Editorial

Invasion and egress cascade in intracellular protozoa: Part 2 (*T. gondii*)

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As an apicomplexan member, *T. gondii* has a complex life cycle that involves multiplication within vertebrate and invertebrate hosts by specialized cell-invasive and egress life cycle stages, called zoites. They are unique eukaryotic cells with characteristic four main sub-cellular structures. They include a specific inner membrane complex beneath the plasma membrane, an apical "conoid" to sustain parasite micro-tubular cytoskeleton, a plastid responsible for lipids synthesis, and specific secretory organelles, micronemes (MICs), rhoptries (ROs) and dense granules (DGs). The last is involved in maturation of the parasitophorous vacuole (PV), where the parasite multiplies; the first essential step after invasion and before egress. Similar to *Plasmodium* spp., successful invasion and egress cascade depends mainly on efficient rapid invasion without alteration of host cell cytoskeleton, and multiplication within host cells inside its PV. However, *Plasmodium* spp. export proteins into host cell cytoplasm and plasma membrane utilizing PV as a trafficking vehicle. Instead, PV of *T. gondii* zoites utilizes abundantly expressed DGs and ROs proteins to build up the intra-vacuolar membranous network (IVMN) for trafficking. The present editorial aims to clarify the roles of proteins released from MICs, ROs and DGs in invasion and egress cascade of *T. gondii*.

Keywords: AMA1; apical organelles, egress; invasion; *T. gondii*; trafficking network, virulence.

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Abbreviations: AMA: Apical membrane antigen; **DGs:** Dense granules; **GRAS:** DGs expressed proteins; **IVMN:** Intra-vacuolar membranous network; **MICs:** Micronemes; **Myc:** Regulatory genes family coding transcription factors; **MYR:** Myc regulatory protein; **PLPs:** Perforin-like proteins; **PV:** Parasitophorous vacuole; **PVM:** Parasitophorous vacuole membrane; **ROs:** Rhoptries; **ROPs:** Rhoptry bulbous proteins; **RONs:** Rhoptry neck proteins; **STAT:** Signal transducer and activator of transcription protein.

Apical secretory organelles

Micronemes (MICs): These organelles are restricted to the apical third of *T. gondii* life cycle stages. Their released proteins are essential for host cell invasion and actin-based gliding motility. The latter is activated by elevated cytosolic calcium concentrations. Meanwhile, mobilization of intracellular calcium ions also triggers release of MICs proteins that mediate parasite attachment. Besides, MICs released proteins are distributed on zoites surface triggering the sub-membranous acto-myosin motor required for gliding motility. Essential MICs proteins established for host cell attachment include *Tg*MICs 1-4 that contribute to the establishment of connection between host cell surface receptors and zoites cytoskeleton machinery activating the gliding motility[1]. The transmembrane *Tg*MIC6 is an example of an escort protein for soluble MIC proteins such as MIC1 and MIC4 forming a three-polymer complex. This was confirmed by deletion of MIC6 preventing the function of these two proteins[2]. Besides, *Tg*MIC8 accompanies *Tg*MIC3, and is also required for RO neck protein (RON4) expression[3]. Other identified *Tg*MICs proteins without adhesive motifs include *Tg*MICs 5, 10 and 11[1].

Apical membrane antigen 1 (*Tg*AMA1) was identified in *T. gondii*. In contrast to *Plasmodium* AMA1, localized as RO antigen, *Tg*AMA1 release was observed from tachyzoites MICs. *In vitro* studies showed significant enhanced secretion with increase of intracellular calcium levels[4]. Later, *Tg*AMA1, displayed on tachyzoite surface, interacts with RON2 secreted in host cytosol, to maintain the structural integrity of the moving junction through which invading tachyzoites penetrate host cell. However, deletion of the genes encoding *Tg*AMA1 and *Tg*RON2 did not lead to failure of intracellular invasion. Accordingly, the investigators hypothesized alternative release of AMA1 homologues (AMAs 2-4) and contribution of RONs 4 and 5 instead of mutant RON2[5]. Additionally, as previously discussed[6], MICs release actinomyosin on zoites surface membrane for gliding motility, as well as catalytic proteases and perforin-like proteins (PLPs) for degradation of host cell membrane[6].

