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Review

The *Toxoplasma* Parasitophorous Vacuole: An Evolving Host–Parasite Frontier

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The parasitophorous vacuole is a unique replicative niche for apicomplexan parasites, including *Toxoplasma gondii*. Derived from host plasma membrane, the vacuole is rendered nonfusogenic with the host endolysosomal system. *Toxoplasma* secretes numerous proteins to modify the forming vacuole, enable nutrient uptake, and set up mechanisms of host subversion. Here we describe the pathways of host–parasite interaction at the parasitophorous vacuole employed by *Toxoplasma* and host, leading to the intricate balance of host defence versus parasite survival.

Toxoplasma and the Host Cell: Living Together Apart

Toxoplasma gondii, the causative agent of toxoplasmosis, is a widespread global infection that is prevalent chronically in 30–50% of humans [1]. Sexual reproduction occurs in the feline gut, from where millions of environmentally resistant sporozoite-containing oocysts are shed in the cat faeces. Transmission to intermediate hosts is through ingestion, whereupon the sporozoites develop into tachyzoite forms. This rapidly replicating form of the parasite is responsible for the acute stage of the infection. Three classical strains are present throughout North America and Europe, types I, II, and III, while a dramatic expansion of strain diversity has been found in South America [2,3]. Under pressure of the host immune system, and depending on strain type, the tachyzoites localise to the deep tissues and brain where they convert into slowly replicating bradyzoites in cysts (Figure 1) [4].

Toxoplasma is an obligate intracellular parasite of the phylum Apicomplexa, infecting most nucleated cells of warm-blooded animals. It resides within a **parasitophorous vacuole (PV)** (see Glossary) in its host cell, physically separated from the host cell cytoplasm. The **parasitophorous vacuole membrane (PVM)** forms the boundary between host and parasite and creates a niche for survival and replication. At the same time, the PV becomes a target for recognition by the host immune defence mechanisms. Recent progress in elucidating host defence mechanisms and the responding parasite's evasion strategies centred at or around the PV has shed light on significant differences between species and cell type. Here we collate these findings and provide a perspective on future challenges to unravel the *Toxoplasma*–host interaction at the PV.

Key Features and Composition of the *Toxoplasma* Parasitophorous Vacuole

Formation of the PV

As an apicomplexan parasite, *Toxoplasma* actively invades the host cell in a process mediated by the sequential secretion of dedicated proteins from specialised apical organelles (Figure 2,

Trends

Toxoplasma gondii is parasite of the phylum Apicomplexa. It resides in a host cell, enclosed in a parasitophorous vacuole.

Classically, host control of *Toxoplasma* proliferation has been studied in mice. However, recent work has highlighted striking differences between human and mouse cells.

In this review we shed light on the pathways of host–parasite interactions at the parasitophorous vacuole membrane interface and point out differences and similarities between both species, especially in the recognition and destruction of the parasitophorous vacuole membrane.

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Invasion, and inset). The PV itself is formed by invagination of the host cell plasma membrane in a process completed within 25–40 s [5]. Once inside the host cell, the parasite replicates rapidly within the PVM, ultimately leading to its rupture and egress of the tachyzoites (Figure 2, Replication and Egress).

The PVM is largely host-derived as evidenced by its lipid composition and capacitance measurements of patch-clamped host cells, indicating that the host cell membrane contributes >85% of the PVM [6]. In corroboration, a fluorescent lipid probe inserted into host membranes internalised with the PVM on invasion [7]. The vacuole is rendered nonfusogenic, thus avoiding lysosomal acidification, by selectively stripping away transmembrane proteins at the **moving junction** between host and parasite, based on their mechanism of membrane association [7,8]. Rhoptry neck proteins, for example, Tg**RON**2 and 4 proteins as well the microneme protein AMA1, are involved in the establishment of the moving junction through which the parasite enters the cell [9,10]. Research by Hakansson *et al.* implies a two-step process of rhoptry secretion and fusion to generate the PV [11]. Firstly, after the formation of a moving junction, part of the parasite rhoptry content is released into the host cell as evacuoles, characterised as being positive for Tg**ROP**1, TgROP2, and negative for Tg**GRA**2. This is thought to occur via a transient break in host cell membrane, rapidly resealing to prevent lysis. Secondly, invasion occurs with the formation of the PVM. The evacuoles are able to fuse with and modify established PVM [11], and secreted proteins from rhoptry and dense granules have been shown to be involved in its development and maintenance (Table 1).

Toxoplasma Proteins Shape the PVM and Host Organelle Recruitment to Enable Parasite Persistence

Rhoptry and dense granule proteins secreted by *Toxoplasma* on invasion modify the PVM. These modifications, in addition to altering the structural environment in and around the PVM [12], adapting the PVM to access the nutrient-rich host cytosol, and facilitating host organelle recruitment, also play a role in combating host defence mechanisms (Table 1). The contributions of these parasite protein-mediated modifications are discussed below in greater detail.

Toxoplasma is auxotrophic for many nutrients, including tryptophan, cholesterol, and iron [13–15]. The parasite has therefore modified the PVM to function as a molecular sieve to permit diffusion of small molecules (<1300 Da) [16]. Subsequent research specified two parasite dense granule proteins, TgGRA17 and TgGRA23, that enable this function. Functionally these proteins enable passive, nonselective and bidirectional diffusion of nutrients and small molecules across the PVM [17]. The mechanism for transport of parasite proteins across the PVM is more complex. A sequence resembling the *Plasmodium* HT/PEXEL (Host Targeting/*Plasmodium* Export ELement) domain was initially described for *Toxoplasma* dense granule proteins TgGRA19, 20, and 21 [18], but unlike *Plasmodium*, this *Toxoplasma* '**TEXEL**' (*Toxoplasma* Export ELement) motif did not permit export across the PVM. TgAsp5 has been shown to be the protease cleaving this sequence with its deletion blocking translocation of TgGRA16 and TgGRA24 to the host nucleus [18–21]. However, whereas TgGRA16 contained an N-terminal 'TEXEL' motif, this was absent in TgGRA24, suggesting that TgGRA24 was not the direct substrate of TgAsp5 [19,21]. A secreted protein, Myr1, cleaved by TgAsp5, was subsequently found to be essential for export of TgGRA16 and TgGRA24 across the PVM into the host cytosol [22]. Thus it appears that both TgAsp5 and Myr1 are required for protein export to host compartments.

Toxoplasma employs a number of strategies to allow the exchange of molecules with the host, including the diffusion pore described above [16,17]. In addition, the parasite is able to recruit host cell mitochondria, microtubule organising centre (**MTOC**) and endoplasmic reticulum (**ER**) to its PVM soon after invasion [23–25]. This ability depends partly upon the host microtubule

Glossary

Autophagy: an intracellular degradation process used to recycle damaged or nonfunctioning components, routing them for lysosomal destruction. Also extended to the clearance of intracellular pathogens (macroautophagy).
ER: endoplasmic reticulum. Reticular network of cell membranes involved in protein and lipid synthesis and transport.
GBP: guanylate-binding proteins. Large 65–75k Da GTPases stimulated by interferons and immune regulators in response to infection or cell insult. Family of seven proteins (seven genes and one pseudogene) in humans residing on chromosome 1. Family of 11 proteins (11 genes and 2 pseudogenes) in mouse cells split between chromosomes 3 and 5. Proteins have high identity within and between species.
GRA: dense granule protein. Proteins secreted from the parasite dense granule organelles after invasion and PV formation.
IRG: immunity-related GTPases, also known as p47 GTPases. Around 47 kDa, and stimulated by interferon gamma in response to infection or cell insult in mouse cells. Only two present in humans and not responsive to interferons.
IVN: intravacuolar network. A network of membranes within the parasitophorous vacuole.
LC3: microtubule-associated protein1 light chain3. An Atg8 protein associated with the forming of autophagosomal membrane. LC3 interacts with autophagy adaptor proteins on the cargo that is to be recycled by autophagy.
MIC: microneme protein. Proteins secreted from the apically located parasite microneme organelles on invasion.
Moving junction: a structure formed at the contact between the apex of the invading parasite and the host membrane. The moving junction moves over the parasite from the anterior to the posterior end during the process of invasion, resulting in the internalization of the parasite within a parasitophorous vacuole.
MTOC: microtubule organizing centre. A structure found in eukaryotic cells serving as a nucleation site for the formation of microtubules.

