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2 # *Toxoplasma gondii* effectors are master regulators of the inflammatory response
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8 Authors: Mariane B. Melo*, Kirk D.C. Jensen* and Jeroen P.J. Saeij.

9 *These authors contributed equally.

10

11 Department of Biology, Massachusetts Institute of Technology, Cambridge, MA

12

13 *Corresponding author:* Saeij, JPJ (jsaeij@mit.edu)

14

1 **ABSTRACT**

2 *Toxoplasma* is a highly successful parasite that establishes a life-long chronic infection. To do
3 this it must carefully regulate immune activation and host cell effector mechanisms. Here we
4 review the latest developments in our understanding of how *Toxoplasma* counteracts the host's
5 immune response, and in some cases provokes it, through the use of specific parasite effector
6 proteins. An emerging theme from these discoveries is that *Toxoplasma* effectors are master
7 regulators of the pro-inflammatory response, which elicits many of the host's toxoplasmacidal
8 mechanisms. We speculate that combinations of these effectors present in certain *Toxoplasma*
9 strains work to maintain an optimal parasite burden in different hosts to ensure parasite
10 transmission.

11

The immune response to *Toxoplasma gondii*

Toxoplasma gondii is an obligate intracellular parasite that can invade and replicate in almost all nucleated cells of warm-blooded animals [1]. It has a world-wide geographic distribution and is known to infect many species of birds and mammals, including approximately one third of humans [2]. Although the majority of infected healthy individuals have no symptoms, in immunocompromised people or in congenitally infected individuals infection can cause severe disease or even death often caused by damage to the brain, eyes or other organs [2].

Toxoplasma host cell invasion is an active, parasite-driven process [3] that leads to the formation of a specialized non-fusogenic compartment, termed the parasitophorous vacuole (PV) [4]. The invasion process is accompanied by a sequential discharge of parasite proteins from apical secretory organelles called micronemes, rhoptries and dense granules [5]. Proteins secreted from the micronemes are involved in the initial attachment and invasion, while dense granule and rhoptry proteins convert the host cell into a suitable environment for parasite growth by modulating a variety of host processes [6]. It is perhaps not surprising that many of the most polymorphic proteins in the *Toxoplasma* genome are secreted factors that interact with the host cell [7]. Over the past five years it has become increasingly clear that these effectors manipulate host resistance mechanisms at multiple points along the pro-inflammatory pathway (Figure 1). Host control of *Toxoplasma* depends on the production of the pro-inflammatory cytokine interleukin 12 (IL-12) [8], which is produced by macrophages and dendritic cells (DCs) in response to Toll like receptor (TLR) recognition of molecular structures broadly conserved across microbial species (Box 1) (recently reviewed in Ref. [9]). IL-12 in turn activates NK and T cells to secrete interferon γ (IFNγ) [10]. The latter activates effector mechanisms for intracellular elimination of *Toxoplasma*, including the activation of interferon-regulated GTPases (IRGs, see Glossary) [11, 12], induction of reactive nitrogen intermediates [13], tryptophan degradation in human cells [14], and autophagy [15, 16] (Figure 1). The inflammasome (Box 1) has recently gained attention, as defects in this pathway are associated with uncontrolled parasite growth

[17, 18]. Inflammasome activation culminates in the release of IL-1 family members, including the pro-inflammatory cytokines IL-1β and IL-18. When produced in excess, pro-inflammatory cytokines end up damaging the host [19-21], showing that a delicate balance between pro- and anti-inflammatory signals is necessary to guarantee survival of both the host and parasite. Our recent understanding of how *Toxoplasma* effectors determine virulence and their mechanism of action reveal that *Toxoplasma* effectors are specifically aimed at modulating inflammatory pathways, which in turn dictate parasite burden and disease.

Strain-specific modulators of host immune responses

The majority of *Toxoplasma* strains found in North America and Europe can be grouped into three main clonal lineages (types I, II and III) that differ genetically by 1% or less [22] (Box 2). Most of what is known regarding the immune response against *Toxoplasma* is based on data obtained from laboratory mice infected with parasites from these three haplogroups. In laboratory mice, type I strains are categorically lethal, with an $LD_{100} = 1$, whereas the LD_{50} of type II and III strains are ~10^3 and 10^5, respectively [22, 23]. Genetic mapping of the virulence of F1 progeny (*Toxoplasma* is a haploid organism) derived from type I x II, I x III and II x III crosses [24-26] has identified the genetic loci that control this phenotype, and subsequent experiments have identified the causative genes within these loci. It is important to note that these analyses could not identify non-polymorphic *Toxoplasma* genes that determine virulence. Furthermore, it is currently unknown whether the identified polymorphic effectors are operative in species other than laboratory mice, as will be discussed below.