Rhoptries (ROs): They are similar to *Plasmodium* secretory lysosomal granules, however, in contrast to *Plasmodium* spp. that possess only two designated paired organelles, several ROs are localized in *T. gondii*. In the exit site of endoplasmic reticulum, ROs are formed in coated vesicles and travel to the Golgi apparatus to be sorted as immature ROs. In contrast to other apical secretory organelles in *T. gondii*, ROs are transiently emptied due to exocytosis of their content through a duct, to be subsequently discharged and localized into nascent PV membrane (PVM). Expression of RO proteins occurs immediately after zoites adhesion to the host cell surface. Being club-shaped organelles, they are connected by thin necks to the apicomplexan apical poles. Accordingly, there are proteins released from the neck and basal portions; termed RONs and ROPs, respectively. Notably, RONs contribute to formation of

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a tight moving junction along the length of the invading tachyzoite, while ROPs contribute in active tachyzoites penetration into the host cell, as well as neutralization of host innate immune responses. Members of ROP2 family (*Tg*ROPs 2, 4, 5, 7, 8 and 18) are formed in immature ROs, while other ROPs are synthesized in mature ROs[1].

Several ROPs were identified and characterized. While *Tg*ROP1 is assigned as the key molecule for zoites penetration, *Tg*ROP2 is inserted in PVM lining during tachyzoites invasion to mediate PVM association with host cell mitochondria. Deletion of the gene encoding *Tg*ROP2 resulted in impaired functions of ROs biogenesis and cytokinesis, reduced sterol uptake from the host cell due to impaired association between host cell mitochondria and PVM, and reduced invasion and replication *in vitro*, as well as reduced virulence in animal models. Other identified *Tg*ROPs including 3, 4, and 8 have overlapping functions[1].

Moreover, ROP kinases (17 and 18) and the pseudo-kinase (ROP5) are released into the host cell cytosol prior to zoites entry into PV. Cytosol ROP proteins are directed to the host nucleus as well as the PVM. These ROPs form a complex that was proved to have an essential role for immunomodulation of host immune response (discussed later). Besides, ROPs 5, 16 and 18 gene polymorphisms are linked to *T. gondii* virulence[7]. In the last decade, ROPs attracted more attention as potential vaccine candidate due to their essential roles in tachyzoites invasion, survival, and virulence. Besides, several ROPs possess either T cell (*Tg*ROP16) or B cell (*Tg*ROP9) epitope or both (*Tg*ROPs 2, 5, 8 and 19). In their review, Foroutan *et al*.[8] claimed that 38 RO proteins were identified in *T. gondii*, among them only 20 were localized to RO organelles; 11 and 9 were assigned to ROPs and RONs, respectively. The reviewers tabulated the common investigated ROPs with their proposed functions linked with: invasion (e.g., *Tg*ROPs 2, 5, 8 and 19); PV and PVM formation and maturation (*Tg*ROPs 2, 4, and 19); as well as virulence (*Tg*ROPs 5, 16 and 18). They also listed two additional ROPs; *Tg*ROPs 38 and 54. The first contributes to expression regulation of host transcription factors. Due to high similarity in gene sequencing among different *Toxoplasma* strains, ROP38 showed high potentiality as vaccine candidate against toxoplasmosis. The second is a RO pseudo-kinase effector contributor to tachyzoites invasion due to its ability to modulate host innate immunity[8].

