

Published in final edited form as:

Nat Rev Microbiol. 2012 November ; 10(11): 766–778. doi:10.1038/nrmicro2858.

Modulation of innate immunity by *Toxoplasma gondii* virulence effectors

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Preface

Toxoplasma gondii is a common parasite of animals and humans that can cause serious opportunistic infections. However, the majority of infections are asymptomatic possibly because the organism has co-evolved with its many vertebrate hosts and has developed multiple strategies to persist asymptotically for the lifetime of the host. Over the past two decades, infection studies in the mouse, combined with forward genetic approaches aimed at unraveling the molecular basis of infection, have revealed that *T. gondii* virulence is mediated, in part, by secretion of effector proteins into the host cell during invasion. Here, we review recent advances that illustrate how these virulence factors disarm innate immunity and promote survival of the parasite.

Introduction

Toxoplasma gondii is a member of the phylum Apicomplexa, a diverse group of primarily intracellular parasites that infect a wide range of hosts, and occasionally cause serious disease in humans and animals. *T. gondii* is one of the most widespread parasites of this group, found in nearly all warm-blooded vertebrates and a frequent cause of infections in wild, domesticated and companion animals¹. Despite enjoying a broad host range, cats are the only host where sexual development is known to take place. Development of gametocytes within intestinal epithelial cells culminates in fertilization and shedding of infectious oocysts in the faeces (Figure 1)². These spore-like particles can contaminate water and food and lead to infection in a wide range of animals (Figure 1)¹. In the intermediate host, the ability to undergo asexual replication, in the form of fast-growing tachyzoites that replicate within nucleated host cells, allows the parasite to rapidly increase in number and disseminate throughout the body. Following a vigorous immune response, the parasite differentiates into semi-dormant tissue cysts that harbor slow growing bradyzoites. Predation and ingestion of tissue cysts by cats completes the cycle (Figure 1). Unlike related parasites, *T. gondii* is unique in being transmitted asexually via carnivorous/omnivorous feeding³, which may increase dissemination between its many vertebrate hosts (Figure 1). Humans do not typically transmit the parasite, yet they readily become infected by ingesting oocysts in water or tissue cysts in undercooked meat^{4,5}. The clinical signs in humans range from mild flu-like symptoms, to severe complications in immunocompromised individuals or following transplacental transmission to the foetus⁶. Human infections show a range of clinical severity, likely a consequence of many factors including host and parasite genotypes, and such associations have been noted in ocular disease⁷, congenital infection^{8,9},

and in patients with AIDS¹⁰. Although most infections do not lead to serious complications, toxoplasmosis is the third leading cause of food borne infections requiring hospitalization in the USA¹¹.

Within its vertebrate hosts, *T. gondii* encounters many challenges in crossing biological barriers, avoiding immune surveillance and establishing its niche as an intracellular parasite. This journey begins in the gut where parasites invade enterocytes and replicate, but they can also cross the epithelial barrier and reach the lamina propria where they encounter resident macrophages, dendritic cells (DCs), and intra-epithelial lymphocytes¹². The infection spreads rapidly to draining lymph nodes, the spleen and eventually all organs. *T. gondii* is equipped with an efficient system for motility that underlies its ability to invade cells and disseminate in the host (Box 1)^{13,14}. Once established in the host cell, *T. gondii* resides in a unique parasitophorous vacuole (PV) that does not fuse with the endolysosomal system¹⁵. Because tachyzoites can infect any cell type and tissue, and replication leads to cell lysis, it has a tremendous potential to cause disease. The innate immune response limits parasite growth and promotes the development of adaptive immunity, which is required for long term resistance to infection¹⁶. The immune response also induces differentiation of the parasite into its chronic semi-dormant form (bradyzoites), potentially averting a lethal showdown between the pathogen and its host. However, the immune response is unable to eradicate the tissue cysts and reactivation of the cysts can cause severe disease in patients with primary or acquired defects in T-cell mediated immunity¹⁷.

Box 1

Host cell invasion by *T. gondii* involves the concerted action of protein secretion along with actin-based motility^{13,14}. An initial wave of secretion from micronemes is required for motility and host cell attachment¹³⁸. During invasion, the parasite invaginates the host cell plasma membrane to create a uniquely modified compartment, called the parasitophorous vacuole (PV)¹⁵. Vacuole formation is initiated by secretion from a bulb-shaped organelle called the rhoptry, which contains a diverse array of proteins¹³⁹ that are released directly into the host cell and into the forming vacuole⁸⁹. Proteins in the rhoptry neck (RONs) are initially secreted into the host cell membrane, where they help mediate formation of a moving junction that is comprised of RON2, RON4 and RON5, together with the micronemal protein AMA1^{140,141}. Proteins in the bulb of the rhoptry (ROPs) are then released into the host cell cytosol, where they are directed to the host cell nucleus (i.e. ROP16, PP2C) or to the surface of the PV (ROP2, ROP18, ROP5)^{137,142}. The PV resists acidification and fusion with endosomes and lysosomes, while it recruits host mitochondria and endoplasmic reticulum, which may aid in nutrient acquisition¹⁵. The extensive modification of the PV during invasion suggests that the parasite modulates many host cell functions as part of its intracellular lifestyle, a prediction supported by the genetic studies described in the main text. After several rounds of mitotic division daughter cells actively egress from the host cell and invade neighbouring cells.

differentiation, motility and potential to cross biological barriers²⁸, disruption of host cell signalling (see below), induction of intestinal pathology during acute infection²⁹ and development of CNS pathology during chronic infection in the mouse³⁰.

The innate immune response to *T. gondii*

For more than 60 years *T. gondii* has been an important model organism for understanding how the immune system promotes resistance to intracellular pathogens¹⁶. Most laboratory studies have used type II strains (intermediate virulence), which has facilitated the study of the immune response in mice during the acute and chronic phases of infection. These *in vivo* studies, combined with the analysis of mice lacking various immune effector genes, have defined important components of innate resistance (Table S1). Collectively, they have revealed that control of *T. gondii* requires the early production of the pro-inflammatory cytokine IL-12, which stimulates natural killer (NK) and CD4⁺ and CD8⁺ T cells to release IFN- γ ^{31,32,33,34} (Figure 2). Although lysis of parasite-infected cells by cytotoxic lymphocytes contributes to resistance, the primary role of these cells is in the production of IFN- γ , which upregulates antimicrobial effector mechanisms to control *T. gondii*³⁵. The importance of IL-12 induction in early infection is evident from studies reporting that monocytes³⁶, CD8 α ⁺ DCs^{37,38}, plasmacytoid DCs^{39,40} and neutrophils⁴¹ all contribute to the production of this activation signal (Figure 2). Selective ablation studies have implicated CD8 α ⁺ DCs³⁸ and inflammatory monocytes⁴² as early sources of IL-12 *in vivo* and consistent with this, recruitment of inflammatory monocytes to the site of infection is essential to control parasite growth^{43,44}. In addition, myeloid differentiation primary response gene 88 (MyD88) — a central adaptor protein required for the function of multiple toll like receptors (TLRs) and several cytokines — is essential for controlling infection⁴⁵ (Table S1). There has also been progress in defining the parasite molecules and host receptors involved in initial recognition, including TLR2 and TLR4 detection of glycosylphosphatidylinositol (GPI) anchored proteins⁴⁶ and TLR11 mediated detection of parasite profilin⁴⁷ (Figure 2). However, deletion of individual TLRs has only modest effects on susceptibility to infection implying that multiple TLRs are involved in the recognition of *T. gondii*⁴⁸. Consistent this idea, triple deficiency (3d) mice, which carry a point mutation in the UNC93B1 protein and are defective in TLR3/7/9 trafficking and TLR11 signalling, are highly susceptible to infection with *T. gondii*^{49,50}.