ROP18

Genetic mapping of virulence in F1 progeny from a I x III cross identified a single locus which encodes a rhoptry protein kinase, ROP18 [24, 25]. ROP18 belongs to the ROP2 family of *Toxoplasma* kinases, and rapidly co-localizes to the parasitophorous vacuole membrane (PVM)

following infection [24, 27]. Type III strains express extremely low levels of ROP18 due to an extra 2.1 kb sequence 85 bp upstream of the ATG start codon, and addition of a type I copy of the *ROP18* locus to the type III strain (III+ROP18$_{I}$) makes it as virulent as type I parasites [24]. Type III+ROP18$_{I}$ parasites have an increased replication rate in human foreskin fibroblasts (HFFs) [27] and are protected from IFNγ-mediated killing by mouse macrophages [28]. ROP18 can phosphorylate the nucleotide binding site of the switch loop 1 of Irga6 and Irgb6, mouse p47 IRGs which coat the PVM and are crucial for IFNγ mediated killing of *Toxoplasma* [28, 29]. Phosphorylation of the critical threonine residues (T102 and T108) destabilizes Irga6 by preventing subunit oligomerization and GTP hydrolysis, which in turn inhibits IRG accumulation on the PVM and protects the parasite from being destroyed [30]. Indeed, type I Δ*rop18* parasites have reduced numbers and delayed virulence in mice compared to the type I strain, but still kill 100% of infected mice [28, 29]. Type II parasites failed to significantly phosphorylate these p47 GTPases in IFNγ activated macrophages [29], which is surprising because ROP18 is functionally expressed in this strain and type II ROP18 can confer virulence to a type III strain. Thus it is likely that ROP18 works in concert with another polymorphic *Toxoplasma* effector (i.e. inactive in type II) to destabilize IRGs at the PVM. It is important to mention that humans have only two orthologous p47 GTPases, IRGC and IRGM, and both lack GAS and ISRE elements in their promoters, so they are not induced by IFNγ [31]. A splice isoform of human IRGM, IRGMd, localizes to the mitochondria and induces organelle fission and autophagy [32], which inhibits the intracellular survival of *Mycobacterium tuberculosis* [33]. Whether human IRGM has a role in *Toxoplasma* killing and is manipulated by ROP18, or whether ROP18 has another target in human cells is unknown.

Along this line, it was recently shown that ROP18 can phosphorylate the host transcription factor ATF6β, which is subsequently targeted for degradation via the proteasome, resulting in reduced ATF6β-mediated gene expression [34]. Importantly, ATF6 orthologs are present in humans, raising the possibility that expression of different ROP18 alleles by

1 *Toxoplasma* strains may also be relevant for the development of human disease. Interestingly,
2 ATF6β deficient mice died faster after infection with type I ∆*rop18* parasites, with kinetics similar
3 to wild type mice infected with the type I strain, suggesting a role for the ROP18-mediated
4 ATF6β degradation in *Toxoplasma* virulence. Although it is not clear how ATF6β affects parasite
5 virulence, the observation that dendritic cells from ATF6β deficient mice have a reduced ability
6 to elicit IFNγ production by CD8+, but not CD4+ T cells suggests that ATF6β may play a pivotal
7 role in controlling the MHCI antigen presentation pathway. This is possibly through ATF6β
8 modulation of expression of ER-associated degradation (ERAD) system components, which
9 have been shown to be necessary for cross-priming of CD8+ T cells [34, 35]. The N-terminal
10 portion of ROP18 (N2), which mediates the interaction with ATF6β, was necessary for full
11 virulence of ROP18, suggesting a role for this region and binding of ATF6β. However,
12 interpretation of this result is difficult because this N-terminal region has also been shown to be
13 essential for ROP18 localization to the PVM [36]. Therefore, abrogated ∆N2-ROP18 PVM
14 localization could result in reduced protection against PVM localized IRGs. Further analysis of
15 ROP18's interaction with its ligands Irga6, Irgb6 and ATFβ, will shed light on its precise
16 mechanism during *Toxoplasma* infection.
17
18 **ROP5**
19 ROP5 represents a cluster of tandem, duplicated polymorphic pseudokinases that
20 dictate virulence in mice [37]. ROP5 accounts for approximately 90% of the variance in
21 virulence between F1 progeny derived from the I x II and II x III crosses [25, 26, 37]. Types I, II
22 and III strains have divergent isoforms present in different numbers, although three major
23 isoforms (A, B and C) appear to exist in each strain [37]. The virulent isoforms are expressed in
24 the virulent type I and avirulent III strains, suggesting that ROP5 requires at least another factor
25 not present in the type III strain to elicit virulence in mice. Although type III strains

complemented with $ROP18_I$ are as virulent as type I strains, ROP5 contributes to virulence in strains that do not express ROP18 [25, 37], suggesting an independent mode of action. Strikingly, knockout of the entire *ROP5* locus makes the highly virulent type I strain avirulent, as 10^6 parasites do not kill any mice [26, 37]. Apparently a single copy of *ROP5* is enough to partially restore virulence in the Δ*rop5* type I strain, as complementation with one copy of *ROP5* (from type III strains) is able to partially restore virulence. Different ROP5 isoforms vary in the magnitude of their effect. Whereas 20% of infected mice survive a dose of 10^3 parasites transgenically expressing two copies of allele $ROP5A_{III}$, *Toxoplasma* expressing one copy of $ROP5A_{III}$ and $ROP5B_{III}$ are 100% lethal [37].

While it is clear that ROP5 is a major virulence determinant for *T. gondii* strains, its function remains to be determined. ROP5 lacks the catalytic 'HRD' motif that is critical for phosphotransferase activity [38], and although polymorphisms in ROP5 cluster in the pseudokinase domain, none of the variants have a predicted active catalytic site. Nevertheless, the ROP5 catalytic loop contains an 'HGB' motif ('B' denoting any basic residue) that is conserved between other *Toxoplasma* pseudokinases [39]. Complementation of ROP5 knockout parasites with a mutant copy of $ROP5A_{III}$ in which the basic residue (Arg) in its HGB motif was replaced by an acidic residue (Asp) only partially restored parasite virulence, suggesting that the conserved 'pseudokinase motif' has functional significance [39]. Differences between alleles are also found in substrate recognition domains of the kinase [26], suggesting that ROP5 variants might have different binding partners, which could possibly be relevant for virulence.