Dense granules (DGs): They are round 0.2 mm organelles distributed throughout *T. gondii* zoites. Ultrastructural observations revealed ~ twenty DG organelles. Two hypotheses were postulated for expression of DGs proteins. First, a regulated mechanism that takes place shortly after invasion ensuring rapid and massive expression of DGs proteins into the PV. Second is the enhanced expression by ADP-ribosylation factor-1 during the intra-vacuolar parasite' development. It is worth mentioning that DGs release their contents from both the anterior and posterior poles of *T. gondii* life cycle stages after PV formation. Multi-lamellar vesicles are secreted from a specialized invagination at the posterior end 10-20 min post-invasion. A spaghetti-like IVMN is formed extending from PVM into PV lumen. It was reported that IVMN proved to have an essential role in the metabolic exchanges occurring between tachyzoites and host cells. Besides, it covers almost 75% of the PVM within 4 h post-invasion[9]. Concerning IVMN, a recent study was conducted and identified two novel molecules released from IVMN microtubules. Both participate in MICs exocytosis, and accordingly affect their secretions for efficient cell invasion. The first (Centrin 2; CEN2) is a calcium-binding phosphoprotein that mediates sub-pellicular microtubules contraction associated with gliding motility. The second (Dynein light chain 8a; DLC8a) is a cytoskeletal motor protein that transports necessary molecules along tachyzoites compartments[10].

According to a review published in 2005[9], 19 proteins were expressed from *T. gondii* DGs with molecular weights ranging from 18 to 67. They are *Tg*GRA (1-14), protease inhibitors (*Tg*PI 1, and *Tg*PI 2), nucleoside triphosphates (*Tg*NTPase-I, and *Tg*NTPase-II), cyclophilins (CyP 18 and 20), and *Tg* 14-3-3 protein. Out of the 14 *Tg*GRAs, 12 were identified in *T. gondii* tachyzoites without sequence homology to each other; while GRAs 11 and 13 are phantom-like. All *Tg*GRA proteins are excretory/secretory antigens. However only *Tg*GRAs 1, 2, and 4 were investigated for protection and potentiality as vaccine candidates, with special emphasis on the latter (*Tg*GRA4). Besides, the latter *Tg*GRA elicits specific humoral and cellular Th1 response in murine toxoplasmosis[9]. Four years later, another review[11] indicated that only *Tg*GRA1 remains primarily in PV lumen, while other *Tg*GRAs remain dispersed within PVM and IVMN. Besides, *Tg*GRAs 2, 4 and 6 participate in the formation of a multimeric protein complex associated with IVMN, while *Tg*GRAs 3, 5, 7, 8 and 10 are detected as PVM-associated proteins. In addition, *Tg*GRA9 associates with IVMN connected to PVM, *Tg*GRA12 associates with IVMN, and *Tg*GRA14 associates with the membranous structures within IVMN[11]. On the other hand, *Tg*PIs and *Tg*NTPases remain with *Tg*GRA1 primarily in PV, CyPs are involved in regulating and assembling of protein complexes within PV, and *Tg* 14-3-3 is a conserved regulatory protein expressed in all eukaryotic cells due to its ability to bind several functionally diverse signaling proteins such as kinases, phosphatases, and transmembrane receptors[9].

Notably, there was a difference between molecular weights of DGs proteins and those calculated from the amino acid sequence, suggesting potential post-translational modifications. Regarding DGs proteins expression in *T. gondii* life cycle stages, it was reported that abundant *Tg*GRAs 1, 2, 6 and 7 were localized in DGs of invasive tachyzoites. *Tg*GRAs 1, 2, 4, 5 and 8

as well as *Tg*NTPases were observed in bradyzoite DGs. Interestingly, DGs proteins that are localized in tachyzoites (*Tg*GRAs 1, 2, and 6) remain associated with the inner layer of tissue cyst wall, while *Tg*GRA7 remains associated with *Tg*GRAs 3 and 5 in the exterior membrane of tissue cysts. Besides, GRA9 expression was noticed in both tissue cysts and bradyzoites[9]. Additionally, three issues were characterized. First, *Tg*GRAs 2 and 7 expressions are linked with *T. gondii* virulence. Second, *Tg*GRAs 4-8 expressions were suggested to constitute the molecular sieve allowing the passage of molecules across PVM. Third, *Tg*NTPases are involved in the cleavage of di- and triphosphate nucleotides utilized from the invaded host cells, required for zoites egress from PV[1].

