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Toxoplasma gondii secretory proteins and their role in invasion and pathogenesis

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ABSTRACT

T. gondii is a major opportunistic pathogen chronically infecting nearly one third of the world's population. Due to the high infection and mortality rates in immunocompromised patients and newborns, the extent or magnitude of *T. gondii* pathogenesis is determined mainly by host-pathogen interactions. *T. gondii* utilizes specialized secretory proteins to modify host cellular factors and facilitate invasion and replication. This review provides update on the recent progress in this field of research with particular emphasis on the *T. gondii* secretory proteins and their role in invasion and pathogenesis.

1. Introduction

Toxoplasma gondii, an obligate intracellular parasite of the apicomplexan family, infects more than two billion people worldwide (Black and Boothroyd, 2000; Tenter et al., 2000).

All members of the apicomplexa (such as *Babesia* spp., *Cryptosporidium parvum*, *Cyclospora cayetanensis, Eimeria* spp., *Neospora caninum, Plasmodium* spp., and *Theileria* spp.) are obligate intracellular parasites infecting a wide range of warm-blooded animals (Lorenzi et al., 2016; Bartošová-Sojková et al., 2015; Checkley et al., 2015). In humans, toxoplasmosis can cause serious complications particularly in fetuses (severe neurologic and ophthalmologic defects) and in immunocompromised patients (encephalitis and a leading cause of death) (Sterkers et al., 2012; Luft et al., 1984).

Members of apicomplexa share similar morphology, most of them harbor apicoplast, an organelle implicated in type II fatty acid synthesis which has been suggested to be a suitable target of novel drugs (Ramakrishnan et al., 2012). Conoids, organelles composed of micronemes (MICs), rhoptries (ROPs), polar rings, and a set of microtubules can be found in the apical regions of many apicomplexa (Tenter et al., 2000). Furthermore, some members of this family secrete a wide variety of organelles located at their apical end during pathogenesis. Recent study revealed that some of the endosomal tethering complexes (CORVET/HOPS complexes) are crucial for the biogenesis of the secretory organelles in *T. gondii* (Morlon-Guyot et al., 2018). As *T. gondii* harbors all the above features and is easily experimentally tractable (Tomita et al., 2013; Fox et al., 2011), it is an excellent model organism to study apicomplexan pathogenesis, whose investigation might shed light also into the biology of other members of this phylum. In this review, we provide an update on the recent progress in the *T. gondii* research with particular emphasis on the *T. gondii* secretory proteins and their role in invasion and pathogenesis. Special attention is paid to the studies in a mouse infection model and recent *in vitro* studies in particular of the tachyzoite stage of infection.

2. Life cycle of *T. gondii*

The life cycle of *T. gondii* is complex and can involve all warm-blooded animals as the intermediate hosts in addition to the definitive hosts (such as cats) (Tenter et al., 2000).

In the intermediate hosts, when under stress conditions, *T. gondii* can switch from the active, rapidly multiplying tachyzoites to the dormant, encysted bradyzoites, which are hard to eradicate by the immune system and drugs (Cleary et al., 2002; Watts et al., 2015). As in the intermediate hosts, *T. gondii* mainly hides in the brain and choroid-retinal region of the eye (Harker et al., 2015; Konradt et al., 2016; Arantes et al., 2015), potential drugs must cross multiple membrane barriers, such as the blood-brain barrier (BBB) and multiple layers of ocular tissues in addition to be able to enter the bradyzoites (Tenter et al., 2000).

In the definitive hosts, *T. gondii* undergoes both sexual and asexual phases while residing along intestinal linings. Following ingestion of

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bradyzoites by the definitive hosts, bradyzoites undergo multiple rounds of asexual reproduction prior to the sexual phase. Sexual reproduction leads to the production of unsporulated oocysts which after their release into the environment and sporulation generate mature oocysts (Dubey et al., 2012; Delgado Betancourt et al., 2019; Blader et al., 2015).

T. gondii can be disseminated by horizontal or vertical transmission. The former involves ingestion of mature oocysts or tissue cysts by the definitive or intermediate hosts, while the latter involves crossing the placenta and infecting the offspring by tachyzoites in definitive or intermediate host (Tenter et al., 2000; Dubey et al., 2012). Horizontal transmission by the ingestion of tissue cysts is particularly important for the infection of cats.

3. *T. gondii* secretory proteins and their role in the host invasion

T. gondii secretes a broad spectrum of proteins to infiltrate the host cells and to regulate the expression of host proteins, including MICs, ROPs and dense granules (GRAs) (Table S1) (Hakimi et al., 2017). MICs, ROPs and GRAs differ in their localization and their time of release from the cell (Alexander et al., 2005).

