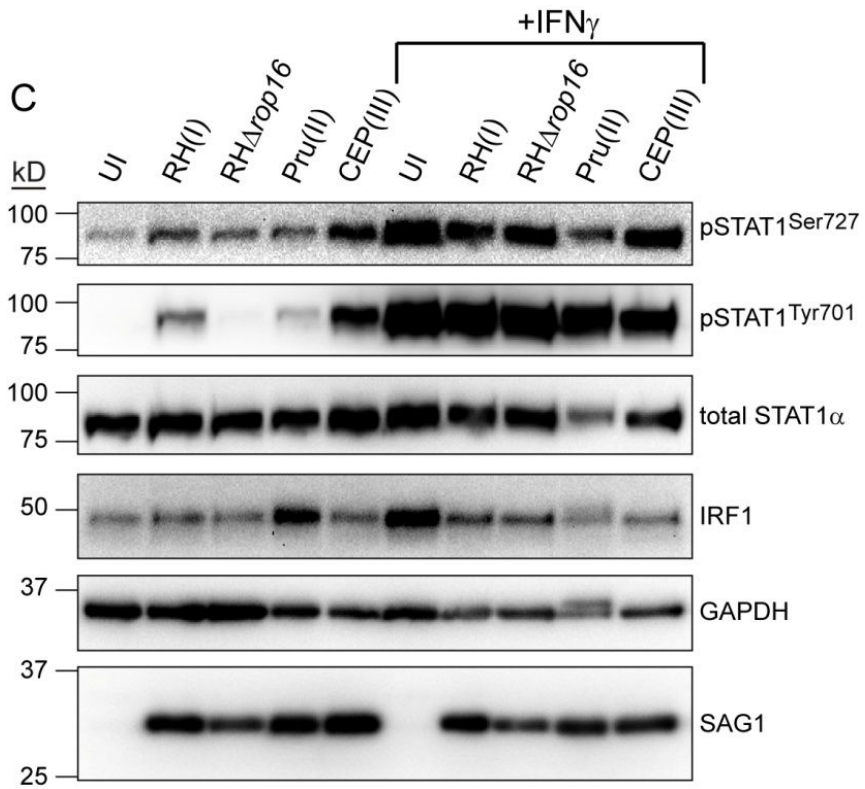

Figure 3. ROP16-activated STAT1 is not transcriptionally active A. HFFs were infected with RH(I) or RH Δ*rop16* parasites at an MOI ~7, or left uninfected, for four hours. Cells were stimulated, or not, with 100 U/ml human IFN-γ for the last hour of infection and cell lysates were collected, run on an SDS-PAGE gel, and Western blotted for phospho-STAT1^{Ser727}, phospho-STAT1^{Tyr701}, total STAT1α, IRF1, GAPDH (host cell loading control) and SAG1 (parasite loading control). This experiment has been performed three times with similar results. **B.** HFFs were infected with RH(I) or RH Δ*rop16* parasites for four hours, stimulated with 100 U/ml IFN-γ for the last two hours of infection, fixed, and stained with α-IRF1 (red), α-phospho-STAT1^{Tyr} (green), and Hoechst dye (nucleus, blue). Parasites also express GFP (green). Scale bar represents 10 μm. This experiment was performed three times with similar results. **C.** HFFs were infected with RH(I), RH Δ*rop16*, Pru(II), or CEP(III) parasites at an MOI ~1, or left uninfected, for four hours. Cells were stimulated, or not, with 100 U/ml human IFN-γ for the last hour of infection and cell lysates were collected, run on an SDS-PAGE gel, and Western blotted for phospho-STAT1^{Ser727}, phospho-STAT1^{Tyr701}, total STAT1α, IRF1, GAPDH (host cell loading control) and SAG1 (parasite loading control). This experiment has been performed two times with similar results.

Type I, II, and III strains all inhibit STAT1 transcriptional activity

While our results demonstrate that type I, II, and III parasites can all inhibit the IFN-γ-induced expression of IRF1, we have also shown that type I, II, and III parasites can differentially modulate specific aspects of the IFN-γ/STAT1 signaling pathway. The type II GRA15 protein induces IRF1 expression independently of STAT1 (Fig. 1D), and the rhoptry kinase ROP16 induces STAT1 tyrosine phosphorylation (Fig. 2, 3). Additionally, the expression of many IFN-γ-regulated genes can be induced by transcription factors other than STAT1; for example the activation of IRF1 by GRA15 via NF-κB (Fig. 1A, B) and the induction of *Socs1* by ROP16 via STAT3 or 6 (Jensen et al., 2011). It is therefore difficult to interpret the modulation of the STAT1 transcriptional response by different *Toxoplasma* strains using the expression of particular genes as a read out. We instead decided to use a stable STAT1 reporter cell line to determine the ability of the *Toxoplasma* clonal lineages to interfere with STAT1's activity in the nucleus. One previous report used two different luciferase reporters to demonstrate that infection with *Toxoplasma* inhibits STAT1 transcriptional activity (Lang et al., 2006). However, one of these reporters was a stable reporter but comprised the entire CIITA pIV promoter, containing binding sites for IRF1, AP-1, and NF-GMa transcription factors and an E-box site as well as a GAS site, making it difficult to determine whether STAT1 activity itself was being affected by *Toxoplasma* infection or if one of the other transcription factors was being affected. The second reporter measured STAT1 activity more clearly as it contained only a minimal GAS site, however the reporter vector was transiently transfected into cells. Given recent results that indicate that chromatin remodeling factors such as Brahma-related gene (BRG)-1 are differentially recruited to GAS sites after *Toxoplasma* infection to inhibit STAT1-mediated transcription (Lang et al., 2012), and that *Toxoplasma* infection can modulate chromatin modifications resulting in changes in gene expression (Leng et al., 2009), a transient plasmid reporter that is not integrated into the genome and does not have a normal chromatin structure also may not be an accurate measure of STAT1 transcriptional activity (Hebbar and Archer, 2008; Smith and Hager, 1997). Additionally, potential strain differences in the inhibition of these reporters were not investigated.

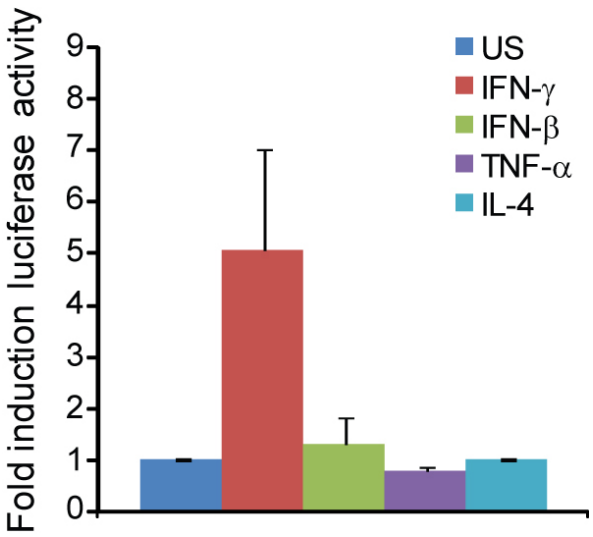

Figure 4. Characterization of HEK293 GAS reporter cell line. A HEK293 GAS luciferase reporter cell line was left unstimulated or stimulated with 100 U/ml IFN-γ, 100 U/ml IFN-β, 20 ng/ml TNF-α, or 50 ng/ml IL-4. Cells were lysed 6-20 hours later and luciferase activity was measured. Average luciferase induction normalized to unstimulated cells from three experiments is shown and error bars represent SEM.

We therefore developed a stable GAS reporter cell line in the easily transduced HEK293 cell line to measure STAT1 transcriptional activity directly. Treatment of this GAS reporter cell line with IFN-γ, but not IFN-β, TNF-α, or IL-4, results in the robust induction of luciferase activity (Fig. 4). We infected this cell line with RH(I), RH Δ*rop16*, Pru(II), Pru Δ*gra15*, or CEP(III) parasites, stimulated the cells with IFN-γ, and measured the induction of luciferase activity. Infection with any of these strains significantly inhibited IFN-γ-induced luciferase activity, and the extent of this inhibition did not vary significantly between the strains (Fig. 5A). These reporter experiments also confirmed that while ROP16 can activate the tyrosine phosphorylation and nuclear translocation of STAT1 (Fig. 2), this STAT1 is not transcriptionally active; infection of this cell line with any of these strains did not result in the induction of luciferase (Fig. 5A). To verify that this ability to inhibit STAT1 transcriptional activity is common to the type I, II, and III clonal lineages and not just RH(I), Pru(II), and CEP(III) strains, we also infected this cell line with other representative strains from these lineages, GT1(I), ME49(II), or VEG(III), as well as RH(I) again (Fig. 5B). Again, all of these strains were able to

inhibit IFN-γ-induced luciferase activity. These results indicate that type I, II, and III strains can all inhibit IFN-γ-induced STAT1 transcriptional activity to a similar extent.

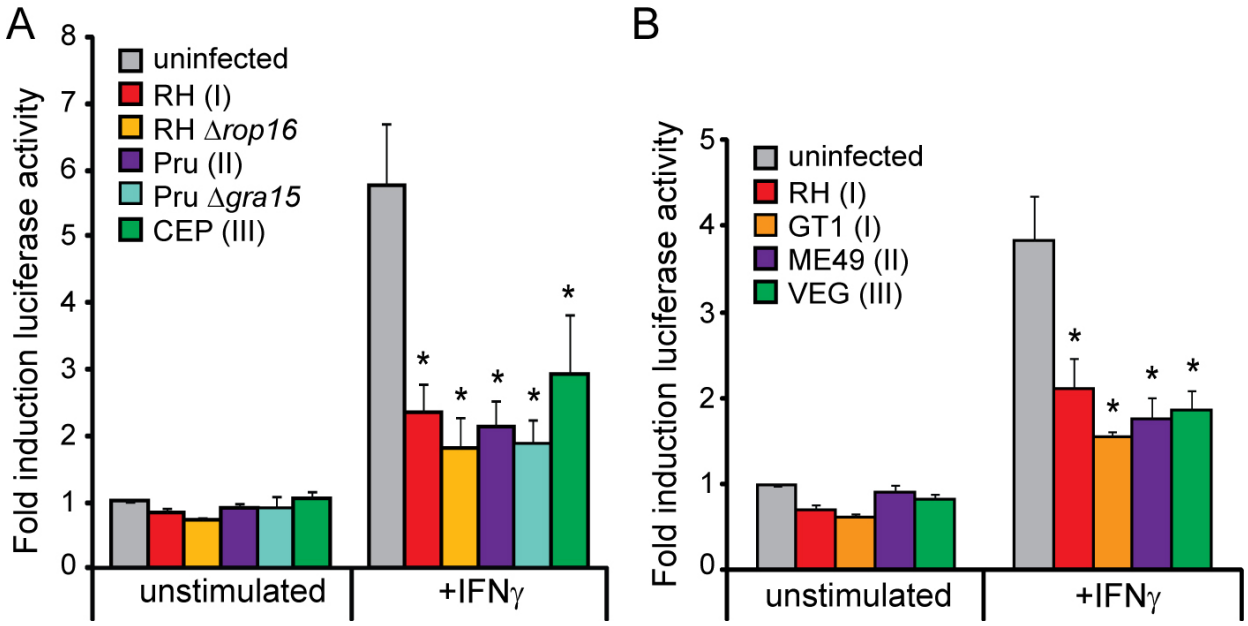

Figure 5. All three clonal lineages of *Toxoplasma* inhibit STAT1-mediated gene expression. A, B. A HEK293 GAS luciferase reporter cell line was infected with RH(I), RH Δ*rop16*, GT1(I), Pru(II), Pru Δ*gra15*, ME49(II), CEP(III), or VEG(III) parasites, or left uninfected, and subsequently stimulated, or not, with 100 U/ml IFN-γ. Cells were then lysed and luciferase activity was measured. Results are from 2-8 experiments per condition, with a pre-infection time of one to five hours followed by a stimulation of 12-24 hours, and represent the average induction over uninfected, unstimulated samples. Error bars represent SEM. Asterisk (*) indicates $p < 0.05$ compared to uninfected cells in the same condition.

Type I, II, and III strains all inhibit global IFN-γ-induced transcription

Although all *Toxoplasma* strains that we have tested inhibit a stable GAS reporter cell line, we have seen that *Toxoplasma* strains can differentially affect particular aspects of the IFN-γ signaling pathway through GRA15 and ROP16, and it is therefore unclear whether the ability to inhibit STAT1 activity corresponds to the ability of type I, II, and III strains to similarly inhibit global IFN-γ induced gene expression. We therefore analyzed the effect of infection with an RH(I), Pru(II), or CEP(III) strain on IFN-γ-induced transcription using microarray analysis. As more genes have been found to be induced by IFN-γ in macrophages than fibroblasts (Kim et al., 2007a; Lang et al., 2012), we pre-infected a murine macrophage cell line (RAW264.7) with

the above strains for 24 hours, adding IFN-γ for the last six hours. We isolated RNA from these cells as well as uninfected control cells, with and without IFN-γ stimulation, and analyzed gene expression with Affymetrix microarrays. In this macrophage cell line, 514 genes were more than 2-fold upregulated by IFN-γ treatment, while the expression of 481 genes was more than 2-fold repressed (Fig. 6). In the pre-infected samples, 431 of the 514 induced genes were at least 2-fold inhibited by at least one strain, with 314 genes being inhibited by all strains (Fig. 6). Interestingly, the expression of genes that are important for control of *Toxoplasma* infection, *Nos2* (Khan et al., 1997), *Iigp1/Irga6* (Liesenfeld et al., 2011; Martens et al., 2005), *Iigp2*/*Irgm2* (Hunn et al., 2008), and *Tgtp*/*Irgb6* (Hunn et al., 2008) were at least 2-fold inhibited by all three strains. Of the 481 IFN-γ-repressed genes, the repression of 312 of them was more than 2-fold inhibited by at least one strain while 147 genes were inhibited by all three strains (Fig. 6). It seems then that while *Toxoplasma* strains may differentially inhibit small subsets of IFN-γ responsive genes, all three of the clonal lineages significantly inhibit global IFN-γ-induced gene expression.

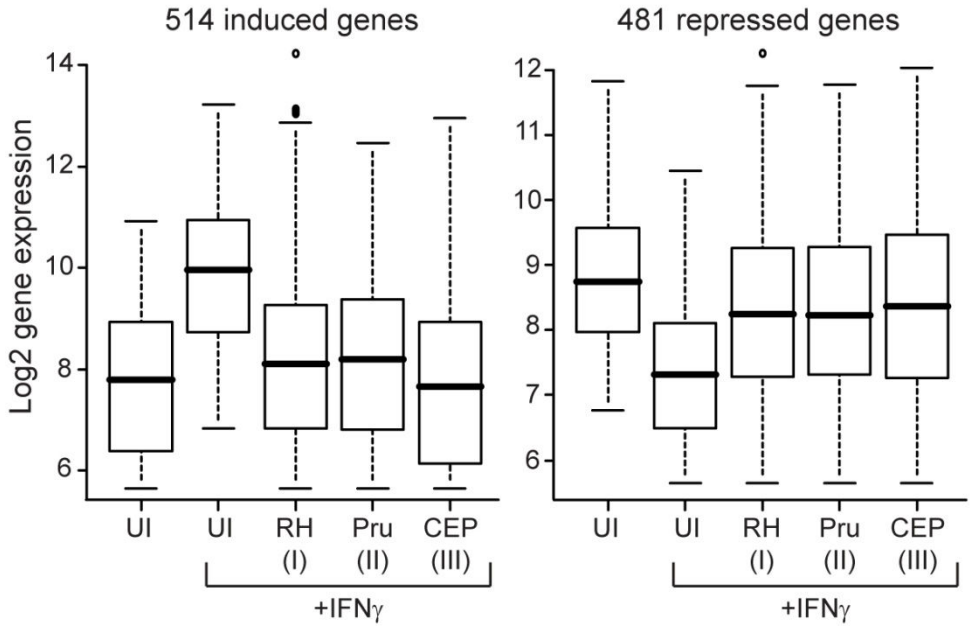

Figure 6. All three clonal lineages of *Toxoplasma* inhibit global IFN-γ-induced gene expression. RAW264.7 macrophages were infected with RH(I), Pru(II), or CEP(III) parasites, or left uninfected (UI) for 24 hours with 100 U/ml IFN-γ added for the last 6 hours of infection, and host gene expression was analyzed by microarray analysis. Greater than 2-fold IFN-γ-induced (left) and repressed (right) genes were determined from the uninfected samples. Boxplots are shown of the $\log_2$ expression of these genes in all samples. Data are from two arrays for the uninfected conditions and one array for each infected sample.

Discussion

The expression level of many genes is regulated by multiple transcription factors allowing more precise control and responsiveness to varying stimuli. While we find that strains representing three *Toxoplasma* clonal lineages, types I, II, and III, can all inhibit IFN-γ-induced STAT1 transcriptional activity, these strains also differentially modulate certain IFN-γ responsive genes through the activity of at least two known polymorphic effectors, GRA15 and ROP16. In studying the ability of *Toxoplasma* to inhibit the IFN-γ response, the choice of readout for IFN-γ-induced gene expression is therefore very important, as some IFN-γ responsive genes are also activated by *Toxoplasma* through GRA15, ROP16, and likely other secreted proteins.

$GRA15_{II}$-mediated activation of NF-κB can induce expression of IRF1, and the levels of IRF1 in Pru(II) infected cells stimulated with IFN-γ are virtually identical to those of Pru(II) infected cells that were not stimulated (Fig. 1A). This indicates that Pru(II) parasites can inhibit IFN-γ-induced expression of IRF1, even though they induce IRF1 through GRA15-mediated activation of NF-κB (Fig. 1A, D). Similarly, $ROP16_{I/III}$ induces *Socs1* expression by 10-fold in murine BMDM (Jensen et al., 2011), likely through STAT3 or STAT6. But, our microarray data from the murine macrophage RAW264.7 cell line shows that pre-infection with RH(I) parasites can still inhibit IFN-γ-induced *Socs1* transcript by two-fold. Thus, although *Toxoplasma* is able to inhibit the STAT1-mediated induction of genes such as *IRF1* and *Socs1*, it does not inhibit the expression of these genes activated by other transcription factors. This indicates that whatever mechanism *Toxoplasma* employs to inhibit the IFN-γ-induced transcriptional response must specifically target STAT1-mediated transcriptional activation of genes.

While neither GRA15 nor ROP16 affects the ability of *Toxoplasma* strains to inhibit the STAT1-mediated global induction of IFN-γ responsive gene expression, it is unclear how large of an effect GRA15 and ROP16 have on subsets of IFN-γ responsive genes as our experiments were done in a different cell line than previous transcriptional analyses on GRA15 and ROP16. However, IRF1 is an important secondary transcription factor in the response to IFN-γ. Additionally, NF-κB is likely to co-regulate other IFN-γ responsive genes besides IRF1. A significant number of genes induced by both IFN-γ and Pru(II) infection are GRA15-regulated (Supp. data). While one microarray analysis in HFFs found that IFN-γ responsive genes that

were also induced by Pru(II) infection alone were associated with TNF-α signaling and included many NF-κB target genes (Kim et al., 2007a), another microarray analysis in murine BMDM did not find an enrichment in NF-κB target genes among genes induced by both IFN-γ and another type II strain, NTE (Lang et al., 2012). However, it is unknown whether the NTE(II) strain has an active copy of GRA15 and activates NF-κB.

The strongest effect of ROP16 on IFN-γ signaling seems be on the phosphorylation status of STAT1 (Fig. 2). Since ROP16 directly tyrosine phosphorylates STAT3 and STAT6 (Ong et al., 2010; Yamamoto et al., 2009), it is likely that tyrosine 701 on STAT1 is also a direct target. It seems that either the affinity or catalytic efficiency of ROP16 for STAT1 is lower than for at least STAT6 because clear phospho-STAT1^{Tyr} activation was only observed in cells infected with at least three parasites, whereas only one parasite needs to inject its rhoptry contents into a host cell to activate STAT6 (Koshy et al., 2012).

It is still unclear why we observe a higher level of IFN-γ-induced phospho-STAT1^{Tyr} after pre-infection with any strain of *Toxoplasma* (Fig. 2A). This phenotype is not dependent on ROP16 as it also occurs in RHΔ*rop16* infected cells. The transcripts of the main components of this pathway, IFN-γ receptor 1 and 2, JAK1 and 2, and STAT1, are not upregulated by type I *Toxoplasma* infection in HFFs, according to previous microarray data (Rosowski et al., 2011). Additionally, SOCS proteins that can downregulate JAK and STAT1 phosphorylation are actually induced by *Toxoplasma* infection (Zimmermann et al., 2006), and the expression of the protein tyrosine phosphatases (PTPs) that are known to dephosphorylate JAK1, JAK2, or STAT1 (Shuai and Liu, 2003) are not downregulated by infection alone (Rosowski et al., 2011).

Our data suggest that the type I, II, and III strains use a similar mechanism to inhibit STAT1 transcriptional activity in the nucleus. This inhibition is independent of how STAT1 is activated; *Toxoplasma* can also inhibit the activity of ROP16-induced phospho-STAT1^{Tyr}, and this interference is specific for STAT1, as ROP16-activated STAT3 and STAT6 are transcriptionally active (Jensen et al., 2011; Saeij et al., 2007). A recent study, which focused mainly on the IFN-γ induced expression of CIITA and MHC class II genes, concluded that *Toxoplasma* inhibits IFN-γ-induced gene expression through impaired BRG-1 chromatin remodeling (Lang et al., 2012). Although that may be the mechanism for CIITA, the IFN-γ-induced expression of IRF1 does not require BRG-1 remodeling (Wang et al., 2011). It is

therefore important for future studies to determine the mechanism by which *Toxoplasma* inhibits the STAT1-mediated induction of primary response genes such as *IRF1*.

Materials and Methods

Parasites and cells

Parasites were maintained *in vitro* by serial passage on monolayers of human foreskin fibroblasts (HFFs), as described previously (Rosowski et al., 2011). RH or GT1 were used as representative type I strains, Pru or ME49 as representative type II strains and CEP or VEG as representative type III strains. A Pru strain engineered to express firefly luciferase and GFP (Pru Δ*hxgprt* A7) (Kim et al., 2007b), and CEP and RH strains engineered to express clickbeetle luciferase and GFP (CEP *hxgprt*$^-$ C22 and RH 1-1) (Boyle et al., 2007) have been described previously. An RH Δ*rop16* strain (provided by John Boothroyd, Stanford University) (Ong et al., 2010), an RH Δ*rop16* strain expressing firefly luciferase and GFP (clone 1A2) (Jensen et al., 2011), a PruA7 $ROP16_I$ strain (Jensen et al., 2011), and Pru Δ*gra15*, PruA7 Δ*gra15*, and RH $GRA15_{II}$ strains (Rosowski et al., 2011) have been described previously. HFFs (provided by John Boothroyd, Stanford University) and RAW264.7 (ATCC) cells were grown as described previously (Jensen et al., 2011; Rosowski et al., 2011). 293FT and HEK293 cells were grown with additional 10 mM HEPES. U3A STAT1-null cells (McKendry et al., 1991; Müller et al., 1993) (provided by George Stark, Cleveland Clinic Foundation Research Institute, Ohio) were grown with 10 mM HEPES, 1 mM sodium pyruvate, and MEM non-essential amino acids. All parasite strains and cell lines were routinely checked for *Mycoplasma* contamination and it was never detected.

Reagents

Antibodies against IRF1 (BD Biosciences #612046), phospho-STAT1^{Tyr701} 58D6 (Cell Signaling #9167), phospho-STAT1^{Ser727} (Cell Signaling #9177), total STAT1α p91 (C-24) (Santa Cruz #345), GAPDH (6C5) (Santa Cruz #32233), and *Toxoplasma* surface antigen (SAG)-1 (kindly provided by John Boothroyd, Stanford University) were used in immunofluorescence and Western blot assays. Secondary antibodies coupled with either Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes) for immunofluorescence assay or conjugated to peroxidase (Kirkegaard

& Perry Laboratories) for Western blots were used. Recombinant human IFN-γ (100 U/ml, AbD serotec) and murine IFN-γ (100 U/ml, Calbiochem) were used to stimulate cells.

Immunofluorescence assay

Immunofluorescence assay was performed as described previously (Rosowski et al., 2011). Briefly, cells were fixed with 3% formaldehyde, permeabilized with 100% ethanol and/or 0.2% Triton-X 100, and blocked with 3% BSA and 5% goat serum. Coverslips were incubated with primary antibody at 4°C, and fluorescent secondary antibodies and Hoechst dye were used for antigen and DNA visualization, respectively. Photographs were taken using NIS-Elements software (Nikon) and a digital camera (Coolsnap EZ; Roper Scientific) connected to an inverted fluorescence microscope (model eclipse Ti-S; Nikon). Quantification of nuclear signal was performed by randomly selecting cells in each condition and measuring the average signal intensity per nucleus using the NIS-Elements software and Hoechst dye to define nuclei. The minimum number of cells measured is indicated in the figure legends for each experiment.

Western blot

Western blots were performed as described previously (Rosowski et al., 2011). Briefly, HFFs were left uninfected or infected with RH Δ*hxgprt*, RH 1-1, RH Δ*rop16*, RH Δ*rop16* 1A2, Pru Δ*hxgprt* A7, or CEP *hxgprt*$^-$ C22 parasites for three hours. Samples were subsequently stimulated with human IFN-γ for one hour, or left unstimulated, and then lysed in buffer containing sodium dodecyl sulfate (SDS) and either β-mercaptoethanol (βME) or dithiothreitol (DTT). After immunoblotting, membranes were stripped with boiling 2% SDS and 0.7% βME and reprobed.

Reporter cell line construction

A GAS (TR027PA-1, 5'-AGTTTCATATTACTCTAAATC -3') pGreenFire1 (pGF1) lentiviral reporter vector containing a Neo selection cassette and a minimal CMV promoter followed by four tandem consensus GAS sites driving the expression of Firefly luciferase was purchased from System Biosciences. The vector was co-transfected into 293FT cells with vectors containing gag, pol, and VSV-G proteins using FuGENE reagent (Roche) according to the

manufacturer's protocol. Supernatant containing virus was collected two and three days after transfection, filtered with a 0.45 μm surfactant-free cellulose acetate filter (Nalgene), and added to HEK293 cells (ATCC) with 8 μg/ml polybrene (Sigma). HEK293 cells containing the pGF1 construct were then selected with 750 μg/ml Geneticin (Invitrogen). Cells were cloned by limiting dilution and were confirmed to be responsive to IFN-γ but not to IFN-β, TNF-α, or IL-4.