ROP5 does not seem to affect invasion, PV formation, nutrient acquisition, parasite replication or egress, as no detectable growth phenotype was observed during *in vitro* cultivation of Δ*rop5* parasites [26, 37]. Since ROP5 is secreted during *Toxoplasma* invasion and associated with the cytosolic face of the PV [40], it may serve as a scaffold or an adaptor protein, bridging together enzymes and substrates that modulate cell signaling pathways,

perhaps even associating with other parasite effector proteins. The presence of tandem copies of different alleles of ROP5 might also affect the ability of the parasite to adapt to hosts other than mice.

ROP16

Whereas the aforementioned virulence factors have not been shown to affect the host transcriptional response, polymorphisms in the rhoptry kinase ROP16 and the dense granule protein GRA15 (see below) together account for approximately 50% of the transcriptional response differences of HFFs or mouse macrophages infected with type II or III strains [41-43]. ROP16 was inferred as a virulence determinant by mapping virulence QTLs of the II x III F1 progeny and was the major determinant controlling the transcriptional response differences of HFFs to type II and III infections [41]. Subsequent bioinformatic analyses indicated that ROP16 might influence the JAK/STAT pathway, and indeed, *in vitro* studies determined that type I and III ROP16, but not type II ROP16, can maintain constitutive activation of STAT3 and STAT6. Recently, direct (Tyr 641) tyrosine phosphorylation of recombinant STAT6 by recombinant ROP16 was clearly observed [44]. Similarly, STAT3 Tyr705, but not the Ser727 residue, was phosphorylated by ROP16 in an *in vitro* kinase assay [45]. Interestingly, a single ROP16 polymorphism at position 503 (Leu to Ser) renders type II ROP16 unable to efficiently activate STAT3. *In silico* modeling of this mutation predicts that the serine residue would narrow the cavity of the ROP16 kinase pocket and possibly weaken interactions with its substrate [45]. The region between amino acids 220 and 303 is required for ROP16 binding to STAT3 by immunoprecipitation [45] and represents one of the most polymorphic regions of ROP16 (http://toxodb.org/). It is possible that ROP16 has other targets since it is found in the nucleus of the host cell, and many of the genes regulated by ROP16 lack known STAT transcription factor binding elements. Following oral infection in susceptible mice, type II strains that transgenically express the type III or I versions of ROP16 quell intestinal inflammation which normally occurs

following infection with the parental type II strain [43]. How ROP16 affects virulence is currently unclear. However, ROP16 can suppress the IL-12 response of infected macrophages stimulated with the Toll-like receptor 4 (TLR4) agonist lipopolysaccharide (LPS) [41] and inhibit NF-κB transcriptional activity [42]. Whether this inhibition reflects the ability of ROP16 to activate STAT3, a known inhibitor of NF-κB activation, remains to be determined. A down-stream consequence of STAT6 activation in infected macrophages is the induction of the alternative activation program [43], which importantly, can inhibit pro-inflammatory responses.

GRA15

Like many pro-inflammatory cytokines, IL-12 synthesis requires the activity of the transcription factor NF-κB [46]. NF-κB modulation by *Toxoplasma* has been the focus of several studies (reviewed in Ref. [47]). Important strain-specific differences in NF-κB signaling were observed in murine bone marrow-derived macrophages and peritoneal exudate cells; type II parasites were shown to induce higher levels of NF-κB activation and IL-12 production, compared to type I strains [48]. These findings were recently expanded upon by showing that type III parasites are also weak inducers of NF-κB and strain differences in NF-κB activation can be reproduced in a variety of murine, human and rat cell lines [42]. Using the F1 progeny derived from the type II x III cross, the locus responsible for this difference was mapped and found to encode a secreted dense granule protein named GRA15 [42]. Type II GRA15 mediates RelA/p50 NF-κB heterodimer translocation into the host nucleus, ultimately activating transcription of genes involved in pro-inflammatory responses. Proof that GRA15 is indeed responsible for strain-specific NF-κB activation was obtained by generating a variety of $GRA15_{II}$ knockout and transgenic parasite strains and observing that NF-κB activation followed the expression of $GRA15_{II}$ in these strains. In fact, HeLa cells transfected with $GRA15_{II}$ induced the activation of host NF-κB demonstrating that $GRA15_{II}$ does not require other *Toxoplasma* effectors or the PVM to mediate NF-κB activation [42]. Furthermore, type II GRA15 knockout

1 parasites induced significantly less IL-12 production, both *in vitro* and *in vivo*, and have a growth
2 advantage when compared with wild type parasites, most likely through reduced induction of
3 IFNγ [42]. The host cell interaction partner of GRA15 has not yet been identified, but its ability to
4 activate NF-κB requires the IKK complex, TRAF6 and is independent of MyD88, signifying the
5 direct activation of this pathway is independent of TLR recognition.
6
7 **ROP38**

