THE CELL BIOLOGY OF PROTIST PARASITE-HOST INTERFACES

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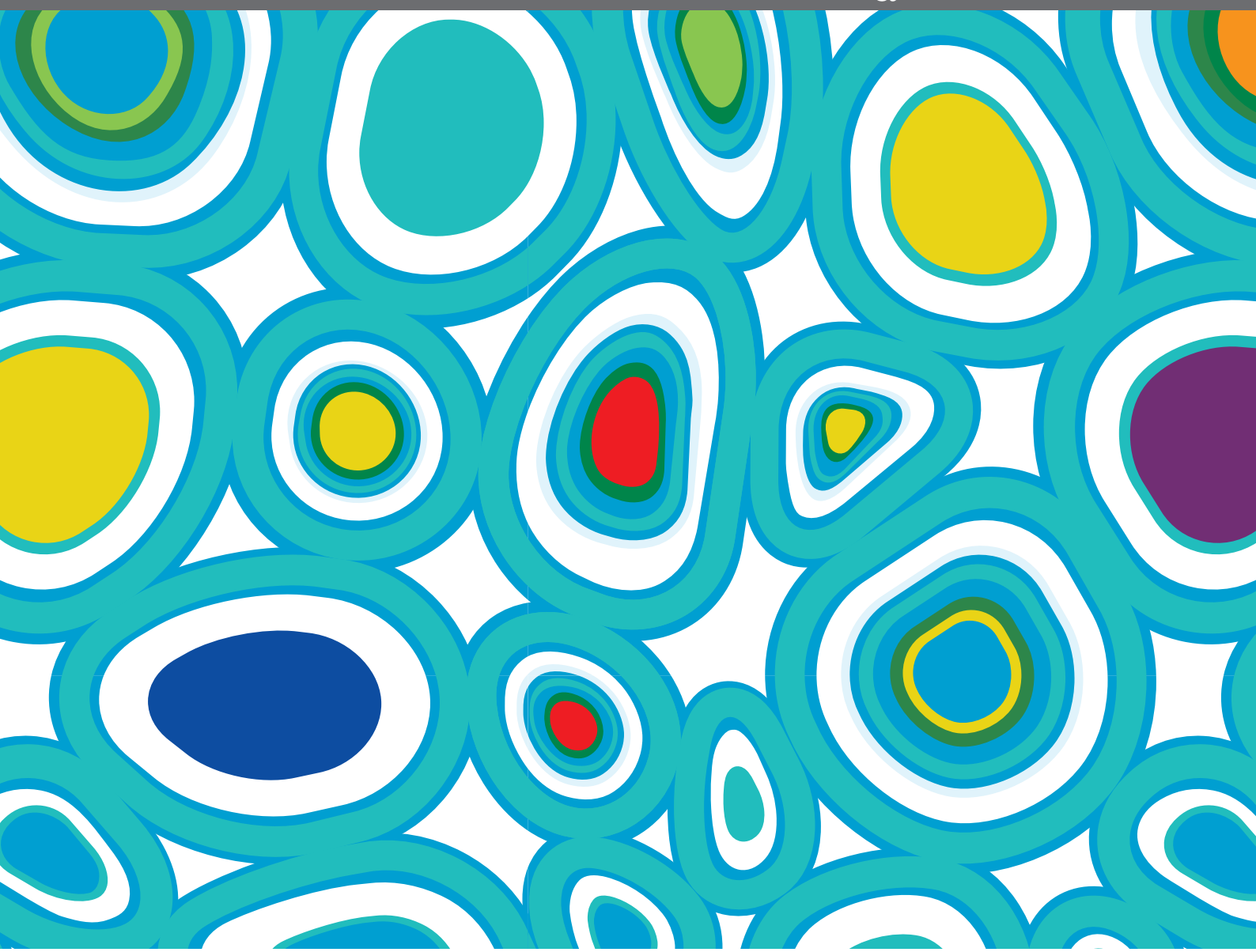

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THE CELL BIOLOGY OF PROTIST PARASITE-HOST INTERFACES

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EDITORIAL
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Editorial: The Cell Biology of Protist Parasite-Host Interfaces

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Keywords: parasite-host interactions, protist, cell-invasion, protein-trafficking, immune-evasion strategies, parasite nutrition

Editorial on the Research Topic

The Cell Biology of Protist Parasite-Host Interfaces

Molecular exchange at the parasite-host interface is key to parasite survival since these eukaryotic species feed off of host-acquired nutrients and often complete key stages of their life-cycles within them. The focus of this special issue is on our level of mechanistic understanding of the complex communication established between a parasite and a host, with a focus on protist parasites which cause significant morbidity and disability worldwide in both medical and veterinary contexts. Protist parasites have evolved an incredibly diverse range of strategies to invade their hosts and ensure survival and the cell biology underlying interactions and protein/metabolite trafficking at protist parasite-host interfaces, whether extra- or intra- cellular, is a field of intense investigation.

In this special issue, contributions from experts in Plasmodium, Toxoplasma, Theileria, Giardia, Leishmania, Trypanosoma and Entamoeba cell biology provide critical reviews and experimental data on essential topics such as parasite nutrition, parasite surface remodeling, protein trafficking and parasite biology in terms of invasion and niche establishment.

Focusing on parasite cell biology, Zeeshan et al. report on the essentiality of parasite kinesin 5 for the production of infectious *Plasmodium berghei* sporozoites. They are joined by Thomas et al. in their investigation of the role for *Gl*Rac in defining progression and maturation of organelles essential for infectious cyst formation in *Giardia lamblia*. A more "omics"-based approach enables Sun et al. to investigate global proteomics changes in both host and parasite cells during *Toxoplasma gondii* infection while König et al. compare and contrast the predicted proteome of *Entamoeba histolytica* and its sister species to discern lineage-specific genes which may help explain virulence patterns in otherwise closely-related species. With a focus on Leishmania pathogenesis, Salloum et al. discuss insight from RNA-Seq based analyses and present an exciting outlook for investigation which includes the host's/vector's microbiota in the equation for a parasite's pathobiology.

Critical reviews on parasite-dependent hijacking of host cell-specific pathways include an in-depth discussion and appreciation for the role of autophagy in *Toxoplasma gondii* infection and establishment in nervous tissue by Subauste, joined by Woods et al. for a discussion of strategies Theileria parasites to invade and immortalize leukocytes. For a stronger focus on parasite nutrition, Counihan et al. discuss recent advances in our understanding of how Plasmodium parasites take up nutrients from the host cell milieu, including the role for endocytosis in ferrying hemoglobin to the parasitophorous vacuole. Endocytosis and protein trafficking routes involved in parasite virulence are discussed in three manuscripts. Link et al. discuss secretory and endosomal pathways in trypanosomes with a focus on VSG proteins, antigenic variation and its important role in immune evasion. They are joined by Borges et al. for a targeted appraisal of the GPI anchor as

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a "universal evolutionary building block" for a diverse set of surface molecules in trypanosomes. Finally, Balmer and Faso review and place the limited data on unconventional protein secretion pathways involved in protist parasite virulence in a wider context.

Structures and processes at the host-parasite interface are notoriously difficult to analyze because the complex orchestration of interactions which have evolved with specific purposes requires investigating two very different organisms simultaneously. The growing community of scientists who explore molecular communications between pathogens and their hosts is addressing this challenge by driving the implementation of rapidly advancing technological developments. This allows researchers to study developmental processes and host-parasite interactions far beyond the mere uptake of nutrients and building blocks by parasites. Specifically, modern -omics approaches and cutting-edge microscopy have been very successful in bringing the term host-pathogen co-evolution to life on a molecular level, revealing the seemingly limitless ingenuity of both parasites and hosts in their perpetual arms race to evolve interaction strategies for colonizing or fending off invasion. However, omics-approaches depend heavily on the quality of parasite genome annotations which are wanting due to the large proportion of highly diverged genes and the richness of new inventions not present in well characterized model organism. The upshot is that a third or more returns from high-content -omics experiments with parasites remain non-interpretable which detracts significantly from the value of the investment in time and materials. Moreover, researchers tend to focus on the "known", ignoring the big chunk of uncharted "dark data" in which the truly novel discoveries remain hidden, thus preventing a more complete understanding of structural and dynamic aspects of host-pathogen interactions. Therefore, despite producing comprehensive landscapes of gene and protein regulation which describing these interactions, the insights remain unsatisfyingly incomplete. Nevertheless, the power of genetic manipulation tools, especially CRISPR-Cas9 (when applicable), in combination with phenotype analysis, holds the promise of closing these gaps while assigning functions to hitherto uncharacterized parasite gene products. This may lead to the identification of novel therapeutic and diagnostic leads which would otherwise remain completely unknown.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication

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ORIGINAL RESEARCH
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Plasmodium berghei Kinesin-5 Associates With the Spindle Apparatus During Cell Division and Is Important for Efficient Production of Infectious Sporozoites

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Kinesin-5 motors play essential roles in spindle apparatus assembly during cell division, by generating forces to establish and maintain the spindle bipolarity essential for proper chromosome segregation. Kinesin-5 is largely conserved structurally and functionally in model eukaryotes, but its role is unknown in the *Plasmodium* parasite, an evolutionarily divergent organism with several atypical features of both mitotic and meiotic cell division. We have investigated the function and subcellular location of kinesin-5 during cell division throughout the *Plasmodium berghei* life cycle. Deletion of *kinesin-5* had little visible effect at any proliferative stage except sporozoite production in oocysts, resulting in a significant decrease in the number of motile sporozoites in mosquito salivary glands, which were able to infect a new vertebrate host. Live-cell imaging showed kinesin-5-GFP located on the spindle and at spindle poles during both atypical mitosis and meiosis. Fixed-cell immunofluorescence assays revealed kinesin-5 co-localized with α-tubulin and centrin-2 and a partial overlap with kinetochore marker NDC80 during early blood stage schizogony. Dual-color live-cell imaging showed that kinesin-5 is closely associated with NDC80 during male gametogony, but not with kinesin-8B, a marker of the basal body and axonemes of the forming flagella. Treatment of gametocytes with microtubule-specific inhibitors confirmed kinesin-5 association with nuclear spindles and not cytoplasmic axonemal microtubules. Altogether, our results demonstrate that kinesin-5 is associated with the spindle apparatus, expressed in proliferating parasite stages, and important for efficient production of infectious sporozoites.