Table 1. Rhoptry Proteins (ROPs) and Dense Granule Proteins (GRAs) Associated with the *Toxoplasma* Parasitophorous Vacuole

Protein	Attributes	Refs
TgROP1	Associated with luminal face of PVM, shortly after invasion, but nondetectable 12–24 h postinvasion. TgROP1 is suggested to be glycosylated.	[105–109]
TgROP2	Appears in PVM shortly after invasion. N-terminus exposed to host cell cytosol. Pseudokinase that associates with TgGRA7. TgROP2 was thought to recruit mitochondria but this has been disproven. TgROP2 is suggested to be glycosylated.	[30,105, 109–112]
TgROP4	Appears in PVM shortly after invasion. Phosphorylated in infected cells. Associates with TgGRA7. TgROP4 is suggested to be glycosylated.	[105,109, 113,114]
TgROP5	Pseudokinase acting as a cofactor for TgROP18 enhancing its activity. TgROP5 is suggested to be glycosylated. Localises to host cytosolic face of PVM. Binds directly to host IRGs. Virulence factor in mice.	[79,83, 88,90,109, 110,115]
TgROP7	Appears in PVM shortly after invasion. Exposed to cell cytoplasm. TgROP7 is suggested to be glycosylated.	[105,109,116]
TgROP8	Pseudokinase. Function not known.	[117]
TgROP14	Multiple transmembrane domains.	[25,118]
TgROP17	Forms a complex and synergises with TgROP5/TgROP18. TgROP17 is autophosphorylated.	[83]
TgROP18	Kinase localises to PVM via Arg repeat regions. Pro-protein cleaved by TgSUB2. Highly expressed in types I and II, but not expressed in type III due to an insertion in the promoter. Virulence factor in mice, prevents IRGs and mGBPs binding. TgROP18 is autophosphorylated and suggested to be glycosylated.	[78,81–83, 88,90,109,111]
TgROP19 TgROP21 TgROP25 TgROP39	All predicted to have kinase activity, having catalytic triad and all have signal sequence for secretion. Function unknown.	[119]
TgROP20 TgROP22 TgROP23 TgROP24 TgROP40	All predicted to not have kinase activity, lacking catalytic triad and all have signal sequence for secretion. Function unknown.	[119]
TgROP38	Kinase differentially expressed between strains, highest in type III. Significantly alters expression of >1000 host genes. Downregulates MAPK signalling and control of apoptosis and proliferation in host.	[119]
TgROP54	Pseudokinase localises to cytoplasmic face of PVM. Important in virulence in mice and mGBP2 loading on PVM.	[120]
TgGRA3	When released into vacuolar space TgGRA3 spontaneously inserts into PVM. Also associated with the intravacuolar network (**IVN**). TgGRA3 is suggested to be glycosylated. Host ER recruitment.	[25,109,121]
TgGRA5	Putative TM domain and localises to PVM. N-terminus exposed to host cell cytosol. Host ER recruitment.	[25,122]
TgGRA7	Associates with TgROP2, TgROP4, TgGRA1 and TgGRA3. TgGRA7 is phosphorylated and glycosylated. Forms strands. Also associated with IVN. Involved in nutrient acquisition. Associates with TgROP5/TgROP18 complex in preventing recruitment of host GTPases to PVM. Associates with Irga6.	[86,109, 114,123]
TgGRA8	Released into vacuole shortly after invasion and associates with vacuole periphery. Proline-rich (24%).	[124]
TgGRA14	Type I transmembrane protein, C-terminus faces host cytosol. Localises on PVM extensions, connecting to neighbouring PVs. Also associated with IVN.	[125]
TgGRA17	With TgGRA23, facilitates diffusion of small molecules across the PVM.	[17]
TgGRA23	With TgGRA17, facilitates diffusion of small molecules across the PVM.	[17,126]
TgGRA33	Localised to PVM. Function unknown.	[127]

PV: parasitophorous vacuole. A compartment formed on invasion of a host cell by the parasite which encapsulates the parasite inside the host cell and is bounded by the PVM.
PVM: parasitophorous vacuole membrane, which bounds the parasitophorous vacuole (PV) surrounding the intracellular parasite.
RON: rhoptry neck protein. Proteins secreted from the neck of the flask-shaped, apically located parasite rhoptry organelles on invasion.
ROP: rhoptry body protein. Proteins secreted from the bulb or body of the apically located rhoptry organelles of the parasite on invasion.
TEXEL: *Toxoplasma* EXport ELement. A conserved pentameric motif (RxLxD/E), similar to the *Plasmodium* PEXEL motif, that is required for transport of proteins to PVM and some to host cell compartments.
Ubiquitin: a small protein found 'ubiquitously', and conserved from yeast to mammals, which is added to a substrate protein to regulate its activity, degradation, interactions, or location. Ubiquitination of proteins is a multistep process requiring an E1 activating enzyme, a conjugation step mediated by an E2 enzyme, and a final process of ligation onto the substrate protein by an E3 enzyme.