Later, additional DGs proteins were identified in a review published by Hakimi *et al*.[7]. They include *Tg*GRAs 15-17, 19-21, 23-25 and an inhibitor of the signal transducer and activator of transcription protein (STAT), named as *Tg*IST. It is worth mentioning that missed *Tg*GRAs (18, and 22) were not reported suggesting that they are phantom DGs proteins similar to GRAs 11 and 13. Being able to form pores within PVM, *Tg*GRAs 17 and 23 contribute to nutrients trafficking between the host cytosol and PV lumen. The reviewers claimed that although significant progress was achieved in identifying new DGs proteins, yet there are several proteins without assigned biological characters and functions. In the same year, expression of a novel protein (*Tg*GRA41) was identified in PV, and its main function was assigned to regulation of calcium homeostasis to initiate non-induced egress[12]. Later, an interesting feature was described in invaded tachyzoites that involved the release of *Tg*GRAs 3, 7, and 14 in structures called beads on a string (BOAS). Their function is unknown; however, they are frequently noticed between multiple PVs in the same host cell, or between a PV and the host cell nucleus[13].

Secretory organelles and PVM: Formation of *T. gondii* PVM depends mainly on expression of ROPs lipids and proteins to form small vesicles that collapse within the invaginated host cell membrane. However, during the first hour after cell invasion, ROPs 2, 4 and 8 contribute with DGs proteins (GRAs 1, 2, 4, and 6) for the possession of new intra-membrane vesicles. Both RO and DGs proteins contribute also during the first hour after invasion in building up the IVMN[9].

Secretory organelles and host immune system: Host immunity is manipulated by *T. gondii* through controlling host gene transcription and dysregulating signaling pathways involved in cytokines release and host cell apoptosis. Several mechanisms described how proteins released from both ROs and DGs act as effector proteins to evade host innate immunity. Among them are: 1) ROP complex (ROPs 5/17/18) prevents the accumulation of immunity-related GTPases (IRGs) on the PVM. It is known that GTPases are a large family of hydrolases that bind to the nucleotide guanosine triphosphate (GTP) to be converted to guanosine diphosphate (GDP). This conversion is essential in many fundamental cellular processes. By phosphorylation, this complex prevents GTP hydrolysis. 2) Involved in disruption of IRGs system, *Tg*GRA7 was reported to interact with ROP complex. 3) ROP kinase (*Tg*ROP16) activates STAT3, and STAT6, and host transcription factors required for the release of cytokines and growth factors. 4) *Tg*GRA15 localized in PVM causes release of pro-inflammatory cytokines, including IL-12. It also induces IL-1β production by host macrophages *via* caspase-1 activation. 5) *Tg*GRA24 elicits a strong inflammatory response through increasing production of pro-inflammatory cytokines, e.g., IL-12 and monocyte chemoattractant protein 1 (MCP-1), increasing macrophage phagocytic activity. 6) *Tg*IST was observed blocking the signaling of INF-1 that promotes *T. gondii* survival and multiplication[7,14].

Recently, low virulent *T. gondii* strains were investigated for their role in establishing chronic toxoplasmosis *in vitro*. Ten types of mutant parasites lacked expression of *Tg*GRAs 3, 7, 8, 14, and 15, and *Tg*GRAs 2, 4, 6, 9, and 12 as PVM- and IVMN-associated GRA proteins, respectively. Results showed that deletion of both associated GRA proteins induced severe defects in the development of chronic-stage cysts. Besides, *Tg*GRA12 was established as *Toxoplasma* virulence factor due to its high resistance to host IFN-γ-activated innate immunity[15].

Secretory organelles and intracellular invasion in *T. gondii*: It can be deduced that release of MIC proteins mediates host cell attachment *via* engagement of its surface receptors. A moving junction is formed by RONs, mainly RON2, and its structural integrity is maintained by *Tg*AMA1-RON2 complex or alternative pathways *Tg*AMAs 1/2/3/4-RONs 2/4/6. Utilizing gliding motility, tachyzoites actively enter host cells through the moving junction using actin-myosin motor. This is followed by encapsulation of tachyzoites by PVM. To form and attain a firm attachment required for tachyzoites motility and invasion, host lipid membrane surface receptor-ligand interactions are essential to overcome host cell repulsive force. Gliding motility is essentially required for tachyzoites invasion that should be preceded by formation of a moving junction all over the host cell membrane. This is followed by secretion of DGs proteins that either remain soluble in PV lumen or associate within the PVM, and the IVMN trafficking network. Besides, RO proteins and GRAs control host signaling as well as transcription of host cytokines to favor intracellular survival, and multiplication.