Keywords: malaria, kinesin-5/eg5, Plasmodium, spindle, sporozoite

INTRODUCTION

Kinesin-5 proteins are a family of molecular motors that is structurally and functionally conserved throughout eukaryotes (Wojcik et al., 2013; Waitzman and Rice, 2014; Mann and Wadsworth, 2019). They are involved in spindle pole separation and are considered essential for mitosis in the vast majority of eukaryotes (Bannigan et al., 2007; Ferenz et al., 2010), except *Caenorhabditis elegans* (Bishop et al., 2005), *Dictyostelium discoideum* (Tikhonenko et al., 2008), and *Candida albicans* (Shoukat et al., 2019). Kinesin-5 contains an N-terminal kinesin motor domain, a central stalk and a C-terminal tail domain (Wojcik et al., 2013), and forms a bipolar homotetramer that cross-bridges and slides on parallel and anti-parallel microtubules (MTs) (Kapitein et al., 2008). The motor domain binds microtubules (MTs) and hydrolyzes ATP, which are conserved functions, while the tail region can also bind MTs, regulate motor activity (Bodrug et al., 2020) and help localization during mitosis (Weinger et al., 2011; Bodrug et al., 2020). The stalk contains a coiled-coil region and neck linker that promote oligomerization and direction of movement, respectively (Hesse et al., 2013). The kinesin-5 motor domain is conserved across eukaryotes including *Plasmodium* (**Supplementary Figure 1A**), but the tail region is highly variable except for a short, conserved region called the BimC box, which contains consensus sites for phosphorylation by cyclin dependent kinase 1 (Cdk1) in most eukaryotes (Sharp et al., 1999; Bishop et al., 2005; Chee and Haase, 2010).

Kinesin-5 is located at spindle MTs and spindle poles during cell division and is distributed diffusely in the cytoplasm during interphase in most eukaryotic cells (Ferenz et al., 2010; Waitzman and Rice, 2014). The essential roles of kinesin-5 in spindle assembly and spindle pole separation can be blocked by specific inhibitors or antibodies, resulting in collapse of bipolar spindles into monopoles (Sharp et al., 1999; Kapoor et al., 2000; Ferenz et al., 2010; Mann and Wadsworth, 2019). Kinesin-5 is required for maintenance of a bipolar spindle in fungi, Xenopus, and Drosophila (Hoyt et al., 1992; Sharp et al., 1999; Kapoor et al., 2000). In budding yeast the kinesin-5 proteins, Cin8, and Kip1, are also present at the kinetochores and help in chromosome alignment during metaphase (Tytell and Sorger, 2006).

Malaria is a deadly vector-borne infectious disease, caused by a unicellular protozoan parasite of the genus *Plasmodium*, which infects many vertebrate hosts including humans, and is transmitted by female Anopheles mosquitoes (WHO, 2019). During the complex life cycle two unique phases of atypical closed mitotic division occur. The first type of mitosis occurs during asexual proliferation, with multiple asynchronous nuclear divisions producing up to 32 nuclei (during schizogony in the blood of the vertebrate host) or more than 1,000 nuclei (during schizogony in the liver of the vertebrate host and sporogony in the mosquito gut), with cytokinesis occurring only after nuclear division is complete, to produce haploid progeny cells (Sinden et al., 1976; Sinden, 1991; Gerald et al., 2011; Francia and Striepen, 2014). It is important to note that the final round of nuclear division is asynchronous, in contrast to what was thought previously (Rudlaff et al., 2020). The second type of mitosis is during male gametogony (part of the sexual stage in the mosquito gut) where there are three rapid rounds of DNA replication from 1N to 8N within 10 to 15 min, followed by karyokinesis and cytokinesis to produce eight flagellate haploid male gametes (Sinden et al., 1976; Zeeshan et al., 2019a). The first phase of meiotic division occurs following fertilization as the zygote differentiates into a motile ookinete in the mosquito gut. The 2N genome is duplicated and recombination occurs (Sinden, 1991); then the final reductive division likely occurs in the oocyst that is formed following ookinete penetration of the mosquito gut wall, leading to the formation of haploid sporozoites. Thus, *Plasmodium* has unusual ways to divide and survive under different physiological conditions in different hosts. As cell division in *Plasmodium* is atypical, so the molecules regulating cell division are also divergent from those of higher eukaryotes (Tewari et al., 2010; Guttery et al., 2014; Roques et al., 2015; Wall et al., 2018). The large family of kinesins includes molecular motors that are essential for several processes during cell division (Wordeman, 2010; Yount et al., 2015), and in *Plasmodium berghei* there are nine kinesin genes, including two kinesin-8 genes that are important in cell division and male gamete formation (Zeeshan et al., 2019a; Zeeshan et al., 2019b). There is a single *Plasmodium* kinesin-5, and since this protein plays an important role during cell proliferation in many eukaryotes, and is considered as a strong target for the development of therapeutics against many diseases including malaria (Liu et al., 2014), we decided to study its role during cell division in the malaria parasite.

In the present study we examined the spatiotemporal dynamics and functional role of kinesin-5 during the proliferative stages of *P. berghei*. Unlike kinesin-5 in many other eukaryotes, *P. berghei* (Pb) kinesin-5 is dispensable for parasite growth and proliferation, although deletion of the gene results in a remarkable decrease in the number of mosquito salivary gland sporozoites. Live cell imaging shows that kinesin-5 is located both on the spindle and at spindle poles during mitotic and meiotic divisions in the parasite life cycle, co-localizing with centrosome marker centrin and kinetochore marker NDC80. Treatment of gametocytes with microtubule inhibitors confirmed the nuclear localization and association with spindle microtubules of kinesin-5 during male gametogony. Altogether, our results show that kinesin-5 is associated with the spindle apparatus, which can be inhibited by microtubule inhibitors, and that the protein is important for efficient production of infectious sporozoites.

MATERIAL AND METHODS

Ethics Statement

The animal work performed in this study has passed an ethical review process and was approved by the United Kingdom Home Office. Work was carried out in accordance with the United Kingdom 'Animals (Scientific Procedures) Act 1986' for the protection of animals used for experimental purposes under Licence number 40/3344. Six to 8-week-old Tuck's Original (TO) (Harlan) outbred mice were used for all experiments.

Generation of Transgenic Parasites

The gene-deletion targeting vector for *Pbkinesin-5* (PBANKA_0807700) *was* constructed using the pBS-DHFR plasmid, which contains polylinker sites flanking a *T. gondii dhfr/ts* expression cassette conferring resistance to pyrimethamine, as described previously (Tewari et al., 2010). PCR primers N1061 and N1062 were used to generate a 995 bp fragment of *kinesin-5* 5′ upstream sequence from genomic DNA, which was inserted into *Apa*I and *Hin*dIII restriction sites upstream of the *dhfr/ts* cassette of pBS-DHFR. A 1008 bp fragment generated with primers N1063 and N1064 from the 3′ flanking region of *kinesin-5* was then inserted downstream of the *dhfr/ts* cassette using *Eco*RI and *Xba*I restriction sites. The linear targeting sequence was released using *Apa*I/*Xba*I. A schematic representation of the endogenous *Pbkinesin-5* locus, the construct and the recombined *kinesin-5* locus can be found in **Supplementary Figure 1**.

To generate kinesin-5-GFP, a region of *kinesin-5* gene downstream of the ATG start codon was amplified using primers T1921 and T1922 and ligated to p277 vector, and transfected as described previously (Guttery et al., 2012). The p277 vector contains the human *dhfr* cassette, conveying resistance to pyrimethamine. Pbkinesin-5 was tagged with GFP at the C-terminus by single crossover homologous recombination. A schematic representation of the endogenous gene locus, the constructs and the recombined gene locus can be found in **Supplementary Figure 2**. The oligonucleotides used to generate the mutant parasite lines can be found in **Supplementary Table 1**. *P. berghei* ANKA line 2.34 (for GFP-tagging) or ANKA line 507cl1 expressing GFP (for gene deletion) parasites were transfected by electroporation (Janse et al., 2006).

Genotypic Analysis of Parasites

For the gene knockout parasites, diagnostic PCR was used with primer 1 (IntN106) and primer 2 (ol248) to confirm integration of the targeting construct, and primer 3 (N106 KO1) and primer 4 (N106 KO2) were used to confirm deletion of the *kinesin-5* gene (**Supplementary Figure 1**). For the parasites expressing a C-terminal GFP-tagged kinesin-5 protein, diagnostic PCR was used with primer 1 (IntT192) and primer 2 (ol492) to confirm integration of the GFP targeting construct (**Supplementary Figure 2**).

Phenotypic Analyses

To initiate infections, blood containing approximately 60,000 parasites of *Δkinesin-5* line was injected intraperitoneally (i.p.) into mice. Asexual stages and gametocyte production were monitored on Giemsa-stained thin smears. Four to five days post infection, exflagellation and ookinete conversion were examined as described previously (Guttery et al., 2012) with a Zeiss AxioImager M2 microscope (Carl Zeiss, Inc) fitted with an AxioCam ICc1 digital camera. To analyze mosquito transmission, 50-60 *Anopheles stephensi* SD 500 mosquitoes were allowed to feed for 20 min on anaesthetized, infected mice whose asexual parasitemia had reached up to 15% and were carrying comparable numbers of gametocytes as determined on Giemsa-stained blood films. To assess mid-gut infection, approximately 15 guts were dissected from mosquitoes on day 14 post feeding and oocysts were counted on a Zeiss AxioImager M2 microscope using 10x and 63x oil immersion objectives. On day 21 post-feeding, another 20 mosquitoes were dissected, and their guts and salivary glands crushed separately in a loosely fitting homogenizer to release sporozoites, which were then quantified using a haemocytometer or used for imaging and motility assays. Mosquito bite back experiments were performed 21-day post-feeding using naive mice; 15–20 mosquitoes infected with WT-GFP or *Δkinesin-5* parasites were fed for at least 20 min on naive CD1 outbred mice and then infection was monitored after 3 days by examining a blood smear stained with Giemsa's reagent. For comparison between *Δkinesin-5* and WT-GFP, an unpaired Student's *t*-test was used.

Culture and Gradient Purification of Schizonts and Gametocytes

Blood cells obtained from infected mice (day 4–5 post-infection) were placed in culture for 8–10 and 24 h at 37°C (with rotation at 100 rpm) and schizonts were purified on a 60% v/v NycoDenz (in PBS) gradient, harvested from the interface and washed (NycoDenz stock solution: 27.6% w/v NycoDenz in 5 mM Tris-HCl, pH 7.20, 3 mM KCl, 0.3 mM EDTA). Purification of gametocytes was achieved using a protocol as described previously (Beetsma et al., 1998) with some modifications. Briefly, parasites were injected into phenylhydrazine treated mice and enriched by sulfadiazine treatment after 2 days of infection. The blood was collected on day 4 after infection and gametocyte-infected cells were purified on a 48% v/v NycoDenz (in PBS) gradient. (NycoDenz stock solution: 27.6% w/v NycoDenz in 5 mM Tris-HCl, pH 7.20, 3 mM KCl, 0.3 mM EDTA). The gametocytes were harvested from the interface and washed.

Live-Cell and Time-Lapse Imaging

Different developmental stages of parasite during schizogony, zygote to ookinete transformation and sporogony were analyzed for kinesin-5-GFP expression and localization using a 63x oil immersion objective on a Zeiss Axio Imager M2 microscope. Kinesin-5 expression and location was examined during different developmental stages of the parasite life cycle. Purified gametocytes were examined for GFP expression and localization at different time points (0, 1–15 min) after activation in ookinete medium. Images were captured using a 63x oil immersion objective on the same microscope. Time-lapse videos (1 frame every 5 s for 15–20 cycles) were taken with a 63x objective lens on the same microscope and analyzed with the AxioVision 4.8.2 software.