8 Based on genome-wide expression profiling of tachyzoites, *ROP38* gene expression
9 was reported to be considerably higher in the type II and III strains (eightfold) when compared
10 to the type I strain [7]. ROP38 is a putative functional kinase with a predicted signal peptide,
11 and was observed both inside rhoptries and associated with the PVM [7]. A type I strain was
12 generated with an additional copy of *ROP38* under the control of the β-tubulin promoter; thus
13 expression of ROP38 became similar to that observed in the type III strain. Transgenic
14 parasites displayed a markedly reduced ability to upregulate host gene expression *in vitro* when
15 compared to the wild type strain, suggesting that ROP38 may have an inhibitory effect on host
16 cell transcription [7]. Although several of the affected genes are modulated by mitogen-
17 activated protein kinase (MAPK) signaling cascades, a direct correlation between ROP38 and
18 MAPK function remains to be evaluated.
19
20 **Other *Toxoplasma* proteins that influence virulence**

21 Another set of proteins that are potentially important for determining *Toxoplasma*
22 virulence are the secreted nucleoside triphosphate hydrolases (NTPases). These enzymes are
23 immunogenic antigens in both humans and mice [49, 50], and exist in two isoforms [51]. Virulent
24 strains of *Toxoplasma* have both NTPase-I and II isoforms, while nonvirulent strains have only
25 isoform II [51]. The role of these enzymes in immune modulation has not been investigated. It is
26 known that binding of ATP to the purinergic receptor $P2X_7$ and the subsequent efflux of

intracellular K^+ are necessary for the activation of the Nlrp3 inflammasome and assembly of the pyroptosome [52, 53]. Activation of the $P2X_7$ receptor *in vitro* triggers the elimination of intracellular parasites by infected macrophages [18, 54], possibly through induction of pyroptosis [55]. Based on this data it is reasonable to infer that the *T. gondii* NTPases [51] can be tools secreted by the parasite to dampen inflammasome activation, thereby inhibiting pyroptosis-mediated parasite killing and reducing levels of the pro-inflammatory cytokines IL-1β and IL-18.

Based on the assumption that not all virulence factors are polymorphic, a forward genetic screen of a library of insertional mutants was used to find determinants that subvert host effector mechanisms. From these efforts a patatin-like phospholipase protein, that localized to an unidentified structure within the parasite cytoplasm, protected *Toxoplasma* from the degradative effects of nitrogen oxide (NO) in activated macrophages [56]. Similarly, a transmembrane protein expressed on the outer membrane of *Toxoplasma* also inhibited the toxoplasmacidal effects of NO and promoted cyst formation [57]. Other factors that promote cyst formation include the GRA6 and GRA4 dense granule proteins, possibly through their effect on the nanotubular network present inside the PVM [58]. Furthermore, in a search for parasite factors responsible for IL-12 induction in murine DCs, a stimulatory molecule from parasite extracts was isolated and identified as a profilin-like protein, TgPRF, which is recognized by TLR11 [59]. TgPFR, is conserved between type I, II and III strains (http://toxodb.org/), which suggests that activation of DCs by profilin is not involved in strain-specific modulation of immune responses. Nevertheless, because profilin is an intracellular actin-binding protein that likely gets released after destruction of intracellular parasites, for example by the IRGs, strain differences in resistance to host toxoplasmacidal activities might result in relative differences of profilin release and subsequent differences in TLR11 stimulation and immune activation.

***Toxoplasma* effectors modulate pro-inflammatory responses**

So how might the aforementioned *Toxoplasma* effectors work to achieve chronic infection? At a glance, it appears that most of these effectors are either directly involved with inhibiting downstream toxoplasmacidal mechanisms of IFNγ or at least capable of manipulating Th1 responses through regulation of IL-12 (Figure 1). Added to these observations is the known phenomenon that cells infected with *Toxoplasma* cannot be stimulated with IFNγ to activate STAT1 [60], an IFNγ-activated transcription factor that induces many of the genes involved with killing *Toxoplasma* (iNOS, IRGs, autophagy, etc.). This inhibition is independent of the strain type and the parasite factors involved have remained elusive. Although the mechanism of ROP5 remains unclear, ROP5 likely controls some aspect of the host's toxoplasmacidal mechanisms since ∆*rop5* strains are rapidly cleared from the host. Inhibition is not the only mode of inflammatory regulation by *Toxoplasma*, as $GRA15_{II}$ and TgPFR actually provoke the IL-12 response.

Therefore, different combinations of parasite factors that inhibit IFNγ-induced toxoplasmacidal mechanisms, such as ROP18 [28], as well as effectors that modulate signaling pathways which regulate cytokine production, such as STAT3/6 activation by ROP16 [41], NF-κB activation by GRA15 [42] and MAPK activation by ROP38 [7], could have profound implications for *Toxoplasma*-induced pathologies and strain-specific differences in parasite burden (Figure 2). Why certain strains have unique combinations of effectors that promote or inhibit inflammation remains an important and unresolved question, but likely reflects limitations of the mouse model and our narrow understanding of the driving forces that determine strain selection in nature (see below).