Additional factors for intracellular invasion in *T. gondii*: It was observed that tachyzoites utilize Myc regulatory (MYR) proteins for c-Myc regulation. It is worth mentioning that regulation of the central regulator (c-Myc) to coordinate host myriad cellular

functions, is an effector pathway for manipulation of the invaded host cell. Tachyzoites MYR proteins (MYRs 1-3) are responsible for *Tg*GRAs translocation within PV, PVM, as well as host cell cytosol and nucleus. Accordingly, *Tg*MYR proteins are additional factors in *T. gondii* invasion[13]. More recently, a new additional function was assigned to *Tg*GRA16, as an effector protein. Being localized in the host cytosol, *Tg*GRA16 reaches host cell nucleus to upregulate host c-Myc and remodel cellular functions. This allows invaded tachyzoites to access nutrients, prevent apoptosis and block specific and nonspecific host immune response[16].

Gliding motility: As a protein component of actomyosin, actin participates in several essential cellular processes in *T. gondii*; motility and cell division, cytokinesis, and maintenance of the moving junction during invasion. However, *T. gondii* tachyzoites possess a low amount of actin filaments to achieve those essential functions. Accordingly, they possess an additional actin-binding protein; toxofilin. Its localization during gliding motility differs in invading tachyzoites in which it is distributed in the whole cytoplasm; while in egressed tachyzoites, especially from the PV, it is restricted to the apical end. In addition, gliding motility occurs by moving the position of contact between *T. gondii* zoites and the invaded host cell longitudinally in the same orientation of the subpellicular microtubules. This is responsible for the corkscrew gliding motility, in addition to the zoites crescent shape and spiral cytoskeleton[17].

Intracellular egress in *T. gondii*

Abscisic acid (ABA), synthetized in the apicoplast, was established as a natural stimulus for *T. gondii* egress. It was observed that ABA induces calcium-dependent protein secretion from MICs of tachyzoites. Metabolic block of ABA biosynthesis significantly delayed egress, resulting in chronic toxoplasmosis. Meanwhile, *in vitro* studies showed that calcium significantly induced MICs secretion and tachyzoites egress, as well as increased gliding motility[18].

Prior to egress, MICs release *Tg*PLP1 to be expressed in PV degrading its membrane. Then, it is transported to host cell membrane, through the endoplasmic reticulum and Golgi apparatus, to bind with the host receptors promoting egress. Activity of *Tg*PLP1 is regulated by its interaction with host membrane receptors (specific phospholipids), and it is significantly limited or increased according to absence or availability of these receptors, respectively. In addition, tachyzoites egress is dependent on PV acidification, which promotes binding of *Tg*PLP1 to host cell membrane. It is worth mentioning that *Tg*PLP2 plays a role in gametocyte egress in the feline host's intestine. Three additional egress factors are addressed: first, a putative double C2 domain protein (*Tg*DOC2.1), released from MICs to facilitate their fusion with the apical membrane within the protrusible cytoskeletal conoid. Second, *T. gondii* conoid releases apical complex lysine methyltransferase (*Tg*AKMT) required for initiating gliding motility[19]. Third, lecithin [cholesterol acyltransferase (*Tg*LCAT)] is suggested to participate in calcium-independent tachyzoites egress. It is stored in DGs and utilizes a substrate (phosphatidylcholine) in PVM[18]. These additional factors increase host cell permeability facilitating membrane degradation.