Generation of Dual Tagged Parasite Lines

The kinesin-5-GFP parasites were mixed with either NDC80-cherry or kinesin-8B-cherry parasites in equal numbers and injected into a mouse. Mosquitoes were fed on this mouse 4 to 5 days after infection when gametocyte parasitaemia was high. These mosquitoes were checked for oocyst development and sporozoite formation at day 14 and day 21 after feeding. Infected mosquitoes were then allowed to feed on naïve mice and after 4–5 days these mice were examined for blood stage parasitaemia by

microscopy with Giemsa-stained blood smears. In this way, some parasites expressed both kinesin-5-GFP and NDC80-cherry or kinesin-5-GFP and kinesin-8B-cherry in the resultant schizonts and gametocytes, and these were purified, and fluorescence microscopy images were collected as described above.

Inhibitor Studies

Gametocytes from the parasites expressing kinesin-5-GFP and NDC80-cherry were purified as above and treated with Taxol (Sigma) and an antimalarial molecule (DDD01028076) (Zeeshan et al., 2019a) at 1 mpa and then fixed with 4% paraformaldehyde (PFA, Sigma) at 8 min after activation. Dimethyl sulfoxide (DMSO) was used as a control treatment. These fixed gametocytes were then examined on a Zeiss AxioImager M2 microscope.

Fixed Immunofluorescence Assay

The purified schizonts from PbKinesin-5-GFP parasites were fixed in 2% paraformaldehyde (PFA) and smeared on poly-L-lysine coated slides. Purified gametocytes were activated in ookinete medium then fixed at 1, 2, 4, 6, 8, and 15 min post-activation with 4% PFA diluted in microtubule stabilizing buffer (MTSB) for 10–15 min and added to poly-L-lysine coated eight-well slides. Immunocytochemistry was performed using primary GFP-specific rabbit monoclonal antibody (mAb) (Invitrogen-A1122; used at 1:250) and primary mouse anti-α tubulin mAb (Sigma-T9026; used at 1:1000) or mouse anti-centrin mAb (Millipore-04-1624, used at 1:500) or anti-NDC80 (polyclonal sera, a kind gift from Marc-Jan Gubbels). Secondary antibodies were Alexa 488 conjugated anti-mouse IgG (Invitrogen-A11004) and Alexa 568 conjugated anti-rabbit IgG (Invitrogen-A11034) (used at 1 in 1000). The slides were then mounted in Vectashield 19 with DAPI (Vector Labs) for fluorescence microscopy. Parasites were visualized on a Zeiss AxioImager M2 microscope. Pearson's colocalization coefficient (R) was calculated using image J software (version 1.44).

Sporozoite Motility Assays

Sporozoites were isolated from salivary glands of mosquitoes infected with WT-GFP and *Δkinesin-5* parasites on day 21 post-infection. Isolated sporozoites in RPMI 1640 containing 3% bovine serum albumin (Fisher Scientific) were pelleted (5 min, 5,000 rpm, 4°C) and used for motility assays as described previously (Wall et al., 2019). Briefly, a drop (6 µl) of sporozoites was transferred onto a microscope glass slide with a cover slip. Time-lapse videos of sporozoites (one frame every 1 s for 100 cycles) were taken using the differential interference contrast settings with a 63x objective lens on a Zeiss AxioImager M2 microscope and analyzed with the AxioVision 4.8.2 software. The motility was also analyzed by using Matrigel. A small volume (20 µl) of sporozoites, isolated as above was mixed with Matrigel (Corning). The mixture (6 µl) was transferred on a microscope slide with a cover slip and sealed with nail polish. After identifying a field containing sporozoites, time-lapse videos (one frame every 2 s for 100 cycles) were taken using the differential interference contrast settings with a 63x objective lens.

Liver Stage Parasite Imaging

For *P. berghei* liver stage parasites, 100,000 HeLa cells were seeded in glass-bottomed imaging dishes. Salivary glands of female *A. stephensi* mosquitoes infected with kinesin-5-GFP parasites were isolated and disrupted using a pestle to release sporozoites, which were pipetted gently onto the seeded HeLa cells and incubated at 37°C in 5% CO_2 in complete minimum Eagle's medium containing 2.5 µg/ml amphotericin B (PAA). Medium was changed 3 h after initial infection and once a day thereafter. For live cell imaging, Hoechst 33342 (Molecular Probes) was added to a final concentration of 1 µg/ml, and parasites were imaged at 24, 48, 55 h post-infection using a Leica TCS SP8 confocal microscope with the HC PL APO 63x/1.40 oil objective and the Leica Application Suite X software.

qRT-PCR Analysis

RNA was isolated from different stages of parasites including all asexual stages, schizonts, gametocytes, ookinete and sporozoites using an RNA purification kit (Stratagene). cDNA was synthesized using an RNA-to-cDNA kit (Applied Biosystems). Gene expression was quantified from 80 ng of total RNA using SYBR green fast master mix kit (Applied Biosystems). All of the primers were designed using primer3 (Primer-blast, NCBI), and amplified a region of 150–200 bp. Analysis was conducted using an Applied Biosystems 7500 fast machine with the following cycling conditions: 95°C for 20 s followed by 40 cycles of 95°C for 3 s; 60°C for 30 s. Three technical replicates and three biological replicates were performed for each assayed gene. The *hsp70* (PBANKA_081890) and *arginyl-t RNA synthetase* (PBANKA_143420) genes were used as endogenous control reference genes. The primers used for qPCR can be found in **Supplementary Table 1**.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software). For qRT-PCR, an unpaired t-test was conducted to examine significant differences between wild-type and mutant strains.

RESULTS

Pbkinesin-5 Is Expressed at Multiple Proliferative Stages in the Parasite Life Cycle

To quantify the expression of kinesin-5 at different stages of the parasite life cycle, we isolated RNA and performed qRT-PCR. *Kinesin-5* is expressed constitutively throughout the blood and mosquito stages of parasite development, with the highest level in gametocytes, followed by schizonts and ookinetes (**Figure 1A**).

Pbkinesin-5 Is Important for Efficient Production of Infectious Sporozoites

The *Plasmodium* life cycle has two unusual mitotic processes that occur during schizogony/sporogony and male gametogony, and

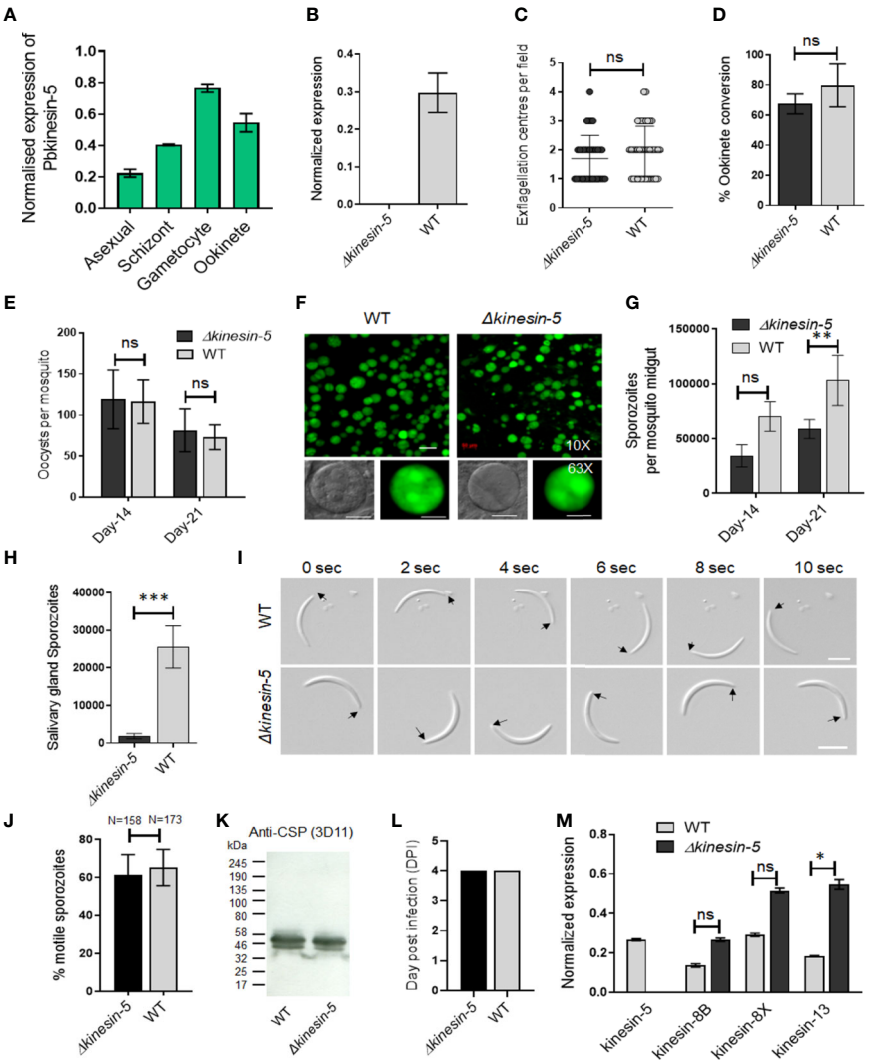

FIGURE 1 | The *Pb*kinesin-5 gene is dispensable for parasite transmission but has a role in sporozoite development. **(A)** Transcript levels of kinesin-5 revealed by qRT-PCR, normalized against two endogenous control genes, arginine-tRNA synthetase and hsp70. Each bar is the mean of three biological replicates ± SD. **(B)** qRT-PCR analysis of *kinesin-5* transcription in *Δkinesin-5* and WT-GFP parasites, showing the complete depletion of *kinesin-5*. Each bar is the mean of three biological replicates ± SD. **(C)** Male gametogony (exflagellation) of *Δkinesin-5* line (black bar) and WT-GFP line (grey bar) measured as the number of exflagellation centres per field. Mean ± SD; n=6 independent experiments. **(D)** Ookinete conversion as a percentage for *Δkinesin-5* (black bar) and WT-GFP (grey bar) parasites. Ookinetes were identified using 13.1 antibody as a surface marker and defined as those cells that differentiated successfully into elongated 'banana shaped' ookinetes. Mean ± SD; n=6 independent experiments. **(E)** Total number of GFP-positive oocysts per infected mosquito in *Δkinesin-5* (black bar) and WT-GFP (grey bar) parasites at 14- and 21-day post infection (dpi). Mean ± SD; n=5 independent experiments. **(F)** Mosquito mid guts at 10x and 63x magnification showing oocysts of *Δkinesin-5* and WT-GFP lines at 14 dpi. Scale bar = 50 µm in 10x and 20 µm in 63x. **(G)** Total number of sporozoites in oocysts of *Δkinesin-5* (black bar) and WT-GFP (grey bar) parasites at 14 and 21 dpi. Mean ± SD; n=4 independent experiments. **(H)** Total number of sporozoites in salivary glands of *Δkinesin-5* (black bar) and WT-GFP (grey bar) parasites. Bar diagram shows mean ± SD; n=4 independent experiments. **(I)** Differential interference contrast (DIC) time-lapse image sequences showing motile *Δkinesin-5* and WT-GFP sporozoites isolated from salivary glands. Arrow indicates apical end of sporozoites. Scale bar = 5 µm. **(J)** Quantitative data for motile sporozoites from salivary glands for *Δkinesin-5* and WT-GFP based on two independent experiments. **(K)** Western blot analysis of WT-GFP and *Δkinesin-5* parasites. Lysates from midgut sporozoites were probed using a monoclonal antibody specific for circumsporozoite protein (CSP)-repeat region (mAb 3D11). Molecular mass markers (kDa) are shown on the left of the gel. **(L)** Bite back experiments show successful transmission of *Δkinesin-5* parasites (black bar) from mosquito to mice, similar to WT-GFP parasites (grey bar). Mean ± SD; n= 3 independent experiments. **(M)** qRT-PCR analysis of other Pbkinesin genes comparing transcript levels in WT-GFP and *Δkinesin-5* parasites. Error bar = ±SD; n=3. Unpaired t-test was performed for statistical analysis. *p < 0.05, **p < 0.01, ***p < 0.001, ns = non-significant.