Concluding remarks

Although in the last decade there was a great advance in understanding how *T. gondii* modulates immune responses in the mouse model, little is known regarding the role of strain-

1 specific virulence factors in other hosts. *Toxoplasma*'s world-wide distribution and its ability to
2 chronically infect multiple animal species, including birds and mammals, raises the question of
3 how this unicellular organism manages to control immune responses of such different species.
4 In order to survive and propagate itself *Toxoplasma* has to convert to the encysted bradyzoite
5 stage, meaning that high virulence leading to killing of its host before chronic infection is not
6 advantageous from an evolutionary point of view. It has been argued that different *Toxoplasma*
7 strains and their effectors have co-evolved with different hosts in different niches [61]. For
8 instance, type I strains of *T. gondii* are super virulent in laboratory mice, which is attributed in
9 part to the evasion from IRG-mediated killing mechanisms by *Toxoplasma* type I ROP18.
10 However, IRG genes have high sequence diversity both within and between species, and some
11 genes were lost during evolution [31]. In nature, it is possible that type I and other super-virulent
12 parasites co-evolved in hosts which are naturally resistant to *Toxoplasma* or less dependent on
13 IRG mediated parasite-killing, such that the super-virulence observed in laboratory mice might
14 be an 'artifact' of its selection in another host. Alternatively, super-virulence might be a trait that
15 was selected to allow superinfection of chronically infected animals. This would increase the
16 chance of a feline becoming infected simultaneously with cysts from two or more strains by
17 eating a single super-infected animal resulting in recombinant F1 progeny, possibly with greater
18 fitness than either parent.
19 Complimenting this hypothesis, we recently argued that the host's macrophage
20 response is a niche that selects for strain-specific combinations of different *Toxoplasma*
21 effectors. For example, there is considerable mouse strain variation in the ability to generate
22 classically (M1) or alternatively (M2) activated macrophages [62]. M1 macrophages are driven
23 by Th1 type cytokines (e.g. IFNγ) and are implicated in cytotoxic and antimicrobial functions
24 against intracellular pathogens, including *T. gondii* [63, 64]. In contrast, M2 macrophages
25 develop in a Th2 cytokine environment (IL-4, IL-13) and secrete anti-inflammatory molecules
26 that can down-regulate Th1 immune responses [62]. Since GRA15 and ROP16 induce M1 and

M2 activation, respectively [43], strain-specific expression of these effectors may have evolved to counteract a host's predisposition to certain types of macrophage responses and toxoplasmacidal activities.

In the same line of reasoning, it was recently shown that human *NLRP1* single nucleotide polymorphisms (SNPs) are associated with susceptibility to congenital toxoplasmosis [17]. Downregulation of Nlrp1 expression in human cells leads to increased parasite numbers and cell death after *in vitro* infection with *T. gondii* [17]. Similarly, the innate resistance of the Lewis rat strain to toxoplasmosis is determined by a genomic locus that includes the *Nlrp1* gene [65, 66]. Another possible receptor involved in activation of the inflammasome following *T. gondii* infection is the $P2X_7$ receptor, as polymorphisms in the human *P2XR7* gene are associated with susceptibility to congenital toxoplasmosis [67]. Activation of the inflammasome may have different consequences depending on the host, as it seems to be deleterious in the mouse model [68], but may help to eliminate the parasite in rats and humans [18, 54, 66]. Besides host differences, it remains to be established if there are also strain specific *Toxoplasma* virulence factors that regulate inflammasome activation.

In conclusion, ending up in the wrong host could result in failure to establish chronic infection due to death of the parasite, as observed in resistant animal models, including Lewis rats [65], or death to the host, as observed in C57BL/6 following type II infection, but not in resistant BALB/c mice [69]. Under-activation of the immune response could also result in death of the host by excessive parasite burden, as observed in mice challenged with type I strains (Figure 2). Furthermore, relatively little is known regarding *Toxoplasma* effectors of the South American strain types IV through XIV, which should be an active avenue of future research. Even less is known regarding how any of these effectors interact with the parasite's definitive host, the cat. For example, do *Toxoplasma* effectors promote parasite sexual reproduction or feline survival following infection? From a clinical point of view it will also be important to establish if any of the aforementioned polymorphic effectors play a role in determining severity

1 of toxoplasmosis in humans. This might be achieved if polymorphic peptides from these
2 effectors contain B-cell epitopes which can be used to serotype strain-specific infections in
3 human patients [70]. The future of *Toxoplasma* research should reveal more interesting
4 parasite effectors that modulate the host's inflammatory responses and disease.

5

6

Box 1. Innate immune requirements for *Toxoplasma* infection.

The innate immune system is armed with a variety of receptors that recognize structures conserved among microbial species or released by damaged cells, named pathogen-associated or damage-associated molecular patterns (PAMPs or DAMPs) [71]. One class of these receptors, the Toll-like receptor (TLR) family, was initially claimed to be a major player in innate immune recognition of protozoan parasite infections, including toxoplasmosis [72]. Mice deficient in MyD88, an adaptor protein necessary for the function of all TLRs but TLR3 [71], display a complete loss in acute resistance to systemic and oral infection with *T. gondii* which was hypothesized to be due to defective IL-12 production [73, 74]. The primary source of IL-12 during systemic murine infection with *Toxoplasma* is dendritic cells (DCs) [75]. However, T cell expression of MyD88 is essential for resistance to *Toxoplasma*, and injection of IL-12 in mice that lack MyD88 in T cells does not rescue susceptibility [76]. Similarly, although 100% mortality is achieved in mice that lack MyD88 in their DCs, they die three weeks later than MyD88$^{-/-}$ animals following infection. Furthermore, none of the TLR single or double knockout mice tested to date are very susceptible to intraperitoneal (i.p.) *Toxoplasma* infection [77], suggesting another cell type (non-DC or -macrophage), and MyD88 associated receptors (non-TLR) might be similarly necessary for early host resistance to *Toxoplasma*.