According to Friedrich *et al.*[20], tachyzoites egress is an ultimate solution for their response to a life-threatening environment (e.g., apoptosis). In these instances, death receptor-mediated apoptosis, induced by cytotoxic T lymphocytes or activated macrophage, stimulates egress. Accordingly, there are two proposed egress pathways, either induced or non-induced. The first utilizes *Tg*PLP1 and gliding motility, while the second relies upon increased vacuolar pressure. This explains that *Tg*PLP1-mutant parasites display delayed egress and become avirulent to mice, but *in vitro* egress is due to increased vacuolar pressure. On the other hand, the induced pathway may result in premature egress due to tachyzoites' ability to react to external stimuli (host cell apoptosis). Whereas non-induced egress responds to intrinsic stimuli related to molecules released for parasite replication. In their review, Friedrich *et al.*[20] claimed that the terms "proliferation-dependent", due to intrinsic stimuli, and "premature egress" due to extrinsic stimuli are suggested nowadays for acute toxoplasmosis. Tachyzoites undergo rapid egress and subsequent *de novo* invasion of new macrophages. Hence, two strategies for tachyzoites egress were hypothesized: either proliferative or premature. In acute toxoplasmosis, the first one is the default strategy. When tachyzoites sense induction of premature egress *via* activated macrophages, they have to decide what to do. This depends on their number. In low numbers (mild infection), the theory of "let's die to let others survive" is initiated with apoptosis of the proliferated tachyzoites, and premature egress starts. In colonies with high numbers of tachyzoites, proliferative egress continues without initiation of immature egress. Besides, both host cell calpains, and calcium-dependent cysteine proteases participate in tachyzoites egress *via* destabilization of host cell cytoskeleton, and plasma membrane. Occasionally, bradyzoites utilize gliding motility to exit and invade neighboring cells without host cell membrane lysis, resulting in chronic toxoplasmosis[20].

CONCLUDING REMARKS

1. Host cell invasion, replication and egress are essential subsequent events in *T. gondii*. To achieve that, molecules released from the apical secretory organelles participate in host cell invasion, PV formation and maturation, IVMN establishment, as well as zoites egress.
2. Localization of MICs is restricted to the apical third of *T. gondii* zoites, and their released proteins are essential for host cell attachment and invasion utilizing actin-based gliding motility. In addition to MIC proteins,

release of *Tg*AMA1 is required for maintaining the structural integrity of the moving junction (invasion), and PLPs are required for tachyzoites egress.

3. Two types of ROs proteins are identified: RONs and ROPs. While RONs form the tight moving junction, ROPs contribute in active zoites invasion and host immune response immunomodulation. Due to their essential roles in invasion, ROPs are investigated as potential vaccines. In addition, several functions were proposed: for invasion (*Tg*ROPs 2, 5, 8 and 19); PV and PVM formation and maturation (*Tg*ROPs 2, 4, and 19): and virulence (*Tg*ROPs 5, 16 and 18).
4. The main function of DG proteins that follows zoites invasion, is to modify PV environment suitable for zoites survival and replication, and establishment of the IVMN. In addition to several GRAs, DGs proteins include *Tg*PIs, *Tg*NTPases, CyPs, and *Tg* 14-3-3 protein. Some remain in PV (*Tg*GRA1, *Tg*PIs, and *Tg*NTPases), while other *Tg*GRAs are either associated within PVM or IVMN. Novel structures (BOAS) were described between multiple PVs, or between a PV and host cell nucleus releasing *Tg*GRAs 3, 7, and 14.
5. Regarding host immunomodulation, several mechanisms were described for the essential roles played by *Tg*ROPs and *Tg*GRAs, as effector proteins, in controlling host gene transcription and impairment of signaling pathways involved in cytokines release and host cell apoptosis.
6. For manipulation of the invaded host cell, tachyzoites utilize MYRs 1-3 to control host cell central regulator (c-Myc). They are responsible for *Tg*GRAs translocation within PV, PVM, host cell cytosol and nucleus.
7. Before invasion, two steps are established. First, MICs proteins are released for host cell recognition and attachment mainly by AMA1. Second, AMA1-RON2 complex or alternative pathways form a moving junction for gliding motility. During invasion, RONs are released for PV formation, followed by ROPs for several functions inside host cell nucleus and cytosol, and PVM. After invasion, DGs proteins are released for PV maturation and establishment of IVMN.
8. Two terms are used for tachyzoites egress in acute toxoplasmosis; proliferation-dependent due to intrinsic stimuli (molecules released for replication), and premature egress due to extrinsic stimuli (host cell apoptosis).

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