IFN- γ is the major mediator of resistance to *T. gondii* and is crucial for the activation of a variety of antimicrobial activities in haematopoietic and non-haematopoietic cells that limit parasite replication^{51,52} (Figure 2, Table S1). For example, IFN- γ alters cell metabolism, which can lead to tryptophan in fibroblasts⁵³ and iron starvation in enterocytes⁵⁴. This cytokine also stimulates professional phagocytes to produce reactive oxygen and nitrogen intermediates, which can lead to parasite damage and impede growth in macrophages^{55,56}. Studies using inducible nitric oxide synthase (iNOS) deficient mice have demonstrated the existence of an IFN- γ dependent, iNOS independent mechanism of resistance to *T. gondii* that operates during the acute phase of infection⁵⁷. This pathway relies on immunity related GTPases (IRGs) that are induced by IFN- γ and contribute to clearance of *T. gondii* in multiple cell types (Figure 2, Table S1)^{58,59}. Although the precise mechanisms are not understood, recruitment of IRGs to the parasite-containing vacuole leads to vesiculation, vacuole rupture and digestion of the parasite within the cytosol⁶⁰. A related family of GTPases known as the p67 guanylate binding proteins (GBPs) were recently shown to contribute to control of toxoplasmosis in the mouse⁶¹. This family of proteins is more widely conserved among vertebrates⁶² and they are also implicated in the control of *Listeria* spp. and mycobacteria⁶³, consistent with a more general role in host resistance to infection⁶⁴.

Although innate immune responses to *T. gondii* have been examined in detail, how these processes lead to the stimulation of adaptive immunity, including the ability of DCs to access antigens for priming of CD4⁺ and CD8⁺ T cells, are less well understood. Within the PV, the parasite is shielded from the major pathways for antigen processing and presentation, as discussed recently⁶⁵. Moreover, infection of host cells is associated with reduced expression of major histocompatibility complex (MHC) molecules⁶⁶. Despite these mechanisms of avoidance, infection with type II strains of *T. gondii* leads to the activation and expansion of DCs and a strong CD8⁺ T cell response, while infection with virulent type I strains induces a weaker response⁶⁷. Several prominent endogenous antigens that are presented on class I MHC molecules include the dense granule proteins GRA6⁶⁸ and GRA4, and the rhoptry protein ROP7⁶⁹. These antigens are polymorphic between strains, suggesting that they may be involved in strain-dependent evasion mechanisms that also influence adaptive immunity. Other studies have highlighted the importance of highly immunogenic surface antigens (SAGs) and SAG-related surface antigen (*SRS*) in stimulating the adaptive immune response⁷⁰.

Alterations in host signalling and immune evasion

Many innate immune effectors are under the control of transcription factors that enhance or regulate the overall immune response to invading microorganisms. In turn, successful pathogens have developed strategies to undermine important host cell immune pathways. Infection of mammalian cells with *T. gondii* induces many changes in host cell gene transcription, including those genes involved in energy metabolism, immune responses and signalling⁷¹. Here, we will focus specifically on how the parasite alters the immune response, as this is crucial for our understanding of how the infection is controlled, and as seen below, the host immune response is a major target of *T. gondii* virulence factors.

Infection with *T. gondii* inhibits host cell signalling pathways involved in protective immunity, for example by blocking the transcription factors signal transducer and activator of transcription 1 (STAT1)⁷² and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B)^{73,74,75}. Fibroblasts that are already infected with any of the three major natural strain types of *T. gondii* (i.e. strain types I, II and III) are unresponsive to the antimicrobial effects of IFN- γ and this appears to be due to defective STAT1 signalling⁷⁶. In *T. gondii*-infected bone marrow derived macrophages, this occurs due to modifications in chromatin structure that disrupt STAT1 binding to nuclear promoters⁷⁷. Infection with *T. gondii* also upregulates anti-inflammatory pathways including those involving the suppressor of cytokine signalling protein (SOCS) 1^{78,79}, SOCS3⁸⁰ and STAT3⁸¹, potentially compromising host mechanisms of parasite control.

Although IFN- γ promotes antimicrobial activities in many cell types, in macrophages it is not typically sufficient for the control of *T. gondii* as a second signal is required to fully activate killing. The best-characterized second signals that promote control of *T. gondii* *in vitro* are provided by TNF- α or signals through CD40^{82,83,84}, both of which utilize the NF- κ B signalling pathway. The NF- κ B family of transcription factors have an important role in the immune response to *T. gondii*, which is expected considering their evolutionarily conserved role in the regulation of many aspects of innate and adaptive immune functions^{85,86}. Notably, the activation of NF κ B by type II strains is associated with higher levels of IL-12 production³⁶, an effect that contributes to early control of infection.

Mapping and identification of virulence loci

Based on the pioneering studies of Elmer Pfefferkorn, who first demonstrated the potential for classical genetics in *T. gondii*⁸⁷, a number of pair-wise genetic crosses were undertaken to map the genetic loci responsible for major phenotypic differences between the clonal

lineages (Box 2). These forward genetic studies have been extremely effective in identifying the underlying genetic basis for some of the most striking phenotypic differences between lineages I, II and III, notably differences in acute virulence and induction of host cell signaling⁸⁸.

Box 2

Classical genetic crosses between the three main clonal strains have been useful for defining virulence determinants of *T. gondii*. Crosses were conducted by tagging strains with drug resistance markers and co-feeding cats with a mixture of cysts obtained from mice (which were infected with two different parasite strains) to generate recombinant oocysts (see Figure 1)¹⁴³. Three pair-wise genetic crosses were conducted (see table) between the different strains and single cell F1 progeny isolated from the oocysts were used to generate genetic linkage maps¹⁴⁴ and to identify phenotypic traits (as described in more detail previously^{23,143}). Analysis of these genetic crosses revealed that the genome of *T. gondii*, is approximately 65 x10⁶ bp in size and is organized into 14 linear chromosomes that range in size from 2->10 Mbp¹⁴⁴. Recombination rates are relatively low, with a centimorgan (1% recombination rate) corresponding to roughly 100 Kbp¹⁴⁴. These parameters predict that it should be relatively easy to map a given trait to a region of one chromosome, but more challenging to fine map and identify the gene of interest. Additional criteria that are useful in narrowing down the candidate list include differential expression levels, high levels of allelic polymorphism and the allocation of genes to specific functional pathways, such as those encoding surface or secretory proteins, which might be in contact with the host and hence more likely to contribute to pathogenesis. Genetic crosses between the three lineages have been used to analyze the segregation of several traits including acute virulence^{90,91,94,95}, growth⁹⁰, migration potential⁹⁰ and effects on host cell transcription⁹³. Analysis of complex traits has been facilitated by the use of quantitative trait locus (QTL) mapping, which provides a statistical means of evaluating the contribution of multiple loci to a given phenotype and for examining interactions between them¹⁴⁵. QTL analysis of growth differences between type I (fast growth) and type III (slow growth) strains of *T. gondii* revealed that 4 or more major loci control this trait⁹⁰, which has thus far been refractory to the identification of individual genes due to the small contribution they each make. Likewise, differences in migration potential⁹⁰, which may contribute to virulence¹⁴⁶, map to a broad region of chromosome VIIa, but has not yet been localized to a specific gene(s). Genes that have been identified by mapping and their known targets are summarized in the table.