ROPs and MICs localize in the apical end of *T. gondii*, while GRAs are dispersed. ROPs can be further divided into two subclasses, namely the rhoptry neck proteins (RONs) and the rhoptry proteins (ROPs) based on their localization in rhoptry neck and rhoptry bulb, respectively (Gubbels and Duraisingh, 2012).

MICs play a role in motility and adhesion of *T. gondii*. MIC1, MIC2, MIC4, MIC6 and particularly MIC3 were shown to bind to a broad spectrum of targets, such as adolase, glucose, ICAM-1, lactose, and heparin (Azzouz et al., 2013; Meissner et al., 2002; Lourenço et al., 2001). Furthermore, secreted MIC2 is crucial for the motility of *T. gondii*.

Attachment of the parasite to the host by MICs is a highly synergistic process, where MIC1, MIC4, and MIC6 initially form a complex from which MIC6 is later cleaved to ensure MIC1 and MIC4 trafficking. MIC2 forms complex with MIC2-associated protein (M2AP), which are responsible for its delivery. Furthermore, MIC8 is a chaperone of MIC3 (Reiss et al., 2001; Jewett and Sibley, 2004). Synergy has been also shown between MICs and RONs, namely in the formation of the moving junction, a physical link which ensures internalization of *T. gondii* in the parasitophorous vacuole (Besteiro et al., 2011). MIC mainly implicated in this process dubbed the apical membrane antigen 1 (AMA1) interacts with RON2 to form a moving junction with RON2, RON4, RON5, and RON8 (Besteiro et al., 2011; Straub et al., 2011; Lamarque et al., 2011; Zhang et al., 2015).

ROPs are involved in the formation of the parasitophorous vacuole (Fig. 1) as evidenced by the localization of ROP2, ROP4, ROP5, ROP7, ROP8, and ROP18 at the parasitophorous vacuolar membrane (PVM) (Boothroyd and Dubremetz, 2008; Hajj et al., 2006). ROP2 has been implicated in the acquisition association of PVM with host's mitochondria (Sinai and Joiner, 2001). The association between mitochondria and the PV was shown to be dependent on the host cell type (Magno et al., 2005).

ROPs and GRAs are involved in the fine-tuning of immunological pathways with a significant impact on the virulence of various *T. gondii* strains (Fig. 2) (Behnke et al., 2016; Fox et al., 2016). Most *T. gondii* strains isolated in Europe and North America can be classified into three clonal lineages, named types I-III (Saeij et al., 2005). Type I *T. gondii* includes TgRsCr1, TgDogCo17, TgCtCo5, TgCkCr1, TgCkCr10, TgCATBr9, TgCatBr26, TgCatBr72, TgCkBr141, BOF, FOU, GT1, RH, RH-88, RH-JSR, and CAST strains. Type II *T. gondii* includes strains TgCat_PRC2, Guy-2004-JAG1, GUY-2004-ABE, SOU, Beverley, COUG, PRU, ARI, RAY, B41, B73 and ME49. Type III *T. gondii* includes strains TgCatBr3, TgShUS28, TgCatBr15, TgCkGy2, VEG p89, and ROD. Types I-III *T. gondii* lineages differ in their virulence, with type I being the most and type III the least virulent in a mouse infection model. In addition to lineages I-III, a number of other *T. gondii* strains were isolated (e.g. TgCatBR5, TgPxd, MAS, NT, and NY11) mainly in Asia, and South America and Africa (Tait et al., 2010) (Table S2).

ROP2 secretory protein superfamily includes pseudokinases ROP2, ROP4, ROP5, ROP7, and ROP8 and kinases ROP11, ROP16, ROP17, and ROP18 (Talevich and Kannan, 2013; Behnke et al., 2012; Qiu et al., 2009) which in combination with GRAs, in particular GRA15, modulate the innate immunity (Fig. 2).

The presence of the active or inactive pseudokinase ROP5 in the *T. gondii* types I and II, respectively, plays a crucial role in the determination of their virulence level (Etheridge et al., 2014; Shwab et al., 2016; Behnke et al., 2015). ROP5 has been shown as an allosteric inhibitor of the immunity-related GTPases (Reese et al., 2014).