The IL-1 family members could possibly fulfill this requirement. The IL-1 response requires the activation of a family of cytoplasmic innate NOD-like receptors (NLRs) that recognize different classes of DAMPs and PAMPs altogether (reviewed in Refs. [52, 53]). Some members of the NLR family, including Nlrp1, Nlrp3 and Nlrc4 (Ipaf), are known to be involved in the activation of caspase-1 through the formation of large multimolecular complexes called inflammasomes [52, 53]. Caspase-1 proteolytically converts the proforms of IL-1β, IL-18 and IL-33 into the bioactive cytokines [78]. Both IL-1β and IL-18 receptors use MyD88 as an adaptor, leading to NF-κB and MAPK activation, and subsequent IL-12 production by antigen-presenting cells [79]. In the presence of IL-12, both IL-1β and IL-18 potentiate NK-cell production of IFNγ

1 during *T. gondii* infection [80, 81]. Notably, IL-18 deficient animals experienced less morbidity
2 and intestinal pathology after oral infection [21], suggesting that parasite-induced IL-18
3 contributes to the immunopathology. IL-33 also appears to be produced upon infection with *T.*
4 *gondii* [82]. This cytokine is produced mainly by fibroblasts and endothelial cells, and its
5 receptor also transduces signal through a MyD88-dependent pathway, but instead of inducing
6 pro-inflammatory cytokines, it drives a Th2-type immune response [78]. Animal knockouts for
7 the IL-33 receptor protein ST2 develop greater brain pathology after *T. gondii* infection due to
8 augmented parasite burden and increased production of IFNγ, TNFα and induced nitric oxide
9 synthase (iNOS) [82]. Thus, it is clear that the ability of the parasite to initiate both pro- and anti-
10 inflammatory innate immune responses (through IL-1β and IL-18 or IL-33, respectively) can
11 determine the fate of the host.
12
13 **Box 2. Population structure of *Toxoplasma*.**
14 *Toxoplasma* is unique among the apicomplexans in that tissue cysts generated in
15 intermediate hosts are infectious to other intermediate hosts. Therefore sex in its definitive host,
16 members of feline species, is not obligatory. Moreover, because *Toxoplasma* is haploid and a
17 single strain can generate both micro- and macrogametes, self mating is very likely as the vast
18 majority of intermediate hosts harbor cysts from just a single strain. However, in the rare
19 occasion a feline gets infected with two distinct *Toxoplasma* strains sexual recombination can
20 occur, and up to 100 million highly stable oocysts can be generated. Animals ingesting these
21 oocysts can subsequently function to select the most successful of these genotypes. As
22 discussed in this review an important part of this selection is likely determined by both the exact
23 allelic combination of *Toxoplasma* effectors and the specifics of the immune system of the host
24 species. In Europe and North America the majority of isolates from humans and domesticated
25 animals belong to three clonal lineages, named type I, II and III [23]. Genotypes not belonging to
26 these three main lineages are predominant in South America [83-85] and are also often isolated

from non-domesticated animals [86]. Recently, a phylogenetic analysis of multiple *Toxoplasma* strains from different continents clustered the strains into 11 distinct haplogroups, including the type I, II and III strains. Remarkably, the three main clonal lineages, as well as many of the less common 'atypical' strains, appear to be the result of mixing of just four ancestral genotypes, resulting in limited allelic polymorphism among these strains [87].

Part of the variability of disease outcome in human infections may also be tied to the type of strain that causes the infection. In North America and Europe most human cases are due to type II strains. Type III strains appear to be more common in animals, and in general are not associated with disease while a relative oversampling of type I strains has been observed in severe congenital infections and in AIDS patients (reviewed in Ref. [88]). Interestingly, in South America *Toxoplasma* can cause disease, especially ocular disease, in otherwise healthy individuals resulting in a high prevalence of ocular toxoplasmosis [89, 90]. There is some evidence that this is associated with specific strains, mainly present in South America. Also in North America, only type I or atypical strains were found in non-immunosuppressed patients suffering from severe ocular toxoplasmosis [84, 91]. Thus different *Toxoplasma* strains seem to cause different pathology both in mice and humans. The molecular basis for these differences in mice is slowly being unraveled but remains largely unexplored in humans and other animal species. As indicated in this review the vast majority of our current knowledge of *Toxoplasma*'s interaction with the host immune system comes from infections of mice with the Euro American strain types. Certainly new and interesting biology will be discovered when the interaction between the non-canonical *Toxoplasma* strains and the immune system is studied.

Glossary

Autophagy – A cellular process by which the cell removes large damaged organelles, particulates and possibly *Toxoplasma* containing vacuoles for degradation via the lysosome.

CD4+ T cell – T cells that recognize peptides presented on MHCI, a complex which specializes in presenting peptides derived from extracellular antigens targeted to the lysosome via the phagocytic pathway.

CD8+ T cell – T cells that recognize peptides presented on MHCI, a complex which specializes in presenting peptides derived from intercellular compartments and pathogens.

GAS – Interferon gamma-activated sequence: promoter element.

GTPase – family of hydrolase enzymes that can bind and hydrolyze guanosine triphosphate (GTP).

IKK – IκB kinase, an enzyme complex that is part of the upstream NF-κB signaling cascade.

Inflammasome – a multiprotein oligomer responsible for the activation of pro-inflammatory caspases, including caspase-1.

IRG – Interferon-regulated guanine triphosphatases, a family of proteins that has been implicated in resistance to intracellular pathogens; sometimes called p47 GTPases to reflect their molecular weight.