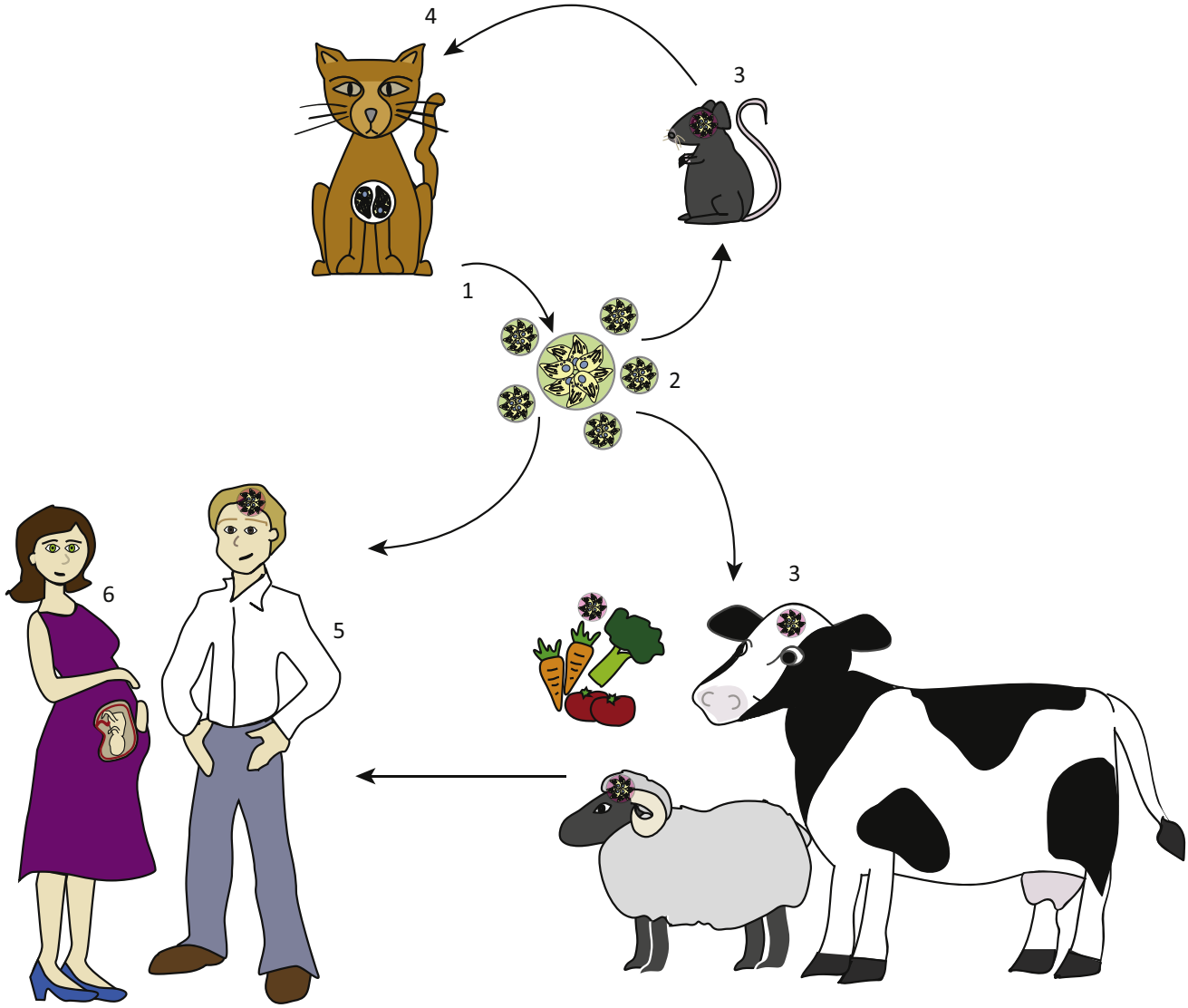

Figure 1. The Life Cycle of *Toxoplasma gondii*. In *Toxoplasma* infections, the definitive hosts are felids (members of the cat family). (1) The infected cat sheds many oocysts in the faeces. (2) Within ~5 days in the environment, the oocysts sporulate and become infectious to intermediate hosts, which can be any warm-blooded animal. Once ingested by the intermediate host, the sporulated oocysts develop into tachyzoites, a rapidly dividing intracellular form of the parasite. (3) Tachyzoites then migrate to the deep tissues and brain of the infected host. Here they convert into the slower replicating bradyzoite form and persist as cysts in these immune-privileged sites for the lifetime of the host. (4) The cycle is perpetuated when a cat eats an infected intermediate host, usually a rodent. (5) In humans, infections occur by eating undercooked meat from infected intermediate hosts or from consumption of food or water contaminated by cat faeces. Other sources of infection include organ transplant or blood transfusion. (6) *Toxoplasma* can also be transmitted from mother to foetus during a primary infection.

network, although this appears not to be the case for host ER–PVM association [24]. The proximity of host organelles to the parasite (12–18 nm) and tight association (resisting dissociation during subcellular fractionation), suggests the potential for materials/nutrients to be transferred to the parasite [24]. Host intermediate filaments and microtubules (MT) are reordered around the PVM and may participate in locating the PV close to the host nucleus [26]. Furthermore, the host MTOC is repositioned from the host nuclear membrane to the PVM [12]. Lysosomes and endocytic vesicles also migrate close to the PVM and may facilitate cholesterol uptake by the parasite [12,14]. Deep infoldings on the PVM into the vacuole caused by host MT