a single meiotic stage during zygote to ookinete transformation (Zeeshan et al., 2020b). To examine any functional role of kinesin-5 during these processes, we deleted the gene from the *P. berghei* genome using a double crossover homologous recombination strategy in a parasite line constitutively expressing green fluorescent protein (GFP) at all stages of the parasite life cycle (**Supplementary Figure 1B**) (Janse et al., 2006). Diagnostic PCR, to show successful integration of the

targeting construct at the *kinesin-5* locus (**Supplementary Figure 1C**), and quantitative real time PCR (qRT-PCR), to show lack of *kinesin-5* expression in gametocytes, confirmed the complete deletion of the *kinesin-5* gene (**Figure 1B**). Successful creation of this transgenic parasite indicated that the gene is not essential for mitosis during asexual blood stage schizogony. Further phenotypic analysis of the *Δkinesin-5* parasite was carried out at other stages of the life cycle, comparing the parental parasite (WT-GFP) with two independent gene-knockout parasite clones (clones 3 and 5) generated by two independent transfections. Both gene-knockout clones had the same phenotype and data presented here are the combined results from both clones. Since *Δkinesin-5* parasites underwent asexual blood stage development, exhibiting no change in morphology, number of progeny merozoites or parasitemia, an essential role for kinesin-5 is unlikely during these stages that cause the disease in the mammalian host.

The transgenic parasites also produced gametocytes in mice, and therefore next we analyzed male and female gametocyte differentiation following activation in the exflagellation/ookinete medium that mimics the mosquito gut environment. Male and female gametes emerge from the infected erythrocyte, and in the case of male gamete development this is preceded by three rapid rounds of genome duplication, resulting in eight flagellate male gametes (Sinden et al., 1976; Zeeshan et al., 2019a). There was no defect in male gamete exflagellation for either of the *Δkinesin-5* parasite clones, with the same exflagellation frequency as WT-GFP parasites (**Figure 1C**). Fertilization, zygote formation and ookinete differentiation, when meiosis occurs (Sinden, 1991), were not significantly different in *Δkinesin-5* parasites from these processes in parental parasites (**Figure 1D**).

To investigate the role of kinesin-5 in oocyst development and sporogony, *Anopheles stephensi* mosquitoes were fed on mice infected with *Δkinesin-5* parasites and WT-GFP parasites as a control. The number of GFP-positive oocysts on the mosquito gut wall was counted on days 14 and 21 post-infection. There was no significant difference in the number of *Δkinesin-5* and WT-GFP oocysts (**Figure 1E**), and the size of the oocysts was similar for both parasites (**Figure 1F**). However, we observed a 40 to 50% decrease in the number of sporozoites in each oocyst at days 14 and 21 post-infection in *Δkinesin-5* parasites compared to WT-GFP parasites (**Figure 1G**) indicating the important role of kinesin-5 during sporogony. In comparison to the WT-GFP parasite, a significant decrease in the number of *Δkinesin-5* sporozoites in salivary glands was also observed (**Figure 1H**), but the shape, size, and motility of these sporozoites were indistinguishable from WT-GFP parasites (**Figures 1I, J**). Furthermore, western blot analysis of circumsporozoite protein (CSP) showed that proteolytic processing of CSP in *Δkinesin-5* sporozoites, an indicator of normal sporozoite maturation, was also not affected (**Figure 1K**). The infected mosquitoes were used for bite back experiments to ascertain the infectivity of *Δkinesin-5* sporozoites in mice; a blood stage infection was observed after 4 days with both *Δkinesin-5* and WT-GFP sporozoites (**Figure 1L**).

The loss of kinesin-5 may have been compensated for by the over-expression of another member of the kinesin family, therefore we analyzed the transcript level of kinesin-8B, kinesin-8X and kinesin-13 in gametocytes of *Δkinesin-5* parasites. These kinesins are highly expressed in gametocytes and have important role during male gametogony (Zeeshan et al., 2019a; Zeeshan et al., 2019b). We found that the kinesin-13 transcript level was significantly upregulated (**Figure 1M**), suggesting that higher levels of kinesin-13 may - in some way - compensate for the kinesin-5 deletion.

Pbkinesin-5 Is Located at the Spindle Apparatus During Mitotic Stages of Asexual Blood Stage Schizogony

To examine expression at the protein level and study the real-time dynamic location of kinesin-5 during cell division, we generated a kinesin-5-GFP transgenic *P. berghei* line expressing kinesin-5 with a C-terminal GFP tag, by inserting an in-frame *gfp* coding sequence at the 3' end of the endogenous *kinesin-5* locus using single homologous recombination (**Supplementary Figure 2A**). Successful insertion was confirmed by diagnostic PCR (**Supplementary Figure 2B**). Western blot analysis of a schizont protein extract using an anti-GFP antibody revealed kinesin-5-GFP protein at the expected size of 198 kDa compared to the 29 kDa GFP (**Supplementary Figure 2C**). This kinesin-5-GFP transgenic line was used to examine the spatiotemporal profile of kinesin-5-GFP protein expression and location by live cell imaging during the whole parasite life cycle, initially during asexual blood stage development in erythrocytes.

After haploid merozoite invasion of an erythrocyte, the initial ring and trophozoite stages are followed by schizogony, which results in the formation of further merozoites that invade fresh erythrocytes. During schizogony there are several independent asynchronous rounds of closed mitosis to produce a multi-nucleate coenocyte, followed by cytokinesis and egress of mature merozoites from the infected erythrocyte. Kinesin-5 expression was not detected in the ring and early trophozoite stages that are considered as interphase or G_0 in the cell cycle (Arnot and Gull, 1998). A very low and diffuse expression of kinesin-5 throughout the cytoplasm was observed in older trophozoites, a stage similar to G1 phase in higher eukaryotes (Arnot and Gull, 1998; Arnot et al., 2011) when preparation for DNA replication begins. Late trophozoites mark the transition into early S phase when DNA synthesis starts (Arnot et al., 2011) and schizogony is marked by the presence of three or more nuclei (Rudlaff et al., 2020). Kinesin-5 was observed as strong foci adjacent to the Hoechst-stained DNA in early schizonts when nuclear division had commenced, and representing the first M phase of the cell cycle (Arnot and Gull, 1998; Arnot et al., 2011) (**Figure 2A**). Each kinesin-5-GFP focus elongated and split into two foci that migrated away from each other, remaining adjacent to the nuclear DNA that then separated into two nuclear masses (**Figure 2A**). Alternating repeated S/M phases followed the division of individual nuclei, accompanied by repeated elongation and duplication into multiple points of these kinesin-5-GFP foci, showing the asynchronous pattern of nuclear division. Following completion of schizogony, kinesin-5 expression was almost undetectable in merozoites (**Figure 2A**).

To compare the location of kinesin-5 with that of other mitotic protein markers, including α-tubulin (spindle MTs), centrin-2 [putative centrosome/spindle pole body (SPB)/MT organising centre (MTOC)] and NDC80 (kinetochores), we used indirect immunofluorescence (IFA)-based co-localization assays with anti-GFP antibodies and other antibodies specific for the marker proteins. We observed co-localization of kinesin-5 with α-tubulin at the early stages of schizogony, both at spindle MTs and the putative MTOC with a Pearson's colocalization coefficient (R) of more than 0.7 (**Figure 2B**). Similarly, kinesin-5 co-localised with centrin-2 with a Pearson's colocalization coefficient (R) of more than 0.7, confirming its location close to or at the putative MTOC (**Figure 2C**). Using anti-GFP with anti-NDC80 antibodies revealed that kinesin-5-GFP is located in close proximity to, and partially overlapping, the kinetochores with a Pearson's colocalization coefficient (R) of less than 0.7 (**Figure 2D**); this location was confirmed by live cell imaging with a dual color parasite line expressing kinesin-5-GFP and NDC80-mCherry (**Figures 2E, F**).

Spatiotemporal Dynamics of Pbkinesin-5 Reveal Its Location on the Spindle Apparatus During Male Gametogony

In order to study the dynamics of kinesin-5 during the rapid genome replication in male gametogony we examined its