An active threonine kinase ROP 18, whose expression is increased in the type I and II lineages plays an important role in the phosphorylation of the immunity related GTPases (IRGs), blocking their recruitment, and prevention of macrophage clearance of intracellular parasites (Fentress et al., 2010; Niedelman et al., 2012; Müller and Howard, 2016).

Pseudokinase ROP5 forms a complex with ROP18 and ROP17 to modulate IRGs (Etheridge et al., 2014). The polymorphism of *ROP5* was shown to play a role in the increased virulence of the type I and III lineages compared to that of type II (Shwab et al., 2016; Behnke et al., 2011). This is mainly due to the ability of ROP5 of the type I and III lineages to block oligomerisation of IRGs, thus allowing phosphorylation of their threonine residues by ROP18 (Reese et al., 2014).

Polymorphic kinases ROP16 and ROP18 were shown to be the key virulence factors of *T. gondii* involved in the difference in the virulence between types I and III lineages (Shwab et al., 2016; Behnke et al., 2015; Saeij et al., 2006; Sánchez et al., 2014). A single amino acid substitution at position 503 was shown to be responsible for the ROP6-mediated difference between *T. gondii* lineages. In types I and III lineages ROP6 was shown to constitutively phosphorylate and activate STAT3 and STAT6, enhance production of interleukin-4 (IL-4) and inhibit production pro-inflammatory IL-12 (Hunter and Sibley, 2012; Ong et al., 2010). Although in type II lineage, ROP16 also activates STAT3 and STAT6, type II variant is unable to sustain this response (Hunter and Sibley, 2012; Ong et al., 2010). Furthermore, type II lineage GRA15 variant is able to activate tumour necrosis factor receptor-associated factor 6 (TRAF6), which in turn activates IκB kinase leading to NF-κB (IκB) degradation inhibition. The resulting increased production of NF-κB enhances production of IL-12 production leading to the classical (M1) activation of macrophages (Rosowski and Saeij, 2012; Rosowski et al., 2011).

The three different *T. gondii* type strains modulate the host immune pathways by different mechanisms (Saeij et al., 2005; Hunter and Sibley, 2012). Type I parasites induce uncontrolled parasite growth and acute death. In this case, truncated and non-functional $GRA15_{I/III}$ does not activate NF-κB and pro-inflammatory cytokines such as IL-12. STAT3 and STAT6 are constitutively activated by $ROP16_{I/III}$, thus antagonising type I cytokines production (Rosowski et al., 2011). Notably, $ROP5_{I/III}$ is associated with high virulence. $ROP18_{I}$ of the type I parasites phosphorylates IRGs, thus blocking IRGs recruitment to parasitophorous vacuoles and inhibiting parasite clearance. Type II parasites express functional ROP18 but avirulent ROP5 which leads to ineffective phosphorylation of IRGs and parasite clearance prevention. In type II parasites, $GRA15_{II}$ activates NF-κB and subsequently expression of type I cytokines (Rosowski et al., 2011). STAT3 and STAT6 in type II parasites are not constitutively activated by $ROP16_{II}$ leading to pro-inflammatory NF-κB –mediated acute death. Type III parasites express ROP5 and ROP16 variant constitutively activating STAT3 and STAT6. $ROP18_{III}$ of type III parasites does not phosphorylate IRGs which are in turn recruited by parasitophorous vacuoles thus leading to chronic infection (Saeij et al., 2005; Hunter and Sibley, 2012).

A number of *T. gondii* secretory proteins (e.g. MIC1, ROP1, ROP2, ROP4, GRA1, GRA5, and GRA8) were shown to be involved in the