Luciferase assay

HEK293 pGF1-GAS cells were plated in 96-well plates, 3.5-4x10^4 cells/well, and grown for four to 20 hours. Cells were then infected with RH Δ*hxgprt*, RH Δ*rop16*, GT1, Pru Δ*hxgprt*, Pru Δ*gra15*, ME49, CEP Δ*hxgprt*, or VEG parasites at varying MOIs for one to four hours, and subsequently stimulated with human IFN-γ for 12-24 hours. Cells were lysed with 20 μl Cell Culture Lysis Reagent (Promega) containing 1x protease inhibitors (Roche), and plates were frozen at -80°C. Luciferase activity in plates was detected using a Varioskan Flash Reader (Roche) after addition of 100 μl Luciferase assay substrate (Promega), according to the manufacturer's instructions. Data were normalized to the uninfected, unstimulated sample and averaged over at least two experiments per condition.

Microarray

1.5x10^6 RAW264.7 cells were plated in 6-well plates and grown for 24 hours. The cells were then left uninfected or infected with RH 1-1, Pru Δ*hxgprt* A7, or CEP *hxgprt*$^-$ C22 parasites at an MOI ~5 for 18 hours and subsequently stimulated with murine IFN-γ for six hours. The RH infection was done at one time and Pru and CEP infections were done together at a later time. Uninfected controls were included for both sets of infections. RNA was isolated and microarray analysis, including analysis with the DiRE server, was performed as described previously (Rosowski et al., 2011), with Mouse 430A 2.0 Affymetrix gene chips. Microarray data has been uploaded to NCBI Gene Expression Omnibus and are accessible through GEO Series accession number GSE34913.

Plaque assay

For Western blot, luciferase reporter, and microarray assays, a plaque assay was done to determine the viability of each strain and the actual MOI. One hundred parasites per well were added to confluent HFFs in a 24-well plate and were incubated undisturbed for 5-7 days at 37°C, and the number of plaques was counted. Samples with similar MOIs were then picked for analysis.

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Chapter 4. *Toxoplasma gondii* inhibits IFN-γ- and IFN-β-induced host cell STAT1 transcriptional activity by increasing the association of STAT1 with DNA

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Abstract

The IFN-γ response, mediated by the STAT1 transcription factor, is crucial for host defense against the intracellular pathogen *Toxoplasma gondii*, but prior infection with *Toxoplasma* can inhibit this response. Recently it was reported that the *Toxoplasm* NTE strain of *Toxoplasma* prevents the recruitment of chromatin remodeling complexes containing Brahma related gene (BRG)-1 to promoters of IFN-γ-induced secondary response genes such as *CIITA* and MHC class II genes in murine macrophages, thereby inhibiting their expression. Here we report that a type I strain of *Toxoplasma* does not require the activity of histone deacetylases to inhibit the expression of IFN-γ-induced primary response genes such as *IRF1* nor does it inhibit the binding of STAT1 to promoters of either primary or secondary response genes in human fibroblasts. In fact, infection increases the IFN-γ-induced association of STAT1 with chromatin. We find that *Toxoplasma* infection also inhibits IFN-β-induced interferon-stimulated gene factor (ISGF) 3 mediated gene expression and that this is also associated with increased association of STAT1 with chromatin. The secretion of proteins into the host cell by *Toxoplasma* without complete parasite invasion is not sufficient to block STAT1 induction of IFN-γ-induced gene expression, suggesting that the effector protein responsible for this inhibition is not derived from the rhoptries.

Introduction

Interferon-gamma (IFN-γ) is a critical cytokine in both the innate and adaptive immune response to infection (Boehm et al., 1997; Saha et al., 2010). The cellular response to IFN-γ includes many effector mechanisms that inhibit pathogen growth and survival, especially of intracellular pathogens. These include the p47 immunity-related GTPases (IRGs), p65 guanylate binding proteins (GBPs), iNOS/*Nos2*, indoleamine 2,3-dioxygenase 1 (IDO1), and major histocompatibility complex (MHC) genes (Hunn et al., 2011; Pfefferkorn et al., 1986; Saha et al., 2010; Scharton-Kersten et al., 1997; Yamamoto et al., 2012). Mice deficient in multiple components of the IFN-γ pathway are acutely susceptible to many pathogens, including the parasite *Toxoplasma gondii* (Khan et al., 1996; Lieberman et al., 2004; Scharton-Kersten et al., 1996; Yap and Sher, 1999; Yap et al., 2000). *Toxoplasma gondii* is an obligate intracellular

protozoan parasite which infects virtually all warm-blooded animals, including mice and humans (Sibley et al., 2009).

It is well-documented that *Toxoplasma* actively inhibits the cellular response to IFN-γ in infected cells (Kim et al., 2007a; Lang et al., 2006, 2012; Rosowski and Saeij, 2012), and it is thought that this inhibition is required for survival of the parasite and conversion to the chronic cyst stage. IFN-γ stimulation activates the signal transducer and activator of transcription (STAT) 1 transcription factor and induces a broad transcriptional program (Platanias, 2005), however, pre-infection with *Toxoplasma* parasites globally inhibits the induction of this program in multiple cell types of multiple species, including human foreskin fibroblasts (HFF) (Kim et al., 2007a), murine bone-marrow derived macrophages (BMDM) (Lang et al., 2012), and RAW264.7 murine macrophages (Rosowski and Saeij, 2012). The mechanism by which this inhibition occurs remains a matter of contention. A recent study showed that in murine macrophages at IFN-γ-induced secondary response genes, such as class II transactivator (*CIITA*) and MHC class II genes, brahma related gene (BRG)-1-mediated chromatin remodeling is impaired by *Toxoplasma* infection leading to inhibition of gene expression (Lang et al., 2012). Treatment of these cells with histone deacetylase inhibitors decreased the inhibition of secondary response genes by the type II NTE strain of *Toxoplasma* (Lang et al., 2012). However, the IFN-γ-induced expression of primary response genes such as *IRF1* does not require BRG-1-mediated remodeling (Wang et al., 2011), suggesting that *Toxoplasma* utilizes a different mechanism to inhibit the expression of primary STAT1 induced genes.

In this study we have further characterized the mechanism of this direct inhibition of STAT1 transcriptional activity by measuring each step of IFN-γ-induced STAT1 activation to determine where in the pathway *Toxoplasma* acts. Since three clonal lineages of *Toxoplasma*, types I, II, and III, all equally inhibit STAT1 transcriptional activity (Rosowski and Saeij, 2012), it is likely that all of these strains utilize a similar mechanism of inhibition and we have focused our study on just one of these strains, the type I RH strain. *Toxoplasma* infection also equally inhibits STAT1 activity and IFN-γ-induced primary (*IRF1*) gene expression in a variety of cell types, including HFFs, HEK293 cells, murine macrophages, and murine dendritic cells (Rosowski and Saeij, 2012; Schneider et al., 2013), and we have therefore focused our study on

two readouts of primary STAT1-induced gene expression: HEK293 STAT1 luciferase reporter cell lines and the expression of IRF1 in human fibroblasts.

We find that *Toxoplasma* infection increases the association of IFN-γ-induced STAT1 with chromatin and with the promoters of primary IFN-γ response genes such as *IRF1* in particular. *Toxoplasma* infection can also inhibit IFN-β-induced gene expression, likely through a similar mechanism involving increased association of STAT1, STAT2, and IRF9 with chromatin. In contrast to findings by Lang et al. on the role of histone deacetlyases (HDAC) in the inhibition of IFN-γ secondary response genes by *Toxoplasma*, we find that *Toxoplasma* can still inhibit the expression of IFN-γ primary response genes in the presence of various HDAC inhibitors. The *Toxoplasma* factor responsible for this inhibition is also unknown, however our results indicate that it is unlikely to be secreted into the host cell from the rhoptry secretory organelle. While the exact mechanism by which *Toxoplasma* inhibits the expression of IFN-γ-induced primary response genes is still not clear, it is likely that this mechanism acts directly on DNA-bound STAT1 and is distinct from the mechanism by which secondary response genes are inhibited in murine macrophages.

Results

Toxoplasma infection does not interfere with IFN-γ-induced STAT1 dimerization

The pathway by which IFN-γ activates STAT1 transcriptional activity is well described. When IFN-γ binds to its receptors, IFNGR1 and 2, the receptors oligomerize and cause constitutively associated Janus activated kinase (JAK) 1 and JAK2 to be activated (Bach et al., 1997; Darnell et al., 1994). Activated JAKs tyrosine-phosphorylate the IFN-γ receptor, creating a docking site for the transcription factor STAT1. STAT1 is then phosphorylated by the JAKs at tyrosine 701, causing it to homo-dimerize and translocate to the nucleus. Once in the nucleus and stably associated with DNA, STAT1 is serine phosphorylated at residue 727 (Sadzak et al., 2008), and this additional serine phosphorylation is required for maximal STAT1 activity (Varinou et al., 2003). *Toxoplasma* infection does not interfere with STAT1 tyrosine or serine phosphorylation or nuclear translocation (Kim et al., 2007a; Lang et al., 2006; Rosowski and Saeij, 2012), suggesting that homo-dimerization is also not inhibited. However it is possible that

a *Toxoplasma* protein containing a nuclear localization sequence directly binds to single tyrosine phosphorylated STAT1 proteins and carries STAT1 into the nucleus.

To determine the predominant complex(es) within which STAT1 is found in infected cells and therefore distinguish between these two possibilities, we visualized STAT1-containing complexes in non-denaturing conditions by native PAGE and Western blot. We infected HFFs with the RH *Toxoplasma* strain for three hours, subsequently stimulated the cells with IFN-γ for one hour, lysed the cells in non-denaturing conditions, ran the lysates on native PAGE, and blotted for STAT1. In uninfected, unstimulated cells, STAT1α runs at a size between 66 and 146 kD, but upon IFN-γ treatment the majority of STAT1α protein shifts into complex which runs at a size between 146 and 242 kD (Fig. 1). These sizes are consistent with STAT1α monomers (91 kD) and STAT1 homodimers, respectively. Additional blotting for the phospho-tyrosine form of STAT1 also demonstrated that the majority of phosphorylated STAT1 is found in the slower migrating band, consistent with this band representing the dimer as tyrosine phosphorylation is required for STAT1 dimerization (Fig. 1). In cells pre-infected with RH parasites, STAT1α again runs at two different bands of exactly the same size as in uninfected cells, indicating that *Toxoplasma* does not inhibit STAT1 homo-dimerization, and suggesting that a putative *Toxoplasma* effector does not strongly bind STAT1 under these conditions (Fig. 1). In type I *Toxoplasma* strains the rhoptry kinase ROP16 can also phosphorylate and induce the nuclear translocation of STAT1 (Rosowski and Saeij, 2012), and our results also show that this ROP16-activated STAT1 can dimerize (Fig. 1). We therefore also performed this experiment with RH parasites deficient for ROP16 to specifically measure IFN-γ-induced STAT1 dimerization and find that RH Δ*rop16* parasites also do not inhibit the dimerization of STAT1 (Fig. 1).

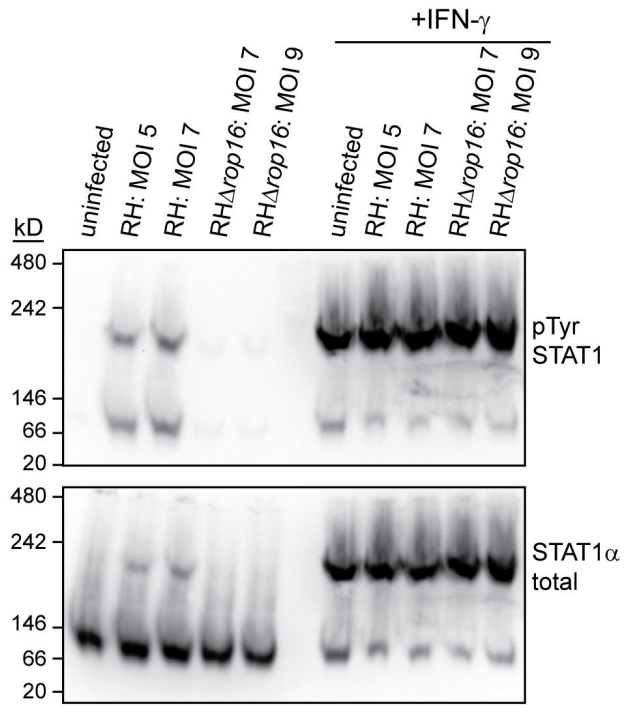

Figure 1. *Toxoplasma* infection does not inhibit IFN-γ-induced STAT1 dimerization. HFFs were infected with RH or RH Δ*rop16* parasites, or left uninfected, for four hours. Cells were then stimulated with 100 U/ml human IFN-γ for the last hour of infection (+IFN-γ) or left unstimulated (US). Cell lysates were collected in non-denaturing buffer and analyzed by native PAGE followed by Western blotting. Cells were infected at two different MOIs and the actual MOIs for each sample calculated after plaque assay are indicated. Blots were probed for phospho-STAT1^{Tyr}, stripped, and reprobed for total STAT1α. This experiment has been performed three times with similar results.

Toxoplasma infection increases IFN-γ-induced STAT1 DNA association

We next wondered whether STAT1 in infected, IFN-γ-stimulated host cells is able to bind DNA, and specifically the gamma-activated sequence (GAS) sites in the genome it normally targets. Previous EMSA experiments suggested that this STAT1 species is still able to bind GAS sites *in vitro*, although in one study the binding was weaker in extracts from infected cells (Lüder et al., 2001) and in two other studies STAT1 seemed to bind with other proteins in an aberrant complex in infected cells compared to uninfected cells (Lang et al., 2012; Schneider et al., 2013). Conversely, *Toxoplasma* was recently reported to inhibit STAT1 binding to the *Irf1* promoter in murine BMDCs (Schneider et al., 2013). To determine whether STAT1 binds GAS

sites in the promoters of IFN-γ-responsive genes in HFFs, we performed STAT1 chromatin immunoprecipitation (ChIP) experiments. We infected HFFs with either RH or RH Δ*rop16* parasites for four hours, or left cells uninfected, and subsequently stimulated cells with IFN-γ for one hour, or left cells unstimulated. Coverslips were included in sample plates to measure STAT1 phosphorylation and the inhibition of IRF1 expression by pre-infection as controls (Fig. S1). In uninfected cells, we detected a significant increase in STAT1 binding after IFN-γ treatment at all loci, except for a negative control locus, *CCND2* (Fig. 2A). Infection with RH parasites, in the absence of IFN-γ treatment, also resulted in a significant increase in STAT1 binding at all except one of these loci (Fig. 2A). We hypothesized that the STAT1 binding to DNA after RH infection was due to ROP16-activated STAT1 and we therefore also infected cells with an RH Δ*rop16* strain. In cells infected with RH Δ*rop16* parasites, STAT1 binding at these loci is not significantly higher than in uninfected cells, suggesting that the STAT1 activated by ROP16 is not only tyrosine phosphorylated, dimerized, and nuclear, but that it is also able to bind to the promoters of IFN-γ-induced genes (Fig. 2A). Most importantly, previous infection with either RH or RH Δ*rop16* parasites did not inhibit STAT1 binding upon IFN-γ stimulation at six of the seven IFN-γ induced loci (Fig. 2A), suggesting that *Toxoplasma* infection inhibits STAT1 activity downstream of STAT1 DNA binding.

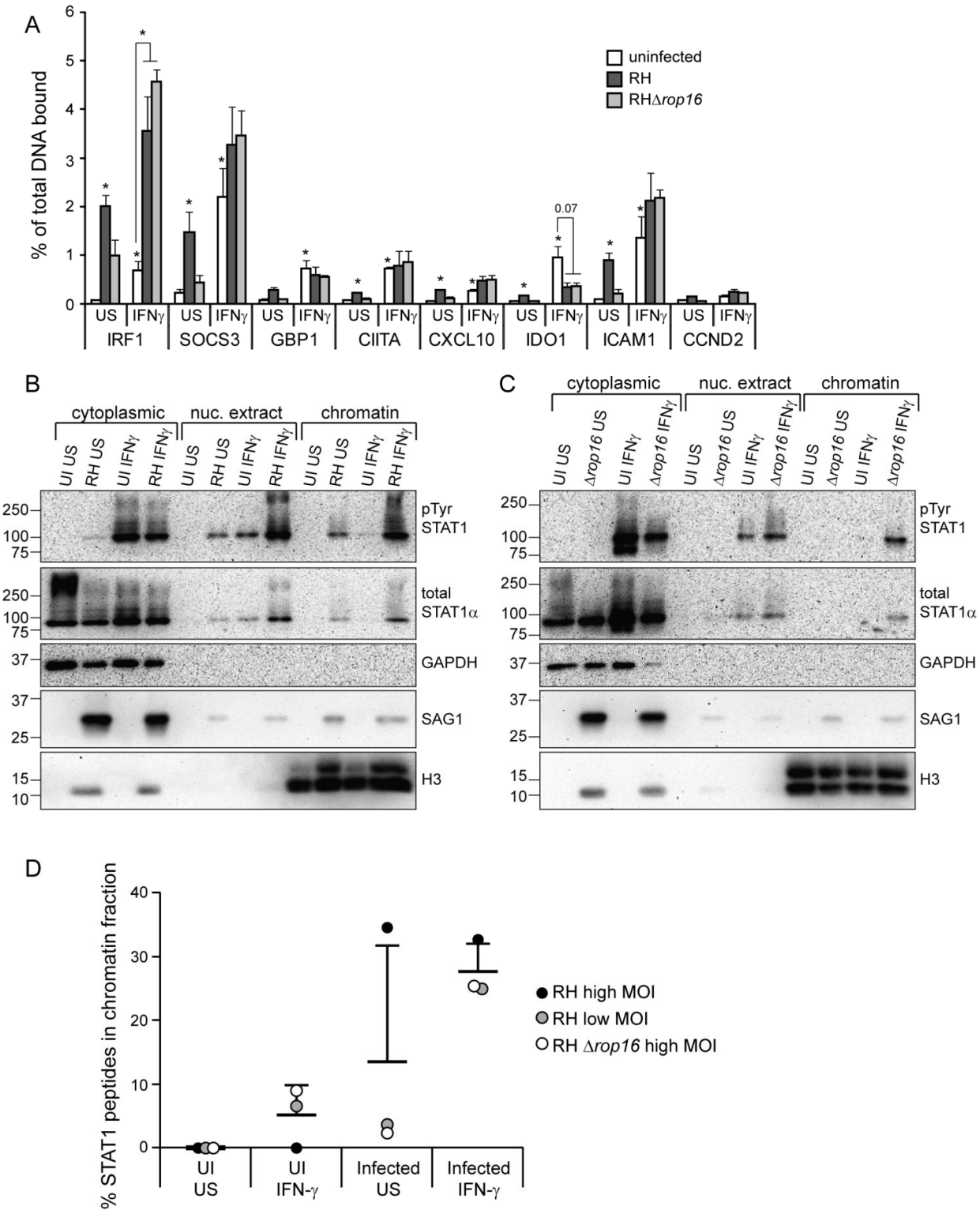
A
% of total DNA bound
uninfected
RH
RHΔrop16
0.07
US
IFNγ
IRF1
SOCS3
GBP1
CIITA
CXCL10
IDO1
ICAM1
CCND2
B
cytoplasmic
nuc. extract
chromatin
UI US
RH US
UI IFNγ
RH IFNγ
pTyr STAT1
total STAT1α
GAPDH
SAG1
H3
C
Δrop16 US
Δrop16 IFNγ
D
% STAT1 peptides in chromatin fraction
RH high MOI
RH low MOI
RH Δrop16 high MOI
UI US
UI IFN-γ
Infected US
Infected IFN-γ

Figure 2. IFN-γ-induced STAT1 DNA association is increased upon infection with *Toxoplasma*. HFFs were infected with RH or RH Δ*rop16* parasites, or left uninfected (UI), for four to five hours. Cells were stimulated with 100 U/ml human IFN-γ for the last hour of infection or left unstimulated (US). **A.** Samples were fixed with 1% formaldehyde and collected for chromatin immunoprecipitation. qPCR of STAT1-binding regions of the promoters of IFN-γ-responsive genes was performed on both the immunoprecipitated STAT1-bound DNA and total input DNA. Percent of the total DNA bound by STAT1 was calculated. A promoter region where STAT1 is not known to bind (CCND2) was also included as a negative control. The average and s.e.m. of three experiments is shown. Average MOI in the three experiments = 8. Asterix (*) indicates $p<0.05$ vs the uninfected, unstimulated sample, or as indicated by bars. **B-D.** Samples were fractionated in cytoplasmic, nuclear extract, and chromatin fractions. A portion of each fraction was diluted in 2x reducing sample buffer, boiled, and protein levels were analyzed by SDS-PAGE and Western blot (B, C). STAT1 was then immunoprecipitated from all three fractions and mass spectrometry was performed. From each sample, the percentage of the total STAT1 peptides that were found in the chromatin fraction was calculated (D). Cells were infected with RH at MOIs of ~8 (B, D) and ~1.5 (D) and with RH Δ*rop16* at an MOI of ~5 (C, D) in three independent experiments.

To confirm that *Toxoplasma* does not inhibit STAT1's association with DNA and chromatin, we again infected HFFs with RH parasites, or left cells uninfected, and subsequently stimulated cells with IFN-γ. We then isolated cytoplasmic, nuclear extract, and chromatin fractions from these cells, and analyzed protein levels in these fractions by SDS-PAGE and Western blot. As expected, in unstimulated, uninfected cells, STAT1 is present exclusively in the cytoplasmic fraction, and upon IFN-γ treatment STAT1 is both tyrosine phosphorylated and present in the nuclear extract (Fig. 2B). Only a very small amount of this STAT1 is stably associated with the chromatin (Fig. 2B). In cells pre-infected with RH and stimulated with IFN-γ, levels of STAT1 in both the nuclear extract and chromatin fraction are significantly higher than in uninfected cells (Fig. 2B). We obtained similar results from cells pre-infected with an RH Δ*rop16* strain (Fig. 2C), suggesting that this increased association is not simply due to ROP16-activated STAT1. This again indicates that *Toxoplasma* infection does not prevent the IFN-γ-induced binding of STAT1 to chromatin and, in fact, infection increases this association. To quantify the relative amount of STAT1 in the chromatin fraction in each of these samples, we immunoprecipitated STAT1 from each of the cytoplasmic, nuclear extract, and chromatin fractions, and performed mass spectrometry on the pulled-down proteins. We then calculated the percent of STAT1 peptides present in the chromatin fraction compared to the total STAT1 peptides measured in each sample. Consistent with our Western blot results, pre-infection with

Toxoplasma resulted in substantially more IFN-γ-induced STAT1 in the chromatin fraction, regardless of the presence of STAT1 on the chromatin in unstimulated cells due to ROP16 (Fig. 2C). Under the conditions of these immunoprecipitations, we did not consistently find any infection-induced change in the human or *Toxoplasma* proteins pulled down with STAT1 in any of the fractions (data not shown).

Toxoplasma infection also inhibits IFN-β-induced STAT1 transactivation activity

Next, we wondered whether *Toxoplasma*'s inhibition of STAT1 transcriptional activity is specific for STAT1 homodimers, the complexes primarily activated by IFN-γ stimulation. From previous research it is clear that the interference is specific for STAT1, as ROP16-activated STAT3 and STAT6 are transcriptionally active (Jensen et al., 2011; Saeij et al., 2007). However, STAT1 can also be present in a complex with STAT2 and IRF9, which is primarily activated by type I interferons, IFN-α and IFN-β (Platanias, 2005). This complex is called interferon-stimulated gene factor 3 (ISGF3) and binds to IFN-stimulated response elements (ISREs) in DNA to induce the expression of a subset of genes that partially overlaps with the set of genes induced by IFN-γ (Platanias, 2005). To test whether *Toxoplasma* infection can inhibit the activity of type I interferon activated STAT1, we developed a lentivirally transduced ISRE reporter cell line in HEK293 cells. We infected this cell line with RH parasites, or left cells uninfected, subsequently stimulated the cells with IFN-β, or left cells unstimulated, and measured the induction of luciferase activity. Treatment with IFN-β led to the induction of luciferase by ~3.5-fold, and pre-infection with RH parasites significantly inhibited this induction (Fig. 3A), suggesting that RH parasites can inhibit IFN-β-induced STAT1 activity. However, this reporter cell line also responds to IFN-γ treatment (Fig. 3A, Fig. S2A), and IFN-β treatment can induce STAT1 homodimers in addition to ISGF3.

To directly test the ability of *Toxoplasma* to inhibit ISGF3-mediated gene expression, we analyzed the expression of genes specifically induced by IFN-β and not by IFN-γ. We infected HFFs with RH parasites, or left cells uninfected, subsequently stimulated the cells with either IFN-γ or IFN-β, or left cells unstimulated, isolated RNA from cells, and analyzed transcript levels by RT-qPCR. *IRF1* expression was induced strongly by IFN-γ treatment but also slightly induced by IFN-β treatment (Fig. 3B), likely via STAT1 homodimers in both conditions. Pre-

infection with RH significantly decreases this expression in both conditions (Fig. 3B). The expression of genes that are specifically induced by IFN-β: *RSAD2*, *MX2*, and *OASL* (Indraccolo et al., 2007), were also significantly inhibited by pre-infection with RH parasites (Fig. 3B). Normalization of qPCR data to a different control gene gave virtually identical results (Fig. S3).