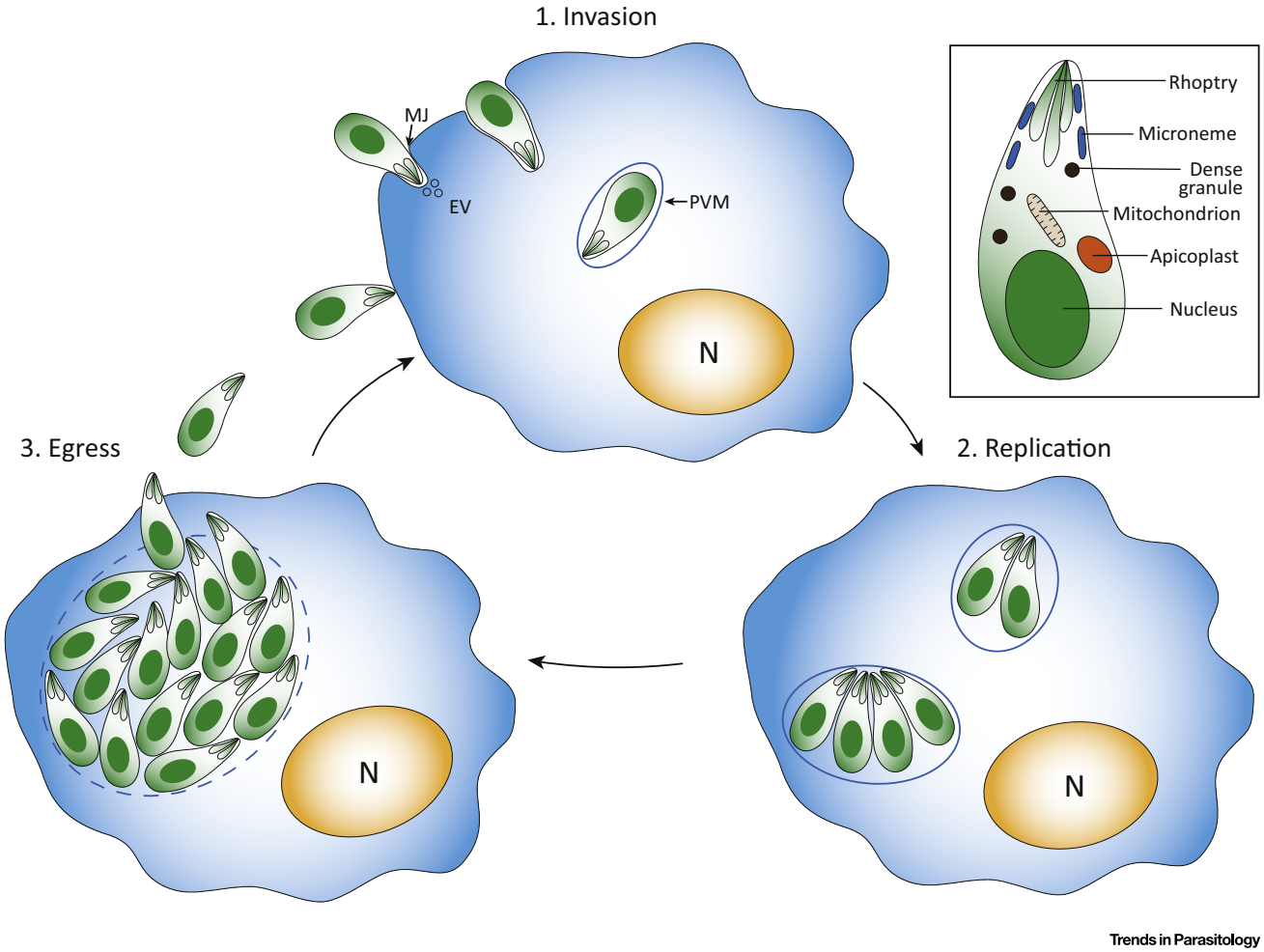

Figure 2. The Lytic Cycle of *Toxoplasma gondii* in Its Host Cell. The *Toxoplasma* lytic cycle comprises the three main stages of invasion, replication and egress. (1) The *Toxoplasma* tachyzoite attaches to and actively invades the host cell through a moving junction between parasite and host cell membrane. During this process the contents of the apical organelles (micronemes and rhoptries) are secreted to help form the host-derived parasitophorous vacuole. Further release of dense granule proteins shapes the parasite's environment (see inset). (2) The parasite resides within the PV where it rapidly replicates by endodyogeny, protected from the host defence machinery, but able to acquire nutrients from the host cytoplasm. (3) After many rounds of replication, the parasites rupture the PV and egress into the extracellular environment. From here the released tachyzoites are able to invade further host cells and continue to proliferate in the infected host. Inset: Structure of a *Toxoplasma* tachyzoite showing parasite organelles. Abbreviatons: PV, parasitophorous vacuole; PVM, parasitophorous vacuolar membrane; MJ, moving junction; EV, evacuoles; N, nucleus.

and stabilised by TgGRA7 are postulated to deliver host endocytic vesicles to the vacuole lumen [12,25].

Despite the parasite synthesising certain phospholipids, it requires host cell choline for the synthesis of phosphatidylcholine [27]. *Toxoplasma* also requires lipoic acid from the host as the amount produced by the parasite is insufficient for its own needs [28]. Since mitochondria are a key source for lipoic acid, the parasite may acquire this from host organelles. The recruitment of host mitochondria was initially attributed to TgROP2 [29]. However, a parasite line lacking the ROP2 locus (including related genes ROP2a, ROP2b, and ROP8) recruited mitochondria to the same levels as wild-type parasites, disproving its need [30]. Mitochondrial association with the PVM is specific for type I and type III parasites and was shown to depend on the parasite protein MAF1 [31]. The *MAF1* locus in *Toxoplasma* contains MAF1 paralogs that differ in their ability to bring about mitochondrial association. In type II parasites, the MAF1b protein is lacking and

correlates with the absence of mitochondrial localisation [32]. Of note, deletion of the parasite protease TgAsp5 causes mislocalisation of MAF1 and a consequent reduction in host mitochondria–PVM association [19,20]. Since MAF1 does not appear to have a TEXEL motif, TgAsp5 may cleave an interacting partner to bring about mitochondrial recruitment to the PVM [19]. Compared to mitochondria, recruitment of host ER to the PVM is less clear, although dense granule proteins TgGRA3 and TgGRA5 have been implicated by their ability to bind the ER protein calcium modulating ligand (CAMLG) [33]. Additionally, the host ER–parasite PVM interaction has been described to permit antigen cross-presentation in dendritic cells infected with *Toxoplasma* [34].

Toxoplasma infection is able to activate the serine/threonine kinase, mammalian-target-of-rapamycin (mTOR) [35]. This capability has the potential to increase the availability of host cell nutrients for the rapidly replicating parasite. Accumulation of host mTOR was observed in a vesicular pattern around the PVM. The PVM is reported to be enriched in phosphatidic acid (PA), and since PA is a key stimulator of mTOR, this may be the route of mTOR activation [35].

Thus *Toxoplasma* has established multiple mechanisms to ensure its survival and proliferation despite being isolated from the host within its vacuole. No doubt this contributes to its successful parasitism of virtually all nucleated cells in all warm-blooded animals. Nevertheless, probably the best studied role of the *Toxoplasma* PV/PVM is in immune defence, which we cover in the next section of this review.

IFNγ-Dependent Murine and Human Host Recognition of the PV

Interferon gamma (IFNγ) has long been known to stimulate mechanisms combating *Toxoplasma* replication and infectivity [13,15,36]. A multitude of studies have focused on elucidating the host defence mechanisms at the PV after stimulation of the host cells with IFNγ *in vitro* [37].

In the absence of immune pressure, *Toxoplasma* tachyzoites avoid fusion of the PVM with the host endolysosomal system. However, host immunity can destroy this safe haven, in particular, the induction of high levels of IFNγ on infection cascades to hundreds of IFNγ-stimulated genes (ISGs). Proteins such as **ubiquitin**, Immunity-Related GTPases (**IRG**s), guanylate-binding proteins (**GBP**s) and **autophagy** proteins subsequently mediate immune recognition of the parasite.

The mechanism by which the host deals with *Toxoplasma* invasion is species dependent, with parasite strain differences added to the mix. Below, we describe contrasting mechanisms of IFNγ-induced host defence between murine and human cells. In murine cells, autophagy protein and host GTPase-mediated damage of the PVM occurs, while in human cells a ubiquitin-mediated recognition of the PVM followed by endolysosomal elimination or partial autophagy takes place, dependent upon cell type.

Murine Host Destruction of the PVM Mediated by IRGs and GBPs

The murine PVM has been known to break and leak after induction of cells with IFNγ [38]. Many reports have employed murine macrophages, fibroblasts, and astrocytes to demonstrate vacuolar disruption of *Toxoplasma* PVs harbouring type II and III parasites [38–46]. Disruption of the PVM induced by stimulation with IFNγ is only seen in murine cells and not in human cells (Figure 3, Key Figure) [38,40,47,48]. IFNγ upregulates large GTPases that have been shown to be involved in *Toxoplasma* vacuole remodelling: the IRGs and GBPs. In C57BL/6 mice, 23 IRGs are present in the genome, and a cohort of them recognise the PV in IFNγ-stimulated murine cells [38–43,45,46,49,50]. Most IRG proteins contain a GKS motif in their active nucleotide binding site, while three IRGs possess a GMS motif at this location (Irgm1, Irgm2, Irgm3) [51].