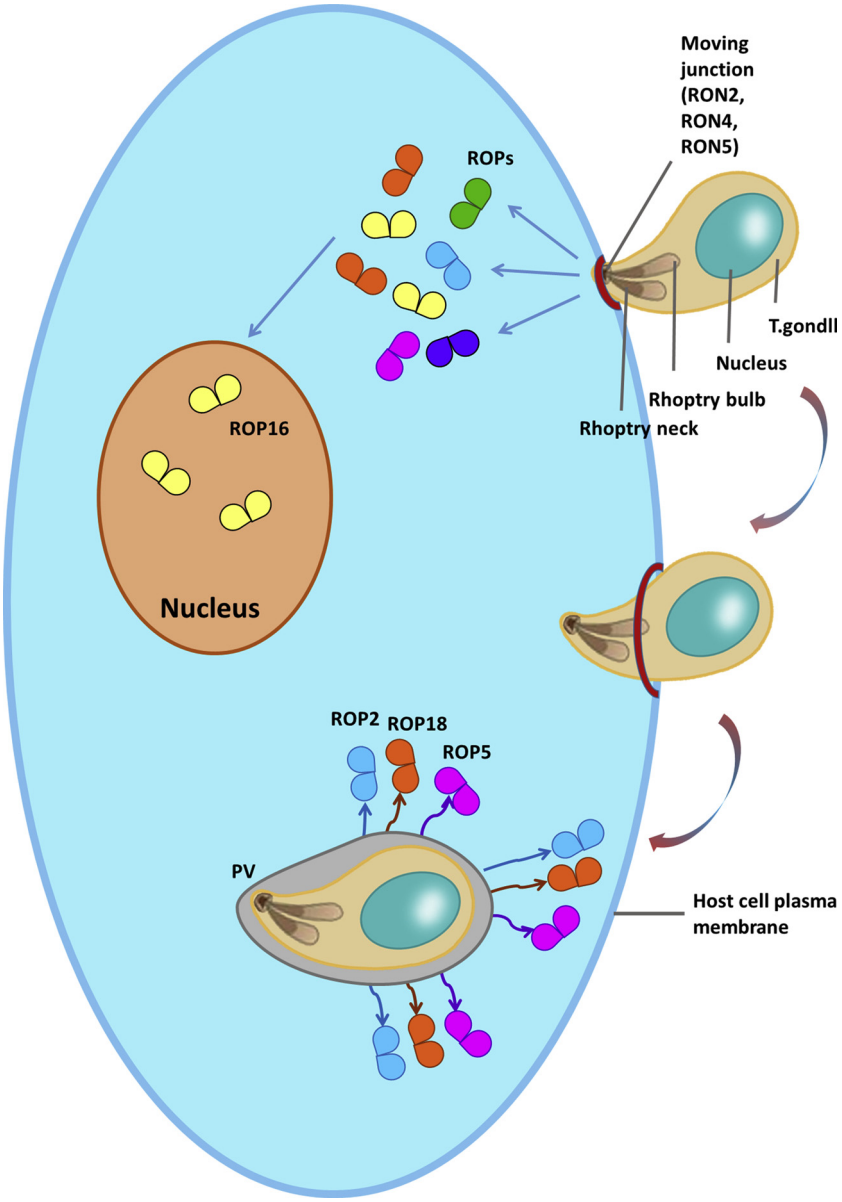

Fig. 1. RONs and ROPs dissemination. Following invasion, most RONs with MICs form the moving junction. Some ROPs (e.g. ROP2, ROP5, ROP18) fuse with the PVM, while others (e.g. ROP16), enter host nucleus to modify immune pathways (e.g. JAK-STAT pathways).

differentiation from tachyzoites to dormant bradyzoites in response to stress by microarrays (Cleary et al., 2002). In tachyzoites, GRA1-8 are all expressed and localised in the PV, while in bradyzoites GRA1, GRA3, GRA5, GRA6, and GRA7 localize at the wall where they associate with bradyzoite pseudokinase 1 (BPK1), which is a known component of the bradyzoite wall, suggesting that they are crucial for its integrity (Adjogble et al., 2004; Ferguson, 2004; Rommereim et al., 2016). Cross-linking, immunofluorescence and affinity chromatography studies showed that analogous to MICs and RONs synergies exist also between GRAs and other secretory proteins. This includes GRA4, GRA6 and GRA2 complex shown to associate with the intravacuolar network of parasitophorous vacuole, GRA1, GRA3 and GRA7 complex associating with ROP2 and ROP4 and GRA2, GRA5 associating with GRA3, GRA6, and GRA7 in parasite and PV (Dunn et al., 2008; Labruyere et al., 1999; Braun et al., 2008; Gold et al., 2015; Ma et al., 2014). This shows that interactions are common between *T. gondii* MICs, ROPs, and GRAs.

T. gondii invasion is a multistep process involving contact, attachment, parasite motility and penetration of the host cell (Jones et al., 2017a). The contact to the host cell is initiated by recognition of surface receptors by GPI-anchored surface antigens (SAGs) (Hehl et al., 2015). This reversible phase which allows *T. gondii* to survey for an optimal invasion condition is followed by apical attachment which involves discharge of adhesive proteins from *T. gondii* apical organelles (modulated by the increased calcium levels within *T. gondii*) (Nagamune et al., 2008). In this phase, MICs accumulate at the parasites' apical surface and RONs are secreted and associate with microneme-derived apical membrane antigen (AMA1) to form the moving junction (Sibley, 2011). Furthermore, ROPs are secreted and injected into PV and host cytoplasm (Boothroyd and Dubremetz, 2008). Adhesins and actin–myosin motor play a role in the motility of *T. gondii* in the host cell (Egarter et al., 2014; Frénal and Soldati-Favre, 2015; Drewry and Sibley, 2015). Asexual replication of *T. gondii* is localized into PV. Exit of *T. gondii* from the host cell is modulated by the increased calcium levels, powered by actin–myosin motor and accompanied by the secretion of MICs, RONs and ROPs (Sibley, 2011).