In the ISGF3 complex, IRF9 contributes most of the DNA binding activity (Sadzak et al., 2008; Veals et al., 1992) while STAT1's transactivation domain is necessary for strong induction of target gene expression (Pilz et al., 2003). Our data indicate that *Toxoplasma* does not inhibit STAT1 DNA binding activity but instead acts downstream, targeting STAT1 transactivation, suggesting that *Toxoplasma* infection could inhibit IFN-β- and IFN-γ-induced gene expression by similar mechanisms. To assess the similarity of these mechanisms, we first determined whether *Toxoplasma* inhibits IFN-β-induced phosphorylation of STAT1. We infected HFFs with RH parasites, or left cells uninfected, for three hours and subsequently stimulated cells with IFN-γ or IFN-β for one hour, or left cells unstimulated. As measured by SDS-PAGE and Western blot, infection with RH parasites does not inhibit IFN-β-induced STAT1 tyrosine or serine phosphorylation, even at an MOI which inhibits IFN-γ-induced IRF1 induction (Fig. 3C). Next, we measured the association of the ISGF3 complex with chromatin by isolating cytoplasmic, nuclear extract, and chromatin fractions from these cells and analzying protein levels by SDS-PAGE and Western blot. The nuclear translocation and chromatin association of IFN-β-induced STAT1 and STAT2 was not inhibited by prior infection with *Toxoplasma*, and in fact we observed an increase in IFN-β-induced STAT1 in the chromatin fraction after infection (Fig. 3D). IRF9 was found in the nuclear extract fraction in all samples, but we observed a slight increase in chromatin association after IFN-γ or IFN-β stimulation or RH infection, and the combination of RH infection and IFN-β treatment together led to a strong increase in the chromatin association of IRF9 (Fig. 3C). These results indicate that as with IFN-γ-induced STAT1, *Toxoplasma* infection does not inhibit the association of IFN-β-induced STAT1, STAT2, or IRF9 with host cell chromatin.

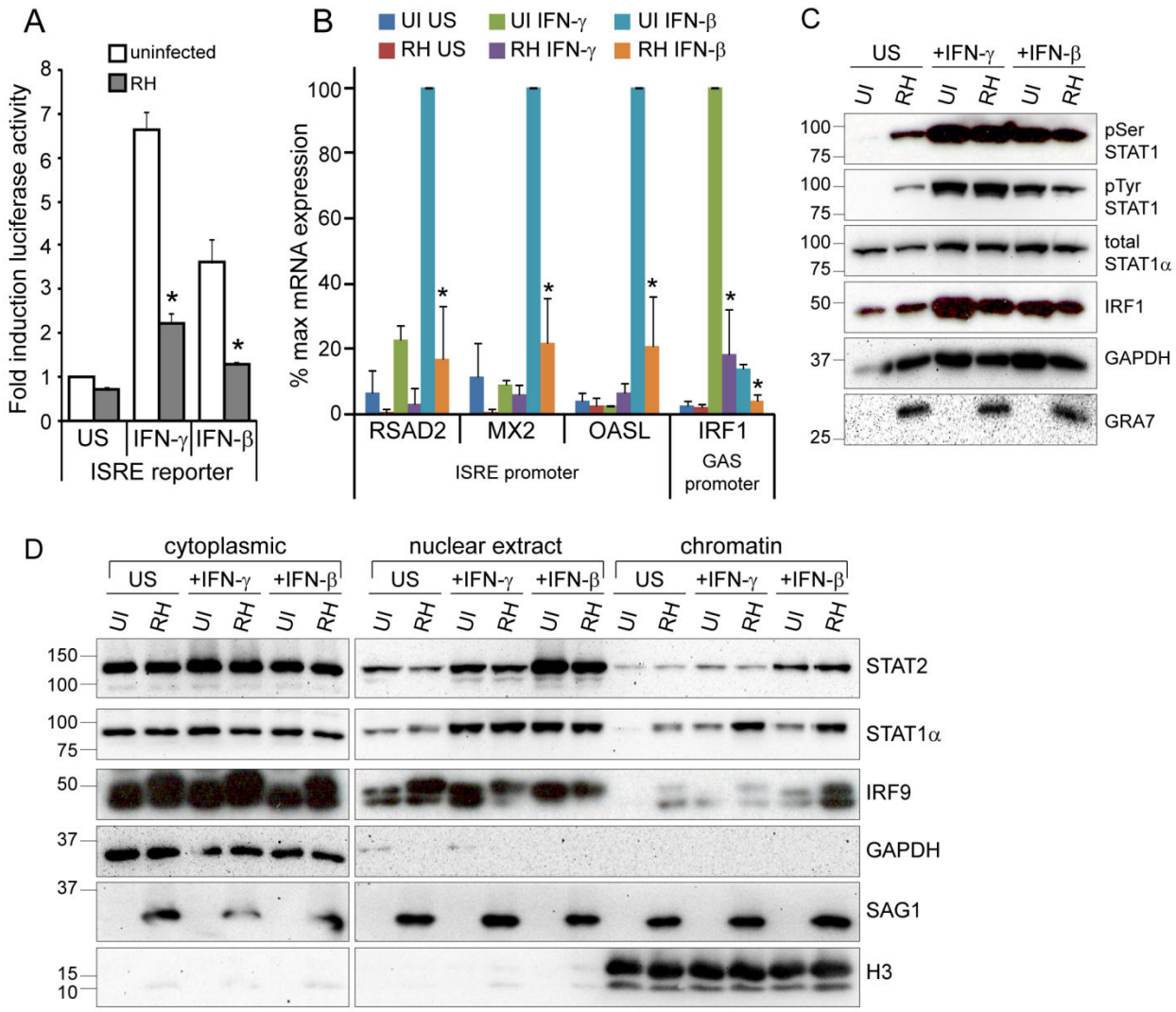

Figure 3. *Toxoplasma* also inhibits IFN-β-induced gene expression through a similar mechanism. A. A HEK293 ISRE reporter cell line was infected with RH parasites for three to five hours, or left uninfected, subsequently stimulated with 100 U/ml IFN-γ, 100 U/ml IFN-β, or left unstimulated (US) for 14-17 hours, lysed, and luciferase activity was measured. Data were normalized within each experiment to the uninfected, unstimulated sample and data shown are the average fold induction and s.e.m. from three experiments. Asterix (*) indicates $p<0.05$. **B.** HFFs were plated in 6-well plates, infected with RH parasites for four hours, and subsequently stimulated with 100 U/ml IFN-γ or 100 U/ml IFN-β for 15-20 hours. Cells were also left uninfected (UI) and unstimulated (US). Transcript levels of three ISGF3-induced (ISRE promoter) genes and one STAT1 homodimer-induced (GAS promoter) gene were analyzed by RT-qPCR and normalized to *ACTB* levels. Averages of three independent experiments are shown; error bars represent s.e.m.; asterix (*) indicates $p<0.05$ vs uninfected sample with the same stimulation. **C, D.** HFFs were plated in 60 cm dishes, infected with RH parasites for three hours, and subsequently stimulated with 100 U/ml IFN-γ or 100 U/ml IFN-β for one hour. Cells were also left uninfected (UI) and

unstimulated (US). A portion of the sample was lysed, boiled, separated by SDS-PAGE, and subjected to Western blotting (C). The rest of the samples were fractionated into cytoplasmic, nuclear extract, and chromatin fractions, diluted in 2x reducing SDS sample buffer, boiled, and protein levels were analyzed by SDS-PAGE and Western blot (D). These experiments (C,D) were performed twice from two independent infections with similar results.

Histone deacetylase activity is not required for *Toxoplasma* inhibition of IRF1 expression or STAT1 transcriptional activity

We next looked at steps downstream of STAT1 DNA binding where *Toxoplasma* could inhibit STAT1 transcriptional activity. It was recently reported that *Toxoplasma* infection inhibits the expression of IFN-γ-induced secondary response genes such as *CIITA* and MHC class II genes by activating histone deacetylases (HDACs) and preventing the recruitment of chromatin remodeling complexes to gene promoters (Lang et al., 2012). The recruitment of histone acetyltransferases and increased histone H4 and H3 acetylation is associated with the formation of euchromatin and accessibility of DNA to transcription factors and RNA pol II (Kouzarides, 2007), and actively transcribed STAT1 target genes have increased H3 and H4 acetylation upon IFN-γ treatment (Lang et al., 2012). However, many stimulus-induced primary response genes do not require chromatin remodeling to be expressed (Medzhitov and Horng, 2009), and it is unclear if *Toxoplasma* could use this mechanism to inhibit the expression of primary IFN-γ-induced genes. We therefore decided to test whether HDAC inhibitors affect the ability of *Toxoplasma* to inhibit the IFN-γ-induced expression of IRF1 or activation of STAT1 reporter cell lines.

First, we pretreated HFFs in coverslips with trichostatin A (TSA), a class I/II HDAC inhibitor, subsequently infected the cells with RH parasites, and then stimulated the cells with IFN-γ. Cells were then fixed and stained for IRF1 expression and acetylated-histone H4. TSA treatment in all conditions increased the intensity of acetylated-histone H4 staining in the host nucleus indicating that under these conditions it potently inhibits host HDACs (Fig. 4A). However, infection with RH parasites either in the presence or absence of TSA strongly inhibited the IFN-γ induced expression of IRF1, indicating that the activity of class I and class II HDACs is not required for *Toxoplasma*'s inhibition of IRF1 expression (Fig. 4A).

However, IRF1 is just one gene and the chromatin environments of all IFN-γ-induced genes may not be regulated in the same manner. Therefore we tested the ability of *Toxoplasma* infection to inhibit STAT1 activity in two different stable HEK293 reporter cell lines, a "GAS" line and a "STAT1" line. These cell lines contain slightly different consensus STAT1 binding sites, both driving the expression of luciferase. Treatment of either of these reporters with IFN-γ, but not IFN-β, TNF-α, or IL-4, results in the induction of luciferase activity (Rosowski and Saeij, 2012) (Fig. S2B). We pretreated both of these cell lines with a variety of HDAC inhibitors: TSA, MC1568, MS-275, or sodium butyrate, or with DMSO as a control. We then infected the lines with RH parasites, subsequently stimulated the cells with IFN-γ, and measured the induction of luciferase activity. In the DMSO control, prior infection with RH parasites significantly inhibited the IFN-γ-stimulated induction of luciferase activity in both cell lines (Fig. 4B), in agreement with previous results (Rosowski and Saeij, 2012). In the GAS reporter line, pretreatment with MC1568, MS-275, or sodium butyrate did not affect the ability of RH infection to inhibit this induction (Fig. 4B). Treatment with TSA by itself inhibited the induction of luciferase after IFN-γ treatment in this cell line, although prior infection with RH still lowered the IFN-γ induced luciferase activity further (Fig. 4B). Conversely, in the STAT1 reporter line treatment with TSA or MS-275 strongly induced luciferase activity even in the absence of IFN-γ treatment (Fig. 4B). Prior infection with RH still inhibited IFN-γ induced luciferase activity in all conditions (Fig. 4B). These results suggest that the mechanism by which a type I strain of *Toxoplasma* inhibits the expression of STAT1-induced primary response genes is distinct from the mechanism of inhibition of secondary response genes, and does not involve the activation of HDACs. Additionally, we find that inhibition of HDAC activity has both positive and negative effects on basal and IFN-γ-induced STAT1 transcriptional activity, depending on the exact promoter and the different HDACs that are targeted.

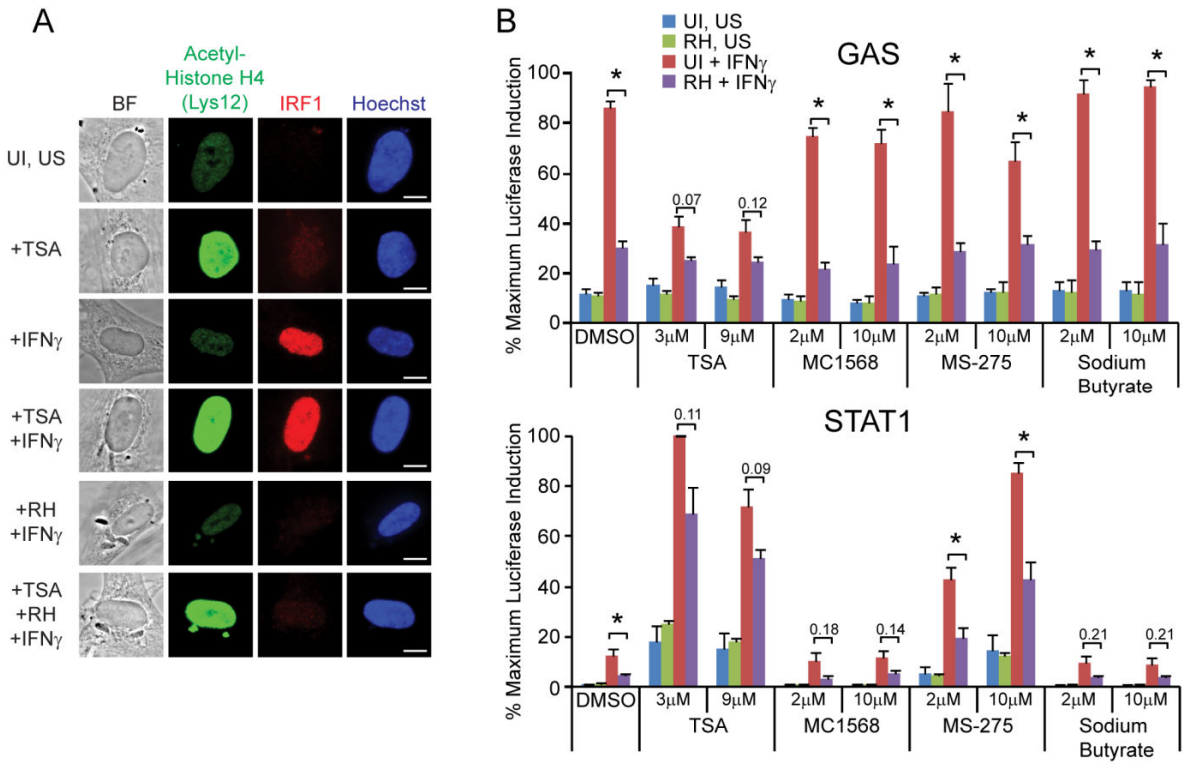

Figure 4. HDAC activity is not required for *Toxoplasma* to inhibit IFN-γ-induced IRF1 expression or induction of IFN-γ-responsive reporter cell lines. A. HFFs were plated on coverslips, pre-treated with 3 μM trichostatin A (TSA), an HDAC inhibitor, for one hour, then infected with RH parasites for one hour, and subsequently stimulated with 100 U/ml IFN-γ for two hours. Control cells were also left unstimulated (US) and/or uninfected (UI). Cells were fixed, permeabilized, and stained with α-acetyl-histone H4 (green), α-IRF1 (red), and with Hoechst dye (nucleus, blue). A representative cell from each condition is shown. Scale bar represents 10 μM. This experiment was performed twice with similar results. **B.** HEK293 GAS (top) or STAT1 (bottom) reporter cell lines were pre-treated with a variety of HDAC inhibitors (TSA, MC1568, MS-275, sodium butyrate) or left untreated (DMSO, vehicle only control) for one hour. Cells were left uninfected (UI) or infected with RH parasites for one to three hours, subsequently stimulated with 100 U/ml IFN-γ for 14-20 hours, or left unstimulated (US), lysed, and luciferase activity was measured. Data was normalized within each experiment to the sample with the maximum luciferase activity and data shown are the average and s.e.m. from three independent experiments. Asterix (*) indicates $p<0.05$ or p-values are shown as indicated by bars.

New host cell protein synthesis is dispensable for the inhibition of IFN-γ-induced primary response gene expression by *Toxoplasma*

Another mechanism by which *Toxoplasma* could inhibit STAT1 transcriptional activity in the nucleus is through the transcriptional or translational activation of host negative regulatory proteins which target STAT1, such as suppressors of cytokine signaling (SOCS) family proteins, protein tyrosine phosphatases (PTPs), and protein inhibitor of activated STAT1 (PIAS) family proteins (Shuai and Liu, 2003). To test the role of proteins whose expression is induced by *Toxoplasma* infection in inhibiting STAT1 activity, we pre-treated HFFs with the protein translation inhibitor cyclohexamide (CHX) for 40 minutes prior to infecting the cells with an RH strain for one hour and subsequently stimulating with IFN-γ for one hour. Under these conditions, infection-induced and IFN-γ-induced protein expression is prevented, and cell viability is not affected (Fig. S4). We then determined IFN-γ-induced *IRF1* mRNA levels by RT-qPCR. IFN-γ treatment increased *IRF1* mRNA levels ~13-fold, and this induction was decreased in samples pre-infected with RH parasites (Fig. 5A). Pre-infection with RH parasites also inhibited IFN-γ-responsive *IRF1* mRNA accumulation in the presence of CHX (Fig. 5A). However, conclusions from the *IRF1* qPCR are complicated by the fact that both CHX treatment (~5-fold) and RH infection combined with CHX treatment (~12-fold) induce *IRF1* mRNA transcription in the absence of IFN-γ (Fig. 5A). We therefore calculated the fold inhibition of *IRF1* expression by RH pre-infection in each of these conditions. The presence of CHX did not significantly alter the ability of RH to inhibit *IRF1* gene expression (Fig. 5B). Similar results were obtained when *IRF1* qPCR data was normalized to a different control gene (Fig. S5).

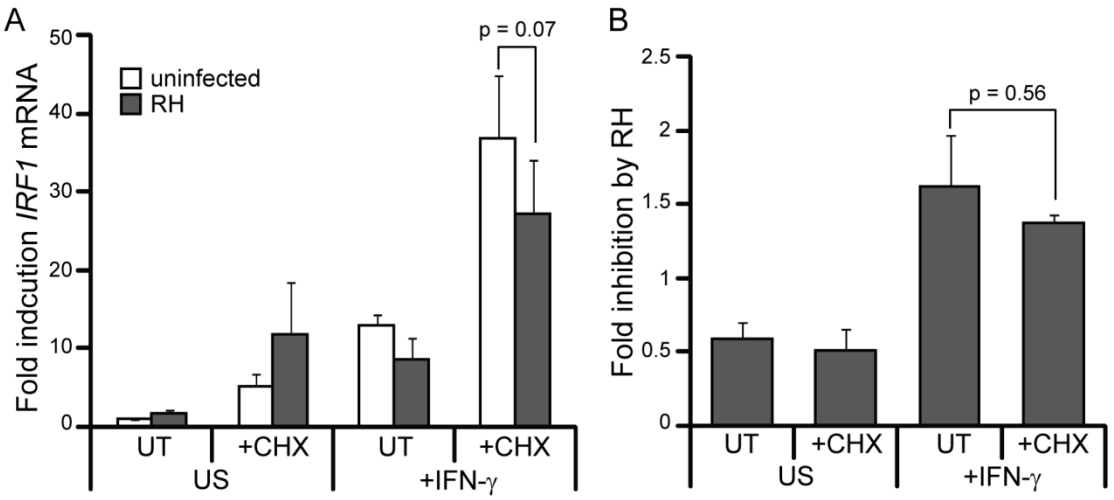

Figure 5. *Toxoplasma* can inhibit IFN-γ-responsive gene expression in the presence of cyclohexamide. HFFs were pre-treated with 50 μg/ml CHX for 40 minutes, infected with an RH strain for one hour, and stimulated with 100 U/ml IFN-γ for one hour. CHX was left on the treated cells for the entire experiment. Cells were also left untreated (UT) and/or uninfected (UI). Induction of *IRF1* mRNA was determined by RT-qPCR analysis and normalized to *ACTB* transcript levels. **A.** Averages of two experiments are shown; error bars represent s.e.m. **B.** Fold inhibition by RH infection in each of the conditions was calculated for each experiment and averages of two experiments are shown; error bars represent s.e.m.

Inhibition of STAT1 activity does not depend on the proteasome

Besides activating histone deacetylases or inducing the expression of STAT1 inhibitory proteins, *Toxoplasma* infection could also cause the degradation of a co-activator that is necessary for STAT1 to recruit general transcription machinery and RNA polymerase II. To determine whether the ability of *Toxoplasma* to inhibit STAT1 transcriptional activity depends on the proteasome, we treated our HEK293 STAT1 and GAS reporter cell lines with MG132, which inhibits the proteolytic activity of the 26S proteasome. We then infected the cell lines with RH parasites, subsequently stimulated the cells with IFN-γ, and measured the induction of luciferase activity. In both the GAS and STAT1 reporter cell lines, treatment with increasing concentrations of MG132 inhibited the induction of luciferase activity even in the absence of infection (Fig. 6). However, in the STAT1 reporter line this inhibition was only partial, and pre-infection with RH parasites significantly inhibited IFN-γ-induced luciferase activity even further (Fig. 6). This result indicates that *Toxoplasma*-induced inhibition of STAT1-mediated gene expression does not require proteolytic activity of the proteasome.

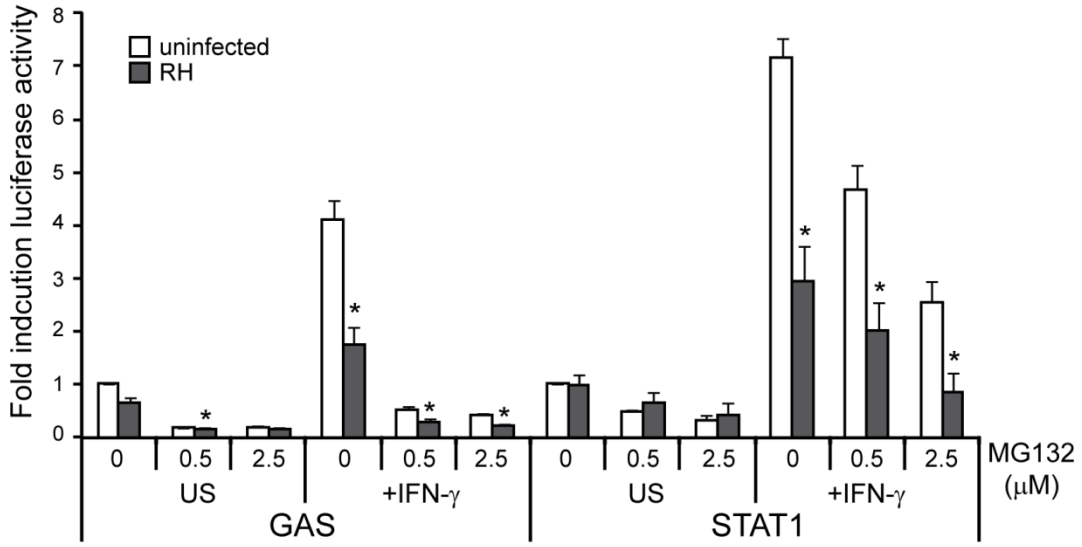

Figure 6. *Toxoplasma* can inhibit IFN-γ-responsive gene expression in the presence of MG132. HEK293 GAS or STAT1 reporter cell lines were pre-treated with MG132 or left untreated for 40 minutes. Cells were then infected with RH parasites for three to five hours, subsequently stimulated with 100 U/ml IFN-γ for 15 hours, lysed, and luciferase activity was measured. MG132 was left on the treated cells for the entire experiment. Data was normalized within each experiment to the uninfected, unstimulated (US) sample and data shown are the average fold induction and s.e.m. from three independent experiments. Asterix (*) indicates $p<0.05$.

Toxoplasma rhoptry secretion is not sufficient for STAT1 inhibition

The *Toxoplasma* effector(s) responsible for the inhibition of IFN-γ-induced, STAT1-mediated primary response gene expression remains unknown. It was previously reported that UV-treated parasites which are unable to replicate can still inhibit the IFN-γ-induced upregulation of MHC class II molecules (Lang et al., 2006). Similarly, we have found that with just three hours of infection, before the parasites have replicated, *Toxoplasma* consistently inhibits the IFN-γ-induced expression of IRF1 (Rosowski and Saeij, 2012) . We therefore wondered if the *Toxoplasma* effector(s) that modulate STAT1 transcriptional activity are secreted factors, which the parasite injects into the host cell upon invasion (Boothroyd and Dubremetz, 2008). To test this hypothesis, RH parasites were pre-treated with cytochalasin D, an inhibitor of actin polymerization which allows parasites to attach to a host cell and secrete rhoptry contents, but inhibits active invasion, which requires *Toxoplasma* actin polymerization (Håkansson et al., 2001). Pre-treated parasites were added to HFFs and allowed to attach for one

and a half hours, after which the cells were stimulated with IFN-γ for 18 hours and the expression of IRF1 was measured by immunofluorescence. Cytochalasin D-treated parasites did not invade the HFF host cells but still attached and secreted rhoptry proteins including ROP16, as demonstrated by the presence of phospho-STAT6 in host cell nuclei (Saeij et al., 2007) (Fig. 7A). However, the injection of rhoptry contents into a cell was not sufficient to inhibit IFN-γ-induced IRF1 expression (Fig. 7A).

Similarly, parasites pre-treated with cytochalasin D were unable to prevent IFN-γ-induced luciferase activity in our GAS reporter cell line (Fig. 7B). Cytochalasin D is a reversible inhibitor and must be kept on the cells for the entire experiment, also inhibiting host actin polymerization. Because it was recently shown that host actin plays a role in chromatin remodeling at IFN-γ-induced promoters (Lang et al., 2012), we also pre-treated parasites with mycalolide B, an irreversible actin depolymerizing agent, which was washed away before parasites were added to host cells. Parasites pre-treated with mycalolide B also were unable to inhibit IFN-γ-induced STAT1 transcriptional activity in the GAS reporter cell line (Fig. 7B). Thus, our results suggest that secretion of rhoptry proteins by a type I parasite into an uninvaded host cell is not sufficient to inhibit IFN-γ-induced STAT1 transcriptional activity.

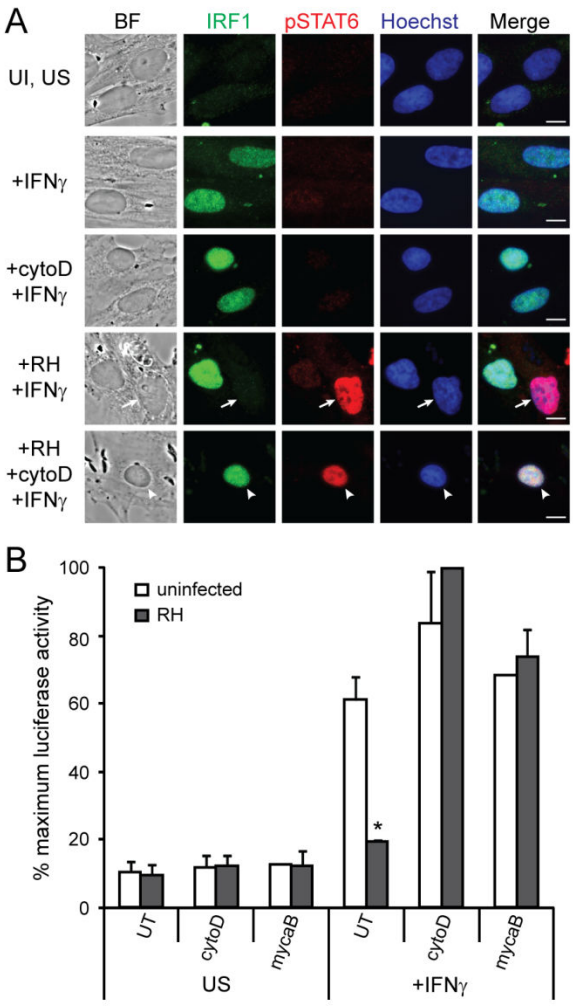

Figure 7. Invasion is required for *Toxoplasma's* ability to inhibit IFN-γ-induced gene expression. RH parasites were pre-treated with 1μM cytochalasin D (cytoD) or 3μM mycalolide B (mycaB) or left untreated (UT) and added to host cells for 1.5 hours. Cells were then stimulated with 100 U/ml IFN-γ, or left unstimulated (US) for 18 hours. **A.** HFFs were fixed and stained for IRF1 (green), phospho-STAT6 (red), and with Hoechst dye (nucleus, blue). Scale bar represents 10 μm. Arrow indicates infected cell, arrowhead indicates uninfected cell with parasites attached and rhoptry proteins secreted. This experiment was performed three times with similar results. **B.** A HEK293 GAS luciferase reporter cell line was then lysed and luciferase activity was measured. Results from two experiments per condition, except for the uninfected mycalolide B treated condition for which only one experiment was done, were normalized to the maximum luciferase activity within the experiment and then averaged. In these experiments, 100% maximum induction represents an average of 10-fold induction over uninfected, unstimulated samples. Error bars represent s.e.m. Asterisk (*) indicates $p<0.05$ compared to uninfected control.