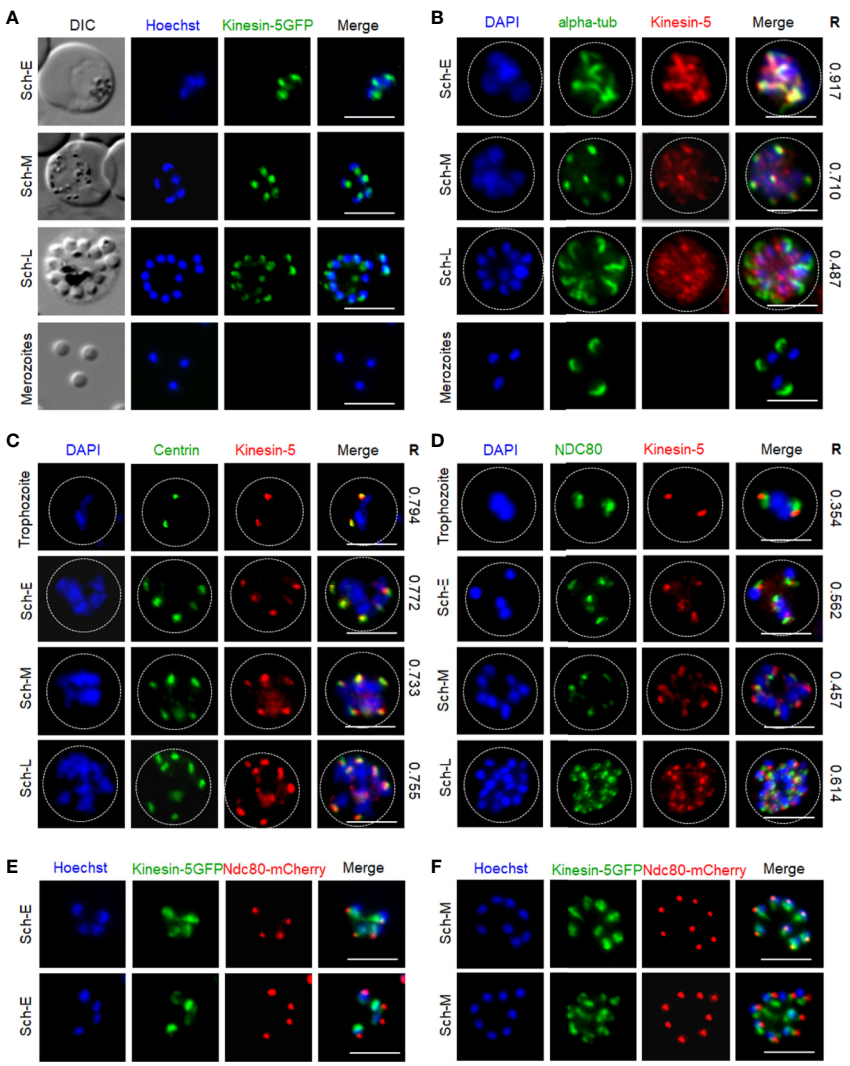

FIGURE 2 | Localization of Pbkinesin-5 on the spindle and spindle pole during asexual blood stage schizogony and its association with other cell division markers. **(A)** Live imaging of *Pb*kinesin-5-GFP (Green) during schizogony within a host erythrocyte, showing its location on a putative MT organizing centre (MTOC) and mitotic spindle during early dividing stages. The protein is diffuse or absent in mature merozoites. **(B–D)** Indirect immunofluorescence assays (IFA) showing the location of *Pb*kinesin-5 (red) in relation to α-tubulin (green, **B**), centrin (green, **C**) and NDC80 (green, **D**). Dotted lines represent the red blood cell membrane. **(E, F)** Live imaging showing the location of kinesin-5–GFP (green) in relation to NDC80-mCherry (red). DIC: Differential interference contrast. Sch-E (Early schizont), Sch-M (Middle schizont), Sch-L (Late schizont), Scale bar = 5 μm, R (Pearson's coefficient for co-localization).

expression by live-cell imaging during the 15-min period following activation of male gametocytes. Both male and female gametocytes express kinesin-5, with a diffuse nuclear location. At the start of male gametogony, kinesin-5 accumulated at one end of the nucleus at a single focal point 1-min post-activation (mpa) (**Figure 3A**). By 2 mpa, this focal point extended to form a bridge across one side of the nucleus, followed by the separation of the two halves of the bridge to produce shorter linear rods that then contracted to two clear single foci by 3 mpa (**Figure 3A**). This process repeated twice, resulting in 8 discrete kinesin-5-GFP foci. These discrete kinesin-5 foci then dispersed just before cytokinesis and exflagellation and the protein remained diffused in the nucleus of the remnant gametocyte (**Figure 3A**). A schematic diagram for this process is shown in the upper panel of **Figure 3A**.

To study the association of kinesin-5 with the mitotic spindle we used immunofluorescence-based co-localization assays with anti-GFP antibodies to stain kinesin-5, and anti-α-tubulin antibodies for MT staining. This analysis showed clear co-localisation of kinesin-5-GFP during early stages (1-3 mpa) of male gametogony with the spindle MT, both on the bridge-like structure and the foci, representing the spindle and spindle pole body, respectively (**Figure 3B**). Because the anti-α-tubulin antibodies recognize both spindle (nuclear) and axonemal (cytoplasmic) MT, spindle MTs are not distinctly visualized during later stages (6–8 mpa) when axoneme assembly is nearly complete. However, it is very clear that the pattern of kinesin-5 staining does not correspond with axonemal MT. Furthermore, kinesin-5 was not present in mature male gametes following their egress (**Figures 3A, B**).

To investigate further whether the location of kinesin-5 is cytoplasmic or nuclear, the kinesin-5-GFP parasite line was genetically crossed with the NDC80-mCherry kinetochore marker line (Zeeshan et al., 2020b) and kinesin-8B-mCherry cytoplasmic axoneme marker line (Zeeshan et al., 2019a), and the crosses were used for live cell imaging of both markers to establish their spatiotemporal relationship. We found that both kinesin-5 and NDC80 were located next to the nuclear DNA, with co-localization on both spindle and spindle poles during different stages of male gametogony (**Figures 4A, B**). In contrast, kinesin-5 did not co-localize with kinesin-8B which is located on the cytoplasmic basal bodies in early stages of male gametogony and later distributes across the axonemes (**Figures 4C, D**).

To study further the association of kinesin-5 with nuclear or cytoplasmic MTs, we examined the effects of tubulin inhibitors specific to nuclear (taxol) and cytoplasmic (DDD01028076) MTs

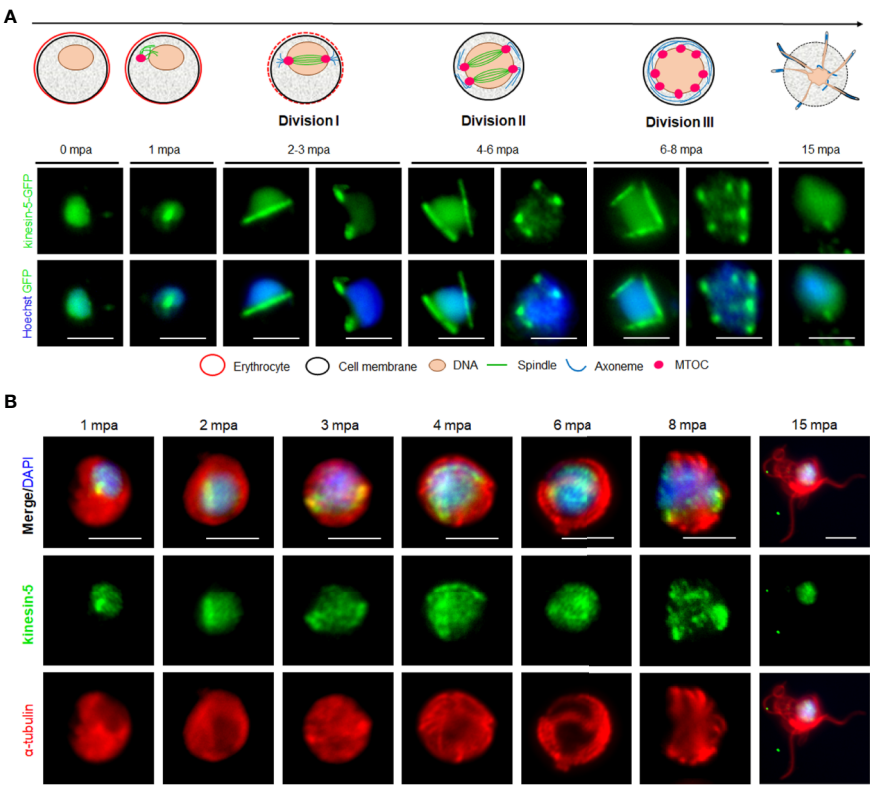

FIGURE 3 | The location of Pbkinesin-5 and its association with MTs during male gametogony. **(A)** Live-cell imaging of Pbkinesin-5-GFP (Green) during male gametogony showing an initial location on a putative MT organizing centre (MTOC) just after activation, and then on spindles and spindle poles in the later three mitotic stages. The schematic shows the principle stages of male gametogony. **(B)** IFA showing location of kinesin-5 (green) and α-tubulin (red) in male gametocytes at 1-, 2-, 3-, 4-, 6-, 8- and 15-min post activation (mpa). Scale bar = 5 μm.

on kinesin-5 organisation during male gametogony (Zeeshan et al., 2019a). Addition of taxol at 1 mpa blocked the dynamic redistribution of both kinesin-5 and NDC80 in more than 70% of male gametocytes, whereas DMSO-treated gametocytes showed normal mitotic progression and kinesin-5 distribution (**Figure 4E**). This result showed that kinesin-5 distribution and localization is associated with spindle dynamics, similar to the behavior of NDC80 (Zeeshan et al., 2020b), and can be blocked by taxol treatment, which binds tubulin and stabilizes MTs by preventing depolymerisation (**Figure 4E**). In contrast, treatment with DDD01028076 had no effect on either kinesin-5 or NDC80 location, consistent with the specificity of this inhibitor for cytoplasmic MT (axonemes) (**Figure 4E**), as shown previously (Zeeshan et al., 2019a).

During Meiosis in Zygote to Ookinete Development, Pbkinesin-5 Location Follows Spindle Dynamics

We studied the location of kinesin-5 in the meiotic stage during zygote differentiation to ookinete over the 24-h period after fertilization.

Kinesin-5-GFP fluorescence was initially diffuse within the zygote nucleus, but after 1.5 to 2 h post-fertilization the GFP signal coalesced to a single focal point adjacent to the DNA (**Figure 5A**). As ookinete development proceeds, with a small apical protrusion (stage I), the intensity of kinesin-5 increased and by stage II to III it was observed on spindles and more prominently on spindle poles (**Figure 5A**). Later, in development stage IV, the polar localization of kinesin-5-GFP was lost and by