Summary of genetic crosses

Genetic Cross	Phenotypes examined	QTLs	Genes identified	Targets
I × II	Acute virulence	XII	ROP5	Activates ROP18 Binds Irga6
II × III	Acute virulence Altered host gene expression IL-12 induction	VIIa, VIIb, XII VIIb X	ROP18, ROP5 ROP16 GRA15	IRGs STAT3/6 NFkB
I × III	Acute virulence Growth Migration	VIIa, Ia VIIa, XI, XII, Ia VIIa	ROP18	IRGs, ATF6b

Identification of rhoptry kinases as mediators of acute virulence

During invasion of host cells, secretory organelles known as rhoptries discharge their contents into the host cell (Box 1), making these primary candidates for modulating host signalling⁸⁹. This prediction was confirmed when ROP18 was identified as a major factor that contributed to strain-specific differences in virulence^{90, 91}. ROP18 is an active serine/threonine (S/T) protein kinase that is secreted into the host cell where it decorates the surface of the parasitophorous vacuole membrane (PVM) (Box 1). It is a polymorphic protein and its expression levels vary from high in type I and II strains to a low level in type III strains. Transgenic expression of ROP18 from type I or type II strains in the type III background greatly enhanced virulence confirming that this locus was responsible for the observed differences in virulence between strains^{90, 91}. This gain of virulence required an active enzyme as transfection with an allele containing an inactive kinase allele failed to enhance virulence⁹⁰. The low expression of ROP18 in type III strains is attributed to the presence of a unique ~ 2kb region upstream of the coding sequence that is absent in type I/II strains⁹². A comparison of this region with the same region in the related parasite *Neospora caninum* suggests that it underwent a rearrangement in the ancestral progenitor to type I and II strains⁹², leading to upregulation of expression of ROP18 in these two lineages and enhanced virulence. Both virulent (encoded by types I and II) and avirulent (encoded by type III) alleles are stable in the population and show evidence of long term stability⁹², implying that the three lineages are adapted for distinct niches.

Genetic crosses between strains II and III also led to the identification of a protein that is responsible for the ability of these strains to differentially affect host gene transcription (Box 2)⁹³. This rhoptry kinase, ROP16, is unusual in that it is targeted to the host nucleus⁹³. Further analysis of differences in host transcription induced by type II (intermediate virulence) and type III strains (avirulent), identified a third locus encoding a polymorphic pseudokinase called ROP5 on chromosome XII (Box 2)⁹⁴. Remarkably, this same *ROP5* locus was the sole quantitative trait locus (QTL) identified in a genetic cross between type I (high virulence) and II (intermediate virulence) strains (Box 2)⁹⁵. In both cases, genetic disruption of the *ROP5* locus in a type I strain led to severe attenuation, which was restored with complementation^{94, 95}. The *ROP5* locus contains a tandem repeat of 6–10 polymorphic genes that differ between strain types, and yet all are predicted to encode catalytically inactive pseudokinases^{94, 95}. Comparison of the three pair-wise crosses predicts that *ROP5* and *ROP18* interact and together they explain nearly 90% of the differences in acute virulence between these three lineages.

The diversity of ROP kinases in *T. gondii*

Protein kinases are expanded in most eukaryotic lineages, where they control a number of important signalling pathways and are defined by a series of conserved domains that fold into common N- and C-terminal lobes⁹⁶. They contain a nucleotide binding pocket characterized by a number of conserved motifs including a catalytic triad that transfers γ -phosphate from ATP to an acceptor hydroxyl group on serine, threonine or tyrosine⁹⁶. Pseudokinases encode intact proteins that typically lack the ability to catalyze phosphate transfer, but they can act as scaffolds to regulate enzymatic processes⁹⁷. The realization that rhoptries contain a family of related polymorphic serine / threonine protein kinases and pseudokinases, led to a bioinformatic search that identified 44 other ROP kinase members, about half of them predicted to be active, while the remainder are predicted to be pseudokinases⁹⁸. The ROP family is highly diverse in *T. gondii* and related apicomplexans, including *N. caninum*, and the abundance of non-synonymous substitutions in these proteins, often accompanied by gene duplication, indicates that they are under strong selective pressure⁹⁸. Most of the predicted ROP kinase family members have not been studied, but several have been validated as *bona fide* rhoptry proteins that are secreted into

the host cell during invasion⁹⁸. In addition ROP38 from type III parasites is implicated in downregulating host MAPK signalling, as expression of this allele in a type I background suppressed this pathway⁹⁸. However the precise role of this kinase in pathogenesis is still uncertain. For the remainder of the review we will focus on the function of the ROP kinases whose functions have been elucidated.

ROP18 and ROP5 belong to a subset of the ROP kinase family typified by the pseudokinase ROP2, the first member of this family predicted to contain a kinase domain⁹⁹. This prediction was borne out when the crystal structures of the kinase domain of ROP2^{100,101} and ROP8¹⁰¹ were solved, which in turn facilitated the generation of a homology model of ROP18¹⁰¹ (Figure 3A). By aligning the sequence of ROP18 to known S/T kinases, all of the conserved residues known to be important in ATP binding and catalysis were identified¹⁰¹. It was also shown that ROP18 has a novel mechanism of activation that relies on phosphorylation of an N-terminal helical domain that wraps around the N-lobe and inhibits kinase activity when it is unphosphorylated¹⁰¹. In contrast to ROP18, the pseudokinases ROP2 and ROP8 have a highly degenerate nucleotide-binding pocket and are not predicted to bind or hydrolyze ATP^{100,101}. Similarly, ROP5 is predicted to be a pseudokinase and although it is able to bind ATP, it is unlikely to carry out phosphate transfer due to an alteration in the central residue of the catalytic triad that is Arg or His instead of Asp¹⁰². Remarkably, ROP2 kinase family members are highly conserved among *T. gondii* strains, however, amino acid differences between strains are clustered in the substrate-binding loop¹⁰¹. This implies that even though they are not catalytically active, they may be capable of binding specific host substrates. ROP2 family members also have an N-terminal extension that contains three amphipathic alpha helical regions that are rich in arginine on one face and hydrophobic on the other¹⁰³. This low complexity region is predicted to be involved in mediating membrane interactions and has been shown to be necessary and sufficient for targeting to the PVM^{103,104} (Figure 3B,C). As recently shown, targeting of ROP18 to the PVM determines access by the kinase to host substrates and is essential for virulence¹⁰⁵.