Discussion

In this study we have further elucidated the mechanism by which type I *Toxoplasma* parasites inhibit STAT1 activity at primary IFN-γ response genes. This mechanism appears to be distinct from how *Toxoplasma* inhibits the expression of secondary IFN-γ response genes, such as *CIITA*, *H2-Eβ*, and *GBP2* (Lang et al., 2012), as it does not require the activity of histone deacetylases (Fig. 4). In fact, treatment of two different STAT1 reporter cell lines with various HDAC inhibitors illustrated that altering histone acetylation can affect both basal and IFN-γ-induced STAT1-mediated gene expression, both positively and negatively (Fig. 4B). This has been found previously; the expression of subsets of IFN-α- and IFN-β-stimulated genes are known to be sensitive to TSA treatment (Chang et al., 2004; Nusinzon and Horvath, 2003; Sakamoto et al., 2004) and IFN-γ-induced MHC class II gene expression can be increased by TSA treatment (Zika et al., 2003). While acetylation in general is thought to increase chromatin accessibility and gene expression, histones are acetylated at multiple residues with each of these modifications having slightly different consequences, and deacetylation of histones can also correlate with increased gene expression (Shahbazian and Grunstein, 2007). While *Toxoplasma* may target histone acetylation to inhibit the expression IFN-γ-induced secondary response genes, our results suggest that it is unlikely that *Toxoplasma* activates histone deacetylases to inhibit IFN-γ-induction of primary response genes such as *IRF1*.

To map out where in the IFN-γ pathway *Toxoplasma* acts to inhibit STAT1 activity, we have measured multiple steps of STAT1 activation: phosphorylation, dimerization, nuclear translocation, and DNA binding (Fig. 8). It was previously demonstrated that *Toxoplasma* infection does not inhibit STAT1 tyrosine phosphorylation or nuclear translocation (Kim et al., 2007a; Lang et al., 2006, 2012; Rosowski and Saeij, 2012). We report that STAT1 dimerization as measured by native PAGE (Fig. 1) and STAT1 DNA binding *in vivo* as measured by ChIP-qPCR (Fig. 2A) are also not inhibited by type I *Toxoplasma* pre-infection. In support of this, previous studies have reported that *Toxoplasma* does not inhibit STAT1 DNA binding activity *in vitro* by EMSA (Lang et al., 2012; Lüder et al., 2001; Schneider et al., 2013). *Toxoplasma* infection also does not inhibit STAT1 serine phosphorylation (Kim et al., 2007a; Rosowski and Saeij, 2012) which can only occur when STAT1 is chromatin-associated (Sadzak et al., 2008). These results differ from a ChIP experiment recently reported in murine BMDCs, where it was

found that pre-infection with type I *Toxoplasma* parasites did inhibit STAT1 binding at the *Irf1* promoter (Schneider et al., 2013). This could be due to differences in the species and cell type tested, although *Toxoplasma* infection can equally inhibit STAT1-mediated transcription in both human fibroblasts and murine macrophages (Rosowski and Saeij, 2012).

At one of the seven STAT1 binding sites we tested in the ChIP assay, the *IDO1* promoter, *Toxoplasma* pre-infection did inhibit IFN-γ-induced STAT1 binding. Many secondary IFN-γ response genes, such as *CIITA* and MHC class I and II genes, require the IRF1 transcription factor in addition to STAT1 for maximal induction (Hobart et al., 1997) and IRF1 can directly contact and recruit RNA polymerase II to promoters (Ramsauer et al., 2007). *IDO1* also requires IRF1 for full expression (Silva et al., 2002), and we hypothesize that at this promoter STAT1 cannot stably bind in the absence of IRF1, whose expression is inhibited by *Toxoplasma* infection. It is also possible that *Toxoplasma* infection dysregulates the binding of STAT1 to DNA across the genome such that binding is inhibited at certain regions and not at others.

We report for the first time that type I *Toxoplasma* infection also inhibits the expression of IFN-β-induced genes (Fig. 3A,B). IFN-β signals through a transcription factor complex, ISGF3, consisting of STAT1, STAT2, and IRF9. Our data suggests that type I *Toxoplasma* infection inhibits STAT1 homodimer and ISGF3 activity by similar mechanisms. Type I *Toxoplasma* infection does not prevent the IFN-β-induced tyrosine or serine phosphorylation of STAT1 (Fig. 3C), or the chromatin-association of STAT1, STAT2, or IRF9 (Fig. 3D). In the ISGF3 complex, IRF9, not STAT1, is responsible for the majority of DNA binding (Sadzak et al., 2008; Veals et al., 1992), supporting the idea that STAT1 DNA binding is not the step at which *Toxoplasma* infection inhibits STAT1 activity.

Toxoplasma likely targets STAT1 transcriptional activity directly in any complex. The requirements for global STAT1-mediated transcription beyond its binding to DNA are not entirely clear, except that STAT1 must recruit RNA polymerase II, possibly through the binding of co-activators. Our data suggests that a *Toxoplasma* effector blocks STAT1 transcriptional activity at this step (Fig. 8). This effector likely specifically targets DNA-bound STAT1, as our native PAGE experiments do not suggest that STAT1 is stably bound to any other proteins upon *Toxoplasma* infection, while in previous EMSA assays a more slowly migrating unknown STAT1 complex was observed (Lang et al., 2012; Schneider et al., 2013). The fact that infection

can also inhibit IFN-β-induced gene expression by a similar mechanism suggests that the effector can target chromatin-associated STAT1 in multiple complexes, making it unlikely that a specific DNA-binding induced STAT1 conformational change is recognized. It is possible that the effector targets serine-phosphorylated STAT1, as STAT1 is serine phosphorylated exclusively on the DNA after both IFN-γ and IFN-β treatment (Sadzak et al., 2008).

Cell fractionation experiments indicate that either type I or type I Δ*rop16 Toxoplasma* infection actually increases overall IFN-γ- and IFN-β-induced association of STAT1 with chromatin (Fig. 2B-D). Specific STAT1 mutants have been identified that enhance STAT1 DNA binding both at GAS sites and at non-canonical sites, and these mutations decrease STAT1 transcriptional activity (Koch et al., 2012). Turnover and degradation of other transcriptional activators has also been linked to increased target gene expression (Geng et al., 2012; Lipford et al., 2005). This suggests that *Toxoplasma* could inhibit STAT1 transcriptional activity simply by inhibiting its disassociation with DNA and/or its degradation.

The *Toxoplasma* effector could directly target STAT1 itself or it could act upon a host protein to indirectly target STAT1. Experiments with a proteasomal inhibitor (MG132) and a protein translation inhibitor (CHX) exclude several possible mechanisms of inhibition via modulation of a host factor. The ability of *Toxoplasma* to inhibit STAT1 activity in the presence of MG132 rules out the possibility that *Toxoplasma* infection induces the proteasomal degradation of a necessary STAT1 co-activator. IFN-γ-induced activation of both of our HEK293 STAT1 reporter cell lines was inhibited by MG132 treatment alone (Fig. 6), which has been observed before (Li and Hassel, 2001), actually arguing for a role of the proteasome in STAT1 activation. *Toxoplasma* also does not require new host protein synthesis to inhibit STAT1-mediated primary response gene expression (Fig. 5), ruling out the transcriptional or translational activation of a host negative regulatory protein or transcriptional repressor as a possible mechanism of inhibition of STAT1 activity. *Toxoplasma* infection does induce the expression of SOCS proteins which regulate STAT activity (Zimmermann et al., 2006), but these proteins target the phosphorylation of the JAK and STAT proteins (Fujimoto and Naka, 2003), while infection inhibits STAT1 activity downstream of these steps (Fig. 8). In this CHX experiment, CHX treatment and CHX treatment in combination with infection resulted in the expression of *IRF1* independent of IFN-γ treatment (Fig. 5A). This expression likely occurs via a

different transcription factor, as IRF1 can also be induced by NF-κB (Robinson et al., 2006; Rosowski and Saeij, 2012; Saha et al., 2010), and CHX treatment leads to the activation of NF-κB by preventing synthesis of inhibitory IκB proteins (Casado and Díaz-Guerra, 1997; Hershko et al., 2004). The activation of another transcription factor such as NF-κB could also explain the synergistic induction of *IRF1* transcript by the combination of CHX and IFN-γ.

The best way to determine the exact mechanism of inhibition may be to find the *Toxoplasma* effector that is responsible. Our results suggest that this factor is not secreted into the host cell upon invasion, as cytochalasin D or mycalolide B treated parasites cannot inhibit IFN-γ-induced gene expression (Fig. 8). Three major possibilities remain for the identity of the *Toxoplasma* effector: 1) a rhoptry or dense granule protein that is secreted into the host cell upon invasion but must traffic back to the PV to be modified or to act, 2) a small molecule or metabolite that can diffuse or be transported into the host cell from the PV that activates a host cell protein such as a nuclear receptor, or 3) a protein that is secreted into the host cell post invasion. Our data suggests that the *Toxoplasma* effector must act within the host nucleus, as STAT1 is inhibited after binding to DNA, making the first possibility seem unlikely. Additionally, in order to traffic back to the exterior of the nascent PVM a secreted protein must contain an arginine-rich amphipathic helix, which only a few *Toxoplasma* proteins possess (Reese and Boothroyd, 2009). These proteins all belong to the ROP2-family, several of which have been extensively characterized and are not known to be involved in STAT1 modulation (Niedelman et al., 2012). It also seems unlikely that infection activates the STAT1-inhibiting nuclear receptors that are best characterized, the liver X receptors (LXRs), as these proteins inhibit STAT1 DNA binding (Lee et al., 2009), which is upstream of where *Toxoplasma* acts. *Neospora caninum* is an apicomplexan parasite related to *Toxoplasma* that does not inhibit IFN-γ-induced gene expression (Kim et al., 2007b) but has a very similar metabolism (Reid et al., 2012), also making it less likely that the effector is a metabolite.

We favor the third hypothesis, that the effector protein is secreted into the host cell post-invasion. In addition to the rhoptries, *Toxoplasma* possesses another type of secretory organelle, the dense granules. Dense granule proteins are known to accumulate in the host cell, including GRA15 which activates the host cell NF-κB pathway (Rosowski et al., 2011) and GRA16 which accumulates in the host cell nucleus post-invasion and targets host HAUSP and PP2A enzymes

(Bougdour et al., 2013). These proteins contain a protein motif similar to one found in *Plasmodium* called a PEXEL motif which targets proteins for export across the PVM into the host cell post invasion (Hiller et al., 2004; Marti et al., 2004). Many other uncharacterized *Toxoplasma* proteins capable of interacting with host cell signaling pathways are likely to be exported into the host cell in this manner, possibly including a protein which can target STAT1 on the host cell DNA.

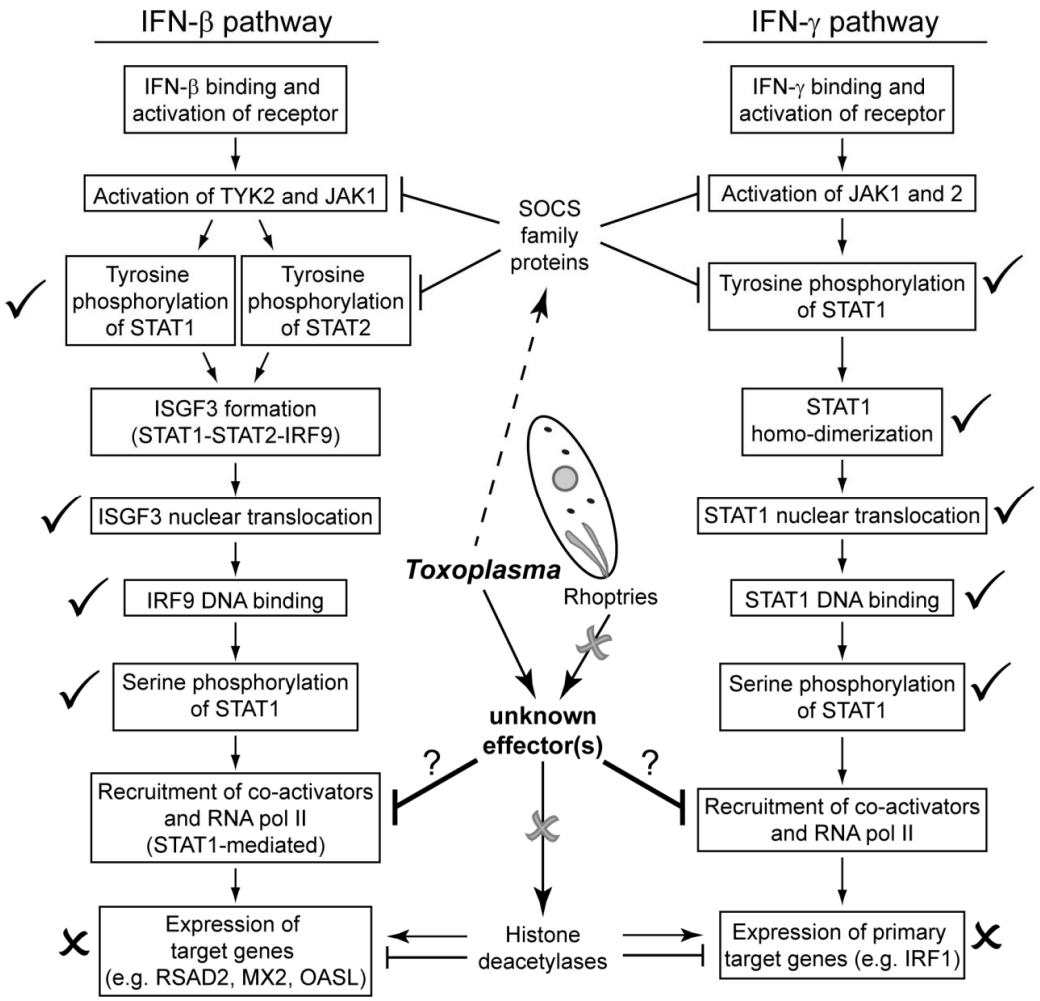

Figure 8. Intersection of IFN-STAT1 pathways and *Toxoplasma*. IFN-β and IFN-γ activate the expression of downstream target genes through ISGF3 and STAT1 homodimer complexes, respectively. The activation pathways of these cytokines are outlined. In a cell pre-infected with *Toxoplasma*, the STAT1 mediated expression of both IFN-γ- and IFN-β-induced target genes is inhibited. We have measured multiple steps of these pathways and indicate here whether each step is inhibited by *Toxoplasma* infection or still occurs in a *Toxoplasma*-infected cell. Arrows indicate activation, inhibitory arrows indicate negative regulation, X marks indicate steps which do not occur in *Toxoplasma*-infected cells, and check marks indicate steps that do still occur in *Toxoplasma*-infected cells. We find that the *Toxoplasma* effector responsible for the inhibition of STAT1 activity and the expression of IFN-γ primary reponse genes is unlikely to be a protein secreted from the *Toxoplasma* rhoptry organelle prior to invasion, and that the unknown effector does not depend on the activity of histone deacetlyases, which can have both negative and positive effects on IFN-stimulated gene expression. While *Toxoplasma* infection induces the expression of SOCS family proteins, which negatively regulate JAK/STAT activation, this induction is not necessary for *Toxoplasma* to inhibit STAT1 mediated gene expression. We hypothesize that *Toxoplasma* infection targets the recruitment of co-activators or RNA polymerase II by STAT1.

Materials and Methods

Parasites and cells

Parasites were maintained *in vitro* by serial passage on monolayers of human foreskin fibroblasts (HFFs), as described previously (Rosowski et al., 2011). An RH strain engineered to express clickbeetle luciferase and GFP (RH 1-1) (Boyle et al., 2007), an RH Δ*rop16* strain (provided by John Boothroyd, Stanford University) (Ong et al., 2010), and an RH Δ*rop16* strain expressing firely luciferase and GFP (Jensen et al., 2011) have been described previously. HFFs were cultured as previously described (Rosowski et al., 2011). 293FT and HEK293 cells were cultured with additional 10 mM HEPES. A HEK293-pGreenFire1-GAS IFN-γ responsive reporter cell line has been previously described (Rosowski and Saeij, 2012). All parasite strains and cell lines were routinely checked for *Mycoplasma* contamination and it was never detected.

Reagents

Antibodies against total STAT1α p91 (C-24) (Santa Cruz #345), phospho-STAT1^{Tyr701} 58D6 (Cell Signaling #9167), phospho-STAT1^{Ser727} (Cell Signaling #9177), STAT2 (H-190) (Santa Cruz #22816), IRF1 (BD Biosciences #612046), IRF9/ISGF-3γ p48 (C-20) (Santa Cruz #496), GAPDH (6C5) (Santa Cruz #32233), *Toxoplasma* surface antigen (SAG)-1 (kindly provided by John Boothroyd, Stanford University), *Toxoplasma* GRA7 (Dunn et al., 2008), Histone H3 (Abcam #1791), Acetyl Histone H4 ^{Lys12} (Cell Signaling #2591), and phospho-STAT6^{Tyr641} (Santa Cruz #11762-R) were used in immunofluorescence and Western blot assays. Secondary antibodies coupled with either Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes) for immunfluorescence assay or conjugated to peroxidase (Kirkegaard & Perry Laboratories) for Western blots were used. Recombinant human IFN-γ (AbD serotec), IFN-β (Peprotech), IL-4 (Peprotech), and TNF-α (GIBCO/Life Technologies) were used to stimulate cells. Cyclohexamide (50 μg/ml, Sigma), cytochalasin D (1 μM, Enzo), mycalolide B (3 μM, Wako), trichostatin A (3-9 μM, Sigma), MS-275 (2-10 μM, Selleck), MC1568 (2-10 μM, Selleck), sodium butryate (2-10 μM, Sigma), and MG132 (0.5-2.5 μM, Sigma) were also used to treat cells.

Native PAGE and Western blot

HFFs were infected with either RH Δ*hxgprt* or RH Δ*rop16* parasites at two different MOIs (actual MOIs = five and seven for RH Δ*hxgprt* and seven and nine for RH Δ*rop16*) for three hours, or left uninfected, and subsequently stimulated with 100 U/ml human IFN-γ for one hour, or left untreated. Cells were then lysed in non-denaturing buffer containing 1% sodium deoxycholate and lysates were run on 7.5% PAGE gels in Tris-glycine buffer with 1% sodium deoxycholate in the cathode chamber, at 4□C. Western transfer and blotting were performed as described previously (Rosowski et al., 2011). Blots were stained with Ponceau S to visualize protein standard (NativeMark, Life Technologies). After immunoblotting, membranes were stripped with 2% SDS and 0.7% β-mercaptoethanol and reprobed.

Chromatin immunoprecipitation (ChIP) and qPCR

ChIP experiments were performed following the protocol of Lee et al. (Lee et al., 2006) with several modifications. HFFs were grown in 15 cm dishes to ~90% confluency (~10^7 cells). Coverslips were placed in dishes to measure nuclear phospho-STAT1^{Tyr} and IRF1 as controls and processed after fixation according to immunofluorescence assay methods below. HFFs were infected with RH Δ*hxgprt* or RH Δ*rop16* parasites for four hours, or left uninfected, subsequently stimulated with 100 U/ml human IFN-γ for one hour, or left unstimulated, and fixed with 1% formaldehyde. ~5x10^6 cells were used for each immunoprecipitation. After cell lysis, DNA was sheared with a Bioruptor (Diagenode). Immunoprecipitation was performed using an IP-Star (Diagenode) and 3 μg antibody (total STAT1α p91 (C-24) (Santa Cruz #345)). After DNA purification, quantitative PCR was performed using SYBR Green reagent (Kapa Biosciences) and a LightCycler 480 II real-time PCR machine (Roche). Primers were designed to amplify STAT1 binding sites in the promoters of IFN-γ-induced genes as well as negative control regions in the promoters of genes unaffected by IFN-γ where STAT1 is not known to bind from published STAT1 ChIP-seq data (Robertson et al., 2007). Primer efficiencies were calculated using Real-time PCR Miner (Zhao and Fernald, 2005) and are listed with primer sequences in Table S1. Percent of total DNA bound by STAT1 was calculated by comparing qPCR results from immunoprecipitated and input samples.

Cell fractionation, STAT1 immunoprecipitation, and mass spectrometry

Cells were fractionated into cytoplasmic, nuclear extract, and chromatin fractions using a Qiagen Qproteome Nuclear Protein Kit according to the manufacturer's instructions. For Western blot analysis, samples were diluted in 2x reducing sample buffer and boiled before SDS-PAGE analysis and Western blotting as described previously (Rosowski et al., 2011). For STAT1 immunoprecipitations, all fractions were diluted to have a final concentration of 150 mM NaCl. Rabbit α-total STAT1α p91 (C-24) (Santa Cruz #345) (~1 μg per 10^6 cells) was crosslinked to protein A Dynabead slurry (Life Technologies) (~20 μl per μg antibody) with 5 mM Bis(Sulfosuccinimidyl) suberate (BS3) (Pierce) as described previously (Niedelman et al., 2012). Samples were incubated with the bead-antibody slurry for 1.5 hr at 4□C, rotating. Beads were then washed three times with IP wash buffer (10 mM HEPES-KOH pH 7.5, 150 mM NaCl, 0.5% NP-40/IGEPAL, 2.5 mM EGTA-KOH, 20 mM β-glycerophosphate), washed two times with HEPES-buffered saline (HBS), and boiled in 2x reducing sample buffer. Mass spectrometry analysis was performed as described previously (Niedelman et al., 2012).

Reporter cell line construction

The construction of a HEK293-pGreenFire1-GAS IFN-γ responsive reporter cell line has been previously described (Rosowski and Saeij, 2012). pGreenFire1-ISRE and STAT1 cell lines were constructed by the same method from ISRE (TR016PA-1, 5'-CAGTTTCACTTTCCCTTT-3') and STAT1 (TR015PA-1, 5'-GATTTCCGGGAAATGGGGAAGG-3') vectors purchased from System Biosciences. Briefly, lentivirus containing the vector was produced in 293FT cells and added to HEK293 cells (ATCC). Cells containing the construct were selected with 750 □g/ml Geneticin (Invitrogen), cloned by limiting dilution, and assayed for responsiveness to IFN-γ, IFN-β, TNF-α, and IL-4 (Fig. S2).

Luciferase assay

Luciferase assay of HEK293 pGF1-GAS, STAT1, or ISRE cells was done as previously described using the Promega Luciferase Assay System (Rosowski and Saeij, 2012). For all experiments, 3.5-4x10^4 cells were plated in 96-well plates for at least four hours before any treatment or infection. Exact treatment and infection times varied slightly between experiments

but we obtained similar results for all time points and present averaged data with standard error. For ISRE experiments, cells were infected with RH parasites at an MOI~1.5 for three to five hours, and subsequently stimulated with 100 U/ml of either IFN-γ or IFN-β for 14-17 hours before lysis. For HDAC inhibitor experiments, cells were pretreated with HDAC inhibitors for one hour, infected with RH parasites at an MOI ~4 for one to three hours, and subsequently stimulated with 100 U/ml IFN-γ for 14-20 hours before lysis. For MG132 experiments, cells were pretreated with MG132 for 40 minutes, infected with RH parasites at an MOI~1.5 for three to five hours, and subsequently stimulated with 100 U/ml IFN-γ for 15 hours before lysis. HDAC inhibitors and MG132 were kept on the cells for the entire experiment. For cytochalasin D experiments, parasites were pre-teated with 1 μM cytochalasin D for 15 minutes, and cytochalasin D was kept on the parasites for the entire experiment. For mycalolide B experiments, parasites were pre-treated with 3 μM mycalolide B for 10 minutes then pelleted and resuspended in normal media. Pre-treated parasites were added to cells for one and a half hours and cells were subsequently stimulated with 100 U/ml IFN-γ for 18 hours before lysis.

RT-qPCR

For IFN-β infections, ~$9x10^5$ HFFs were grown in 6-well plates, infected with RH 1-1 parasites for four hours, and subsequently stimulated with 100 U/ml IFN-γ or IFN-β for 15-20 hours. The exact treatment was between 15 and 20 hours, varying slightly between experiments, but we obtained similar results for all time points and present averaged data with standard error. For CHX infections, 1.5-$2x10^5$ HFFs were grown in 12-well plates, pre-treated with CHX for 40 minutes, infected with RH 1-1 for one hour, and stimulated with human IFN-γ for one hour. Cells were also left untreated and uninfected. RNA was isolated using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. Samples from the CHX experiments were cleaned and concentrated using RNeasy MinElute kit (QIAGEN). Genomic DNA was removed from RNA preparations by DNase I treatment (Invitrogen), and first-strand cDNA was synthesized with SuperScript II or III RT (Invitrogen) and oligo-dT (Ambion), according to the manufacturer's protocol. Quantitative PCR was performed using SYBR Green reagent (Kapa Biosciences) and a LightCycler 480 II real-time PCR machine (Roche) according to the manufacturer's instructions. For IFN-β experiments, genes specifically induced by IFN-β and not

by IFN-γ in human fibroblasts (*RSAD2, MX2, OASL*) were chosen from published microarray results (Indraccolo et al., 2007). Primer efficiencies were calculated using Real-time PCR Miner (Zhao and Fernald, 2005) and are listed with primer sequences in Table S2. Fold change was calculated using the ΔΔCt method (Livak and Schmittgen, 2001), comparing expression to two different control genes that were not affected by *Toxoplasma* infection in previous gene expression analyses, *ACTB* and *NFE2L1* (Rosowski et al., 2011). Similar results were obtained from both normalizations.