Key Figure

IFNγ-Dependent Mechanisms of *Toxoplasma* Control in Mouse and Human Cells

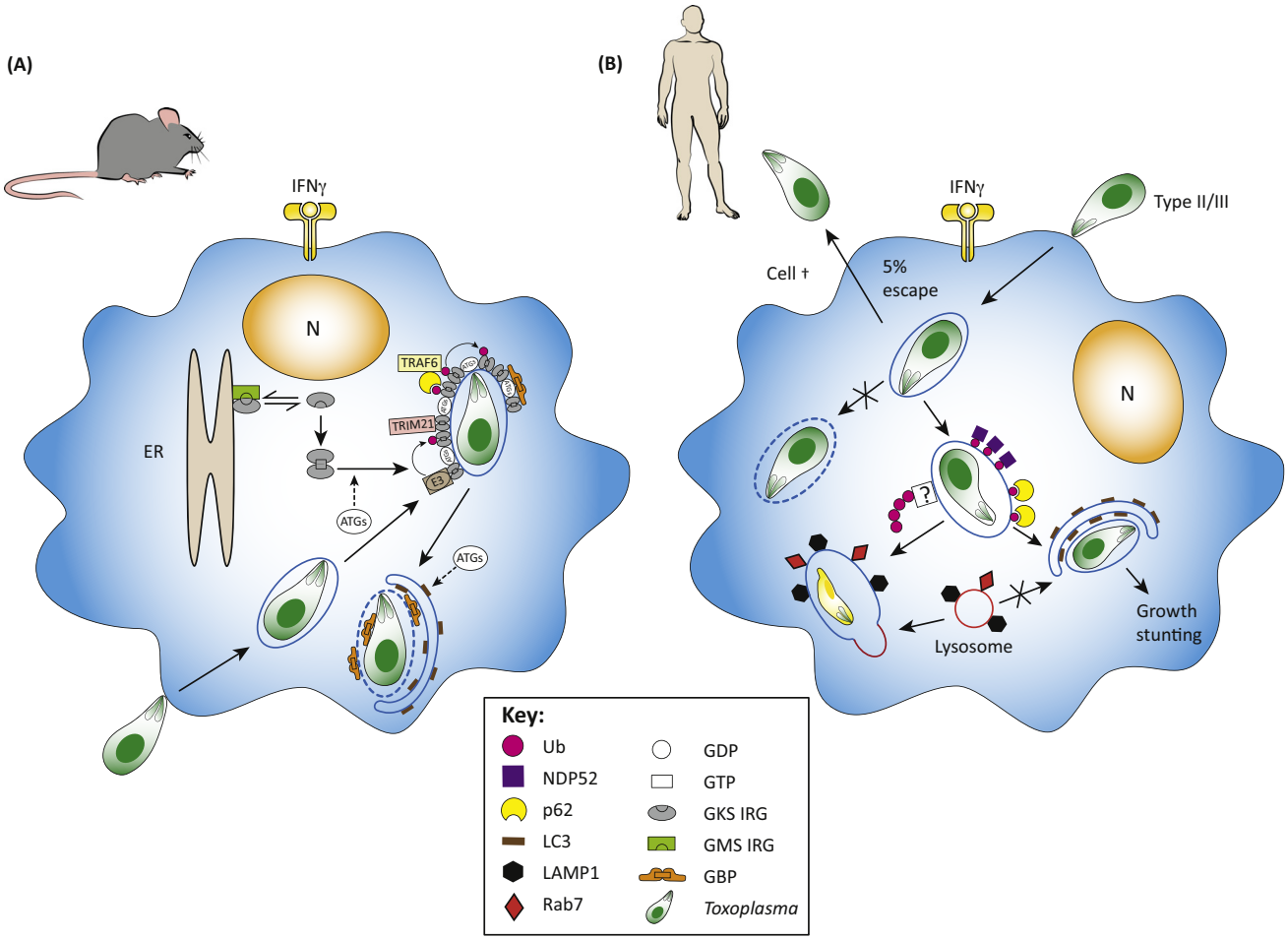

Figure 3. (A) In laboratory murine cells, host GTPases (GKS IRGs) are recruited initially to the PVM of type II and III parasites aided by ATGs. Subsequent recruitment of ubiquitin, mGBPs, TRIM21, TRAF6, and p62 occurs, with TRAF6 and possibly other E3 ligases, mediating further ubiquitination of p62. The resulting disruption of the PVM exposes the parasite's plasma membrane to further attack by mGBP2. Once destroyed, the remains of parasite and PVM are targeted by autophagy. (B) In human cells, no evidence for PVM destruction is reported. Here, type II and III *Toxoplasma* are targeted for elimination by ubiquitination of the PVM, whereupon ubiquitin-binding proteins, p62 and NDP52, interact with ubiquitin at the PVM. The parasites are then routed either for endolysosomal fusion in HUVEC, the PVM becoming LAMP1-positive with some Rab7 accumulation, or are recognised by LC3 and the autophagy machinery in HeLa cells, stopping short of lysosomal fusion. A small proportion of invaded parasites escape from the host cell, which dies in the process. Abbreviations: GTP, guanosine triphosphate; IRG, immunity-related GTPases; PVM, parasitophorous vacuolar membrane; ATG, autophagy protein; GBP, guanylate-binding protein; TRIM, tripartite motif-containing protein; TRAF, TNF receptor-associated factor; E3, E3 ubiquitin ligase; HUVEC, human umbilical vein endothelial cells; ER, endoplasmic reticulum; N, nucleus; IFNγ, interferon gamma; Ub, ubiquitin.

In the murine IFNγ-dependent host defence, the*Toxoplasma* PV is targeted first by the GKS IRGs Irgb6 and Irgb10 [39]. GKS IRGs are rendered inactive by GMS IRGs, both associating with each other on endomembranes in the GDP-bound form. Once released from the membranes and the GMS IRG complex, GKS IRGs can load GTP and recruit to the *Toxoplasma* PVM (Figure 3A) [49,52]. It is by virtue of this that IRGM proteins guard 'self' organelles such as

lipid droplets and prevent the association of GKS IRGs and mGBPs [53,54]. *Toxoplasma* PVs display a 'missing self' phenotype without GMS IRGs and are thus prone to GKS IRG and mGBP targeting [53]. Further details by which mechanism mGBPs are recruited to the PV are unclear. In line with these findings, it was unequivocally shown that in the absence of GMS IRGs, mGBP2 is not found on the vacuole [53]. Thus, one could speculate that IRG recruitment precedes mGBP recruitment to the PV. However, when deleting mGBPs on chromosome 3 in mice, less IRG recruitment was observed [43], and deletion of mGBP1 leads to less Irgb6 2 h postinfection despite initial loading of Irgb6 at the PVM [44]. While IRGMs seem to be the seeding IRGs for the whole pathway [53], it is conceivable that the GKS IRGs and mGBPs operate in feedback loops with each other to coat the PV for ultimate destruction. Knocking out either leads to the PV staying intact in murine cells [41,43]. The interdependence of IRGs and mGBPs for vacuolar recruitment is partially supported by the observation that the negative regulator of mGBP2, RabGDIα, controls Irga6 recruitment to the PVM via mGBP2 [55].

The mechanism by which IRGs and mGBPs lead to the disruption of the murine PVM to expose *Toxoplasma* to the cytoplasm in murine cells is not clear. An elegant study has recently shown that mGBPs are present in two discrete subcellular reservoirs and attack the PVM as large multimers comprised of various combinations of mGBPs 1, 2, 3, 5, and 6. Subsequently, mGBP2 was visualised to localise directly to the plasma membrane of the parasite, presumably disrupting its integrity as well [56]. A newly discovered parasite effector, TgIST, inhibits STAT1-dependent responsiveness of the host cell to IFNγ [57,58], thus blocking IRGs potentially saving the first tachyzoites from destruction [57]. IRGs and mGBPs are therefore essential host defence proteins that target the murine PV for destruction, while hGBPs exert an anti-*Toxoplasma* effect not necessarily dependent on PV targeting [59,60].

Autophagy Proteins and Ubiquitin Control *Toxoplasma* in Murine Cells

Autophagy is a catabolic pathway for cell content recycling that is extended to the clearance of pathogens or macroautophagy, herein called autophagy. In canonical autophagy, the ubiquitin-like machinery of autophagy, including Atg7 (E1-like), Atg3 (E2-like), and the Atg12-Atg5-Atg16L1 (E3-like) complex, bring Atg8 proteins such as **LC3** to the autophagosome isolation membrane [61]. Membrane-bound LC3 associates with the pathogen cargo via autophagy adaptor proteins such as NDP52 and p62 that bind ubiquitinated proteins on the pathogen [62,63]. Autophagosome membranes surround and finally deliver their cargo to lysosomes for destruction. Autophagy proteins are essential in clearance of *Toxoplasma* in murine cells in two distinct ways. Firstly, they recruit host GTPases to the PVM in a nonautophagic capacity. Secondly, after PVM destruction, the observation of autophagic membranes around the exposed parasite implies their participation in a classical autophagic role.