ISRE – Interferon-stimulated response element: promoter element.

JAK – Janus kinases, a family of intracellular non-receptor tyrosine kinases that transduce cytokine-mediated signal.

LD_{100} – dose of parasites necessary to kill 100% of infected animals.

LD_{50} – dose of parasites necessary to kill 50% of infected animals.

MAPK – Mitogen-activated protein kinases, serine-threonine kinases that respond to extracellular stimuli and regulate various cellular activities, such as gene expression, mitosis, differentiation, proliferation, and cell survival/apoptosis.

MyD88 – Myeloid differentiation primary response gene 88, a universal adapter protein as it is used by all TLRs, except TLR3, to activate the transcription factor NF-κB.

1 Nanotubular network – a network of interconnecting nanotubules derived from the membrane of
2 the PVM and is maintained by the dense granule proteins GRA6 and GRA2.
3 NF-κB – Nuclear factor kappa B, a family of transcription factors that includes the proteins RelA
4 (p65), RelB, c-Rel, p50 and p52. These factors play a key role in regulating immune responses.
5 PAMP/DAMP – Pathogen or danger associated molecular patterns, molecular motifs associated
6 with groups of pathogens or non-infectious stimuli, for example cellular debris from dying cells,
7 that are recognized by cells of the innate immune system.
8 Pyroptosome – a large supramolecular complex composed of Pycard dimers that mediates
9 inflammatory programmed cell death (pyroptosis) through caspase-1 activation.
10 QTL – Quantitative trait locus, stretches of DNA containing or linked to the genes that underlie a
11 quantitative trait.
12 STAT – Signal transducers and activators of transcription, transcription factors activated by
13 Janus kinases that regulate several cellular processes, including growth, differentiation and
14 immune activation.
15 Th1 – CD4 T cells that produce IFNγ, IL-12 promotes their development *in vivo*.
16 Th17 – CD4 T cells that produce IL-17; combinations of IL-1β, IL-6, and TGFβ or IL-23 induces
17 their development; IL-17 promotes neutrophil homeostasis.
18 Th2 – CD4 T cells that produce IL-4; IL-4 promotes allergic responses and the host response to
19 worm infections
20 TRAF – TNF receptor-associated factors, a family of proteins primarily involved in the regulation
21 of inflammation, antiviral responses and apoptosis.
22
23

Figure Legends

Figure 1. Host cell responses that can be modulated by *Toxoplasma gondii*. (1) Toll-like receptors (TLRs) are activated upon recognition of pathogen associated molecular patterns (PAMPs). The main TLR ligand identified in *T. gondii* is a parasite profiling-like protein (TgPRF) that can bind to and activate TLR11 [59, 92]. *Toxoplasma* is also armed with molecules of glycosilphosphatidylinositol anchors (GPI) and glycoinositolphospholipids (GIPLs) that can be recognized by TLR2 and TLR4 [93]. (2) TLR engagement triggers MyD88-dependent signaling pathways that culminate with the activation of NF-kB. However, *T. gondii* strains that express the active form of the dense granule protein GRA15 are able to directly activate NF-κB through a MyD88-independent mechanism. (3) NF-κB activation leads to transcription of a series of pro-inflammatory genes, including genes for IL-1β, IL-12, IL-18, induced nitric oxide synthase (iNOS) and some NOD-like receptors (NLRs). Nevertheless, parasite ROP16 is able to suppress the IL-12 response of infected macrophages stimulated with the TLR agonists [41] and to inhibit NF-κB transcriptional activity [42], possibly due to its ability to phosphorylate and activate STAT3/6 [41], which dampens TLR-induced cytokine production. Parasite induced MAPK signaling pathways also modulate IL-12 production [94], and there is evidence that *T. gondii* ROP38 may regulate MAPK function [7]. (4) Binding of ATP to the purinergic receptor $P2X_7$ and the subsequent efflux of intracellular K^+ leads to activation of the inflammasome [52, 53]. Although it is not known if *Toxoplasma* infection affects $P2X_7R$ function, the parasite secretes nucleoside triphosphate hydrolases (NTPases) that could possibly control extracellular levels of ATP. (5) Inflammasome stimulation activates caspase-1, which cleaves the proforms of IL-1β and IL-18 generating bioactive cytokines. Both IL-1β and IL-18 receptors activate NF-κB and MAPK signaling and subsequent pro-inflammatory cytokine production. *Toxoplasma* is known to induce IL-1β and IL-18 secretion, both of which serve to amplify IFNγ production by NK cells [80, 81]. It remains to be elucidated if the parasite can directly activate the

inflammasome or modulate caspase-1 activity. (6) IFNγ binding to its receptor triggers the JAK/STAT pathway, leading to phosphorylation of STAT1. Phosphorylated STAT1 then dimerizes and translocates to the nucleus, leading to transcription of interferon-stimulated genes, including the transcription factor IRF1, class II MHC and interferon regulated GTPases (IRGs). Yet, *Toxoplasma* infected cells display a marked inhibition of STAT1 dependent transcription [60], and parasite secreted kinase ROP18 can phosphorylate and inactivate IRGs (7), preventing its accumulation on the parasitophorous vacuole membrane and protecting the parasite from IRG-dependent intracellular killing [28, 29]. Abbreviations: IRF1, interferon regulatory factor 1; JAK, Janus kinases; MAPK, mitogen-activated protein kinase; STAT, signal transducer and activator of transcription; ROS, reactive oxygen species.