Host targets affected by *T. gondii* secretory proteins

Although multiple host signalling pathways are altered by *T. gondii* (Table S2), we have only recently begun to appreciate the specific parasite proteins that mediate these effects. We will focus on three parasite “effectors” that are known to target the innate immune response of the host.

Alteration of host cell transcription by ROP16

The role of ROP16 in altering host gene transcription was initially identified by analyzing the differences in host gene expression induced by different parasite strains. These studies led to a focus on genes involved in IL-4 and IL-6 responses and implicated changes in the activity of the transcription factors STAT3 and STAT6⁹³. Although all three strains initially induce STAT3 and STAT6 activity, only the type I or III strains (which share the same ROP16 variant, ROP16_{I/III}) sustain this response (Figure 4, Table 1)⁹³. Subsequent studies revealed that ROP16_{I/III} directly phosphorylates Tyr705 in STAT3¹⁰⁶ and Tyr641 in STAT6¹⁰⁷, residues that are required for activation of these transcription factors. Studies using a type I strain carrying a knockout of the *ROP16* allele ($\Delta rop16$) indicate that initial stimulation of STAT3 is independent of ROP16, whereas early activation of STAT6 requires ROP16¹⁰⁸. Comparison of the two ROP16 variants (i.e. the protein from type II strains, ROP16_{II} and the protein common to types I and III, ROP16_{I/III}) revealed that a single amino acid substitution at position 503 is responsible for the marked difference in sustained activation of STATs¹⁰⁶. Prolonged STAT3/6 activation by ROP16_{I/III} down-regulates the induction of IL-12, thus limiting the protective T_H1 cytokine responses⁹³, which might lead

to less inflammation and reduced pathology but also enhanced parasite survival (Figure 4). Consistent with this model, transgenic expression of a type I *ROP16* allele in type II strains inhibits IL-12 production⁹³, while the $\Delta rop16$ type I strain shows increased IL-12 production¹⁰⁸. Additionally, the altered cytokine response of the $\Delta rop16$ type I strain provided further evidence that ROP16 controls the ability of *T. gondii* to block STAT3-dependent production of IL-12 and TNF- α ¹⁰⁸.

Despite having profound effects on host gene expression, ROP16 has only a moderate role in acute virulence in mice. Thus, although the $\Delta rop16$ type I strain shows slightly enhanced parasite replication *in vivo* compared with wild type, deletion of this allele does not reduce host mortality following ip challenge¹⁰⁸. ROP16 may also have a role in reducing inflammation, and thus have important roles in other infection models since transgenic expression of ROP16_{I/III} in a type II strain was shown to reduce pathology caused by oral infection of mice¹⁰⁹. The enhanced growth that was observed in the absence of ROP16 may be explained by the ability of this protein to activate STAT6, which leads to the induction of arginase, an enzyme associated with alternatively activated macrophages¹⁰⁸. It is possible that arginase could have two opposing roles in *T. gondii* growth: enhancing parasite growth by providing polyamines, but limiting access to the essential amino acid arginine and impairing growth¹¹⁰. Induction of arginase is also expected to antagonize the ability of macrophages to kill intracellular parasites since arginine also serves as a substrate for iNOS; consistent with this, elimination of arginase in macrophages promotes resistance to *T. gondii*¹¹¹. Thus, the balance of these two opposing roles for arginine may determine whether the pathogen persists or is eliminated *in vivo*.

Induction of IL-12 production by GRA15

Infection of macrophages with type II parasites results in production of higher levels of IL-12, compared to infection with the other two lineages³⁶. This effect is partly modulated by ROP16 since the allele expressed in type II strains is incapable of sustained phosphorylation of STAT3, as discussed above. However, this is unlikely to be the sole factor responsible for the increased levels of IL-12 during infection with type II strains. Indeed, genetic mapping identified a locus on chromosome X encoding a dense granule protein named GRA15 that is responsible for induction of IL-12 in type II strains and expression of the type II variant (GRA15_{II}) in a type I strain enhances IL-12 production¹¹². The molecular mechanism of GRA15 function is uncertain as it has no homology to any other proteins in the database, nor does it contain any conserved domains. Localization studies revealed that GRA15 is secreted into the host cell together with ROP proteins in small cytoplasmic inclusions known as evacuoles¹¹². GRA15 also localizes to the PVM¹¹², although its topology at this interface has not been established. Deletion of GRA15 in the type II strain ME49 prevents nuclear translocation of NF κ B but the mechanism by which GRA15 activates NF κ B is incompletely understood. However, it acts independently of MyD88, the IL-1 receptor family and TIR-domain-containing adapter-inducing interferon- β (TRIF)¹¹². Since MyD88 and TRIF co-ordinate the main signalling pathways for TLR ligands it seems unlikely that GRA15 is recognized by a TLR. Nonetheless, induction of NF κ B by GRA15 depends on TNF receptor associated factor 6 (TRAF6), which is an adaptor that functions with IL-1R-associated kinase 1 (IRAK1) to mediate signalling downstream of MyD88. Engagement of TRAF6 activates IKK, followed by phosphorylation of I κ B, proteasome degradation, and translocation of NF κ B to the nucleus¹¹² (Figure 4). GRA15 appears to be sufficient to induce this pathway as its expression in HeLa cells results in nuclear translocation of NF κ B¹¹². Although GRA15_{II} can directly drive NF κ B activation and IL-12 production, this is likely to be only one contributing factor as other studies have shown that following infection by type II strains, production of IL-12 is largely Myd88-dependent in macrophages¹¹³ and dendritic cells⁴⁵.

The polymorphic nature of ROP16 and GRA15 in different lineages of *T. gondii* leads to polarizing responses in cells infected with *T. gondii*^{109,112}. Infection of macrophages with parasites expressing ROP16_{I/III} leads to activation of STAT6 and the development of alternatively activated macrophages, while those infected with strains expressing ROP16_{II} show the classical activated phenotype^{108,109}. GRA15_{II} activates NF κ B resulting in high levels of IL-12 production, further enhancing the classical activation pathway in cells infected by type II parasites. Classically activated macrophages typically express chemokines and cytokines that activate cells with antimicrobial activity while alternatively activated macrophages secrete anti-inflammatory molecules that can down-regulate Th1 responses¹¹⁴. Hence, different variants of the effector proteins drive antagonizing responses in infected macrophages, which can have broad effects on antimicrobial effector pathways, inflammation and parasite control.