The factors governing the initial recruitment of Atg proteins to the PVM are unclear. It has been postulated that phosphorylated products of phosphatidylinositol on the PVM may bring the Atg12-Atg5-Atg16L1 complex to the membrane using effector proteins that link phosphoinositides to the Atg complex [64]. Alternatively, the PVM may be recognised by 'missing self' in a manner similar to that described for GMS IRGs above [53,54,64]. This early involvement of Atgs does not lead to canonical autophagy since the Atg proteins do not promote the formation of an isolation membrane at the PVM prior to breakage, shown in activated macrophages and astrocytes [38,41]. Instead, a core set of autophagy proteins has been implicated in the recruitment of IRGs and mGBPs to the PVM described above.

Autophagy proteins, including the E3-like autophagy complex, localise and recruit host IRGs and mGBPs to the PVM, leading to its disruption [39,42,64,65]. Specifically, Atg5 was shown to be essential for recruitment of Irga6 and Irgb6 to the PV in mouse macrophages, fibroblasts, and granulocytes [39,42]. Without Atg5, Irga6, Irgb6, and Irgd formed aggregates in the host

cytoplasm [39,42], thus exhibiting diminished soluble cytoplasmic protein leading to diminished recruitment to the PVM [39]. Similarly, Atg3 expression was required for loading of IRGs and mGBP2 (and possibly other GBPs) on the PVM and control of *Toxoplasma* infection [65,66]. These Atg proteins appear to activate the GTPases, since a GTP-locked, constitutively active, IRG protein mutant was able to overcome the targeting defect in Atg3- and Atg5-deficient cells [66]. A role for Atg7 and Atg16L1 in promoting recruitment of Irgb6 and mGBPs to the vacuole has also been described, with Atg9a and Atg14 being dispensable [67]. Depletion of all LC3 homologues, including GABARAP, GABARAPL1, and GABARAPL2, led to a decrease in the targeting of IFNγ-stimulated GTPases to the PVM [64]. Furthermore, if the Atg12-Atg5-Atg16L1 complex that marks the LC3 conjugation site, was relocated onto alternate target membranes, the host GTPases accumulated at the new target membranes rather than the PVM [64]. A later clearance of *Toxoplasma* by autophagy was shown to occur after PVM disruption and removal of the PVM and parasite plasma membrane, in primed macrophages [41]. Here, a dependence on the IRG Irgm3 was observed, which localises to the autophagosomal membranes enveloping the naked parasite [41].

The E3 ubiquitin ligases TRAF6 and TRIM21 in part mediate the ubiquitination of type II and III PVMs in mouse cells, with other E3 ligases likely involved [68,69]. Following this, the recruitment of p62 and mGBPs leads to PVM disruption [68]. IRGM proteins are critical for targeting of PVM by TRAF6, p62, and ubiquitin, and the autophagy proteins Atg3/5/7 and 16L1 are needed for ubiquitin and p62 recruitment to the murine PVM [68,69]. However, in this instance, p62 does not play a role as an autophagy adaptor, but is key in activation of vacuolar-antigen-specific $CD8^+$ T cells [69]. Of note, macrophages lacking p62 had no impact on IRG or GBP recruitment or on parasite clearance and replication [69]. The substrates of ubiquitination at the PVM have remained elusive, with only one report demonstrating Irga6 itself being ubiquitinated [70].

Human Host-Dependent Recognition of the PVM and Parasite Restriction

In humans, no role for the IFN-stimulated IRGs has been documented. Only one full-length IRG (IRGC) is present and it is non-interferon-inducible and testis specific. Another truncated IRG protein can be found in the human genome (IRGM) [71]. Polymorphisms in IRGM are associated with Crohn's disease, and although it is currently not clear what the link is to intracellular pathogen inclusions or vacuoles, IRGM has been reported as a risk locus for tuberculosis [51,72,73]. In the absence of a full-length IFNγ-inducible human IRG, they are highly unlikely to serve the same function as in mice. Both mice and humans possess IFNγ-inducible GBPs – eleven in mice and seven in humans. In mice, mGBPs recognise type II and III PVs [43,44,74,75]. In humans, hGBP1-5 and hGBP1 were shown to recruit to *Toxoplasma* in HAP1 and mesenchymal stromal cells respectively [60,67]. This is at odds with our own study demonstrating no recruitment of hGBP1 to the *Toxoplasma* PVM in A549 cells [59]. The data may reflect cell-type-specific differences; however, the images documenting localization to the PVM show the entire parasite stained, which is not consistent with murine-like PVM-only localization [60,67]. Of note, the antibody used in the A549 study was confirmed to be hGBP1-specific, whereas the antibodies used in the other studies are commercial hGBP antibodies that have pan-hGBP or unvalidated specificity and could potentially be reactive to other hGBPs.

Ubiquitin also recognises type II and III PVs in human cells [47,48]. The PVM is ubiquitinated by an unknown E3 ligase, followed by p62 and NDP52 binding but no obvious PVM disruption (Figure 3B) [47,48]. Minimal recognition by galectin 8 was found in an IFNγ– and type II parasite-specific manner, potentially indicating permeability of the PVM [47]. A role for autophagy proteins in *Toxoplasma* infection of the human epithelial HeLa cell line has been described [47,48]. Besides ubiquitin, p62, and NDP52, these cells recruited LC3B and membranes to the type II and III PV [48]. Knockouts of autophagy proteins Atg16L1 and Atg7 resulted in increased parasite replication with no membrane encapsulation in this cell line. In addition, the process of

autophagy did not lead to lysosomal fusion, with no evidence for LAMP1 staining, but instead led to parasite growth-restriction by an unknown mechanism [48]. This report contrasted with the IFNγ-dependent parasite clearance in primary-like human endothelial cells (HUVEC). Here, despite an initially similar IFNγ- and type II-specific accumulation of ubiquitin, p62 and NDP52 proteins at the PVM, only low levels of LC3 and GABARAP were recruited for both type I and II parasites with no evidence of autophagosome membranes [47]. Furthermore, knockdown of ATG16L1 did not impact parasite replication or clearance, whereas knockdown of p62 led to a loss of IFNγ-dependent restriction of type II *Toxoplasma*. The parasites were trafficked to the host endolysosomal system for destruction without the involvement of autophagy (Figure 3B). Whether this is a difference in parasite clearance by endothelial versus epithelial cells or whether this is due to the high endogenous autophagic flux previously reported in HeLa cells remains to be determined. However, other studies have shown that the key autophagy mediator Atg5 is not important in restriction of the parasite in human foreskin fibroblast (HFF) cells [76].