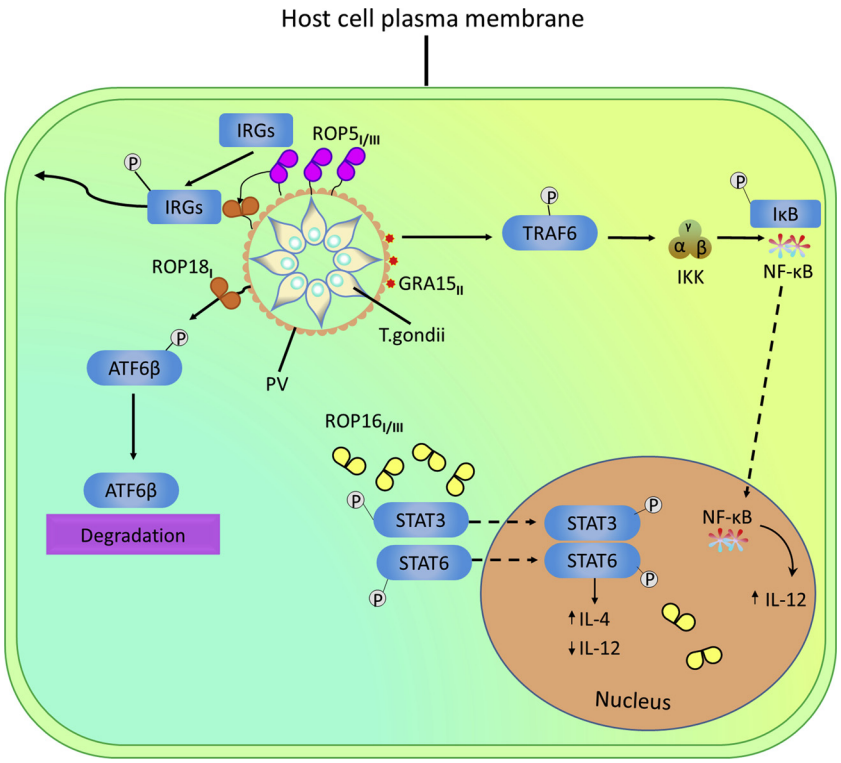

Fig. 2. Modulation of innate immunity by secretory proteins ROP5, ROP16 and ROP18. Phosphorylation of IRGs by ROP18 prevents macrophage clearance. Phosphorylation and activation of STAT3 and STAT6 by ROP16 leads to alternate activation of macrophages. In contrast, GRA15-mediated activation of the tumour necrosis factor receptor-associated factor 6 (TRAF6) activates in turn IκB kinase and leads to classical activation of macrophages.

4. Trafficking of *T. gondii* secretory proteins

T. gondii's secretory pathway is best characterised by its polarisation, where the endoplasmic reticulum (ER) is more concentrated posterior to the parasite nucleus, which is centrally situated. The ER is perinuclear, as the nuclear membrane extends continuously to the ER in close proximity (Sangaré et al., 2016; Ngô et al., 2000).

T. gondii secretory proteins (MICs, ROPs, GRAs) translocate from the lumen of the rough ER through the Golgi to the cell surface via the standard ER-Golgi pathway (Ngô et al., 2000). Translocation of MICs, ROPs and GRAs via ER mediated by their N-terminal signal sequences is followed by their transfer into ER-to-Golgi transport vesicles. In the Golgi apparatus all *T. gondii* secretory proteins translocate via the same pathway from the *cis* to the *trans* face of cisternae (Glick and Malhotra, 1998); however, the following route from the *trans*-Golgi to the parasite cell surface differs between MICs, ROPs, GRAs (Ngô et al., 2000; Venugopal et al., 2017; Dogga et al., 2017; Mercier and Cesbron-Delauw, 2015; Venugopal and Marion, 2018; Hammoudi et al., 2018) (Fig. 3). While GRAs are sorted by protein aggregation, and fusion with plasma membrane is mediated by the NSF/SNAP/SNARE/Rabs machinery, MICs and ROPs are sorted by tyrosine motif/adaptin/clathrin pathways (Fig. 3).