Modulation of acute virulence by ROP18 and ROP5

As noted above, differences in virulence between strains of *T. gondii* are associated with the S/T kinase ROP18 and the related pseudokinase ROP5. Understanding the mechanistic basis for this effect has been aided by recent observation that together this pair of parasite effectors targets host immunity by disrupting IRGs. Induced by IFN- γ , IRGs are crucial for the control of toxoplasmosis in mice^{59,60}. These enzymes are expanded in the mouse system but are less common in other vertebrates (for example humans have only two IRGs.)¹¹⁵. Although the PVM lacks most host cell proteins¹⁵, IRGs are recruited to the PVM surrounding type II and III strains where they cause vesiculation and promote parasite destruction,^{116,117} a process that is also dependent on the autophagy protein Atg5 although the nature of its involvement is unclear¹¹⁸. Unlike strain types II and III, highly virulent type I strains avoid this fate^{116,119}.

In IFN- γ activated cells, several IRGs including Irga6, Irgb6, and Irgb10 are phosphorylated by ROP18 and this is associated with reduced IRG recruitment to the PVM {Fentress, 2010 #6020;Steinfeldt, 2010 #6396}. Transgenic expression of the active form of ROP18 from a type I strain in a type III parasite inhibited IRG recruitment to the PVM, resulting in persistence of the parasite in activated macrophages¹²⁰. RNAi suppression of Irgb6 reversed the enhanced clearance of parasites lacking ROP18¹²⁰. The interplay between ROP18 and the IRGs was revealed by mass spectrometry, which showed that ROP18 phosphorylates key threonine residues in switch region I of Irga6 and Irgb6 {Fentress, 2010 #6020;Steinfeldt, 2010 #6396}. Phosphorylation of Irga6 prevents GTP hydrolysis and oligomerization of this protein {Steinfeldt, 2010 #6396}, steps that are thought to be important for loading of IRGs onto the PVM¹²¹. These findings indicate that ROP18 contributes to virulence by prolonging survival of *T. gondii* in IFN- γ activated macrophages. Moreover, the role of ROP18 in blocking IRG function is not limited to professional phagocytes as similar studies have shown it is important for the avoidance of IRG recruitment to the PVM in activated fibroblasts¹²².

More recently, the transcription factor ATF6 β , which resides in the host ER and is important in the unfolded protein response (Figure 4), was identified as a ROP18 target¹²³. In co-transfected cells, phosphorylation of ATF6 β by ROP18 led to proteosomal degradation of ATF6 β and a consequent reduction in ATF6 β mediated gene expression when the unfolded protein response was induced¹²³. The interaction of ROP18 with ATF6 β in co-transfected cells was mapped to the N-terminus of the kinase and the C-terminus of ATF6 β ¹²³. Since the C-terminus of ATF6 β is in the lumen of the host ER, while the N-terminus of ROP18 is tethered to the PVM, this interaction suggests a precise topological rearrangement of one or both of these proteins within infected cells. A role for ATF6 β *in vivo* was shown by the increased susceptibility of ATF6 β ^{-/-} mice to challenge with an attenuated type I $\Delta rop18$ strain. This deficiency was associated with decreased induction of IFN- γ by CD8⁺ T cells

incubated with DCs from $ATF6\beta^{-/-}$ mice¹²³, suggesting that $ATF6\beta$ might have a previously unrecognized role in antigen presentation.

Although the phenotype of $\Delta rop18$ type I parasites in IFN- γ activated macrophages *in vitro* is highly attenuated, this mutant is only partially attenuated *in vivo*¹²⁰. In contrast, deletion of the pseudokinase ROP5 in the type I background significantly decreases virulence by more than a million fold^{94,95}. However, because the *ROP5* locus encodes a tandem array of predicted pseudokinases, it was initially unclear why this catalytically inactive protein would have such a dramatic effect on virulence. Insights into this question were provided by genetic studies that suggested that ROP5 and ROP18 interact. Firstly, it has been established that ROP5 binds to Irga6 and affects its ability to form GTP-dependent oligomers^{124,125} which would disrupt the loading of Irga6 onto *T. gondii* containing vacuoles. In two separate studies, it was shown that ROP5 enhances ROP18_F-mediated phosphorylation of Irga6¹²⁴ and Irgb6¹²⁶. Since ROP5 does not bind well to Irgb6, the latter result is likely due to enhanced ROP18 catalytic activity in the presence of ROP5, which has been confirmed by *in vitro* studies¹²⁶. This indicates that ROP5 allosterically activates ROP18, as has been recently described for several human kinases^{97,127}. Collectively, these data suggest a model in which ROP5 contributes to acute virulence in the mouse by activating other ROP kinases, as well as disrupting IRG oligomerization, which allows type I strains to evade antimicrobial effector mechanisms.

Overview of strain-dependent mouse virulence in *T. gondii*

Genetic mapping studies have revealed that the marked phenotypic differences between strains of *T. gondii* in mice are mediated by combinations of different alleles at only a few major loci. The acutely virulent type I strains have a highly active ROP18 that functions cooperatively with ROP5 to phosphorylate host IRGs, inhibit IRG loading on the PVM and subsequently block parasite clearance in IFN- γ activated cells (Figure 4, Table 1). ROP18 also targets $ATF6\beta$ and may thus compromise antigen presentation by DCs. Type I strains also express STAT3/6 activating alleles of ROP16 that can dampen T_H1 cytokine responses and an allele of GRA15 that fails to induce the pro-inflammatory cytokine IL-12 (Figure 4, Table 1). The combined activities of these effectors could allow the parasite to delay induction of protective immunity systemically, while escaping innate mechanisms of control within infected cells, ultimately resulting in expansion to very high tissue burdens. Somewhat paradoxically, mice eventually succumb to infection despite producing very high levels of type I cytokines^{128,129}. It is likely that the initial impairment of T_H1 responses (via ROP16, ROP18 and possibly other factors), allows parasite expansion and results in a high parasite burden that causes tissue damage and subsequent activation of danger signals, triggering a secondary response that leads to cytokine-mediated pathology. Although the molecular basis is not fully understood, this strong acute virulence trait is also shared by numerous South American lineages⁹². Although this phenotype leads to rapid death of laboratory mice, it may be an adaptation to survive in hosts such as wild deer mice¹³⁰, rats¹³¹ or chickens¹³², all of which appear innately resistant. In contrast to the extreme virulence of type I strains, type II strains induce protective immunity and exhibit intermediate virulence. Despite having a virulence enhancing ROP18 allele, they express a variant of ROP5 that is considered “avirulent” (Figure 4, Table 1). Consequently, they are less able to avoid IRG clearance within IFN- γ activated cells (Figure 4, Table 1). Although the GRA15 variant expressed by type II strains induces the production of IL-12, the inability of this strain to prolong STAT3/6 activation (due to the particular ROP16 variant it encodes) results in early IL-12 production leading to a type 1 cytokine response and effective control of acute infection (Figure 4, Table 1). Such a strategy is consistent with the high propensity of type II strains to differentiate into bradyzoites and their associated prevalence in chronic infections in animals. Finally, type III strains appear to lack an effective mechanism for

blocking IRGs because they under-express ROP18 by ~ 100 fold compared to types I or II (Figure 4, Table 1). As such, they are readily cleared from IFN- γ activated murine macrophages. Type III strains also induce prolonged phosphorylation of STAT3/6, while not strongly activating NF κ B and hence avoiding induction of IL-12 (Figure 4, Table 1). This strategy may be an adaptation to survive in hosts that lack the IRG pathway or where overproduction of cytokines causes detrimental pathology to the host. Although we do not know what specific hosts each of these phenotypes are adapted for, they are each common in natural isolates and likely reflect successful long-term evolutionary strategies⁹².