Immunofluorescence assay

Immunofluorescence assay was performed as described previously (Rosowski et al., 2011). Quantification of nuclear signal was performed by randomly selecting at least 30 cells per condition and measuring the average signal intensity per nucleus using the NIS-Elements software and Hoechst dye to define nuclei.

Plaque assay

For native PAGE, ChIP, cell fractionation and immunoprecipitation, luciferase reporter, and RT-qPCR experiments, a plaque assay was done to determine parasite viability and the actual MOI. One hundred parasites per well were added to confluent HFFs in a 24-well plate and were incubated undisturbed for 5-7 days at 37°C, and the number of plaques was counted.

Statistical analyses

Two sample t-tests, either paired or unpaired as applicable, were performed to assess statistical significance for ChIP-qPCR, luciferase reporter, and RT-qPCR assays.

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Supplementary Figures

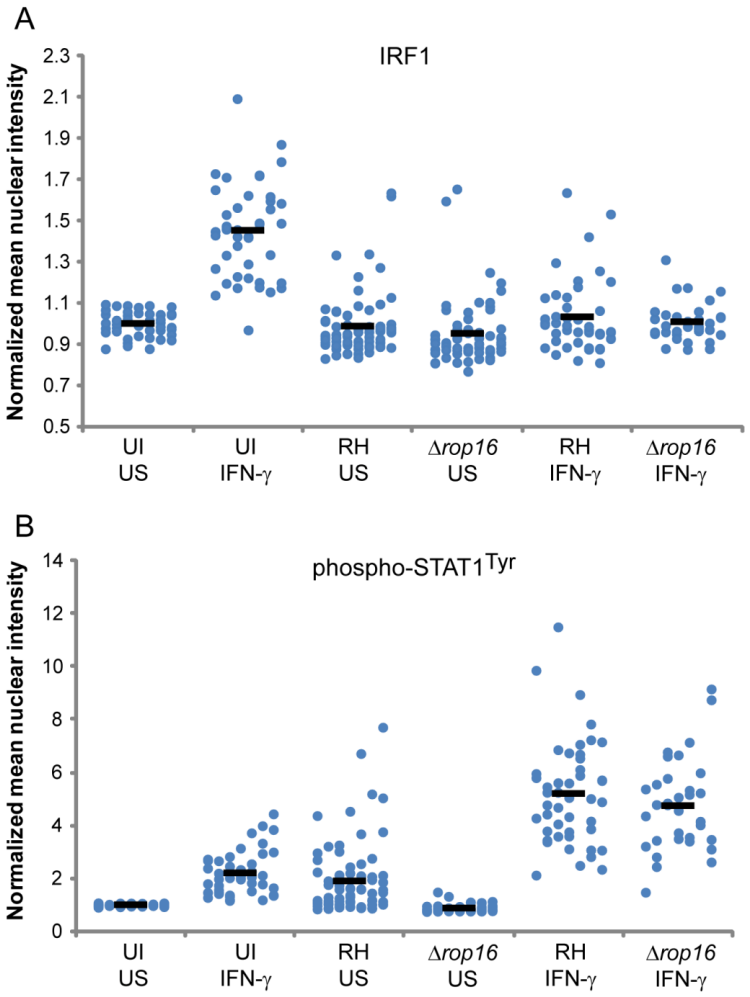

Figure S1. Infection of cells for ChIP inhibits IRF1 expression and activates phosphorylation of STAT1. Coverslips were placed in 15 cm dishes prior to plating of HFFs for the ChIP assay. HFFs were infected with an RH or RH Δ*rop16* strain for five hours, or left uninfected, with 100 U/ml IFN-γ added for the last hour of infection, or cells were left unstimulated. After fixation, coverslips were removed from the plates and the remaining HFFs were used for ChIP assays (Fig. 2). Coverslips were then permeabilized and stained for IRF1 and phospho-STAT1^{Tyr}. The intensity of IRF1 (A) and phospho-STAT1^{Tyr} (B) nuclear staining was quantified in at least 30 cells, regardless of infection status. Each dot represents one cell and black bars represent average staining. This experiment was performed for all three biological replicates of the ChIP assay with similar results, data shown are from one experiment.

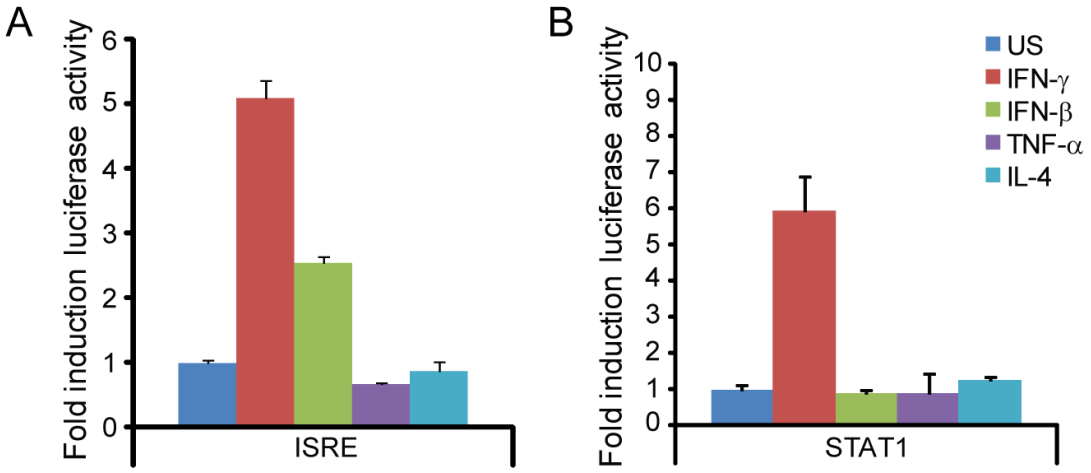

Figure S2. Characterization of HEK293 reporter cell lines. HEK293 luciferase reporter cell lines were left unstimulated or stimulated with 100 U/ml IFN-γ, 100 U/ml IFN-β, 20 ng/ml TNF-α, or 50 ng/ml IL-4. Cells were lysed 6-20 hours later and luciferase activity was measured and normalized to levels in unstimulated cells. **A.** HEK293 ISRE reporter cell line. Data and standard deviation from one experiment are shown. Unstimulated, IFN-γ, and IFN-β conditions have been repeated four times with similar results. **B.** HEK293 STAT1 reporter cell line. Average luciferase induction from three experiments is shown and error bars represent SEM.

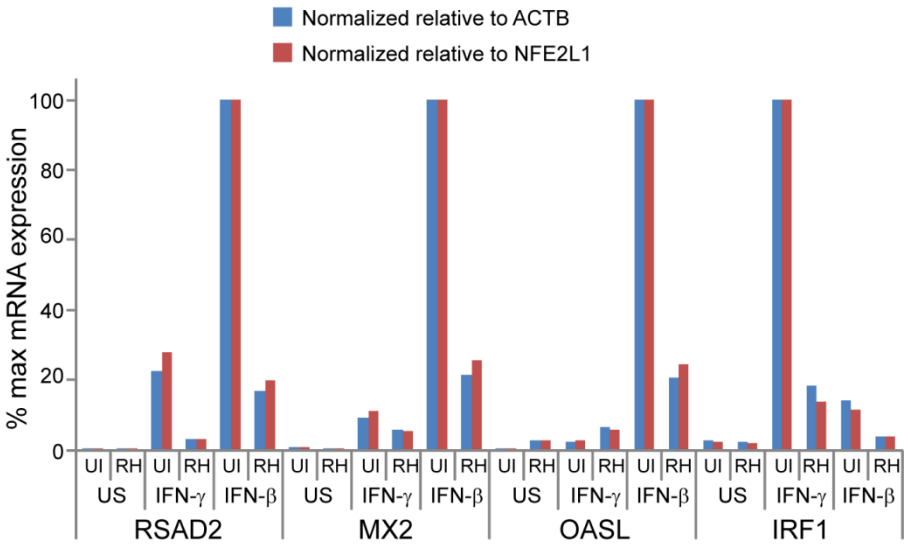

Figure S3. IFNb qPCR data normalized to NFE2L1. RT-qPCR data from one experiment from Fig. 3B are shown normalized to two different control genes, *ACTB* and *NFE2L1*.

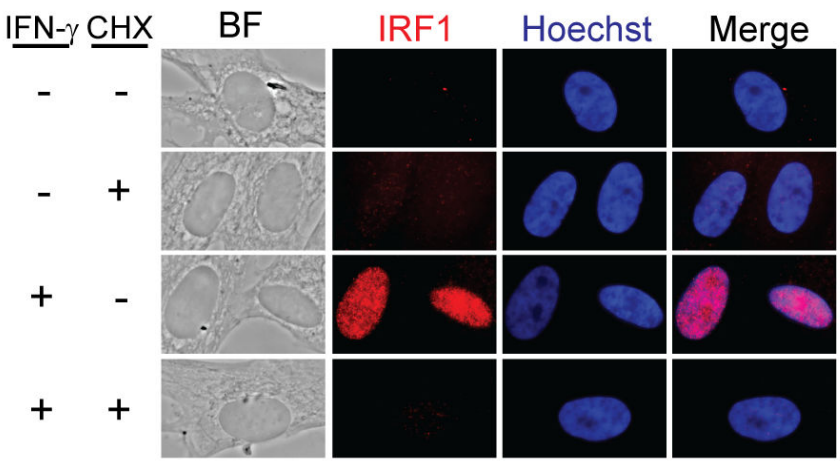

Figure S4. CHX treatment prevents the expression of IFN-γ-induced IRF1. HFFs on coverslips were pre-treated with 50 µg/ml CHX for one hour and 40 minutes and subsequently stimulated with 100 U/ml IFN-γ for two and a half hours. Cells were also left untreated and/or unstimulated. Cells were fixed, permeabilized, and stained for IRF1 (red) and with Hoechst dye (nucleus, blue).

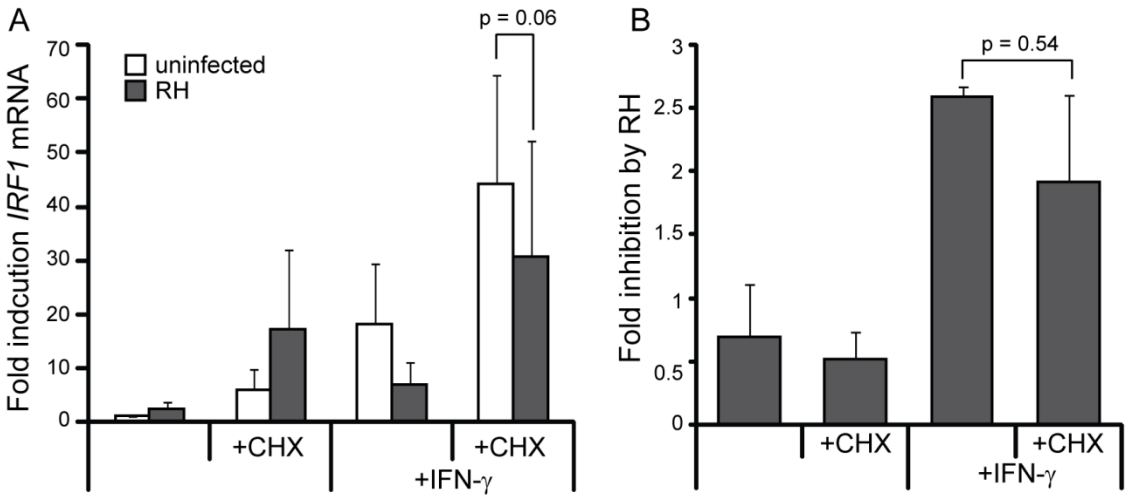

Figure S5. CHX qPCR data normalized to NFE2L1. RT-qPCR data from Fig. 5A,B are shown normalized to a different control genes, *NFE2L1*.

	Forward primer (5' to 3')	Reverse primer (5' to 3')	Calculated Primer Pair Efficiency
IRF1	TTGCCTCGACTAAGGAGTGG	TTCGCCGCTAGCTCTACAAC	89.50%
SOCS3	GGAGCAGGGAGTCCAAGTC	GCGCTCAGCCTTTCTCTG	105.46%
GBP1	TGGACAAATTCGTAGAAAGACTCA	GCACAAAAACTGTCCCCAAC	95.73%
CIITA	ACATTTTTGCCCATGAGGTC	CTCACATCCTAAGGGCCAGA	105.44%
CXCL10	AAAGGAACAGTCTGCCCTGA	GCCCTGCTCTCCCATACTTT	106.46%
IDO1	CACAGTCATTGTATTCTCTTTGCTG	GCATATGGCTTTCGTTACAGTC	107.48%
ICAM1	CCCTGTCAGTCCGGAAATAA	CCATAGCGAGGCTGAGGTT	100.73%
CCND2	TCACCCCCTCCCTTATTTTT	ACCCTCCAACTTTGGCTTCT	107.60%

Table S1. STAT1 ChIP-qPCR primers. Primers were designed to amplify 150-180 bp regions of STAT1 binding sites in the promoters of IFN-γ induced genes as well as a negative control region where STAT1 is not known to bind. Published STAT1 ChIP-seq data was used to determine regions of STAT1 binding (Robertson et al., 2007). Primer pair efficiencies were calculated using Real-time PCR Miner (Zhao and Fernald, 2005).

	Forward primer (5' to 3')	Reverse primer (5' to 3')	Calculated Primer Pair Efficiency
IRF1	GGATTCCAGCCCTGATACCT	CCTGCTCCACCTCCAAGTC	100.50%
RSAD2	GAGCGCCACAAAGAAGTGTC	TCCTTCCGTCCCTTTCTACA	104.44%
MX2	GCATCCACCTGAATGCCTAC	GCTTTCTGCAAGGAGTCACC	93.34%
OASL	AGGGTACAGATGGGACATCG	AAGGGTTCACGATGAGGTTG	98.25%
ACTB	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT	95.41%
NFE2L1	AGCAGCTCTGAAGGCAGTTC	AGCTGGATCCTGGTAGCTCA	99.22%

Table S2. RT-qPCR primers. Primers were designed to amplify 100-300 bp near the 3' ends of genes, spanning intron-exon boundaries if possible. Primer pair efficiencies were calculated using Real-time PCR Miner (Zhao and Fernald, 2005).

Chapter 5. Discussion and Future Directions

The interplay between *Toxoplasma* and its host is a balancing act, not a battle

Host-pathogen interactions are often described as an arms race, with each side evolving better and better strategies to kill or evade the other. But for a parasite such as *Toxoplasma gondii*, which must persist within its host and form a chronic infection, this interaction is actually more of a balancing act. *Toxoplasma* partially evades the innate immune response of its host to survive and grow. At the same time, it cannot grow uncontrollably as it must convert to the slow-growing chronic encysted bradyzoite stage, the only asexual stage of the parasite that can be transmitted to a new host through oral infection. Therefore, *Toxoplasma* must activate the host immune response to some extent and allow its host to survive. Another important factor to consider is the wide host range of *Toxoplasma*, comprising basically every warm-blooded animal, including both mammals and birds. Different animals have different complements of immune mechanisms that are known to act against *Toxoplasma*, including TLRs (Roach et al., 2005), IRGs and GBPs (Hunn et al., 2011), and iNOS (Schneemann and Schoeden, 2006; Schneemann et al., 1993), and a successful *Toxoplasma* strain must be equipped with an array of effectors that can modulate the immune response of each host it encounters. For example, while in mice the IRGs are crucial for survival of an acute *Toxoplasma* infection, humans have a very restricted complement of these GTPases. Humans also do not possess active copies of TLR11 or 12, the two main TLRs that activate NF-κB in response to *Toxoplasma* infection in mice. The genetic diversity of *Toxoplasma* is probably dictated by the different niches that these strains live within, and the population genetics and history of these niches. In North America and Europe, *Toxoplasma* strains are largely clonal, whereas in South America the genetic diversity of *Toxoplasma* is much greater (Minot et al., 2012).

A *Toxoplasma* strain therefore must possess a wide array of effector molecules that drive a partial evasion of the immune response in multiple hosts. Mice are the most well studied host of *Toxoplasma*, and one of the hosts contributing to the parasite niche and *Toxoplasma* evolution in North America and Europe, where the clonal type I, II, and III lineages predominate. In the mouse, the innate immune response to *Toxoplasma* consists of two main phases: 1) the activation of NF-κB and production of the cytokine IL-12 in macrophages and dendritic cells and 2) the production of the cytokine IFN-γ by T cells and NK cells and induction of IFN-γ responsive effector mechanisms in multiple cells types (Chap. 1 Fig. 3). In my work I have studied how

Toxoplasma modulates both phases of this response. We found that type II strains carry an active copy of the novel dense granule protein GRA15, which activates the host innate immune response by directly activating NF-κB, and expression of GRA15 leads to increased cytokine production and decreased parasite growth early in infection (Chap. 2 Figs. 3 and 9). Conversely, type I and III strains of *Toxoplasma* carry an active copy of the rhoptry kinase ROP16 which activates host STAT3, 5, and 6 transcription factors, and acts in opposition to GRA15 to promote alternative activation of macrophages and decreased host inflammation (Jensen et al., 2011, 2013). In the second phase of the innate immune response, the transcriptional response to IFN-γ, some strains of *Toxoplasma* can directly inhibit the activity of a major IFN-γ-induced toxoplasmacidal mechanism, the IRGs, through different combinations of the polymorphic effectors ROP18 and ROP5 (Behnke et al., 2012; Fleckenstein et al., 2012; Niedelman et al., 2012). However, *Toxoplasma* infection can also inhibit the upregulation of IFN-γ-induced genes through direct inhibition of the activity of the transcription factor STAT1, and we found that all three clonal lineages can equally inhibit STAT1 transcriptional activity (Chap. 3 Fig. 5) and have further characterized the mechanism by which this inhibition occurs (Chap. 4). Further research on the mechanism of action of all of these effectors and their functions *in vivo* will provide insights into the strategies each *Toxoplasma* strain has evolved to achieve this balancing act between the host and the pathogen.

Elucidating the role of GRA15 *in vivo*

One major question that is still unanswered is the full role of GRA15 *in vivo*. We found that the expression of GRA15 in type II parasites increases host pro-inflammatory cytokine secretion and decreases parasite growth early in infection (days 1-5), but later in infection these differences seem to disappear, as mouse survival after infection with either a type II or type II Δ*gra15* parasite was virtually identical (Chap. 2 Fig. 9). We hypothesize that early in infection, when the first parasites infect host cells, the presence of GRA15 in type II parasites activates NF-κB earlier than strains that do not carry an active copy of GRA15, leading to an early difference in IL-12 and IFN-γ levels and therefore host control of parasite growth (Fig. 1). Later in infection, after parasites have undergone multiple rounds of growth, host cell lysis, and reinvasion, multiple PAMPs and DAMPs will be released independently of the presence of

GRA15, including the parasite protein profilin which activates TLR11 and 12 (Fig. 1). These PAMPs and DAMPs will strongly activate NF-κB in many cells, not just parasite infected cells, allowing the host to initiate a strong innate immune response and control growth of any parasite strain.

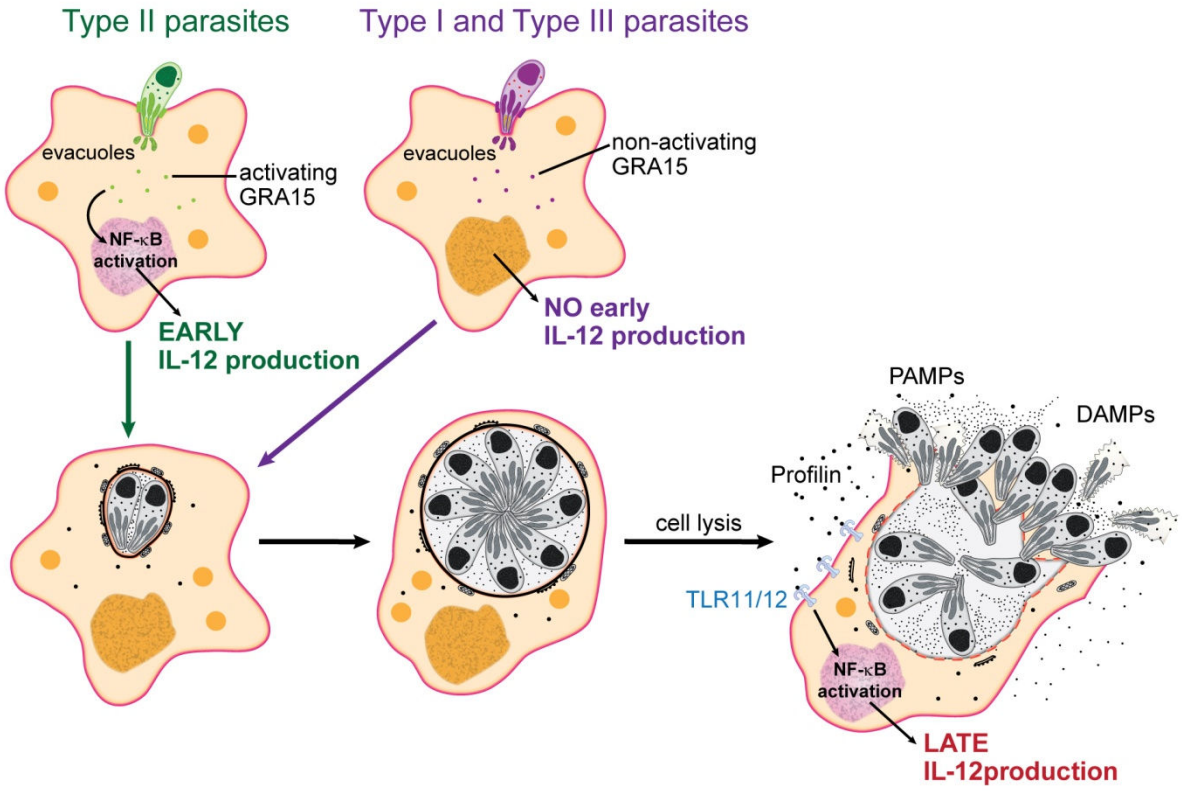

Figure 1. Model of the effect of GRA15 on IL-12 production *in vivo*. Illustration by Mariane Melo.

However, it is likely that GRA15 has other effects *in vivo* that we have not yet studied. For example, NF-κB upregulates the expression of chemokines and chemokine receptors and promotes cell migration (Newton and Dixit, 2012). It is possible that GRA15 increases migration of its host cell, helping the parasite to move throughout the host, a process that is necessary for *Toxoplasma* to form tissue cysts in muscle and brain tissue, regions far removed from the initial infection site, either the intraperitoneal cavity or the small intestine (Tardieux and Ménard, 2008). Studies in the lab have not found any differences in the number of cysts formed in the brain in type II versus type II Δ*gra15* chronic infections (Kirk Jensen, unpublished). This implies that GRA15 does not affect the ability of *Toxoplasma* to cross the blood-brain barrier. But,

GRA15 might still affect cell migration during other stages of infection. I did find a difference in the number of type II versus type II Δ*gra15* parasites in the lung and spleen after an intraperitoneal injection, but it is unclear whether this is due to differences in growth or migration (Chap. 2 Fig. S8). I briefly tried *in vitro* cell migration assays, and I think that optimizing these assays and testing the effect of GRA15 on cell migration in different cell types at least *in vitro* could be an interesting follow-up study. GRA15 might also play a role after a chronic infection has been established, possibly in reactivation of cysts or in the level of inflammation. To investigate this phase, I infected mice with a sublethal dose of either type III or type III $GRA15_{II}$ parasites to allow them to establish a chronic infection. I then immunosuppressed the mice by treating them with dexamethasone, following an established protocol (Saeij et al., 2005b), but I did not detect any parasite reactivation as measured by whole body luciferase imaging. However, the mice did become sick and had enlarged abdomens. I did sacrifice these mice and dissect out their brains for histology but did not follow up on this experiment as I was not convinced that there was any parasite reactivation, but rather the mice likely suffered from a bacterial infection. I am also not sure about the choice of dexamethasone as an immunosuppressant as its mode of action is through the inhibition of NF-κB signaling (Wissink et al., 1998), which could possibly mask any effects of GRA15 on NF-κB.

Toxoplasma can infect a wide range of cell types *in vivo*, including macrophages, dendritic cells, neutrophils, NK cells, T cells, and B cells. It is possible that GRA15 has important effects only within some of these cells, and it would be interesting to look at differences in the numbers of cells infected and the cytokines secreted specifically by each cell type after infection with a type II versus a type II Δ*gra15* strain. Cell-type specific effects could be either cell-intrinsic or -extrinsic. In terms of cell-autonomous responses, we initially found that GRA15 affected growth of parasites *in vitro* in HFFs, but not MEFs (Chap. 2 Fig. S8). At the time, these experiments were repeatable and performed with multiple clones of type II Δ*gra15* and type I $GRA15_{II}$ parasites and heterologous controls expressing the *HXGPRT* selection marker. However, later, neither I nor others in the lab were able to recapitulate these results. Whether this is due to differences in the passage or age of host cells, media composition, growth conditions, or something else, is unknown, but as of now we cannot attribute any effects of GRA15 *in vivo* to such a growth defect.

Work by Kirk Jensen has shown that GRA15, through its activation of NF-κB, and another *Toxoplasma* effector, ROP16, which activates STAT3 and 6, oppositely regulate many of the same genes (Jensen et al., 2011). In an oral infection model, expression of ROP16 decreases infection-induced small intestinal inflammation and increases mouse survival, but interestingly, this effect is dependent on the co-expression of GRA15 (Jensen et al., 2011, 2013). How the combined action of these antagonistic effectors achieves this infection outcome is unknown. It might be that the presence of both effectors creates a general balance between pro- and anti-inflammatory cytokines and effectors in the host. By itself, ROP16 might decrease inflammation too much, and the opposite might be true of GRA15. It is also unknown whether the effects of these proteins that achieve this balance occur cell-autonomously within the infected cell or through secretion of cytokines and chemokines that affect uninfected cells. We do know that ROP16 inhibits GRA15-induced NF-κB activity (Chap. 2 Fig. S9), but this doesn't seem to be through an independent effect of ROP16 on NF-κB, as infection of cells with a type I strain, which possesses an active copy of ROP16, does not inhibit TNF-α- or LPS-mediated activation of NF-κB (Chap. 2 Fig. 2). Perhaps these proteins directly interact within the cell, or perhaps GRA15 activates NF-κB by a fundamentally different mechanism than TNF-α or LPS. This latter possibility seems unlikely as GRA15-mediated activation of NF-κB follows the canonical NF-κB activation pathway through the IKK complex and proteasome-mediated degradation of IκBα (Chap. 2 Fig. 6,7). It might be that continuous activation of NF-κB by GRA15, as opposed to transitory activation by TNF-α or LPS, is more susceptible to ROP16-induced STAT3 and STAT6 downregulation.