Figure 2. Overview of how *Toxoplasma* strains modulate host immune pathways. Modulation of host cell signaling pathways requires the secretion of numerous parasite proteins from specialized secretory organelles called dense granules and rhoptries. At early time points, infection with type I parasites does not activate pro-inflammatory responses. The type I (RH strain) allele of GRA15 results in a truncated and non-functional protein, allowing a 'silent' infection without activation of NF-κB [42]. On the other hand, $ROP16_I$ induces sustained activation of STAT3 and STAT6, dampening the production of IL-12, IL-1β and IL-6 [41]. Together with the ability to reduce pro-inflammatory cytokine production, type I parasites express ROP5 alleles associated with high virulence [26, 37], and $ROP18_I$ phosphorylates IRGs blocking their recruitment to the PV, which is required for parasite destruction, permitting unrestricted parasite growth [28, 29]. Conserved parasite proteins secreted by infected cells, profilin and cyclophylin-18, are recognized by DCs via TLR11 and CCR5 respectively, leading to late NF-κB activation and production of IL-12, which in turn activates NK and T cells to secrete IFNγ [59, 95]. However, type I parasites also prevent activation of DCs [96], and by the time that

the pro-inflammatory response kicks in, host survival is already compromised due to uncontrolled parasite burden. Type II parasites are very effective in activating an early response. These parasites express the active form of GRA15, which activates NF-κB in the infected cells [42], and a less functional form of ROP16, which leads to a transitory activation of STAT3/6 [41]. As a consequence there is a massive production of pro-inflammatory cytokines early after infection. The environment induced by the parasite modulates activation of several T cell subtypes, mainly directing the response towards a Th1 type [97]. Aspects of the Th17 response to *Toxoplasma* seem to have opposite effects on host survival, mainly an IL-23 driven IL-22 response by CD4 T cells has a negative effect [98], while signaling through the IL-17 receptor can have a beneficial effect by lowering parasite burden [99]. Intracellular parasite growth is controlled due to expression of an avirulent form of ROP18, which does not block the recruitment of IRGs to the PV [28, 29], and type II parasites also express ROP5 alleles associated with low virulence [26, 37], but susceptible animals die of severe ileitis [69]. Like type I, type III secreted GRA15 and ROP16 do not activate NF-κB and induce a sustained activation of STAT3/6 respectively, limiting the initial production of pro-inflammatory cytokines [41, 42]. Nevertheless, these parasites express an inactive ROP18, being unable to avoid intracellular killing mediated by IRGs [28, 29]. In this case, late production of IL-12 by DCs triggers a Th1-type response that is sufficient to control parasite burden and induce cyst formation, leading to a chronic infection. CCR5, C-C chemokine receptor type 5; DCs; dendritic cells; GRA, dense granule protein; IRG, interferon-regulated GTPase; NK, natural killer cells; NO, nitric oxide; PV, parasitophorous vacuole; ROP, rhoptry protein; STAT, signal transducer and activator of transcription; ROS, reactive oxygen species, TLR11, Toll-like receptor 11.

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Figure 1

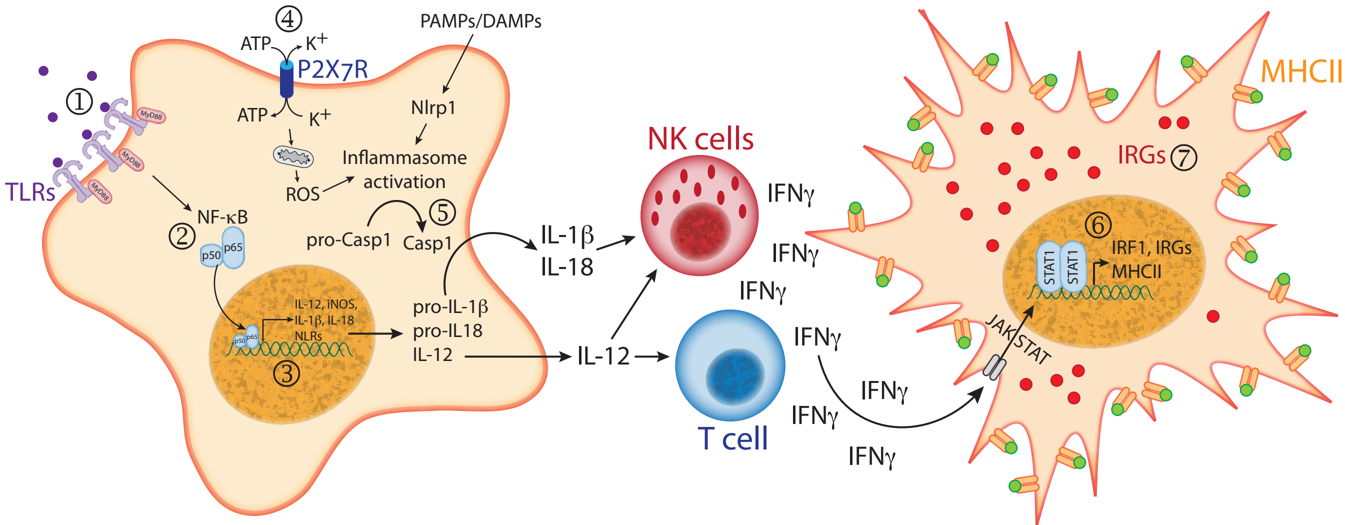

Figure 2