Future directions

Recent advances have revealed that similar to other pathogens that inject virulence factors into host cells^{133,134,135}, *T. gondii* injects rhopty proteins not only into the cells it infects but also into bystander cells¹³⁶. Using this so called “kiss and spit” model¹³⁷, the parasite can modify the cell it resides in, as well as potentially influencing the behavior of adjacent cells¹³⁶. An assessment of the many characterized mutants with advanced imaging techniques should allow further molecular dissection of the interactions between the parasite and its host, in order to reveal the strategies that are central to dissemination and pathogenesis of *T. gondii*. The questions that have received the most focus in the last decade have revolved around understanding differences in virulence between parasite strains and have provided important new perspectives on pathogenesis in the mouse model. Extending these studies to other host species will be crucial to evaluate the contribution of virulence factors to transmission dynamics and disease in natural hosts. Beyond the traits described here, a large number of other differences in host responses have been noted in previous studies, although most of these have not been examined for potential parasite strain-specific or host differences (Table S2). Thus far, most studies have focused only on the dominant strains in North America and Europe. Extension of these approaches to more diverse lineages from South America and other regions²⁴ is likely to reveal additional layers of complexity in parasite virulence. Moreover, the current strategy for mapping of genetic differences is unlikely to uncover conserved mechanisms used by all strains of *T. gondii* to evade the immune response. Additional forward genetics screens will be needed to identify the molecular basis of such virulence activities. As additional more powerful experimental approaches are developed, we can look forward to learning more about the role of parasite secretory proteins in modulating host functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Work in the authors' laboratories is supported by the National Institutes of Health (L.D.S., C.A.H.) and the State of Pennsylvania (C.A.H.). We regret not being able to cite all of the appropriate primary literature due to space limitations.

Glossary

Apicomplexans	Protozoans of the phylum Apicomplexa known for their apical complex consisting of a specialized microtubule organizing center, called the conoid, and secretory organelles involved in host cell invasion. <i>Toxoplasma</i> spp. belong to the tissue-cyst forming coccidian group of the Apicomplexa
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Tachyzoite	The rapidly replicating intracellular form (from the greek <i>tachys</i> , meaning fast) of many tissue cyst-forming coccidians such as <i>T. gondii</i>
Bradyzoite	A slow growing form of the parasite (from the greek <i>bradys</i> , meaning slow) residing within long lived tissue cysts that are associated with chronic infection. One of the parasitic stages that is specialized for transmission if ingested
TLR	toll-like receptors, pattern recognition receptors that mediate immune responses by detecting pathogen associated molecular patterns
iNOS	Inducible nitric oxide synthase (NOS2 gene) A soluble enzyme that produces nitric oxide (NO) from L-arginine. It is upregulated by IFN- γ and TNF- α , and constitutes a major antimicrobial activity of macrophages
IRG	Immunity related GTPases: A family of 45–47 kDa GTPases that are strongly upregulated by IFN- γ and contribute to resistance to intracellular pathogens. IRGs are ubiquitous in the mouse, but are much more restricted in other vertebrates and largely absent in humans
GBP	Guanylate binding proteins: A family of 65 kDa GTPses that are upregulated by IFN- γ and contribute to resistance to intracellular pathogens. Widely distributed in vertebrates indicating that they may play an important general role in resistance to intracellular pathogens
MHC	Major histocompatibility complex: A family of cell surface molecules that allow recognition of epitopes from foreign or self antigens through presentation to T cells
STAT	Signal Transducer and Activator of Transcription Cellular transcription factors: A family of transcription factors that are activated by Janus kinases (JAK) and regulate gene expression by binding to nuclear promoters. Have a role in development and the immune system
SOCS	Suppressor of cytokine signalling: A family of intracellular proteins that regulate cytokine signalling by either direct inhibition of receptors or increased degradation of signalling proteins
TNF-α	Tumor necrosis factor alpha: a cytokine involved in inflammation, tumor suppression and host defence
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells: A complex that controls DNA transcription in response to various signalling inputs. Plays a key role in regulating innate and adaptive immune responses
QTL	Quantitative trait locus, Genetic region (locus) that contributes to a quantitative trait, meaning one that varies by degree. Such traits are typically polygenic, being contributed to by multiple QTLs
Pseudokinase	Protein containing a conserved protein kinase fold but lacking key residues in the nucleotide binding pocket that result in inability to transfer phosphate to a donor substrate. Although not catalytically active, they serve regulatory or scaffolding roles

Arginase	Enzyme in the urea cycle that mediates consumption of arginine, and thus has an antagonistic role to nitric oxide synthase as they rely on the same substrate
Alternatively activated macrophages	Refers to one of two polar phenotypes that macrophages can adopt following stimulation by cytokines. Classically activated macrophages exhibit a TH1 phenotype, promoting inflammation, while alternatively activated macrophages have a Th2 phenotype dampening inflammation and promoting repair. Arginase is typically expressed by alternatively activated macrophages, while nitric oxide synthase is produced by classically activated macrophages
MAPK	Mitogen-activated protein (MAP) kinases: Serine/threonine kinases involved in cellular signalling in response to a diverse array of stimuli
TRIF	TIR-domain-containing adapter-inducing interferon- β ; Adaptor protein for signalling from TLRs that contain TIR domains
TRAF6	TNF receptor associated factor 6: Protein adaptor involved in signalling through TNF, IL-1, and TLRs
IKK	I κ B kinase: a kinase comprised of three subunits α , β and γ , also known as NEMO
IκB	Inhibitory subunit of NF κ B: becomes phosphorylated by IKK, ubiquitination and degradation by the proteasome, allowing active NF κ B to translocate to the nucleus
Unfolded protein response	Cellular stress response triggered by unfolded proteins accumulating in the ER. Leads to stalled translation and upregulation of chaperones for protein folding
Micronemes	Secretory organelles that are discharged in response to elevated cytoplasmic calcium. Microneme proteins (MICs) mediate substrate and host cell adhesion
Centromere	(cM) A genetic unit used for establishing genetic linkage based on recombination frequency in the progeny of a genetic cross. 1 cM is equal to the distance over which 1% recombination occurs. Named in honour of Thomas Hunt Morgan
Oocyst	Diploid stage of parasite development that is the product of the fusion of <i>T. gondii</i> gametes. Oocysts are shed in cat faeces and they contaminate the environment, giving rise to infection by accidental ingestion
GPI anchors	Glycosylphosphatidylinositol linked proteins share a common glycolipid anchor that is covalently attached to the C-terminus of a variety of surface proteins as a post-translational modification

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Biographies

Christopher A. Hunter

Chris Hunter is a graduate of the University of Glasgow where he specialized in Parasitology for his Ph.D. and as a post-doctoral fellow. He was also a fellow with Jack Remington at Stanford University where he focused on the immune response to *Toxoplasma gondii*. He joined the faculty at the University of Pennsylvania in 1994 where he is now Professor and Chair of the Department of Pathobiology and work in his laboratory concerns studies on immune regulation.