One question that follows is, if these effectors work so well together to promote both parasite and host survival in the mouse oral infection model, why do none of the successful clonal lineages have active copies of both? As these strains arose from crosses between parental strains (Boyle et al., 2006), it is almost certain that siblings of these strains carried both effectors, yet somehow were not successful. A possible answer to this question is that although GRA15 and ROP16 together decrease host inflammation and increase host survival, they also decrease parasite numbers in the intestine, and this might decrease later parasite transmission. One other answer is that GRA15 and ROP16 are not required for host control of intestinal infection in all mouse strains, as type II oral infection of A/J mice does not result in intestinal pathology (Jensen

et al., 2013). It seems likely then that the wild hosts that make up the *Toxoplasma* niche in North American and Europe have immune responses that are more similar to A/J mice than B6 mice, the mouse strain in which these effectors synergistically decrease inflammation.

In fact, we don't know what host niche type II parasites, which express an active copy of GRA15, co-evolved to live within, and it is possible that GRA15 has much greater effects on parasite survival and transmission in hosts other than the mouse, such as rats or birds. While humans are usually a dead-end host in the parasite life cycle, human infections are important to consider from a medical standpoint, and type II parasites are the strain commonly found in human infections (Dardé, 2004). These strains therefore might have evolved to live in a species that has a similar immune system to humans. Additionally, based on our model of how GRA15 affects early parasite growth through induction of IL-12 (Fig. 1), we hypothesize that GRA15 has a greater effect on the immune response in hosts, such as humans, that do not have functional copies of TLR 11 and 12. These two TLRs recognize the most prominent *Toxoplasma* TLR ligand, profilin, and strongly activate NF-κB and IL-12 secretion in response (Koblansky et al., 2013; Yarovinsky et al., 2005). Mice that are doubly deficient in TLR 11 and 12 have recently been generated and are extremely susceptible to *Toxoplasma* infection, even with a type II strain ME49 that possesses an active copy of GRA15 (Koblansky et al., 2013). Based on our model, we hypothesize that infection of these mice with a type II Δ*gra15* strain would result in even greater morbidity and mortality. Additionally, in humans, TNF-α signaling, which activates NF-κB, is a much more important pathway for combating *Toxoplasma* infections (Janssen et al., 2002), as opposed to in mice where IFN-γ is the main mediator of resistance (Suzuki et al., 1988).

What role does *Toxoplasma*'s inhibition of STAT1 signaling play *in vivo*?

We also don't know exactly what role the ability of *Toxoplasma* strains to inhibit IFN-γ-induced transcription plays *in vivo*. Mice deficient in IFN-γ, the IFN-γ receptor, STAT1, and IRF1 are acutely susceptible to *Toxoplasma* infection (Khan et al., 1996; Lieberman et al., 2004; Scharton-Kersten et al., 1996; Suzuki et al., 1988; Yap and Sher, 1999). However, mice deficient in single downstream IRG effectors, Igtp (Irgm3) (Taylor et al., 2000) or LRG-47 (Irgm1) (Collazo et al., 2001) are as susceptible as IFN-γ deficient mice in the acute phase. If the IRGs are responsible for the majority of IFN-γ induced killing of *Toxoplasma*, why do *Toxoplasma*

strains that carry active copies of ROP18 and ROP5, which directly inhibit IRG activity, also have an effector to inhibit the expression of these and other IFN-γ induced genes? For one thing, not all animals possess such a diversity of IRG effectors, but the IFN-γ response might still be important for host resistance to *Toxoplasma* in these species. When the *Toxoplasma* STAT1 inhibitor is identified, it will be interesting to determine the phenotype in mice of a parasite that is deficient in this inhibitor but still expresses ROP18 and ROP5. There are several reasons why all of these effectors might also be required in mice. A specific and limited number of copies of ROP18 and ROP5 protein are directly injected into the host cell upon infection and since ROP5 directly interacts with the IRGs and is hypothesized to act as a scaffold, keeping IRGs in their monomeric form (Fleckenstein et al., 2012; Niedelman et al., 2012), perhaps it can only inhibit a limited number of IRG proteins. The STAT1 inhibitor might then be required to keep the expression of these proteins limited. ROP18 and ROP5 might also be most important in cases where *Toxoplasma* infects a cell that has already been exposed to IFN-γ and already upregulated the IRGs. It is possible as well that, at least during the acute phase of infection, these factors act redundantly.

The STAT1 inhibitor also might play a much larger role in survival of *Toxoplasma* during the chronic phase of infection. While the IRGs are the main effector mechanism against *Toxoplasma* infection during the acute stage of infection, in the chronic phase they appear to play a more limited role in mouse survival, while STAT1 is still critical (Collazo et al., 2002). To establish and maintain a chronic infection, *Toxoplasma* must evade the adaptive immune response, which is largely driven by the presentation of antigen to T cells on MHC molecules, and by inhibiting STAT1 activity *Toxoplasma* also inhibits MHC molecule expression. *Toxoplasma* also inhibits other IFN-γ induced mechanisms such as iNOS and NO production that are most important during the chronic phase of infection (Scharton-Kersten et al., 1997; Seabra et al., 2002). However, all of our studies and the studies of others have focused on the ability of *Toxoplasma* tachyzoites to inhibit IFN-γ signaling. It would be interesting to determine whether *Toxoplasma* bradyzoites, which are predominant during chronic infections, can also inhibit STAT1-mediated gene expression.

As with GRA15, we also don't know whether the presence of this STAT1 inhibitor is more or less important in different cell types *in vivo*. IRGs are expressed by and can kill

Toxoplasma in a variety of cell types, including macrophages (Butcher et al., 2005b), fibroblasts (Niedelman et al., 2012), and astrocytes (Halonen et al., 2001), and expression in both hematopoietic and non-hematopoietic compartments is required for mouse resistance (Collazo et al., 2002). In contrast, other IFN-γ induced effector mechanisms are more restricted in the cells types in which they are expressed. MHC class II molecule expression is restricted to professional antigen presenting cells, including macrophages, dendritic cells, and B cells, and iNOS expression is only required in hematopoietic cells to help control *Toxoplasma* infections (Yap and Sher, 1999).

This STAT1 inhibitor may also be more or less important in hosts other than the mouse. In humans, for example, the IFN-γ response is not as important for control of *Toxoplasma* infection as in mice. For one thing, humans do not have many of the IFN-γ induced effector mechanisms that control parasite growth in the mouse. Humans lack both the diverse complement of IRGs that mice have (Hunn et al., 2011), and the strong induction of nitric oxide by macrophages upon IFN-γ treatment (Schneemann and Schoeden, 2006; Schneemann et al., 1993). It is therefore not surprising that human patients that carry mutations in the IFN-γ receptor are not susceptible to Toxoplasmosis, whereas they are more susceptible to infections of Salmonella (Janssen et al., 2002).

What happens after STAT1 DNA binding?

The mechanism by which *Toxoplasma* inhibits STAT1 transcriptional activity remains unknown. However, we have narrowed down where in the activation pathway a *Toxoplasma* effector acts. *Toxoplasma* infection does not inhibit STAT1 DNA binding at promoter target sequences in host cells (Chap. 4 Fig. 2A), it must act downstream of this step. However, it is not very well described in the literature what steps after STAT1 binding are required for productive gene transcription. Therefore it is very difficult to test further specific steps of activation to see if *Toxoplasma* infection inhibits them. For example, how does STAT1 recruit general transcription factors and RNA polymerase II? Could *Toxoplasma* prevent the recruitment of RNA polymerase II by STAT1, or are there primary response genes, such as IRF1, whose expression *Toxoplasma* inhibits, that already have RNA polymerase II basally bound to their promoters? It is probably worthwhile testing the binding of RNA polymerase II to the promoters of both primary and

secondary IFN-γ response genes in uninfected and infected cells, both in unstimulated and IFN-γ stimulated conditions. While *Toxoplasma* does not require histone deacetylase activity to inhibit STAT1 signaling, we could also test whether *Toxoplasma* infection dysregulates specific histone methylation marks that are associated with active genes, such as H3K4me3 at transcription start sites and H3K36me3 in gene bodies towards 3' ends (Barski et al., 2007).

One hypothesis for how *Toxoplasma* inhibits STAT1-mediated transcription is by preventing the association of STAT1 with necessary co-activators. For example, it has been proposed that *Toxoplasma* inhibits the recruitment of the chromatin remodeler BRG-1 to STAT1 target genes such as MHC class II genes and CIITA (Lang et al., 2012). However, chromatin remodeling, and BRG-1 specifically, is not necessary at every IFN-γ induced locus, including at the IRF1 promoter (Wang et al., 2011). In general this seems to be the case for all of the co-activators that have been shown to bind to STAT1 and enhance IFN-γ-induced gene expression, including CBP/p300 (Zhang et al., 1996), MCM5 (Zhang et al., 1998), Nmi (Zhu et al., 1999), and BRCA1 (Ouchi et al., 2000). It is unknown whether these factors 1) are actually required for STAT1 activity, they do not just augment STAT1 activity, 2) are expressed and active in all IFN-γ responsive cell types, and 3) act to increase STAT1-mediated transcription of all target genes, not just a select few. To be a co-activator that *Toxoplasma* could modulate to inhibit STAT1 transcriptional activity, all of these criteria must be met, as *Toxoplasma* infection can inhibit STAT1 activity directly, regardless of the locus, as shown by our experiments with stable STAT1 reporter cell lines (Chap. 3 Fig. 5), and infection can inhibit IFN-γ induced gene expression in multiple disparate cell types, including human embryonic kidney cells (Chap. 3 Fig. 5), human fibroblasts (Chap. 3 Fig. 1), and mouse macrophages (Chap. 3 Fig. 6). It is more likely that *Toxoplasma* directly prevents the association of STAT1 with more general factors, either RNA polymerase II or the Mediator complex. However, recently, the CDK8 module of the Mediator complex was shown to be responsible for the serine phosphorylation of STAT1 (Bancerek et al., 2013), which is not inhibited by *Toxoplasma* infection (Chap. 3 Fig. 3), suggesting that its recruitment is not inhibited.

One experiment that might provide a clue to both the mechanism of STAT1 transactivation and the inhibition of STAT1 activity by *Toxoplasma* is the treatment of a STAT1 reporter cell line with MG132, a proteasome inhibitor (Chap. 4 Fig. 6). Treatment with this

inhibitor alone, in the absence of infection, potently inhibits IFN-γ induced STAT1 mediated transcription. While cells can undergo apoptosis after extended treatment with this drug and this could be a possible explanation for a decrease in gene expression, the proteasome has also been implicated in transcriptional activation by several transcription factors (Geng et al., 2012; Lipford et al., 2005). One model that explains this dependence is that each time a transcription factor binds to its cognate sequence in the DNA, it can recruit one round of general transcription factors and RNA polymerase II. Then it is somehow "used up" and must be removed from the DNA and be degraded before another round of transcription factor can bind and initiate another round of transcription. If this model is correct, it seems clear that *Toxoplasma* infection blocks this recycling (Fig. 2).

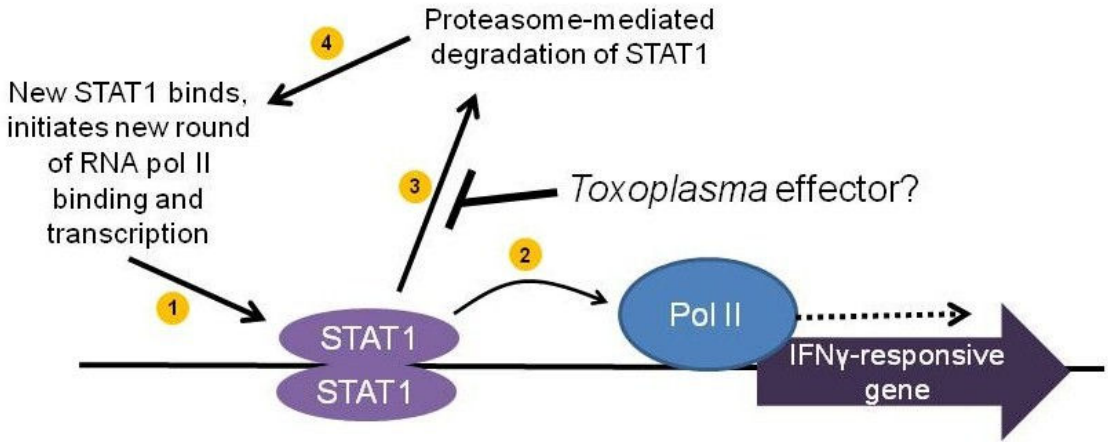

Figure 2. Model of STAT1 recycling and inhibition by *Toxoplasma*. STAT1 binds to target sequences in the DNA (1), recruiting general transcription machinery and RNA polymerase II (2). STAT1 must then be degraded by the proteasome (3), so that a new round of STAT1 can bind to initiate a new round of transcription (4). I hypothesize that a *Toxoplasma* effector inhibits this proteasome-mediated removal of STAT1 from the DNA and degradation.

Multiple lines of evidence point to the fact that STAT1 is actually more bound to DNA after infection plus IFN-γ treatment than after IFN-γ treatment alone. First, we observe higher levels of IFN-γ-induced tyrosine phosphorylated STAT1 in the nucleus after infection (Chap. 3 Fig. 2). Second, in my STAT1 ChIP experiments, not only did *Toxoplasma* infection not inhibit IFN-γ induced STAT1 binding, it actually increased IFN-γ induced STAT1 binding at three loci (*IRF1*, *SOCS3*, and *ICAM1*), even when the infecting strain lacked ROP16 and therefore did not induce STAT1 binding with infection alone (Chap. 4 Fig. 2A). Third, in nuclear and chromatin isolations from uninfected and infected cells stimulated with IFN-γ, we observed this same

phenomenon: the amount STAT1 found in the chromatin fraction by Western blot was greatly increased by infection combined with IFN-γ compared to IFN-γ alone (Chap. 4 Fig. 2B, C). Mass spectrometry results from STAT1 IPs from these fractions gave the same result, the percentage of total STAT1 peptides that were found in the chromatin fraction was greater upon infection plus IFN-γ treatment compared to IFN-γ treatment alone (Chap. 4 Fig. 2D). Another group has observed more IFN-γ induced STAT1 binding to DNA after *Toxoplasma* infection in an EMSA assay as well (Schneider et al., 2013). All of these lines of evidence suggest that infection plus IFN-γ induces a more stable STAT1-DNA interaction than IFN-γ alone, and supports the hypothesis that *Toxoplasma* is somehow inhibiting STAT1's disassociation with the DNA. It will be very interesting to test if STAT1 is ubiquitinated upon IFN-γ treatment and if *Toxoplasma* modulates this ubiquitination. Other experiments could directly measure whether STAT1 recycling is occurring, and whether multiple rounds of STAT1 binding and RNA polymerase II recruitment are occurring. Additionally, all of my experiments have always been done with continuous IFN-γ stimulation, but if *Toxoplasma* is blocking STAT1's dissociation from DNA, differences in STAT1 bound to DNA might be even more pronounced in cells that are only exposed to a pulse of IFN-γ.

In this model of proteasome-dependent recycling of transcriptional activators, these activators must be somehow marked as "used" to signal for their degradation (Geng et al., 2012). This signal is often a phosphorylation event, and many transcription factors have residues that are phosphorylated within their transactivation domains (TADs), and their TADs partially overlap with a degron sequence (Salghetti et al., 2000). One of these factors that is known to have an overlapping TAD and degron is the STAT family member, STAT5. I therefore think that if STAT1 is being recycled, the mark that is most likely to signal for STAT1's degradation is its serine phosphorylation at residue 727 within its TAD. This phosphorylation can only occur when STAT1 is associated with DNA (Sadzak et al., 2008), and as mentioned previously, it can be phosphorylated by the CDK8 protein within the Mediator complex (Bancerek et al., 2013). However, CDK8 is present in a distinct module of the Mediator complex, and the CDK8 complex has been reported to change the conformation of the Mediator complex, preventing its association with RNA polymerase II (Elmlund et al., 2006). In my model, however, these two reports do not necessarily conflict. The CDK8 module could bind only after RNA polymerase II

has been recruited and is elongating along and transcribing the DNA, at which point CDK8 would mark STAT1 for degradation.

The phosphorylation of this serine residue has long been implicated in the full transcriptional activation of STAT1 target genes, as STAT1 S727A mutants induce decreased gene expression upon IFN-γ treatment (Varinou et al., 2003). It has been hypothesized that the serine phosphorylation is required for STAT1 to recruit co-activators and/or RNA polymerase II, however, how this would occur is still unknown. It could be that by allowing STAT1 to more efficiently recycle off of the DNA, the serine phosphorylation would achieve the same result—greater association of STAT1 with co-activators and RNA polymerase II—as new rounds of STAT1 would be able to re-establish these interactions for new rounds of transcription. If this STAT1 recycling is occurring, *Toxoplasma* infection is blocking STAT1 recycling, and the serine phosphorylation of STAT1 is a signal for degradation, we can then ask how *Toxoplasma* is blocking this recycling. Infection does not block STAT1 serine phosphorylation (Chap. 3 Fig. 3), but perhaps it blocks STAT1 ubiquitination, association of STAT1 with a ubiquitin E3 ligase, or association of STAT1 with some other degradation factor. It would be interesting to test what happens to STAT1 serine phosphorylation after only a pulse of IFN-γ. If this model is correct, I would hypothesize that in uninfected cells this phosphorylation would decrease over time, as STAT1 is degraded off of the DNA, but that in infected cells, it would remain indefinitely as STAT1 is kept on the DNA and not degraded.

Does *Neospora caninum* modulate the IFN-γ response?

Neospora caninum, a parasite species also in the phylum Apicomplexa and therefore related to *Toxoplasma*, does not inhibit the expression of IRF1 in host cells after IFN-γ treatment (Kim et al., 2007a) (Appendix 3 Fig. 1A). However, results from experiments with *Neospora* in the STAT1 reporter cell lines that I made have been hard to interpret. Infection of these lines with *Neospora* at a lower MOI does not seem to inhibit the expression of IFN-γ-induced luciferase, giving similar results to the IRF1 IF experiments. In contrast, at these lower MOIs *Toxoplasma* can inhibit STAT1 activity in these lines to some extent. However at much higher MOIs, sometimes I have seen inhibition of these reporters by *Neospora*. It is unclear, however, how much cell death and cell lysis is occurring in these experiments, as these would also

decrease luciferase expression. Ideally, these experiments would be repeated with multiple MOIs of *Neospora* and with cell viability or cell death assays done in parallel. One hypothesis that could explain possible differences between IRF1 IF results and STAT1 reporter results is that *Neospora* activates IRF1 through some other transcription factor, such as NF-κB, just as *Toxoplasma* type II strains do (Chap. 3 Fig. 1). However, in unstimulated cells, I do not observe such IRF1 activation (Appendix 3 Fig. 1A), and *Neospora* infection does not activate our NF-κB reporter cell line (Appendix 3 Fig. 2) or NF-κB p65 nuclear translocation (Herman et al., 2007). Additionally, in a co-infection with the type I *Toxoplasma* RH strain and *Neospora*, RH still potently inhibits IFN-γ-stimulated IRF1 expression, again suggesting that all of this expression is induced by STAT1 and that *Neospora* does not induce IRF1 through some other transcription factor (Appendix 3 Fig. 1B). Therefore, the most likely explanation is that *Neospora* does not inhibit STAT1 activity and that any inhibition I have observed in the STAT1 reporter cell lines is due to cell lysis or death or the presence of anti-inflammatory cytokines in my *Neospora* parasite preparations that are also transferred into the well and have an inhibitory effect on STAT1-mediated expression at high concentrations. Therefore, in thinking about screens and selections for the *Toxoplasma* effector that inhibits STAT1 activity, we could complement *Neospora* with *Toxoplasma* genes instead of mutagenizing *Toxoplasma* itself. This could be done with either of the *Toxoplasma* cosmid libraries that are available (http://toxomap.wustl.edu/cosmid.html), or by testing possible candidate genes.

What is the *Toxoplasma* STAT1 inhibitor?

Our data indicates that the *Toxoplasma* effector(s) responsible for inhibiting IFN-γ-stimulated gene expression is not secreted into the host cell upon infection, as parasites treated with cytochalasin D or mycalolide B, which attach to host cells and secrete their rhoptry contents but do not invade, cannot inhibit this response. We therefore have three main hypotheses for the identity of this effector: 1) a protein that is secreted upon invasion but needs to be activated somehow on the outside of a parasitophorous vacuole (PV), 2) a small molecule or metabolite that diffuses or is transported into the host cell through the parasitophorous vacuole membrane (PVM), or 3) a protein that *Toxoplasma* secretes into the host cell post-invasion across the PVM.

To investigate this first possibility, I pre-infected cells with *Neospora*, which doesn't inhibit IFN-γ induced IRF1 expression but forms a PV very similar to *Toxoplasma*, and then added *Toxoplasma* parasites pre-treated with mycalolide B. In this scenario, we expect that any proteins secreted by *Toxoplasma* into the host cell that normally traffic back to the outside of the PV and associate with the PVM will associate with the *Neospora* PVM. However, this hypothesis should be tested in the future by staining these *Neospora* PVs for a *Toxoplasma* protein such as ROP2 that is known to be secreted into the host cell and then traffic back to the outside of the PV (Reese and Boothroyd, 2009; Sinai and Joiner, 2001). I then stimulated these *Neospora*-infected, *Toxoplasma*-injected cells with IFN-γ. In preliminary results, in both HFFs stained for IRF1 expression and in a STAT1 reporter cell line, I did not observe any inhibition of IFN-γ induced gene expression in these conditions, suggesting that the *Toxoplasma* effector is not a protein that is secreted upon infection that requires the presence of a PV for its activity. However, it is also possible that there are unknown fundamental differences in the composition of *Neospora* and *Toxoplasma* PVMs.

One reason why we think that the *Toxoplasma* effector could be a small molecule or metabolite is the relatively recent finding that STAT1 activity can be controlled by nuclear receptors (Lee et al., 2009; Li et al., 2011). Nuclear receptors regulate transcription and are activated by a variety of small molecule ligands, the most well characterized of which are steroids, through binding of these ligands to a specific receptor domain (Glass and Saijo, 2010). These receptors often interact at promoters with other transcription factors or co-repressor and co-activator complexes (Glass and Saijo, 2010). STAT1 activity can be inhibited by ligands for LXR, RXR, and PPAR nuclear receptors, through the modulation of STAT1 phosphorylation, STAT1 DNA binding, or the recruitment of SUMO E3 ligase complexes to STAT1 (Lee et al., 2009; Li et al., 2011). It is still unclear though whether these pathways are present in all cells or only in inflammatory cells, such as macrophages and astrocytes. I did some preliminary experiments in HFFs, and found that several LXR agonists did not inhibit IRF1 expression after treatment with 100 U/ml of IFN-γ, the concentration of IFN-γ that I generally use in all of my assays. However, in one experiment with only 10 U/ml of IFN-γ and a high concentration of LXR ligand, quantitation of IRF1 in the nucleus did suggest that STAT1 activity was at least somewhat inhibited. Another reason that this hypothesis is appealing is that depending on its size

and structure, the activating ligand from *Toxoplasma* might be able to passively diffuse through the PVM. It might also be transported into the host cell through a pore complex. Recent work in our lab has discovered a *Toxoplasma* protein, GRA17, that seems to comprise at least part of a pore in the PVM that allows small molecules to get into the PV from the host cell (Dan Gold, submitted). However, a *Toxoplasma* strain that is deficient in this gene still inhibits IFN-γ induced IRF1 expression, making the possibility that the *Toxoplasma* effector is a secreted small molecule slightly less likely (Appendix 4 Fig. 3).

Currently, I believe that the best hypothesis we have for the identity of this *Toxoplasma* effector is that it is a protein that is secreted into the host cell post-invasion, most likely from the dense granules. Recently, a dense granule protein, GRA16, was shown to accumulate in the host nucleus with increasing intensity over time post-invasion (Bougdour et al., 2013), and a PEXEL secretion motif known from malaria parasites was shown to also be present in *Toxoplasma* proteins (Hsiao et al., 2013). The main problem with this hypothesis is that I observe strong inhibition of IFN-γ induced IRF1 expression quite early after infection, after less than three hours (Chap. 3 Fig. 1), whereas nuclear accumulation of GRA16 does not peak until 15-24 hours after infection. GRA15, another protein that contains a PEXEL motif and that we suspect is secreted into the host cell via this mechanism, requires four hours of infection to induce observable levels of NF-κB p65 nuclear translocation (Chap. 2 Fig. S4). While in an infection time course the average inhibition of IFN-γ induced IRF1 nuclear protein does increase over time (Fig. 3), these infections were not synchronized and I believe this increase in inhibition over time is at least partially due to some percentage of parasites infecting host cells after IFN-γ was added in the earlier time points. As early as two hours and five minutes after infection, ~1/3 of infected cells have the same level of inhibition as the maximal average inhibition seen at 26 hours after infection (Fig. 3), suggesting that the amount of effector in the host cell at this early time point is sufficient to fully inhibit STAT1 activity. It could be either that only a small amount of the protein secreted into the host cell is enough to inhibit STAT1 activity or that this inhibitor is very highly expressed and a lot of it is secreted into the host cell during only two hours of infection. Depending on how confident we are in this hypothesis, that the *Toxoplasma* effector is a secreted dense granule protein, we can easily form a list of candidate genes using two main criteria: 1) high and non-variable mRNA expression, since while rhoptry proteins are only secreted into the

host cell upon invasion and their expression is synchronized with the *Toxoplasma* cell cycle, dense granule proteins are continuously secreted and therefore continuously expressed, and 2) contains a PEXEL motif, signaling for secretion of the protein into the host cell post-invasion. With the available *Toxoplasma* cosmid libraries, it would be relatively easy to express cosmids containing these genes in *Neospora*, which does not inhibit IFN-γ induced IRF1 expression, and screen for this dominant inhibition phenotype. However, preliminary results suggest that *Neospora* does not secrete proteins into the host cell post-invasion; in one experiment, cells infected with *Neospora* expressing a transgenic copy of GRA16 did not accumulate GRA16 in their nuclei. We would therefore need to first define the composition of this protein translocation machinery and express it in *Neospora*. It is possible that expression of this *Toxoplasma* effector alone in mammalian cells would inhibit STAT1 activity, as transient expression of GRA15 in HeLa cells activates NF-κB p65 nuclear translocation. It could be possible then to clone the coding regions of these candidate genes in mammalian expression vectors, directly transfect them into human or mouse cells, and measure their ability to inhibit IFN-γ-induced gene expression.