Toxoplasma Virulence Factors Defend the PV from the IFNγ–Mediated Attack in Murine Cells

Toxoplasma ROPs and GRAs defend the parasite against the murine host (Figure 4). Several ROP proteins have been identified as key virulence factors that hijack host cellular functions. These proteins belong to a family of rhoptry kinases that have an active kinase or pseudokinase domain [77–80]. TgROP17 and TgROP18 phosphorylate IRG proteins, causing them to fall off the PVM [81–83]. While TgROP18 seems to have a preference for Irga6 over Irgb6 and Irgb10 [81,82,84,85], TgROP17 was shown to target Irgb6 over Irga6 [83]. TgGRA7 regulates TgROP18 acting on Irga6 [85,86]. TgROP18 and TgROP17 are polymorphic, with TgROP18 highly expressed by virulent type I and avirulent type II parasites, and TgROP17 sharing an allele in type II/III that is distinct from type I parasites [83,87]. In order for TgROP18 to efficiently phosphorylate Irga6, it binds to TgROP5, a pseudokinase, and its interaction with Irga6 keeps this GTPase in the inactive GDP-bound form [85,86,88–90]. TgGRA7 is an additional player in this parasite kinase complex [85,86]. Deletion of TgROP5 in RH type I *Toxoplasma* renders this virulent strain completely avirulent in C57BL/6 mice [79,91], while only a double deletion of TgGRA7/TgROP18 lead to an attenuated parasite strain [86]. Interestingly, it is the allelic combination of TgROP5 and TgROP18 in nonclassical *Toxoplasma* strains that determines IRG coating of the PVs and the strain's virulence, not only during primary but also secondary infection [90,92]. In wild-derived mice, the TgROP5/TgROP18 virulence machinery is taken out of commission by highly polymorphic IRG proteins leading to dephosphorylated and thus active Irga6 [50].

These parasite proteins combine to engage in a battle of arms with the murine host's IRG system (Figure 4). In IFNγ-stimulated human cells, parasite virulence factors await discovery, since virulent TgROP5 and TgROP18 have been shown to exert only a minimal effect on parasite survival [90]. Ubiquitin recruitment is also not altered in mutant parasite strains possessing virulence exchanged rhoptry proteins [47,48]. Thus striking differences exist between murine and human hosts in their strategy to control *Toxoplasma*, although, as in mouse cells, in human cells, type I parasites do appear to grow more vigorously than type II or III.

IFNγ-Independent Murine and Human Host-Mediated Destruction of the PV

CD40 Ligation-Dependent Autophagy of the PV

Early reports *in vivo* identified an IFNγ-independent, but tumour necrosis factor (TNFα)-dependent control mechanism of *Toxoplasma* involving the adaptive immune system. Hereby, CD40 ligation on macrophages by CD154 on activated CD4 T cells impacts *in vivo* replication of *Toxoplasma* in the brain [93], restricting parasite growth in peripheral tissues during the acute phase of infection [94]. Subsequently it was found that CD40 ligation induces a pathway for

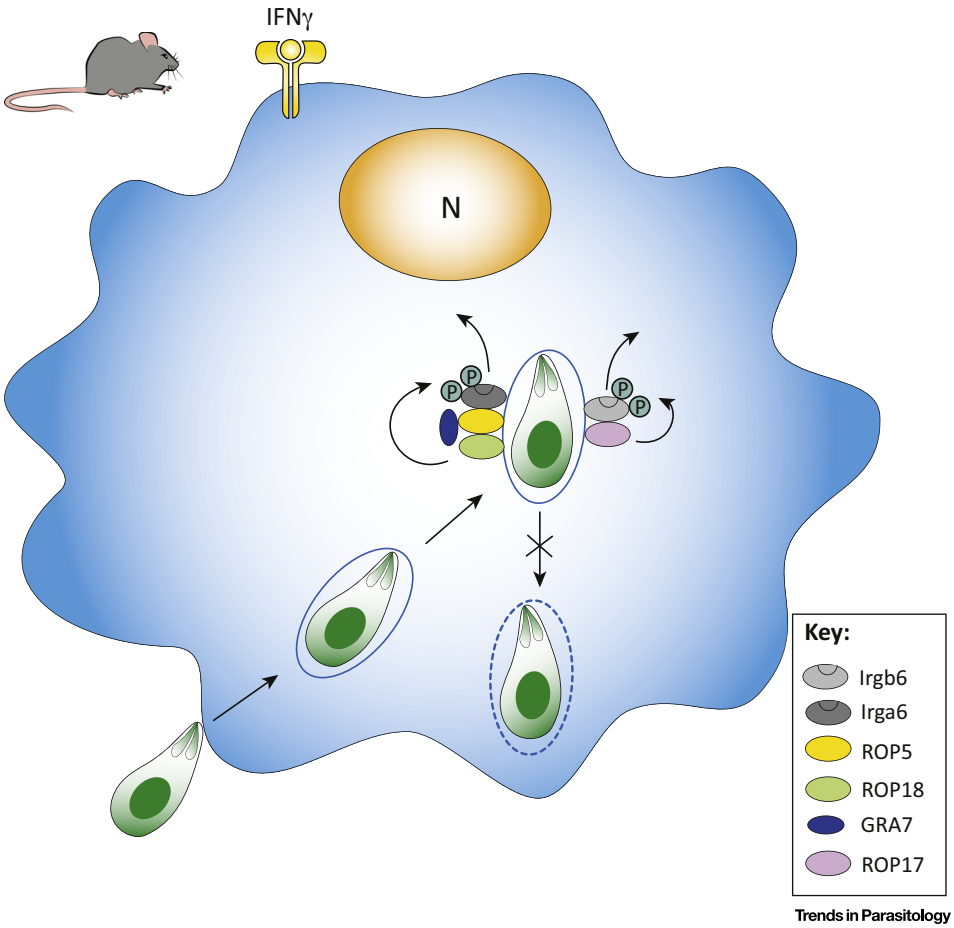

Figure 4. *Toxoplasma* Mechanisms of Defence against IFNγ-Dependent Host Control. In murine cells, rhoptry proteins counteract the IRG defence machinery. TgROP5 forms a complex with TgROP18 and binds directly to Irga6, enabling TgROP18 to phosphorylate Irga6, a process that is essentially facilitated by TgGRA7. TgROP17 phosphorylates Irgb6 independently of TgROP5. Both phosphorylation events lead to the displacement of IRG proteins from the PV and an avoidance of PVM rupture. Abbreviations: IRG, immunity-related GTPases; ROP, rhoptry protein; PV, parasitophorous vacuole; PVM, parasitophorous vacuolar membrane; N, nucleus; P, phosphate.

control of *Toxoplasma* by inducing its autophagic clearance (Figure 5) [95]. The autophagic machinery localises around the PV within 6 h and is comprised of late endolysosomal markers [95]. These findings suggested that CD40 ligation directs the PV to fuse with endolysosomal compartments. It is currently believed that the PVM stays intact throughout; however, more detailed microscopy is required for confirmation. CD40 ligation to kill *Toxoplasma* requires synergy with TNFα [96]. To achieve this, CD40 recruits TRAF6 to an intracellular binding site serving two purposes: it enhances autocrine production of TNFα [97], and TRAF6 signaling downstream of CD40 synergises with TNFα to activate autophagy [98]. While the former uses Beclin1 to induce autophagy, the latter signals through ULK1 to synergistically achieve the same [99]. This mechanism is mostly studied in murine macrophages, with additional evidence of its existence in human macrophages [95] and nonhaematopoietic murine cells [100].