L. David Sibley

L. David Sibley did his undergraduate studies at Oberlin College, Ohio, USA and received his Ph.D. from Louisiana State University. He was a postdoctoral fellow in the laboratory of John Boothroyd at Stanford University and subsequently joined the faculty at Washington University in St. Louis where he currently holds the Alan A and Edith L Wolff Professorship in Molecular Microbiology. His laboratory studies unique cellular adaptations and virulence mechanisms of the parasite *Toxoplasma gondii*.

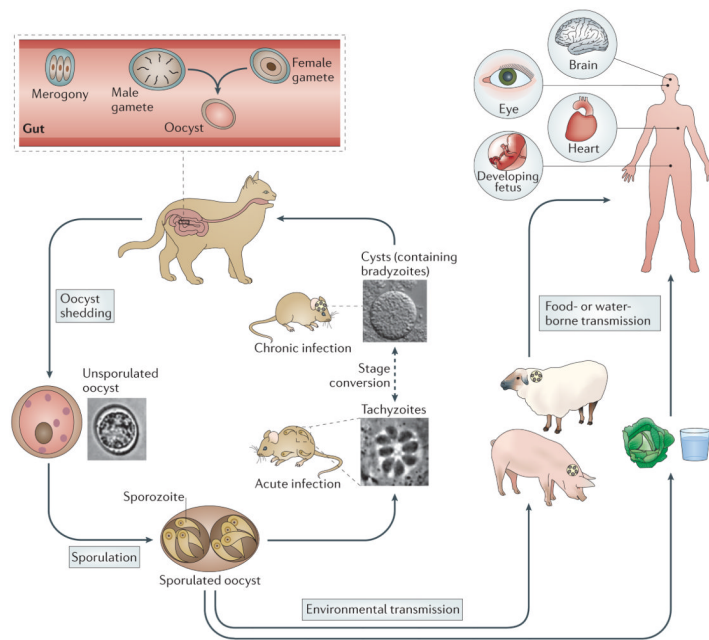


Figure 1. The complex life cycle of *T. gondii*

Cats are the definitive host where sexual replication takes place. Following replication within enterocytes of the gut (a process known as merogony), male and female gametes are formed within the host cell, as described previously². Fusion of gametes leads to the formation of diploid oocysts that are shed in cat faeces and undergo meiosis in the environment for long periods of time and can contaminate food and water, providing a route of infection for intermediate hosts. In the intermediate host (shown here as rodents) asexual replication occurs. Acute infection is characterized by fast replicating tachyzoites that disseminate throughout the body. Differentiation to slow-growing bradyzoites within tissue cysts leads to long-term chronic infection. Ingestion of tissue cysts via omnivorous or carnivorous feeding can lead to transmission to other intermediate hosts or to cats, which re-initiates the sexual phase of the life cycle. Many animals serve as intermediate hosts, including farm animals. Humans become infected by eating undercooked meat containing tissue cysts or by the ingestion of oocysts in contaminated water^{4,5}. Although most infections are mild, toxoplasmosis can cause serious symptoms in the brain and other organs (as indicated) in immunocompromised patients, as well as in the developing foetus following congenital infection.

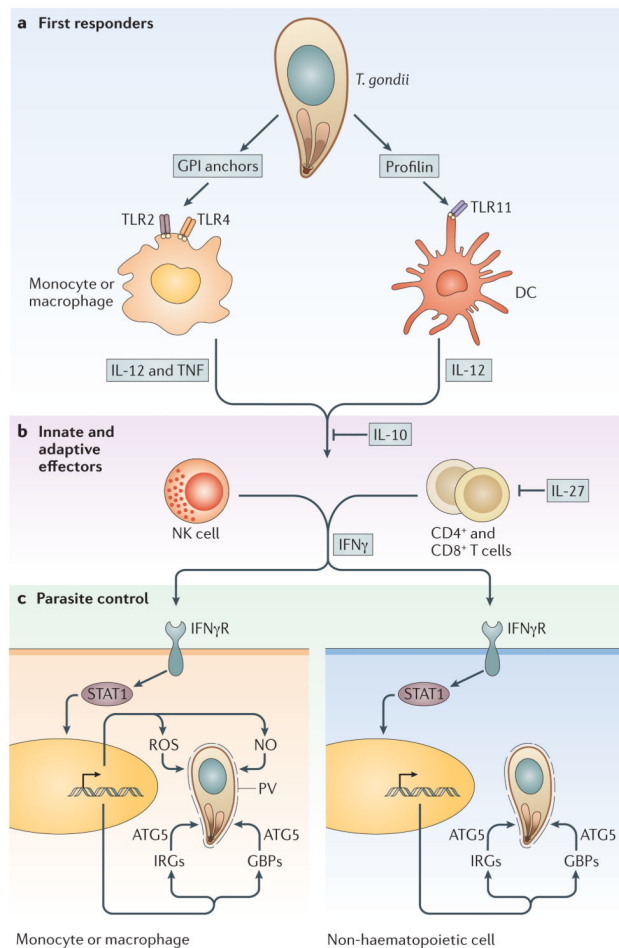


Figure 2. Innate immune responses to *Toxoplasma gondii* during infection

a) Early in infection, the first cells to respond are dendritic cells (DCs) and monocytes/macrophages. Interaction of *T. gondii* profilin with TLR11 on DCs is important for the production of IL-12. In addition to stimulating IL-12 production, macrophages also induce TNF- α , a cofactor in antimicrobial activity, in response to the detection of GPI anchored proteins via TLR2 and TLR4. b) The immune response results in the production of IFN- γ from NK cells through the innate response and eventually from CD4⁺ and CD8⁺ T cells as the adaptive response ensues. IL-10 and IL-27 are key to modulating these pathways and prevent the overproduction of T_H1 cytokines. c) Production of IFN- γ during the innate and adaptive phases is responsible for activating cells to control parasite infection. IFN- γ propagates a signal through a surface receptor (IFN- γ R) to activate STAT1, a nuclear transcription factor that controls the expression of many genes. In response to STAT1 activity, nitric oxide (NO) and reactive oxygen species (ROS) are upregulated in monocytes/macrophages, both of which contribute to the control of intracellular parasites. Both haematopoietic and nonhaematopoietic cells also upregulate two families of defence proteins called immunity related GTPases (IRGs) and guanylate binding proteins (GBPs), which are recruited to pathogen-containing vacuoles and are involved in parasite clearance. The function of IRGs and GBPs depends on the autophagy protein Atg5.