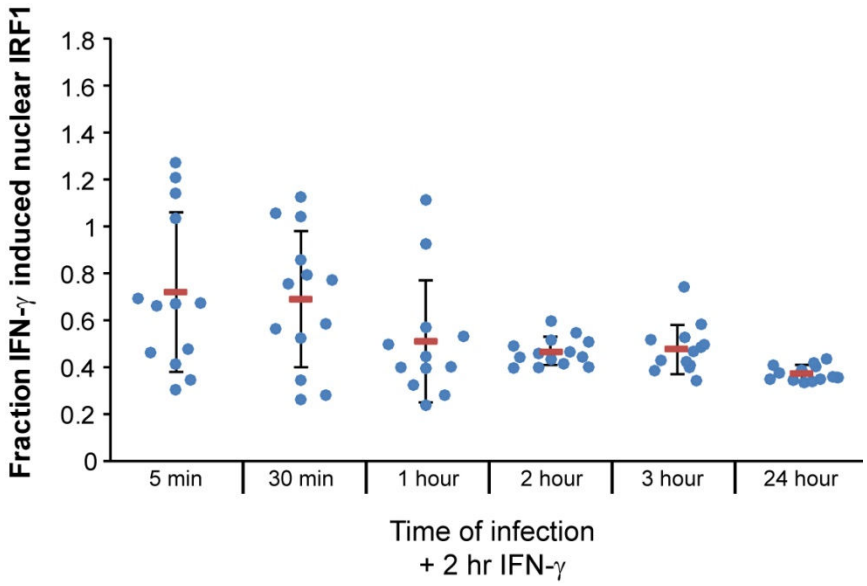

Figure 3. *Toxoplasma* can inhibit IFN-γ-induced IRF1 expression as early as two hours and five minutes after infection. HFFs on coverslips were infected with RH *Toxoplasma* parasites for the indicated lengths of time and subsequently stimulated with 100 U/ml IFN-γ for two hours. Cells were then fixed, permeabilized, and stained for IRF1. The intensity of IRF1 in the nuclei of host cells was quantified and is plotted. Each dot represents one cell. Red bars represent average IRF1 levels and error bars represent standard deviation.

Unbiased methods to determine the mechanism of inhibition and/or the *Toxoplasma* effector

Exactly how STAT1 initiates transcription of downstream target genes is unknown, and while I hypothesize that *Toxoplasma* inhibits the activity of STAT1 by preventing it's recycling on and off of DNA, this is still purely speculation, and there are no reports in the literature that describe this STAT1 recycling even in uninfected cells. Whatever the mechanism of inhibition is, it is likely to directly inhibit STAT1 activity, but determining how an unknown *Toxoplasma* effector inhibits STAT1 activity is virtually impossible when the mechanism of transactivational activity of STAT1 is poorly described. Therefore, in future studies, we would like to pursue more unbiased methods to discover both host cell mechanisms that regulate STAT1 activity and *Toxoplasma* effectors that are necessary for the inhibition of STAT1 activity.

Immunoprecipitation and mass spectrometry

One method that I have been undertaking is the immunoprecipitation of STAT1 and mass spectrometry of co-immunoprecipitating proteins. With this method I can compare what proteins are pulled down with STAT1 in both uninfected and infected cells and in unstimulated and IFN-γ stimulated conditions. Ideally, we would detect either *Toxoplasma* or host cell proteins that specifically associate with STAT1 in certain conditions. For example, we might find a co-activator that specifically interacts with STAT1 in uninfected cells but that is depleted in infected cells. Alternatively, there could be a negative inhibitory repressor that specifically interacts with STAT1 in infected cells.

Initially, I tried immunoprecipitating STAT1 from whole cell extracts. While this was successful in terms of pulling down STAT1, I was never able to detect reported STAT1 interactors, such as p300/CBP or BRG-1, by Western blot, and there was still a lot of STAT1 remaining in flow through samples, indicating that there is a high level of STAT1 expressed in cells. Because of this, I think that by IP-ing total STAT1 from a whole cell extract, I was pulling down mostly cytoplasmic STAT1. But to look for STAT1 interactors that affect it's activity on the DNA, I am most interested in the DNA-bound fraction of STAT1. I tried one IP using an antibody against the tyrosine phosphorylated form of STAT1, instead of total STAT1, which should specifically be in the nucleus, but this antibody is raised against a short peptide of STAT1

containing the phospho-tyrosine residue and if an important factor is interacting with this region of STAT1, that complex might not be pulled down as the region binding to the antibody would be blocked. Additionally, STAT1 proteins associate with each other into homodimers by binding via their phosphorylated tyrosines and SH2 domains, so it is also unclear whether this antibody can even IP STAT1 homodimers, although it does recognize the dimerized form of STAT1 by native PAGE (Chap. 4 Fig. 1) and nuclear STAT1 by IF (Chap. 3 Fig. 2).

Therefore, I decided to use a protocol that includes a cell fractionation procedure prior to STAT1 immunoprecipitation to specifically isolate nuclear fractions. I used the Qiagen Qproteome Nuclear Protein Kit, which isolates very clean cytoplasmic, nuclear extract, and chromatin fractions, based on Western blottings for GAPDH, STAT1, and histone H3. However, the buffers that are used to isolate each of these fractions are very different, so after isolation I diluted all of the fractions in the same buffer to try to make IPs from each of these fractions have a similar efficiency. I was able to IP STAT1 from these different fractions successfully, in a pattern that would be expected in unstimulated, IFN-γ stimulated, and infected cells. However, mass spectrometry of these fractions from three different experiments has not resulted in any promising leads. This could be due to a variety of reasons, but I believe that the most important reason is the high salt concentration used in the chromatin and nuclear extract fractionations. Although the makeup of these extraction buffers is proprietary, Qiagen's technical assistance told me that the sodium chloride concentrations in these buffers is 1000 mM and 350 mM, respectively. It is therefore likely that in these fractions the isolation procedure disrupts many STAT1 interactions that we would like to detect.

In the future, I do think this line of experimentation is worth pursuing, although with changes in the experimental procedures. I do think that it is very important to specifically isolate a nuclear fraction, and even if possible the fraction of STAT1 specifically bound to DNA. Perhaps the best way to do this without losing specific interacting proteins is to fix the cells prior to isolation. I did try one IP experiment where I IP-ed STAT1 using the same protocol that I used in the ChIP assays (fixing the cells with formaldehyde, doing a dirty nuclear preparation, and sonicating prior to the IP), but then boiling the entire IP-ed material in SDS buffer instead of purifying the DNA. However, I concluded that this nuclear preparation is in fact quite dirty and I was still getting a lot of cytoplasmic protein contamination. While for the ChIP assay, in which

you are only interested in the DNA, this doesn't matter, it does matter for our purposes. The best protocol would include a fixation step, a much cleaner nuclear isolation, and either sonication or an enzymatic treatment to cut the DNA to allow the specific IP of DNA-bound STAT1.

One consideration that we might need to think about further is what controls to include. Initially, I thought that having all of the IPs from the different fractions would be a great internal control. A protein that modulates STAT1 activity is likely to be found in the nuclear fractions, while contaminating proteins that are pulled down by the antibody or beads aspecifically might be found in all of the fractions. However, I did some controls with random IgG antibodies, and it seemed that most proteins, including STAT1, were also pulled down by these antibodies, perhaps because STAT1 and many of our other hits are so abundant. It may be worthwhile to use STAT1-deficient host cells, such as the U3A mutant and the parental line 2FTGH (McKendry et al., 1991; Pellegrini et al., 1989), to identify specific STAT1 interactors. We also need to decide what strain of *Toxoplasma* to infect the cells with prior to IFN-γ stimulation and STAT1 IP. The *Toxoplasma* effector ROP16 can induce the phosphorylation (Chap. 3 Fig. 2), nuclear translocation (Chap. 3 Fig. 2), and DNA binding (Chap. 4 Fig. 2) of STAT1 in the absence of IFN-γ treatment, but this STAT1 is not transcriptionally active (Chap. 3 Fig. 3). In some ways this is an appealing condition in which to IP STAT1, because many more proteins might be induced by IFN-γ to bind at STAT1-regulated loci, including secondary transcription factors such as IRF1, but *Toxoplasma* can apparently inhibit ROP16-induced STAT1 activity in the absence of all of these proteins. However, it does not allow for a good control to subtract out *Toxoplasma* proteins that bind aspecifically to the antibody or the beads from the infected, unstimulated nuclear fractions. Therefore it might be best to do side by side infections with both an RH strain and an RH Δ*rop16* strain to get the most information. We should also probably investigate further exactly where the *Toxoplasma* PV and its contents end up during the cell fractionation procedure, as this could affect exactly what *Toxoplasma* proteins are aspecifically pulled down from the different fractions. This could be determined by Western blot of the different fractions for different *Toxoplasma* proteins that have a known localization, for example *Toxoplasma* nuclear, mitochondrial, rhoptry, and dense granule proteins.

Another avenue worth pursuing, and which might be more simple and more promising, is to perform mass spectrometry on STAT1 containing complexes that are pulled down by GAS

oligonucleotides. This would automatically select for interactors that specifically bind to STAT1 on DNA. Additionally, in two recent studies, an aberrant complex, of a greater molecular weight but still containing STAT1, has been observed in an EMSA assay binding to such oligonucleotides (Lang et al., 2012; Schneider et al., 2013). While we do not observe this complex in a native PAGE (Chap. 4 Fig. 1), it is possible it can only form while bound to DNA. It would be very interesting to specifically do mass spectrometry on these complexes.

Using FACS for a genetic screen

An unbiased method that could directly discover *Toxoplasma* genes that are necessary for this inhibition is a screen for a *Toxoplasma* mutant that can not inhibit IFN-γ-induced STAT1 activity. This could be done using the luciferase reporters that we have or IRF1 IF experiments to do a full screen, however, these assays would require a lot of time and effort. For one thing, we would not be able to directly screen a whole population of mutants, but instead would need to clone a mutated population, into either 96- or 384-well plates. The luciferase assay would be more high-throughput but would require some way to account for differences in parasite growth and viability, because the cloned parasites would have to be replica-plated into the reporter cells, and we would not be able to be sure that the infecting MOI was the same across all of the clones. The IRF1 IF assay could get around this MOI dependence, as only one infecting parasite is capable of inhibiting IFN-γ-induced IRF1 expression, and infected and uninfected cells can be individually visualized, however the assay is not high-throughput and would require a lot of time.

The ideal method to perform such a screen would utilize an assay such as FACS that could sort through a whole population of mutant *Toxoplasma* parasites in a high-throughput way and specifically sort out those that cannot inhibit IFN-γ-induced gene expression (Fig. 3). In order to then grow and validate any mutants, these cells cannot be fixed and permeabilized to stain for an intracellular protein such as IRF1. Instead, sorting must be based on a natively fluorescent reporter or the expression of extracellular proteins that can be bound in their native form by fluorescently conjugated antibodies.

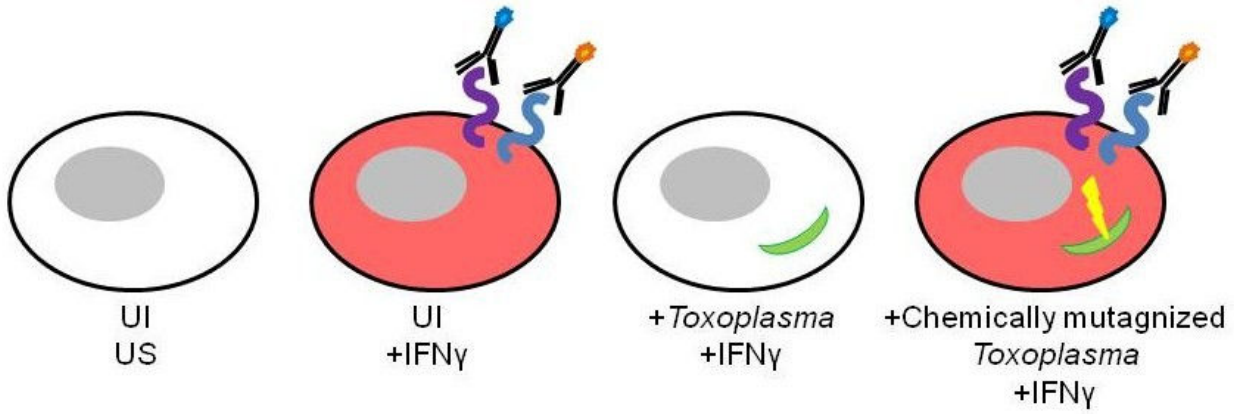

Figure 3. A FACS-based genetic screen. In an ideal screen, IFN-γ stimulation induces the expression of either a fluorescent reporter (red) or extracellularly exposed membrane proteins to which good antibodies exist. A wild-type *Toxoplasma* parasite should then potently inhibit the upregulation of these proteins. We could then screen for parasites within a mutant pool that do not inhibit the expression of these proteins.

Unfortunately, generating a STAT1 reporter line that strongly induces the expression of a fluorescent reporter has not been trivial. The original reporters that I made, both a "STAT1" and "GAS" reporter, made from pGreenFire (pGF) lentiviral vectors purchased from System Biosciences (see Materials and Methods, Chap. 3, 4), drive the expression of one open reading frame, which consists of a destabilized version of GFP (dscGFP) and luciferase. These polypeptides are separated by a T2A self-cleaving peptide, such that both proteins will be produced separately from one transcript. However, while I can detect luciferase expression in these cell lines (Chap. 3 Fig. 4, Chap. 4 Fig. S2), I was never able to detect a significant increase in GFP expression that could be easily used to separate responding versus non-responding cells by flow cytometry (Appendix 1 Fig. 1). The induction of even luciferase in these cell lines is also relatively low (3-6 fold), and I think that this level is just not high enough to see induction of a destabilized version of GFP. Our HEK293 NF-κB reporter cell line, made using the same pGF vector backbone, in which dscGFP induction is robust, has levels of luciferase induction of greater than 1000-fold after TNF-α stimulation and between 200- and 400-fold after infection with a type II strain of *Toxoplasma* which activates NF-κB through GRA15. Additionally, contact with System Biosciences technical assistance indicated that several of the reporter vectors they produce do not induce significant levels of GFP, likely due to weak induction in general. Replacing this destabilized GFP and luciferase cassette with a copy of a more robust

fluorescent protein, tdTomato, did not result in better fluorescent reporter induction (Appendix 1 Fig. 2). Additionally, sorting specific cells within the population for "high-responders" did not work, indicating that differences in how the cells responded were stochastic and not intrinsic to the cell due to more viral integrations or integration of the vector into more highly expressed regions of the genome.

In an effort to design a reporter vector that responds more robustly to IFN-γ stimulation, I cloned into these vectors regions of the IRF1 enhancer/promoter, with the idea that having a more complete sequence where not only STAT1, but also IRF1 itself and histone acetyltransferases such as CBP/p300 can bind would increase transcriptional output. I used ChIP-seq data from the ENCODE project to pick three different regions of the IRF1 promoter where all of these factors have been found to bind upon IFN-γ stimulation, but that are short enough to fit in the pGF1 lentiviral vector (< 3000 bp). However, I have not been able to detect GFP or Tomato induction in these lines either.

An alternative method to a fluorescent reporter is to sort cells based on the expression of IFN-γ-induced extracellular proteins with fluorescently conjugated antibodies. The problem with this method is that IFN-γ-induced proteins can also be controlled by other transcription factors, and different IFN-γ-induced loci may require different co-activators or chromatin remodeling factors. Therefore this method would work best with a panel of IFN-γ-induced genes, such that we could select for mutant parasites that do not inhibit the expression of any of these proteins. To this end, I choose three genes that were well expressed upon IFN-γ stimulation in RAW264.7 murine macrophage cells in previous microarray experiments (Chap. 3 Fig. 6), were not induced by infection with a RH Δ*rop16* strain, and for which there exists commercially available fluorescently conjugated antibodies. We then measured the expression of these proteins upon IFN-γ stimulation and the inhibition of the expression of these proteins in *Toxoplasma* pre-infected cells by flow cytometry (Appendix 2). Unfortunately, however, two of these proteins do not seem to be induced highly enough by IFN-γ treatment in every single cell to be able to use them in such a screen. Some cells that are infected with a parasite mutant in this effector will not express these proteins by chance and would therefore be false-negatives, and we would not select for them and lose them from our population. Additionally, the infected and uninfected cell populations after IFN-γ treatment also have some overlap, and we might have to set the cut-off

for lack of inhibition so low that we would also still sort a large number false-positives—parasites that can still inhibit this response.

How does *Toxoplasma* continuously secrete dense granule proteins across the PVM into the host cell post-invasion?

If the *Toxoplasma* effector that is responsible for STAT1 inhibition is indeed secreted into the host cell post-invasion, then any *Toxoplasma* strain that is mutant in this secretion mechanism would also be mutant in terms of inhibiting IFN-γ. Additionally, we believe that GRA15 also uses this secretion mechanism, and therefore another logical follow-up to this research is to find the identity of this pore/secretion mechanism. We hypothesize that a *Toxoplasma* parasite which has components of this pore mutated will not be able to inhibit STAT1 activity or to activate NF-κB. Therefore, we could use our robustly expressing GFP NF-κB reporter (Chap. 2 Fig. 2) to perform a FACS screen of a mutant pool of parasites that express $GRA15_{II}$ to select for parasites that do not activate NF-κB. Parasites that are mutant for the secretion mechanism will not be able to secrete very much GRA15 into the host cell and should not activate the reporter. Theoretically we could also recover mutants in the *GRA15* gene, however we have RH $GRA15_{II}$ transgenic clones that carry multiple copies of the gene, making this scenario less likely. It would be very interesting to study the virulence of a parasite that lacks this proposed protein secretion mechanism. It is still unknown how many proteins are secreted by this pathway and their relevance *in vivo*.

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Appendix 1. Construction of additional STAT1 reporter cell lines

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KDCJ assisted in all FACS analysis and sorting.

Results

Previously I have described the construction of three IFN-responsive cell lines (GAS, STAT1, and ISRE) in HEK293 cells from lentiviral vectors purchased from System Biosciences (Chapters 3 and 4, Materials and Methods). These cell lines consistently and specifically activate expression of luciferase upon IFN stimulation (Chap. 3 Fig. 4, Chap. 4 Fig. S2). These lines should also express GFP in these conditions, as the transcriptional response elements in these vectors drive the expression of one transcript which codes for a destabilized version of GFP, a T2A self-cleaving peptide, and luciferase all in one polypeptide. However, by FACS analysis, while some induction of GFP can be measured in some of these cell lines after IFN-γ treatment, this induction is not high enough to be able to easily distinguish responding versus non-responding cells in a FACS-based screen (Fig. 1).

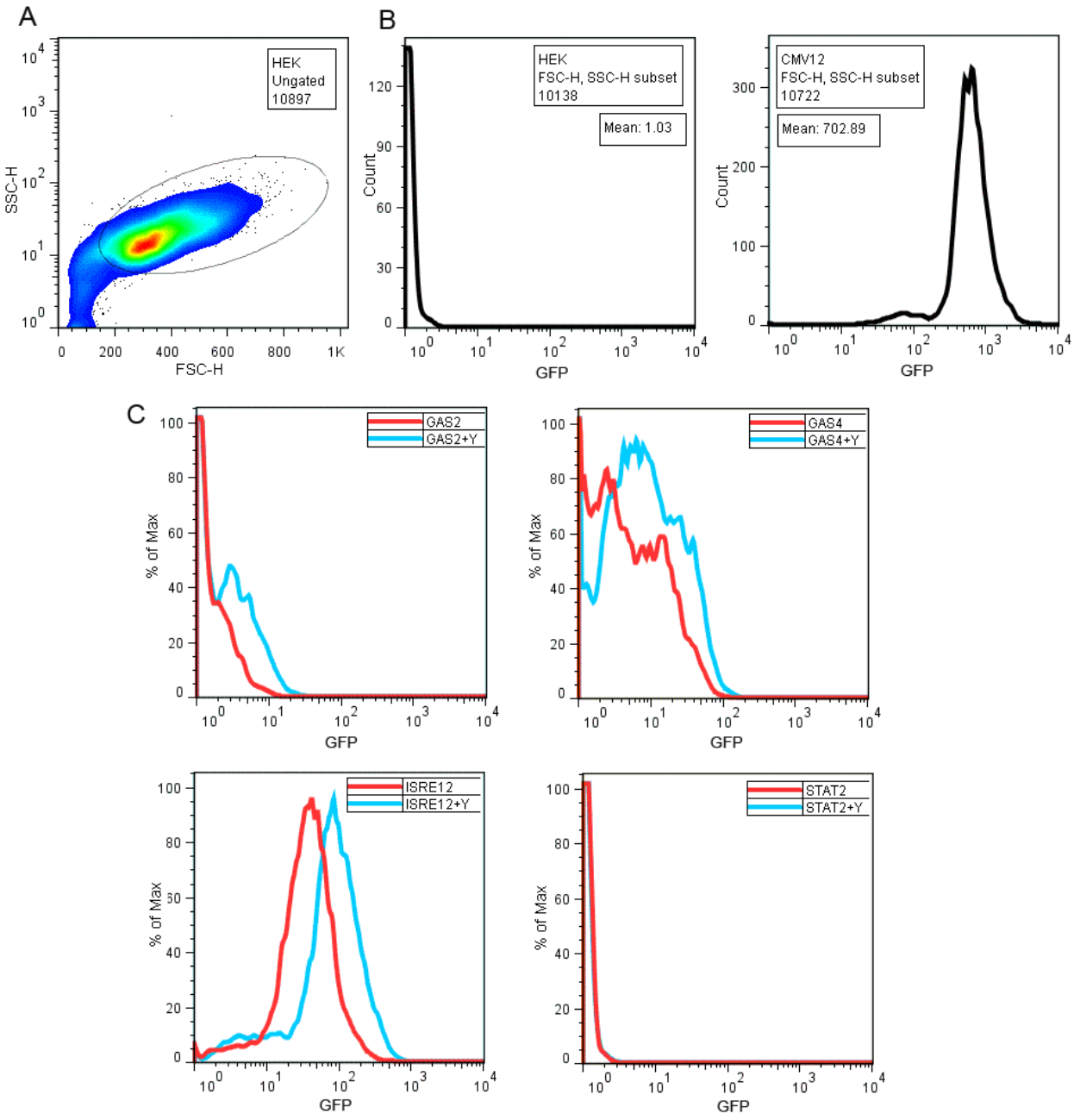

Figure 1. Flow cytometry of GFP expression in IFN-responsive HEK reporters. HEK293 pGF1-CMV, STAT1, GAS, and ISRE lines. This experiment was done twice, with different clones, with similar results. **A.** Gating of cells based on side and forward scatter. **B.** GFP levels in parental HEK cells and positive control CMV-GFP. **C.** GFP levels in unstimulated (red) and IFN-γ stimulated (blue) GAS, ISRE, and STAT1 reporter clones.

In an effort to make a cell line that induces higher levels of a fluorescent reporter in response to IFN-γ stimulation, I replaced the dscGFP-T2A-luciferase coding region in a GAS pGF1 vector with a copy of tdTomato. After transduction and selection of a HEK293 cells with this construct, I stimulated the population with IFN-γ. While a CMV-Tomato vector strongly expressed Tomato fluorescent reporter in comparison to the parental HEK293 line (Fig. 2A), we could not detect significant Tomato expression by eye in the microscope or by flow cytometry (Fig. 2B). We therefore tried sequentially sorting cells from this population that both responded to IFN-γ stimulation with high levels of Tomato expression and that did not express Tomato in a basal unstimulated state (Fig. 2C). The idea was that perhaps some cells had integrated multiple copies of the lentiviral vector or had inserted the construct into more or less "silent" regions of chromatin and that we could sort specifically for these cells. However, re-analysis of these sorted populations showed that the distribution of Tomato expression through the population was virtually identical to that of an unsorted population, indicating that differences in Tomato expression are not due to differences in integration site or number but instead to stochasticity in Tomato expression (data not shown). Additionally, treatment of these populations with increasing concentration of drug did not result in selection of higher responders with more copies of the lentiviral vector (data not shown).

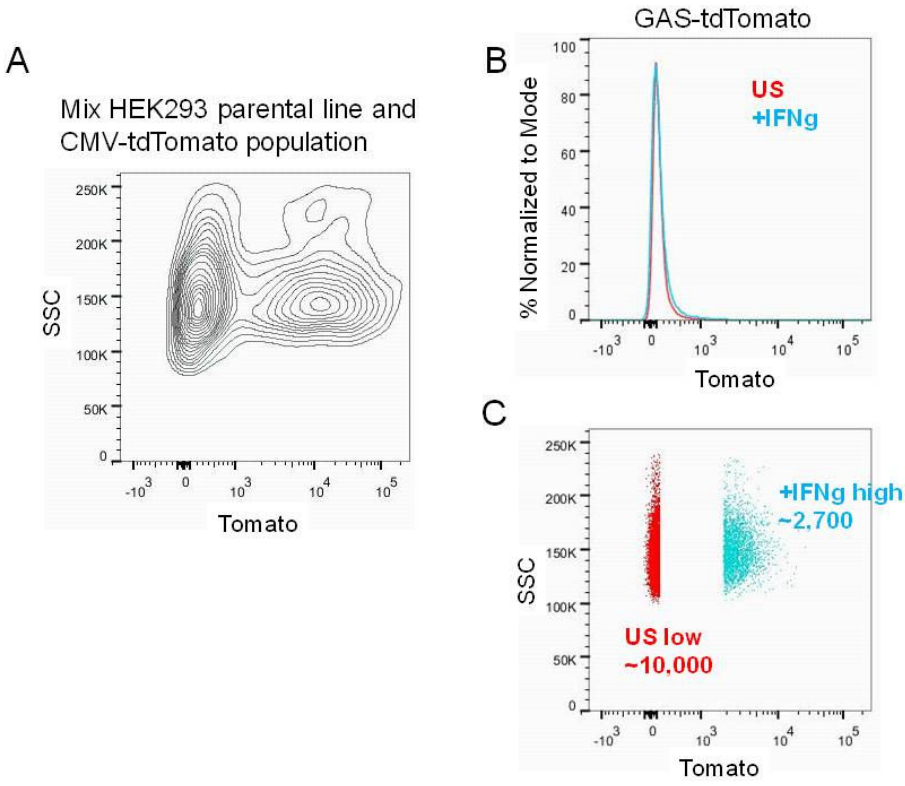

Figure 2. Sorting of HEK GAS-Tomato population for high responders. A. tdTomato levels in a mixed sample of the HEK parental line and HEK cells expressing tdTomato under the control of a CMV promoter. **B.** tdTomato levels in populations of unstimulated (red) and IFN-γ stimulated (blue) GAS-tdTomato cells. **C.** "High responders" were sorted from the IFN-γ stimulated sample (blue) and "low expressors" were sorted from the unstimulated sample (red).