Toxoplasma-Mediated EGFR Activation to Prevent Autophagy of the PV

To ensure tachyzoite survival, *Toxoplasma* has to maintain the nonfusogenic property of the PV. To this end, infection with *Toxoplasma* leads to the activation of epidermal growth factor receptor (EGFR) Akt signalling in host cells, preventing the targeting of the PVM by the

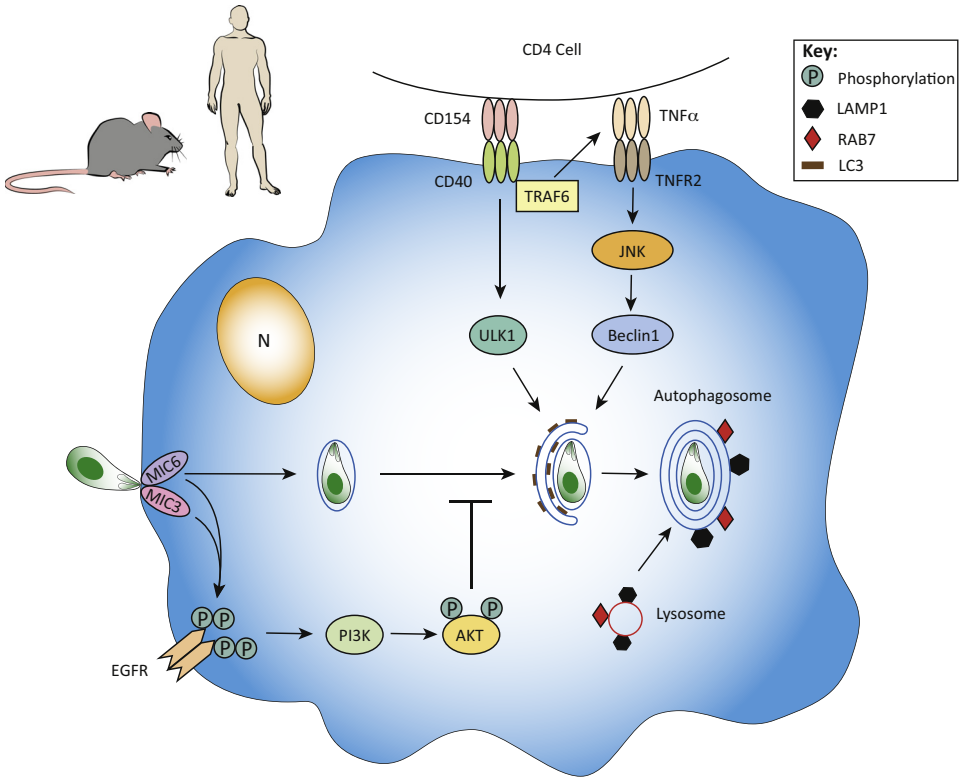

Figure 5. Non-IFNγ-Dependent Mechanisms of *Toxoplasma* control. Scheme of CD40-dependent autophagy, a balance between host and parasite. Interaction of *Toxoplasma*-infected macrophages with $CD4^+$ T cells leads to expression of CD154 on the T cell surface. Subsequent CD40-CD154 binding permits recruitment of TRAF6 to the cytoplasmic portion of CD40. Consequently, TNFα-expression is increased. Synergy between TRAF6 signaling via ULK1, and TNFα signaling via JNK and Beclin1, triggers autophagy. The destruction of the parasite is completed by the Rab7-dependent deployment of lysosomes to the autophagosome. *Toxoplasma* is able to modulate this autophagy-mediated parasite destruction by activation of host EGFR. On invasion, the parasite secretes microneme proteins, MIC3 and 6, which promote EGFR activation leading to activation of host AKT by PI3K-dependent phosphorylation. Activated AKT is then able to prevent recruitment of LC3 and autophagy membranes to the parasite. Abbreviations: CD40, cluster of differentiation 40; CD154, CD40 ligand; TNFα, tumour necrosis factor alpha; TNFR2, tumour necrosis factor receptor 2; JNK, Jun N-terminal kinase; ULK1, Unk51-like autophagy activating kinase 1; MIC, microneme protein; EGFR, epidermal growth factor receptor; AKT, protein kinase B; PI3K, phosphoinositide 3-kinase; N, nucleus; P, phosphate; TRAF6, TNF receptor associated factor 6.

autophagy protein LC3 and thus avoiding Beclin1- and Atg7-dependent autophagic clearance (Figure 5) [101]. Phosphorylation of Akt increased in response to parasite infection, requiring viable parasites, and was IFNγ-independent. This occurs in many cell types, including human brain endothelial cells and retinal cells as well as mouse endothelial cells, microglial cells, and macrophages [101]. Avoidance of autophagy was observed for both type I and type II parasites. When Akt or EGFR were depleted by siRNA or chemical inhibition, LC3 was found to accumulate around the PVM and a decrease in tachyzoites per 100 cells recorded 24 h postinfection [101]. It is important to note that, in this pathway, overall autophagy was not inhibited by *Toxoplasma* infection, but rather a targeted recruitment of LC3 to the PVM was blocked by activation of EGFR. It was found that the two parasite microneme (**MIC**) proteins containing EGF domains, MIC3 and MIC6, were important contributors to this process [101]. Another study confirmed that Gefitinib, an EGFR inhibitor, halted parasite replication in HeLa cells, as measured by the number of type I tachyzoites per PV [102]. It is noteworthy that the

inhibitor was added 1 h postinfection and thus may have exerted its effect on related parasite tyrosine kinases.

Although, as we discuss in earlier sections of this review, the predominant murine mechanism for parasite clearance is IFNγ-dependent, the CD40-dependent pathway serves as an alternative way to eliminate *Toxoplasma* and operates in mouse and human cell types of different origin. The parasite response appears similarly universal, with both type I and II *Toxoplasma* activating EGFR-Akt to avoid autophagic elimination.

Conclusion and Future Perspectives

The PVM is an essential barrier between *Toxoplasma* and host. Communication across the PVM provides the parasite with the means to survive and replicate by being able to access nutrients and larger molecules. On the flipside, the host can attack and kill the parasite, with the PVM being the recognition surface in murine cells. This leads to an arms race between host and parasite, with different molecules interacting across the border.

Other apicomplexans avoid host recognition by alternative means. *Theileria*, for example, passively enters the host cell by endocytosis, zippering its way in through ligand–receptor interactions with the cell membrane. Once inside, the parasite dispenses with its endocytic vacuolar membrane within minutes accompanied by microneme secretion, to avoid fusion with the host endolysosomal apparatus [103].

Of interest, patients with partial IFNγR1 deficiency do not suffer from toxoplasmosis, even though being *Toxoplasma* seropositive [104]. TNFα stimulation of these patient's macrophages could compensate for the absent IFNγ-dependent *Toxoplasma* killing. It remains to be investigated what the mechanism of TNFα-dependent *Toxoplasma* control at the PV are in primary human macrophages. Equally, the parasite's strategy for circumventing host attack in immune-stimulated human cells remains unknown and will be important to address (see Outstanding Questions).

Outstanding Questions

Is transport through the newly identified pore (TgGRA23/TgGRA17) in the PVM regulated?

How are exported proteins not containing a conserved TEXEL motif translocated to the host?

What is the function of organelle recruitment to the PVM, and why is it parasite-strain-specific for mitochondria?

Is IFNγ the most important cytokine mediating immune protection of acute-phase *Toxoplasma* infection in humans?

What are the *Toxoplasma* virulence factors that control evasion of immune destruction of the PVM in human cells?

What are the substrates of ubiquitination at the PVM in human cells?

Do any hGBPs recruit to the PVM in human infections?

Does the difference in parasite control in HUVEC and HeLa cells reflect a difference in mechanism between endothelial and epithelial cells?

What are the defence mechanisms occurring at the PVM in human macrophages?

What is the pathway of vacuolar acidification in IFNγ-stimulated human cells? At what stage does the PVM become committed to the endolysosomal pathway?

How are growth-stunted *Toxoplasma* cleared in HeLa cells?

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