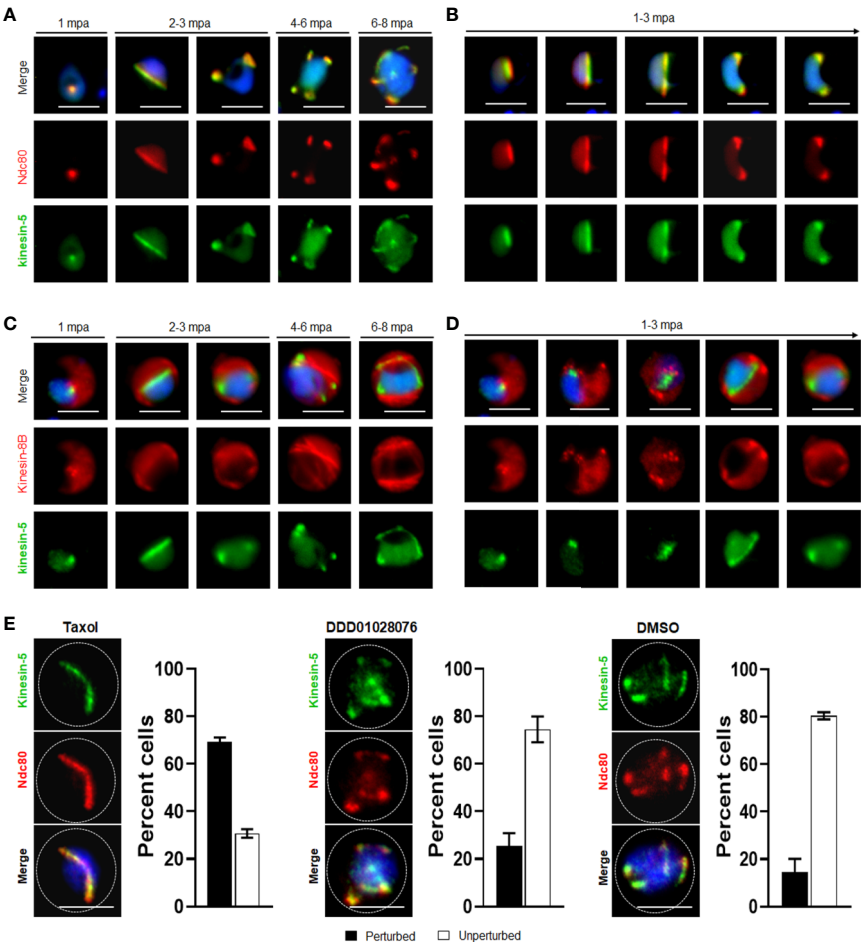

FIGURE 4 | Spatiotemporal dynamics of Pbkinesin-5-GFP with kinetochore marker (NDC80); and basal body and axoneme marker (kinesin-8B), and the effect of MT inhibitors on kinesin-5 distribution. **(A)** The location of kinesin-5-GFP (green) in relation to the kinetochore marker, NDC80-mCherry (red) during male gametogony. **(B)** The dynamic location of kinesin-5-GFP and NDC80-mCherry during the first round of mitosis (1-3 mpa) in male gametogony. **(C)** The location of Pbkinesin-5 in relation to kinesin-8B, a basal body and axonemal marker. The nuclear location of kinesin-5-GFP contrasts with the cytoplasmic location of Pbkinesin-8B during male gametogony. **(D)** The dynamic location of Pbkinesin-5-GFP and kinesin-8B-mCherry during the first round of mitosis (1-3 mpa.) in male gametogony. **(E)** The MT-stabilizing drug Taxol blocks the dynamic distribution of kinesin-5-GFP and NDC80-mCherry; the resulting phenotype of compound addition at 1 mpa is shown. The antimalarial molecule (DDD010128706) had no significant effect on the dynamic location of kinesin-5-GFP and NDC80-mCherry. Inhibitors were added to gametocytes at 1 mpa and cells were fixed at 8 mpa. Scale bar = 5 μm. Bar diagram shows mean ± SEM. n=3.

stage V to VI, it again became diffuse in the nucleus with some less-prominent foci (**Figure 5A**).

Pbkinesin-5-GFP Exhibits Multiple Nuclear Foci During Oocyst Development and in Liver Stage Schizogony

Mitosis during oocyst development (sporogony) resembles that of schizogony within the mammalian host, and, as in exoerythrocytic schizogony (in hepatocytes), with many nuclei. Each oocyst contains multiple lobes and produces hundreds of sporozoites (Zeeshan et al., 2020b). Kinesin-5-GFP fluorescence was observed as multiple foci representing a location at the putative MTOC/nuclear poles, from very early in development (day 7) to late stages (at day 14) of oocyst maturation (**Figure 5B**), similar to the pattern for PbCEN-4 as described previously (Roques et al., 2019). Many arc-shaped GFP signals were also observed that may represent the distribution of kinesin-5 on mitotic spindles (**Figure 5B**). This mirrors what has been seen in electron microscopy studies: nuclear spindles radiate from the nuclear poles, with attached kinetochores within an intact nuclear membrane during oocyst development (Zeeshan et al., 2019b; Zeeshan et al., 2020b). Interestingly, with maturation of oocyst from day-14 (sporulation started) to day-21 (fully sporulated) kinesin-5 fluorescence started decreasing and in mature oocysts (day 21), fluorescence was restricted to the residual body of the oocyst and was absent from mature sporozoites, suggesting that once nuclear division is completed kinesin-5-GFP is degraded (**Figure 5B**, **Supplementary Figure 3**).

Sporozoites produced in oocysts move to the mosquito salivary glands and, when transmitted by mosquito bite, infect the new host and migrate to the liver. As a model to study expression and location of kinesin-5 during mitosis in liver cells, we infected HeLa cells with sporozoites *in vitro*. The pattern of kinesin-5 distribution in these cells was similar to that of other asexual proliferative stages showing multiple foci of kinesin-5GFP next to DNA staining (**Figure 5C**).

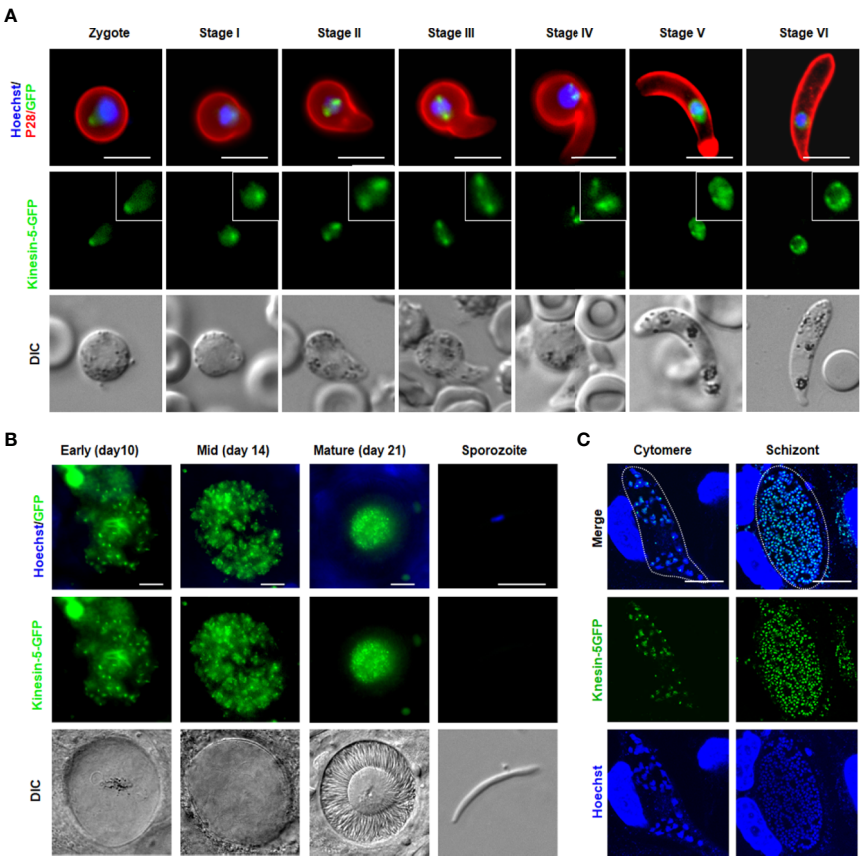

FIGURE 5 | *Pb*kinesin-5 localizes to a spindle and spindle poles during ookinete development, sporogony and liver schizogony **(A)** Live cell imaging showing *Pb*kinesin-5-GFP location during ookinete development. A cy3-conjugated antibody, 13.1, which recognizes the P28 protein on the surface of activated female gametes, zygotes and ookinetes was used to mark these stages (red). Panels: DIC (differential interference contrast), kinesin-5-GFP (green, GFP), Merged: Hoechst (blue, DNA), kinesin-5-GFP (green, GFP) and P28 (red). Scale bar=5 µm. Insets show the zoom of kinesin-5-GFP signal **(B)** Live cell imaging of Pbkinesin-5-GFP in developing oocysts in mosquito guts at 7-, 10-, 14- and 21-days post-infection and in a sporozoite. Panels: DIC (differential interference contrast), kinesin-5-GFP (green, GFP), Merged: Hoechst (blue, DNA) and kinesin-5-GFP (green, GFP). Scale bar = 5 µm **(C)** Expression of the kinesin-5 in early (cytomere) and late liver schizonts detected by live cell imaging. Merge = DAPI and GFP. Dotted lines represent the host cell membrane. Scale bar = 5 µm.

DISCUSSION

Spindle apparatus assembly and chromosome segregation are key processes of nuclear division that require forces generated by MT-based motor proteins (Wordeman, 2010; Kull and Endow, 2013). In most eukaryotes kinesin-5 is the major mitotic motor protein that crosslinks anti-parallel spindle MTs and drives bipolar spindle formation (Ferenz et al., 2010; Shirasugi and Sato, 2019). Any defect in kinesin-5 function results in the failure of spindle pole separation and prevents successful nuclear division (Sharp et al., 1999; Kapoor et al., 2000; Mann and Wadsworth, 2019). In the present study we show by deletion of *kinesin-5* that this protein is not essential for either mitotic or meiotic division during *P. berghei* parasite proliferation but it has an important role in production of infectious sporozoites.

The decrease in number of sporozoites was seen in the oocyst, suggesting an important role for kinesin-5 in sporozoite production in oocysts. Kinesins are MT-based motor proteins and it is not evident if Pbkinesin-5 has any role in regulation of MT function. A recent study has shown that in the absence of MT, chromosome segregation in oocyst proceeds but the MT number and size affect the shape and infectivity of Plasmodium sporozoites (Spreng et al., 2019). The reasons for the drastic decrease in sporozoites in the salivary glands are unclear, but it may result from several factors (Graumans et al., 2020). Either sporozoite egress from the oocyst is impaired or released sporozoites may be unable to reach the salivary glands if they are trapped in other tissues or degraded in the haemolymph by phagocytes (Golenda et al., 1990; Hillyer et al., 2007; Raddi et al., 2020). Another reason is that entry of sporozoites into the salivary glands may be affected, and this depends on the interaction of sporozoite surface proteins, mainly circumsporozoite protein (CSP), thrombospondin related anonymous proteins (TRAP), TRAP related protein (TREP) and apical membrane antigen/erythrocyte binding like protein (MAEBL), with salivary gland proteins such as CSP binding protein (CSPBP), salivary gland surface protein (SGS1) and Saglin protein (Kariu et al., 2002; Combe et al., 2009; Ghosh et al., 2009; Wang et al., 2013). Currently, it is not clear whether kinesin-5 has any role in these events of sporozoite egress or movement or infectivity to salivary glands.

Although the number of sporozoites in salivary glands decreased significantly, they were able to infect a new host. This is likely because even in the absence of kinesin-5, the sporozoite inoculum, number of mosquito bites, or the sporozoite infectivity to liver cells are sufficient to cause infection (Aleshnick et al., 2020; Graumans et al., 2020). Thus, more broadly, the absence of kinesin-5 does not completely block the transmission of parasite.