Next, I decided to use a more complete promoter instead of four tandem STAT1 binding sites to drive the expression of the reporter, with the idea that perhaps STAT1 by itself is not a strong enough inducer but at a complete promoter, other factors such as histone acetyltransferases and secondary transcription factors such as IRF1, would also be able to bind and help induce a higher level of expression. I chose regions of the IRF1 promoter at which STAT1, IRF1, and/or p300 have been found to bind, using data available online from the ENCODE project (http://genome.ucsc.edu/ENCODE), amplified them, and inserted them into both pGF1 vectors driving the dual expression of luciferase and GFP or the expression of tdTomato. I then infected HEK293 cells with virus containing these lentiviral vectors and

selected for transduced cells. However, treatment of this population of cells also did not result in visible induction of either GFP or Tomato expression by eye in the microscope.

Materials and Methods

Construction of pGF1-Neo-GAS-Tomato

The tdTomato coding region was amplified by PCR from ptub-tdTomato-CAT (received from Mariane from UMass), incorporating BamHI and PacI restriction sites (italicized in primers) at the 5' and 3' ends of the amplicon, respectively, with the following primers: tdTomato_F_BamHI_2: 5'-cacc*ggatcc*gacatccaccaaacggtgtt-3', tdTomato_R_PacI_2: 5'-cc*ttaattaa*gatccctccgaaaagagagg-3', and cloned into pENTR/D-TOPO (Invitrogen). pGF1-Neo-GAS, STAT1, ISRE, and CMV vectors (System Biosciences) were digested with BamHI and PacI to cut out the dscGFP-T2A-luciferase cassette, and BamHI-tdTomato-PacI was ligated into the cut vector.

Construction of pGF1-Neo Gateway compatible vectors

pGF1-Neo-mCMV, pGF1-Neo-GAS, and pGF1-Neo-GAS-tdTomato were digested with SpeI which cuts once in these vectors, just before the mCMV sequence. After isopropanol precipitation, vectors were then blunted with T4 DNA polymerase, and the Gateway cassette C.1 insert (Invitrogen) was ligated into the vector, to make pGF1-Neo-mCMV-att, pGF1-Neo-GAS-att, and pGF1-Neo-GAS-att-tdTomato. Vectors were digested and sequenced to check that the cassette had ligated in the correct direction.

Construction of pGF1-Neo-IRF1promoter vectors

Regions of the IRF1 promoter were amplified by PCR from HFF genomic DNA, with primers listed in Table 1 (complementary regions to genome shown in uppercase), and cloned into pENTR/D-TOPO (Invitrogen). These constructs were then recombined into pGF1-Neo-GAS-att and pGF1-Neo-GAS-att-tdTomato by LR recombination with LR clonase II Plus (Invitrogen).

IRF1 promoter construct	F primer (5' to 3')	R primer (5' to 3')
IRF1prom1_1400	caccactagtGTAGGGGCCTTACCAAGGAG	atgctagcggtggcCGAGTGGAAGAGGGAAGAAG
IRF1prom1_3000	caccactagtAACCCAGGCTTTCAGACTCA	atgctagcggtggcCGAGTGGAAGAGGGAAGAAG
IRF1prom2_3000	caccactagtAGGTTGAGTCACCAGGGTTG	atgctagcggtggcCCAGGTTTGGTTCTGGAGAT

Table 1. Primers for amplifying IRF1 promoter constructs.

Appendix 2. FACS analysis of IFN-γ-induced protein expression in RAW264.7 murine macrophages.

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KDCJ assisted in all FACS analysis and sorting.

Results

Another method by which to analyze IFN-γ-induced gene expression by FACS in unfixed cells is to use antibodies that recognize cell surface proteins. Using previous microarray data (Chap. 3 Fig. 6) (Rosowski and Saeij, 2012), I chose three proteins that were highly induced by IFN-γ in RAW264.7 murine macrophages, were also significantly inhibited by prior infection with an RH Δ*rop16* strain, are expressed on the cell surface, and have good commercially available fluorescent conjugated antibodies designed for FACS analysis. These proteins are ICAM1, B7-H1, and MHC class II.

I then pre-infected RAW264.7 murine macrophages with GFP-expressing RH Δ*rop16* parasites at an MOI < 1, stimulated with murine IFN-γ, and stained for these proteins (Fig. 1). One of these proteins, ICAM1, was already basally expressed at a high level in uninfected cells, although voltage settings might be able to be changed to separate the expression in uninfected and infected cells better. The expression of another of these proteins, MHC class II proteins, was only induced in a subset (~30%) of cells. B7-H1 was induced well by IFN-γ. The IFN-γ induced expression of all of these proteins was inhibited on a population level by *Toxoplasma* infection, however, in each case there were also individual infected cells which expressed normal levels of the proteins.

Similar results were also obtained in infected, IFN-γ-stimulated B6 BMDMs.

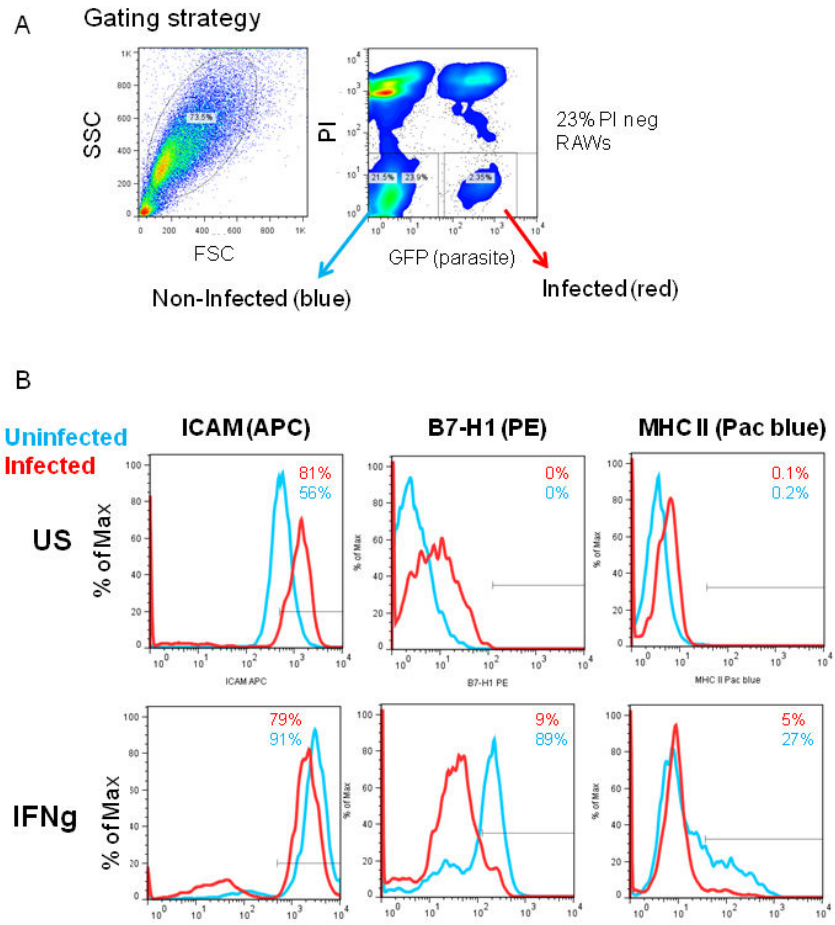

Figure 1. FACS analysis of IFN-induced expression in RAW264.7 murine macrophages. A. Gating strategy based on forward and side scatter, PI staining, and presence of GFP labeled parasite. **B.** Expression of ICAM1, B7-H1, and MHC class II proteins in unstimulated and IFN-γ stimulated cells. Uninfected cells (blue) and infected cells (red) within the sample are both shown.

Materials and Methods

Antibodies

The following antibodies were used to stain cells: anti-mouse MHC Class II (I-A/I-E) eFluor450, clone M5/114.15.2 (eBioscience); anti-mouse CD54 (ICAM-1) APC, clone 3E2 (BD Pharmingen); and anti-mouse CD274 (B7-H1) PE, clone MIH5 (BD Pharmingen).

Cell infection, isolation, and staining

$8x10^5$/well RAW264.7 murine macrophages were plated in 12-well plates, 18 hours before infection. Cells were infected with RH Δ*rop16* gfp luc 1A2 parasites thoroughly washed in PBS

at an intended MOI = 0.5 for 3.5 hours, and subsequently stimulated with 100 U/ml murine IFN-γ. After 18 hours of stimulation, cells were scraped from plates, collected and washed in cold PBS, and strained over a 70 μm filter. Cells were blocked in FACS buffer (2% FBS) containing 10% normal hamster serum and 1% Fc block. Antibodies were then incubated with cells in the same FACS buffer at a dilution of 1:100 for 25 minutes and excess antibody was washed off prior to flow cytometry analysis.

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Appendix 3. Effect of *Neospora caninum* on host IFN-γ/STAT1 and NF-κB pathways

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Results

Neospora caninum is another Apicomplexan species related to *Toxoplasma gondii* which has a very similar life cycle to *Toxoplasma*. *Neospora* can also infect human cells in tissue culture, however, unlike *Toxoplasma*, *Neospora* was reported to not inhibit the expression of IRF1 after IFN-γ treatment (Kim et al., 2007a). In our search for the *Toxoplasma* effector responsible for this inhibition, we thought that knowing more about *Neospora*'s ability, or lack of ability, to inhibit this response could give us more information. For example, comparative genetic analyses might show whether *Toxoplasma* candidate genes are also present in *Neospora*.

To investigate the ability of *Neospora* to inhibit IFN-γ induced IRF1 expression in host cells further, I pre-infected HFFs on coverslips with *Neospora* or *Toxoplasma* RH(I) strain parasites and subsequently stimulated the cells with IFN-γ. I then fixed the cells and stained for IRF1 protein expression. This experiment confirmed previously published results, that infection with *Neospora* does not inhibit host cell expression of IRF1 in response to IFN-γ, while cells infected with RH(I) *Toxoplasma* parasites had much lower levels of IRF1 in their nuclei (Fig. 1A).

However, Pru parasites, a type II strain of *Toxoplasma*, can inhibit the IFN-γ induced expression of IRF1 but also activate IRF1 expression via activation of another transcription factor, NF-κB (Chap. 3 Fig. 1). While *Neospora* infection alone, in unstimulated cells, does not result in IRF1 expression (Fig. 1A), I also wanted to be sure that *Neospora* does not also activate NF-κB family transcription factors. *Neospora* has been reported to not induce NF-κB p65 nuclear translocation (Herman et al., 2007), but to check *Neospora*'s ability to induce NF-κB-meditated gene expression through other NF-κB family members, I infected a stable HEK293 NF-κB reporter cell line, which expresses luciferase upon NF-κB activation (Chap. 2 Materials and Methods), with either *Neospora* parasites or Pru(II) *Toxoplasma* parasites (Fig. 2). TNF-α stimulated and uninfected, unstimulated controls were also included. However, I did not detect any significant luciferase activity induction upon *Neospora* infection, while TNF-α induced

luciferase activity > 1000-fold over uninfected, unstimulated cells and Pru(I) infection induced luciferase activity 200-400 fold over controls, with increasing activation by higher MOIs (Fig. 2).

It therefore seems that *Neospora* is not inducing IRF1 expression via NF-κB activation, but what about other transcription factors? In a co-infection of RH(I) *Toxoplasma* parasites and *Neospora*, the presence of even one RH vacuole potently inhibited IFN-γ stimulated IRF1 expression (Fig. 1B). Since RH is only known to inhibit STAT1-mediated transcription, this suggests that all of the IRF1 expressed is through STAT1 and that *Neospora* is not inducing IRF1 through some other transcription factor (Fig. 1B).

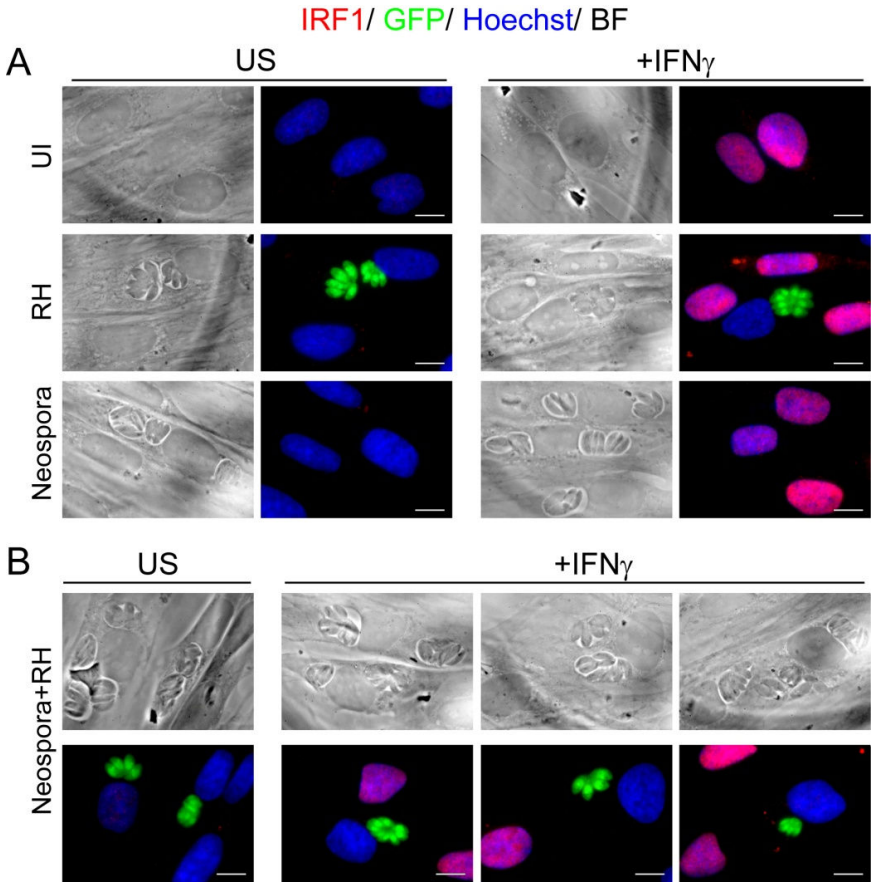

Figure 1. *Neospora* does not inhibit IFN-γ-induced IRF1 expression. HFFs were infected with *Neospora* or RH (A) or *Neospora* and RH in a co-infection (B). Cells were then stimulated, or not, with IFN-γ, fixed, and stained for IRF1 (red) and with Hoechst dye (blue). RH parasites express GFP (green). IRF1 IFA after *Neospora* infection has been repeated more than 3 times with similar results. *Neospora* + RH co-infection plus IFN-γ has been done twice with similar results.

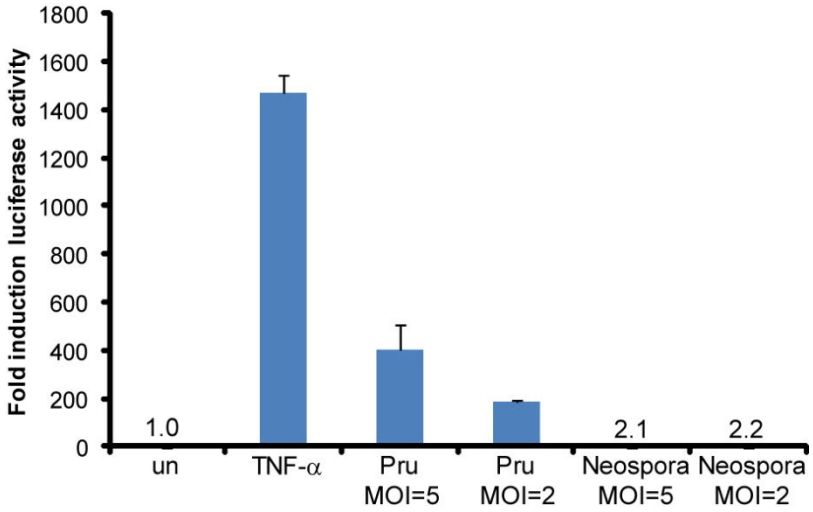

Figure 2. *Neospora* does not activate NF-κB mediated gene expression. A HEK293 NF-κB luciferase reporter cell line was infected with Pru(II) *Toxoplasma* parasites or *Neospora* parasites at two different MOIs, or treated with TNF-α. Cells were then lysed and luciferase activity was measured. This experiment has been performed twice with no detectable NF-κB activation by *Neospora* infection.

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Appendix 4. *Toxoplasma* genes that are dispensable for inhibition of IFN-γ induced IRF1 expression

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DL, SL, and DAG constructed the ROP47 knockout, DAG constructed the GRA17 knockout, and AC constructed the tgd057 knockout.

Results

One approach that we have taken to try to identify any *Toxoplasma* effectors that might inhibit IFN-γ-induced, STAT1 mediated transcription is to test any *Toxoplasma* gene knockouts that are made in the lab and seem like they could plausibly affect this response. Previously, I have reported that two effectors, ROP16 and GRA15, are dispensable for inhibition of luciferase expression in a STAT1 reporter cell line (Chap. 3 Fig. 5). The South American strain BOF does not express the ROP5 pseudokinase (Mariane Melo, in preparation), but it still inhibits IRF1 expression to the same level as an RH strain which expresses high levels of ROP5, indicating that ROP5 is not necessary for this inhibition (Fig. 1). We also tested parasites deficient in GRA2 (Fig. 2), ROP47 (Fig. 2), GRA17 (Fig. 3), or tgd057 (Fig. 4). All of these knockout strains were able to inhibit IFN-γ induced IRF1 expression as well as a wild-type RH strain.

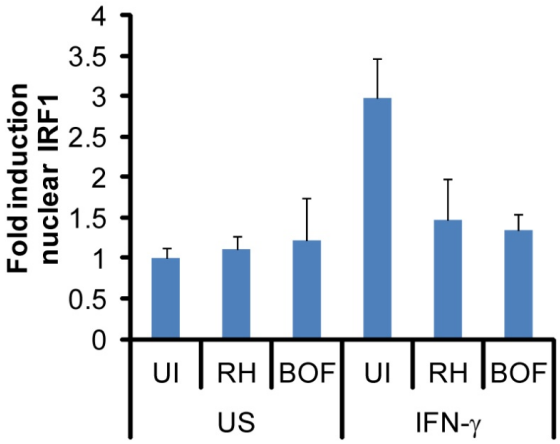

Figure 1. BOF inhibits IFN-γ-induced IRF1 expression. HFFs were infected with either RH or BOF *Toxoplasma* parasites, subsequently stimulated with IFN-γ, fixed, and stained for IRF1. Nuclear levels of IRF1 were quantitated in at least ten cells in each condition and average induction of IRF1 expression was normalized to the UI, US sample. Error bars represent standard deviation. This experiment has been done twice with similar results.

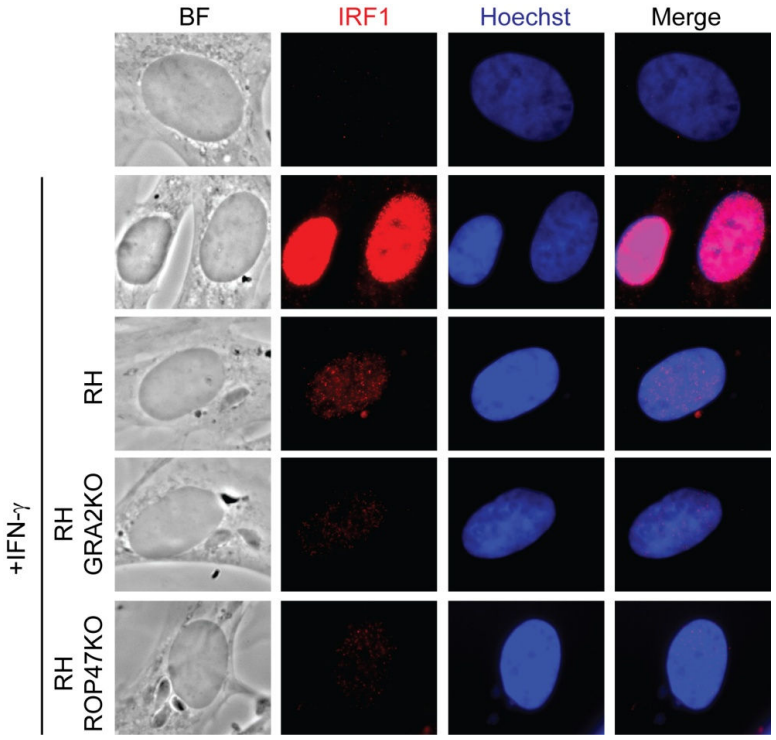

Figure 2. RH strains deficient in GRA2 or ROP47 inhibit IFN-γ-induced IRF1 expression. HFFs were infected with *Toxoplasma* strains, subsequently stimulated with IFN-γ, fixed, and stained for IRF1 (red) and with Hoechst dye (blue).

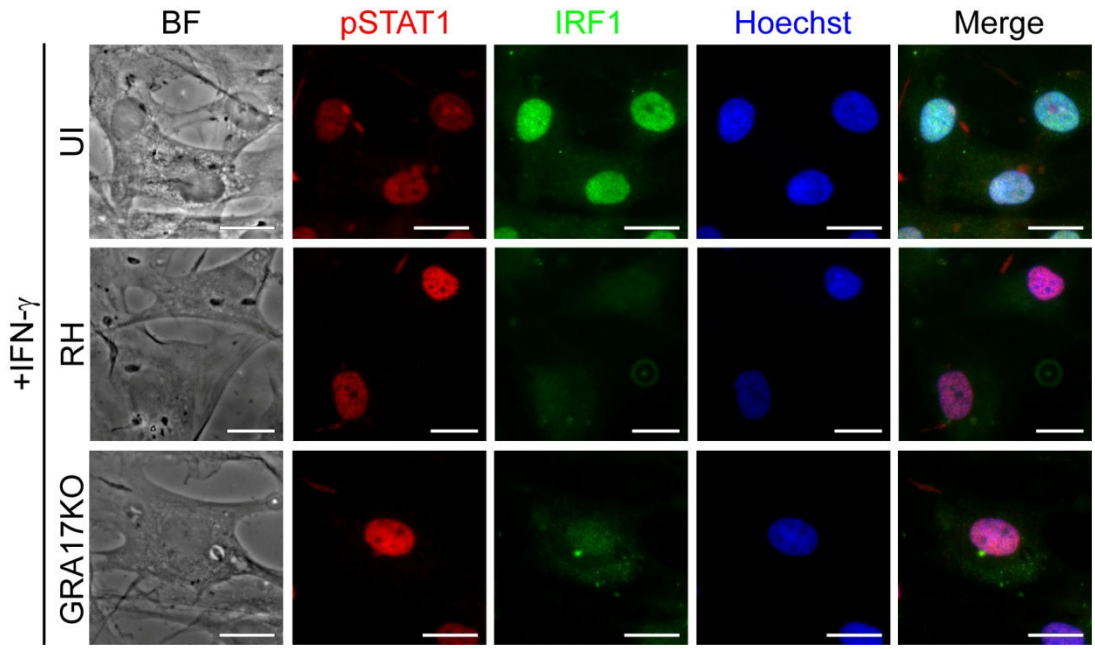

Figure 3. An RH strain deficient in GRA17 inhibits IFN-γ-induced IRF1 expression. HFFs were infected with *Toxoplasma* parasites, subsequently stimulated with IFN-γ, fixed, and stained for IRF1 (green), pSTAT1 (red), and with Hoechst dye (blue). Scale bar represents 20μm.

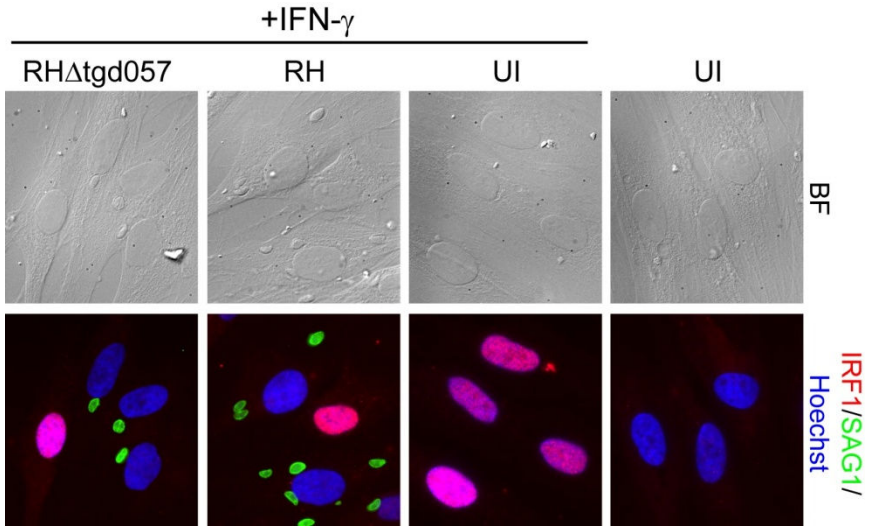

Figure 4. An RH strain deficient in tgd057 inhibits IFN-γ-induced IRF1 expression. HFFs were infected with *Toxoplasma* parasites, subsequently stimulated with IFN-γ, fixed, and stained for IRF1 (red), SAG1 (green), and with Hoechst dye (blue).