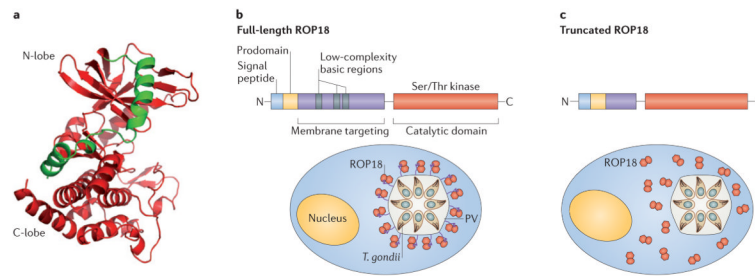


Figure 3. Structure and function of ROP2 family members

a) Model of the kinase domain of ROP18 based on homology to the X-ray crystal structure of the pseudokinase ROP8 (pdb 3BYV). The two N-terminal helices (green) are important for stabilization of the kinase domain (red) and are also involved in auto-activation of ROP18. The N- and C-lobes of the kinase domain are labelled, while the N-terminal extension involved in membrane tethering, is absent from the structure. Provided by Ray Hui, Structural Genomics Consortium, Toronto. b) Domain structure of ROP2 family members showing the signal peptide and prodomain, both of which are processed prior to secretion (arrows). The N-terminal half of the mature protein (green) contains a series of three amphipathic alpha helical regions with low sequence complexity (grey boxes) that mediate membrane targeting. The C-terminal region contains a S/T kinase domain (red). Localization of full-length ROP18 on the membrane of the parasite containing vacuole is mediated by the N-terminal amphipathic region (green). Deletion of this region disrupts vacuole association of the kinase domain (red), as shown for the truncated version of ROP18.

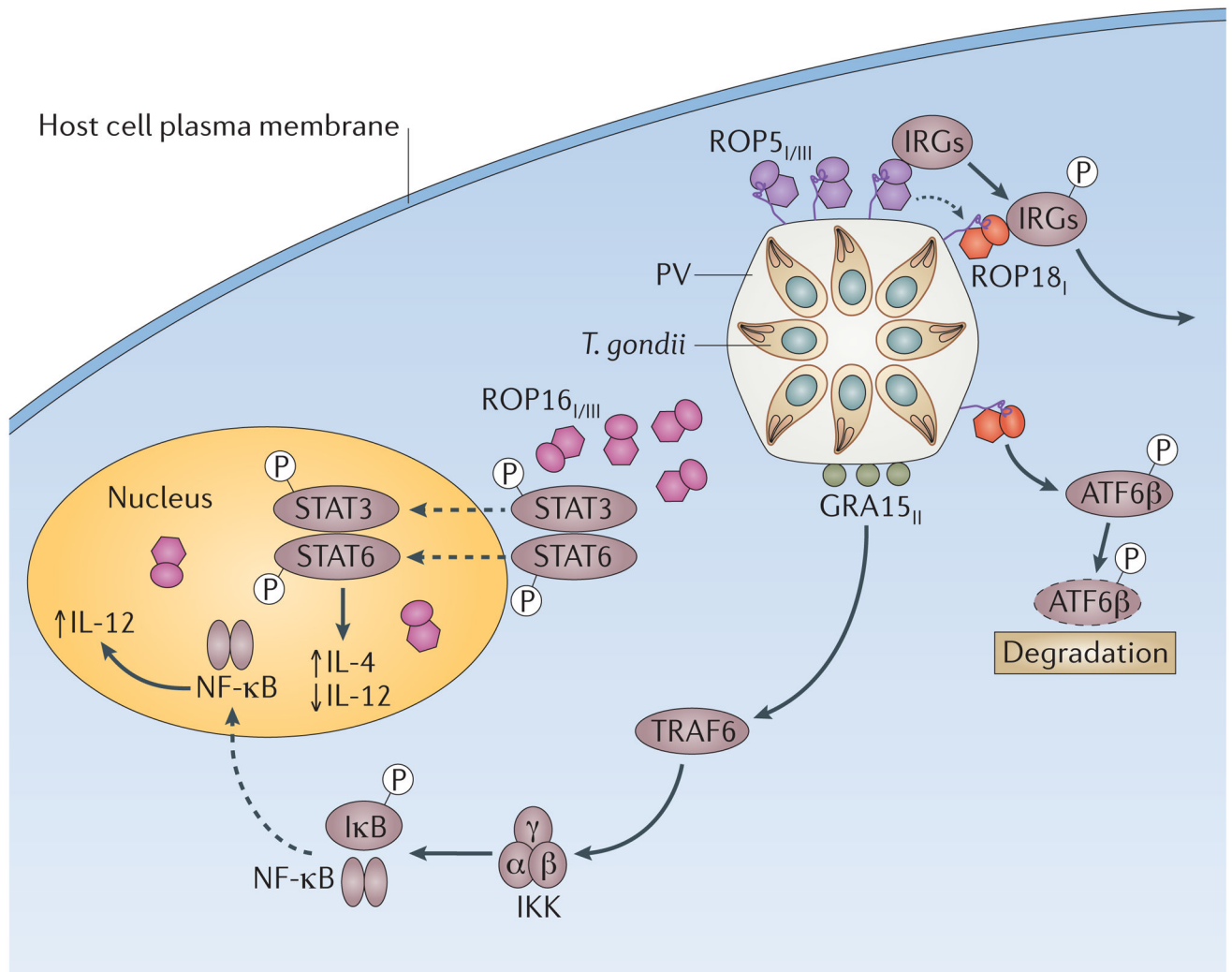


Figure 4. The role of *T. gondii* virulence factors in modulating immune signalling in the host ROP16_{I/III} (pink) phosphorylates STAT3 and STAT6 resulting in prolonged activation of these two transcription factors and subsequent upregulation of IL-4 while antagonizing induction of IL-12. The dense granule protein GRA15_{II} (green) activates TRAF6, which in turn activates IKK, leading to phosphorylation of IκB and the release of NFκB following proteasomal degradation. NFκB migrates to the nucleus and drives the production of IL-12. ROP18_I (red) is localized on the cytoplasmic side of the parasite containing vacuole (PV). ROP18_I has been shown to phosphorylate immunity related GTPases (IRGs), thus blocking their accumulation on the vacuole and protecting the parasite from destruction. ROP18_I also phosphorylates the host transcription factor ATF6β, which is involved in the unfolded protein response and may also be important for efficient antigen presentation by DCs. Genetic evidence suggests that ROP5_{I/III} (purple), a pseudokinase important for virulence, regulates the functions of ROP18_I. Allele types are shown in subscript (i.e. ROP_I denotes the allele in type I strains).

Table 1

Strain-specific virulence factors and their effects on the mouse immune response

Strain	ROP18	ROP5	ROP16	GRA15	Effects on host	Phenotype
I	Type I Virulence enhancing	Type I/III Virulence enhancing	Type I/III Prolonged activation of STAT3/6	Type I/III Non-activating	Prolonged STAT3/6 activation Reduced IL-12 production Avoidance of parasite clearance	High virulence
II	Type II Virulence enhancing	Type II Avirulent	Type II Non-activating	Type II Activates NFκB	Non-sustained STAT3/6 activation High levels of IL-12 Enhanced parasite clearance	Intermediate virulence
III	Type III Hypomorphic	Type I/III Virulence enhancing	Type I/III Prolonged activation of STAT3/6	Type I/III Non-activating	Prolonged STAT3/6 activation Reduced IL-12 production Enhanced parasite clearance	Low virulence