5. Conclusions and future directions

T. gondii is a major obligate intracellular pathogen infecting more than two billion people worldwide. MICs, ROPs and GRAs presented in this review play a crucial role in the pathogenicity of *T. gondii*. They were shown to modify the host's cellular functions to facilitate the invasion and replication of *T. gondii*. A number of ROPs (e.g. ROP11, ROP16, ROP17, ROP19/29/38, ROP20, ROP21/27, ROP25, ROP28, ROP30, ROP31, ROP32, ROP33, ROP34, ROP35, ROP39, ROP41 and ROP46) are proved or predicted to be active kinases, while others (e.g. ROP2/8, ROP4/7, ROP5, ROP22, ROP23, ROP26, ROP37, ROP40, ROP42/43/44, ROP47, ROP49 and ROP50) are predicted inactive pseudokinases (Talevich and Kannan, 2013; Qiu et al., 2009; Zhou et al., 2016; Ning et al., 2015).

ROP pseudokinases such as ROP2, ROP5 and ROP8 were shown to play a crucial role in the *T. gondii* virulence by interacting with other proteins (Talevich and Kannan, 2013). ROP5 was shown to adopt kinase activity by binding ATP in a non-canonical conformation, while the catalytic Asp in kinase-conserved "HRD" motif is replaced with a

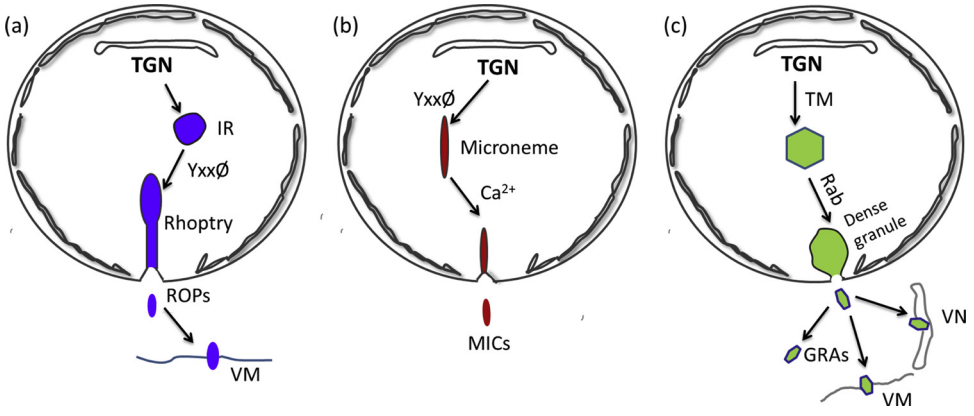

Fig. 3. Putative mechanisms of targeting, sorting, and secretion of dense granules, micronemes and rhoptries. Rhoptries (a) and micronemes (b) are sorted by tyrosine-based cytoplasmic signals (YxxØ); however, while the secretion of micronemes is calcium-dependent, that of rhoptries is not. Dense granules (c) are sorted by protein aggregation, and fused with plasma membrane with the help of the NSF/SNAP/SNARE/Rabs machinery. IR (immature rhoptry); MICs (micronemes); Rab (NSF/SNAP/SNARE/Rab); GRAs (dense granules); ROPs (rhoptries); TGN (*trans*-Golgi network); VM (vacuole membrane); VN (vacuole network).

basic residue in ROP4/7 (HGK), ROP5 (HG[R/K/H]), ROP22 (HTH), ROP36 (HGH), ROP40 (LRR) and ROP42–43-44 (HGK) (Talevich and Kannan, 2013). Furthermore, ROP5 and ROP18 share about 30% sequence identity (El Hajj et al., 2007).

Development of new detection tools and techniques targeting MICs, ROPs and GRAs will be crucial for the illumination of the *T. gondii* – host cell interactions. Recent studies suggest that *T. gondii* secretory proteins are promising targets for the development of novel vaccines and therapeutics (Chen et al., 2015; Naserifar et al., 2015; Rashid et al., 2017; Nabi et al., 2017; Zhang et al., 2016; Foroutan et al., 2019; Ahmadpour et al., 2017; Wang et al., 2019). As *T. gondii* secretory proteins play key roles in the *T. gondii* pathogenicity, developing new drugs targeting MICs, ROPs and GRAs will likely have profound therapeutic consequences *in vivo*.

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Declaration of interest

The authors report no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.06.003.

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