The biology of malaria parasite development is very different from that of many model eukaryotes, from which it is evolutionarily distinct, with different modes of cell division at different stages. Asexual proliferation is by asynchronous closed mitosis to produce a multinucleate coenocyte that undergoes cytokinesis at the end of the cell cycle to produce haploid extracellular progeny. This cell division lacks several classical regulators such as polo-like kinases, group 1 cyclins and many components of the anaphase promoting complex (Tewari et al., 2010; Solyakov et al., 2011; Guttery et al., 2014; Roques et al., 2015; Wall et al., 2018). The classical cell division kinase, cyclin dependent kinase 1 (CDK1) is not essential for cell division in *Plasmodium* (Tewari et al., 2010), but the parasite does possess other divergent and apicomplexan specific kinases that may be important in this process (Ward et al., 2004; Tewari et al., 2010; Fang et al., 2017). The likely unusual regulatory mechanisms of cell division in *Plasmodium* are consistent with the observation that kinesin-5 is non-essential during most stages of the life cycle except sporozoite production, since there may be alternative, non-classical, ways to mediate and regulate mitosis. Alternatively or additionally, other kinesin motors in *Plasmodium* may have a compensatory role in the absence of kinesin-5; this is seen in budding yeast, where the two kinesin-5s can at least partially complement each other (Hoyt et al., 1992; Roof et al., 1992). Although kinesin-5s and kinesin-13s have very different in vitro activities (Wordeman, 2010), our transcript analysis of *Δkinesin-5* parasites revealed a significant upregulation of kinesin-13 expression suggesting that this is one mechanistic route by which loss of kinesin-5 in spindle apparatus function can be complemented. Our preliminary data show a similar pattern of expression and localization for kinesin-13-GFP (Zeeshan et al, unpublished), consistent with a compensatory role.

Since *Plasmodium* kinesin-5 is not essential for parasite survival but has an important role in sporozoite production, we were intrigued to see its expression and location during the different mitotic/meiotic stages of the parasite life cycle. Spatiotemporal, dynamic localization of the protein during blood stage schizogony revealed that kinesin-5 starts to coalesce adjacent to the nuclear DNA in the late trophozoite stage. This is the time when the centriolar plaque/spindle pole body appears for the first time before the start of mitosis, and serves as a putative MTOC (Arnot et al., 2011; Roques et al., 2019). The location of kinesin-5 on this putative MTOC was confirmed by co-localization with centrin-2 and α-tubulin, which have been shown to track MTOC (Arnot et al., 2011; Gerald et al., 2011; Roques et al., 2019). With the progression of nuclear division during schizogony, kinesin-5 showed its characteristic location on spindles, similar to the situation in other eukaryotes, where it helps in spindle assembly and chromosome segregation (Ferenz et al., 2010; Arnot et al., 2011). Kinesin-5 has the same pattern of localization as α-tubulin, showing its association with spindles and MTOCs in consecutive nuclear divisions (Ferenz et al., 2010; Arnot et al., 2011). A similar location of kinesin-5 was observed during the other asexual mitotic stages: liver schizogony and sporogony in the mosquito gut. Kinesin-5 expression was not detected in mature and extracellular merozoites, male gametes and sporozoites, indicating that once its role during mitosis is over it is degraded or discarded. The location of kinesin-5 in the residual body of mature oocysts following release of sporozoites, suggests that kinesin-5 is actively involved during mitosis in oocysts and then it is discarded at the end of endomitotic cell division. A similar fate was also observed for another molecular motor, myosin J (MyoJ) that also accumulates in the residual body during sporogony (Wall et al., 2019).

The residual bodies play important roles in Toxoplasma during organization of developing progeny inside the parasitophorous vacuole and promote their orderly and

efficient externalization after maturation (Muniz-Hernandez et al., 2011). A defect in Toxoplasma MyoF molecular motor function results in enlarged residual bodies with accumulation of intact organelles (Jacot et al., 2013), but Pbkinesin-5 deletion did not show any such phenotype. Although the number of *Δkinesin-5* sporozoites in salivary glands is reduced, they are as motile and infective as normal sporozoites, transmitting the parasite.

Plasmodium male gametogony is a very rapid process and completed within 15 min, producing eight gametes. It involves three rounds of DNA replication (with 8-fold chromosome replication before nuclear division), along with basal body formation and axoneme assembly in the cytoplasm, followed by chromosome condensation, karyokinesis, and cytokinesis leading to the emergence of motile flagellated gametes (Sinden et al., 1976; Zeeshan et al., 2019a). Live cell imaging of kinesin-GFP and fixed immunofluorescence assays using antibodies against GFP and α-tubulin showed that kinesin-5 associates with spindle MTs and spindle poles during the mitotic divisions in male gametogony. The association of kinesin-5 with spindle MTs was further confirmed by gametocyte treatment with Taxol, a spindle MT-specific inhibitor, which inhibited the dynamic relocation of kinesin-5. We investigated further this association of kinesin-5 exclusively with spindle MTs and not with axonemal MTs by live cell imaging of a parasite line expressing both kinesin-5-GFP and kinesin-8B-mCherry. Kinesin-8B is associated with cytoplasmic MTs (axonemes) and not present in the nuclear compartment of male gametocytes (Zeeshan et al., 2019a). Kinesin-5 also associates with kinetochores and this dynamic location of *Plasmodium* kinesin-5 and NDC80 is consistent with a study in yeast, showing that kinesin-5 is recruited to the kinetochore and plays an important role in its organisation (Tytell and Sorger, 2006).

In conclusion, this is the first study to explore the real-time dynamics and functional role of the kinesin-5 molecular motor in the mitotic and meiotic cell division cycles of the different stages of *Plasmodium* development, and to show an important role of kinesin-5 in sporozoite production.

AUTHOR'S NOTE

This manuscript has been released as a preprint at bioRxiv (Zeeshan et al., 2020a).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal work performed in this study has passed an ethical review process and was approved by the United Kingdom Home Office. Work was carried out in accordance with the United Kingdom 'Animals (Scientific Procedures) Act 1986' for the protection of animals used for experimental purposes under Licence number 40/3344.

AUTHOR CONTRIBUTIONS

MZ: formal analysis, investigation, methodology, validation, visualization, writing—original draft, writing—review and editing. DB: methodology, validation. RRS: methodology, validation. CAM: writing—review and editing. AAH: writing—review and editing. RT: project conceived, formal analysis, investigation, methodology, validation, visualization, writing—review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.583812/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Generation and genotype analysis of *Δkinesin-5* parasites. **(A)** Schematic of *Plasmodium berghei* kinesin-5 protein (1-1440 aa) showing different domains. Approximate length of different domains is indicated. aa; amino acids. **(B)** Schematic representation of the endogenous *kinesin-5* locus, the targeting gene deletion construct and the recombined kinesin-5 locus following double homologous recombination. **(B)** Integration PCR showing correct integration with expected size of bands and deletion of kinesin-5 gene from knockout (mut).

SUPPLEMENTARY FIGURE 2 | Generation and genotypic analysis of kinesin-5-GFP parasites. **(A)** Schematic representation for 3'-tagging of *kinesin-5* gene with green fluorescent protein (GFP) sequence via single homologous recombination. **(B)** Integration PCR showing correct integration of tagging construct. **(C)** Western blot showing expected size of kinesin-5-GFP protein.

SUPPLEMENTARY FIGURE 3 | Expression and localization of kinesin-5 during sporulation in oocysts. Live cell imaging showing the kinesin-5-GFP fluorescence in

a developing oocyst between day 14 (sporulation starts) and day 21 (completely sporulated) after infection.

SUPPLEMENTARY TABLE 1 | Oligonucleotides used in this study.

SUPPLEMENTARY VIDEO 1 | Gliding motility of WT-GFP salivary gland sporozoite.

SUPPLEMENTARY VIDEO 2 | Gliding motility *Δkinesin-5* salivary gland sporozoite.

SUPPLEMENTARY VIDEO 3 | Gliding motility of WT-GFP salivary gland sporozoite on matrigel.

SUPPLEMENTARY VIDEO 4 | Gliding motility *Δkinesin-5* salivary gland sporozoite on matrigel.

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REVIEW
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Taxon-Specific Proteins of the Pathogenic *Entamoeba* Species *E. histolytica* and *E. nuttalli*

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The human protozoan parasite *Entamoeba histolytica* can live in the human intestine for months or years without generating any symptoms in the host. For unknown reasons, amoebae can suddenly destroy the intestinal mucosa and become invasive. This can lead to amoebic colitis or extraintestinal amoebiasis whereby the amoebae spread to other organs *via* the blood vessels, most commonly the liver where abscesses develop. *Entamoeba nuttalli* is the closest genetic relative of *E. histolytica* and is found in wild macaques. Another close relative is *E. dispar*, which asyptomatically infects the human intestine. Although all three species are closely related, only *E. histolytica* and *E. nuttalli* are able to penetrate their host's intestinal epithelium. Lineage-specific genes and gene families may hold the key to understanding differences in virulence among species. Here we discuss those genes found in *E. histolytica* that have relatives in only one or neither of its sister species, with particular focus on the peptidase, AIG, Ariel, and BspA families.

Keywords: ***Entamoeba*, peptidases, virulence, AIG, Ariel, BspA**

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INTRODUCTION

The intestinal protozoan *Entamoeba histolytica* is an important human parasite. Recent data clearly indicate that the life-threating amoebic liver abscess (ALA) continues to be a common clinical complication of amoebiasis infection in Asian, African and Latin American countries with estimated 26700 death in 2016 (Collaborators, 2018; Shirley et al., 2019). *E. histolytica* can become invasive and cause amoebic colitis or amoebic liver abscess (ALA) formation. The life cycle of this parasite consists of infectious cysts that survive outside the host and vegetative trophozoites that proliferate in the human gut. In general, trophozoites persist asymptomatically for months or years in the human intestine. However, in 10% of cases, the trophozoites become, under as yet unknown circumstances, invasive and induce extraintestinal amoebiasis. Invasion into the intestinal mucosa can lead to induction of amoebic colitis, whereas dissemination to the liver can result in ALA formation (Blessmann et al., 2003). A related species, *E. dispar*, is microscopically indistinguishable from *E. histolytica* and occurs only as a harmless commensal in the human

intestine. A key question in amoebic research is the elucidation of the mechanisms of *E. histolytica* invasion and tissue destruction. For decades, attempts have been made to identify the virulence factors of *E. histolytica* by comparative studies of both organisms at both biological and molecular level. These studies, and those that compared pathogenic and non-pathogenic *E. histolytica* isolates, have led to the identification of a number of virulence factors.

Three protein families (Gal/GalNAc lectins, cysteine peptidases and amoebapores) are of particular interest. The first step in the invasion process is to overcome the protective mucus barrier. Here, cysteine peptidases play an important role, along with a number of other molecules (Lidell et al., 2006; Thibeaux et al., 2013). Subsequently, the adhesion of amoebae to target epithelial cells *via* the galactose/N-acetyl-D-galactosamine lectin (Gal/GalNAc) (Tannich et al., 1991; Petri and Schnaar, 1995). The Gal/GalNAc lectin is a 260 kDa heterodimer consisting of a disulfide-linked 170 kDa heavy chain (Hgl) and a GPI-anchored 35 kDa light chain (Lgl) (Petri et al., 2002). After establishing contact, amoebae can secrete amoebapores. These mediate a contact-dependent lysis of the target cells (Leippe, 1997). *E. histolytica* has three amoebapores (A, B and C), which are all characterized by a pore-forming activity. They are also capable of killing gram-positive bacteria by destroying bacterial cytoplasmic membranes. Thus, the functionality of these molecules is two-fold: they confer cytolytic activity to amoebae as well as an intracellular antimicrobial effect against phagocytosed bacteria (Leippe et al., 1994). Amoebae whose amoebapore expression is inhibited have a reduced antimicrobial activity and are non-pathogenic, i.e. they are not, or are only to a small extent, able to form ALAs in hamsters (Bracha et al., 1999; Bracha et al., 2003). Important for mucus degradation, invasion, as well as for the process of tissue degradation, are the cysteine peptidases (CPs) of *E. histolytica*. In the genome of *E. histolytica* 35 genes coding for CPs of the C1 papain superfamily can be identified. However, only four CPs (EhCP-A1, -A2, -A5, -A7) can be detected at the protein level in the trophozoite stage (Tillack et al., 2007; Irmer et al., 2009). These have previously been located in lysosomal-like vesicles, and some of them were found to be membrane-associated (Jacobs et al., 1998). The importance of CPs, particularly EhCP-A5, in ALA formation is evident in infections of laboratory animals, where overexpression of CPs leads to an increase in ALA size (Tillack et al., 2006). Conversely, reduced CP activity leads to a decrease in ALA formation ability in *E. histolytica* (Li et al., 1995; Stanley et al., 1995). In addition, an increase in the expression of some *ehcp* genes during ALA formation has previously been described, while non-pathogenic amoebae can be converted to pathogenic amoebae, simply by overexpression of some of specific *ehcp* genes (Matthiesen et al., 2013).

Homologs of the Gal/GalNAc lectins, cysteine peptidases and amoebapores are also present in non-pathogenic *E. dispar* (Nickel et al., 1999; Tillack et al., 2007; Weedall et al., 2011). Nevertheless, it has been shown that the CP activity of *E. dispar* is about 10-1000 times lower than that of *E. histolytica* and that genes corresponding to *ehcp-a1* and *ehcp-a5* are absent or degenerated in *E. dispar* (Bruchhaus et al., 1996; Willhoeft et al., 1999b; Que and Reed, 2000). A lower amount of amoebapores and therefore reduced pore-forming activity was found in *E. dispar* in comparison to *E. histolytica* (Nickel et al., 1999). However, it is not yet clear whether these differences alone determine pathogenicity, whether additional genetic differences are involved, or whether pathogenicity is a result of complex interactions between various proteins.

In addition to the cysteine peptidases and the amoebapores, the AIG, BspA and Ariel families are always mentioned in connection with the virulence of *E. histolytica*. For members of the AIG family, a differential expression between pathogenic and non-pathogenic *E. histolytica* isolates could be shown (Biller et al., 2010). BspA-like molecules have also been described in *T. vaginalis* and are thought to play an important role in pathogenesis (Noel et al., 2010). The Ariel proteins are an *E. histolytica*-specific protein family that has not yet been found in any other *Entamoeba* species so far. Therefore, it is postulated that these molecules may have a role in virulence. However, the functions of the members of all three protein families are largely unclear.

Besides the characterization of individual proteins, one straightforward method for the identification of pathogenicity factors is a direct comparison of pathogenic and non-pathogenic *E. histolytica* isolates using comparative genomic, transcriptomic and proteomic approaches (Davis et al., 2006a; MacFarlane and Singh, 2006; Ehrenkaufer et al., 2007; Biller et al., 2010; Meyer et al., 2016; Nakada-Tsukui et al., 2018). Unfortunately, two isolates with very different genetic backgrounds were used in the majority of the studies cited (pathogenic isolate HM-1:IMSS and non-pathogenic isolate Rahman) (Davis et al., 2006a; MacFarlane and Singh, 2006; Davis et al., 2007; Ehrenkaufer et al., 2007). The non-pathogenic Rahman isolate has several serious functional defects (e.g. defective phagocytosis, reduced cytotoxicity, inability to grow in animals, and a truncated glycan chain of the proteophosphoglycan coating surface) (Davis et al., 2006a). The genomic differences between HM-1:IMSS and Rahman are small, however, DNA fragment duplications have been detected (Weedall et al., 2012). An alternative approach compared the transcriptomes of pathogenic and non-pathogenic clones derived from the isolate HM-1:IMSS were compared (Biller et al., 2009; Biller et al., 2010; Meyer et al., 2016). In total, approximately 90 genes are differentially expressed between the investigated non-pathogenic and pathogenic clones (Meyer et al., 2016). Based on transfectants, in which the identified genes were either overexpressed or silenced, it was possible to identify another pathogenicity factor, namely the hypothetical protein EHI_127670. When EHI_127670 is silenced in pathogenic amoebae their ability to form ALAs is reduced. On the other hand, overexpression of EHI_127670 in non-pathogenic amoebae leads to restoration of ALA formation ability (Meyer et al., 2016; Matthiesen et al., 2019). However, nothing is yet known about the function of the protein and it is not yet known with certainty that the RNA actually encodes a protein.

Using a transcriptome approach, Naiyer and colleagues were able to identify downstream regulatory motifs in very highly expressed *E. histolytica* genes that are very likely important for gene expression. These motifs were also detected in various genes encoding virulence factors, which are also highly expressed under axenic conditions. It can therefore be assumed that corresponding encoded proteins are important for optimal growth, but in addition also play a role in tissue invasion and virulence (Naiyer et al., 2019b). Comparative transcriptome analysis also makes it possible to better understand the biology of *E. histolytica* in general. With the help of this method, it is possible to identify molecules that play a role in phagocytosis, the stress response, the enzyme station and the excystation, among others [for review (Naiyer et al., 2019a)].

Besides *E. histolytica* and *E. dispar*, the genus *Entamoeba* includes many other species, some of which colonize the human intestine (*E. moshkovskii, E. bangladeshi, E. polecki, E. coli*, and *E. hartmanni*). However, in humans a severe course of disease with extraintestinal abscesses has so far only been described for *E. histolytica*. Pathogenic amoebae have also been described in reptiles (*E. invadens*) and macaques (*E. nuttalli*). *E. nuttalli* is the species most closely related to *E. histolytica*. Different species of wild macaques, as well as other non-human primates kept in captivity, have been identified as hosts for *E. nuttalli* (Tachibana et al., 2007; Tachibana et al., 2009; Levecke et al., 2010; Wei et al., 2018; Tanaka et al., 2019). In addition to the genomes of *E. histolytica* and *E. dispar*, the genome of *E. nuttalli* was recently sequenced and all genomes are available on AmoebaDB (Tanaka et al., 2019). The ability to compare the genomes of *E. histolytica*, *E. dispar* and *E. nuttalli* now opens up the possibility of identifying additional molecules responsible for the development of extraintestinal amoebiasis in human or non-human primates.

IDENTIFICATION OF HOMOLOGOUS PROTEINS UNIQUE TO PATHOGENIC *E. HISTOLYTICA* AND *E. NUTTALLI*

Recently, using an OrthoMCL approach, Wilson and colleagues studied the genetic diversity and gene family expansions for *E. histolytica*, *E.dispar*, *E. moshkovskii* and *E. invadens*. They identified 984 genes that were unique to *E. histolytica* (**Table S8**) (Wilson et al., 2019). Here, this list was used as a basis for a BlastP approach to analyze whether homologous proteins are found in *E. nuttalli*. Additionally, *E. dispar* was again included in this analysis. Pseudogenes were not included in the analysis and a sequence identity of ≥ 50% was assumed to be a functionally homologous protein. In a BlastP analysis of all 927 translatable "unique" *E. histolytica* genes, 309 of them had homologs in *E. dispar* and *E. nuttalli* with an identity of ≥ 50% (**Figure 1**, **Table S7**) (Wilson et al., 2019). Another 376 "unique" *E. histolytica* genes encode for proteins with homologs in *E. dispar* but not in *E. nuttalli* (**Figure 1**, **Table S3**). A further 67 putative proteins share ≥ 50% identity only with *E. nuttalli* proteins (**Figure 1**, **Table S6**). Only 175 genes remain, whose encoded proteins do not show homology with proteins of *E. dispar* or *E. nuttalli* (**Figure 1**, **Table S1**). That many of the proteins encoded by the "unique" *E. histolytica* genes were also found in *E. dispar* was very surprising. However, looking at the genomic organization on AmoebaDB, one finds that many of the "unique" *E. histolytica* genes are located at the edge of contigs and therefore do not contain the complete gene sequence (Wilson et al., 2019).

PROTEINS THAT COULD ONLY BE DETECTED IN *E. HISTOLYTICA*

Almost all 175 proteins found to be unique for *E. histolytica* are hypothetical proteins (**Table S1**). However, 18 genes encoding the asparagine-rich *E. histolytica* antigens (Ariel) could be identified (including two genes with an incomplete sequence) (**Figure 1**, **Table S2**) (Mai et al., 2000; Wilson et al., 2019). The fact that Ariel proteins are encoded by an *E. histolytica*-specific multicopy gene family was described by Willhoeft and colleagues 20 years ago (Willhoeft et al., 1999a). No homologous proteins can be detected in *E. dispar* or *E. invadens* (Wilson et al., 2019). However, one homologous protein (ENU1_012640) is found in *E. nuttalli*, with 92% identity to EHI_005260/EHI_188600. Except for the Ariel proteins EHI_057430 and EHI_172730, which did not contain a signal peptide, and EHI_131360 and EHI_185110 which did not contain a transmembrane domain, an N-terminal signal peptide and a C-terminal transmembrane domain can be predicted for all other Ariel proteins. Therefore, a surface localization can be postulated, however it is unknown whether the protein is a deterministic factor in the virulence of *E. histolytica*.

Two further genes unique to *E. histolytica* unique genes (EHI_107560 and EHI_157010) encode for proteins with approximately 55% sequence identity to aldo-keto reductases of plant chloroplasts and are annotated as alcohol dehydrogenases. However, both genes are located at the edges of contigs and are thus only partially represented in the genome assembly. EHI_107560 (187 aa) is located at the edge of contig DS571548, while EHI_157010 (158 aa) is located at the edge of contig DS571869 (AmoebaDB, release 48 beta, 20 Aug 2020). BlastP analysis of these proteins revealed two genes (EHI_029620, EHI_039190) in the genome of *E. histolytica* encoding the same protein sequence of 305 amino acids, termed aldolase reductase. If these proteins are used as a template for a search in the *Entamoeba* genomes, a protein with a sequence identity of 55% was also found in pathogenic *E. invadens* (EIN_497000), along with one with 73% sequence identity in *E. dispar* (EDI_260680). A phylogenetic analysis using the online tool Clustal Omega with the respective protein sequences as input (Sievers et al., 2011), showed the closest relationship to *Dictyostelium discoideum* and plant chloroplastic-like aldo-keto reductases (**Figure 2**). In various Kinetoplastidae such as *Trypanosoma* and *Leishmania*, genes encoding chloroplast-like proteins were identified. It is assumed that the organelles were taken up by endosymbiosis before divergence. Later, the organelles were lost, but several genes

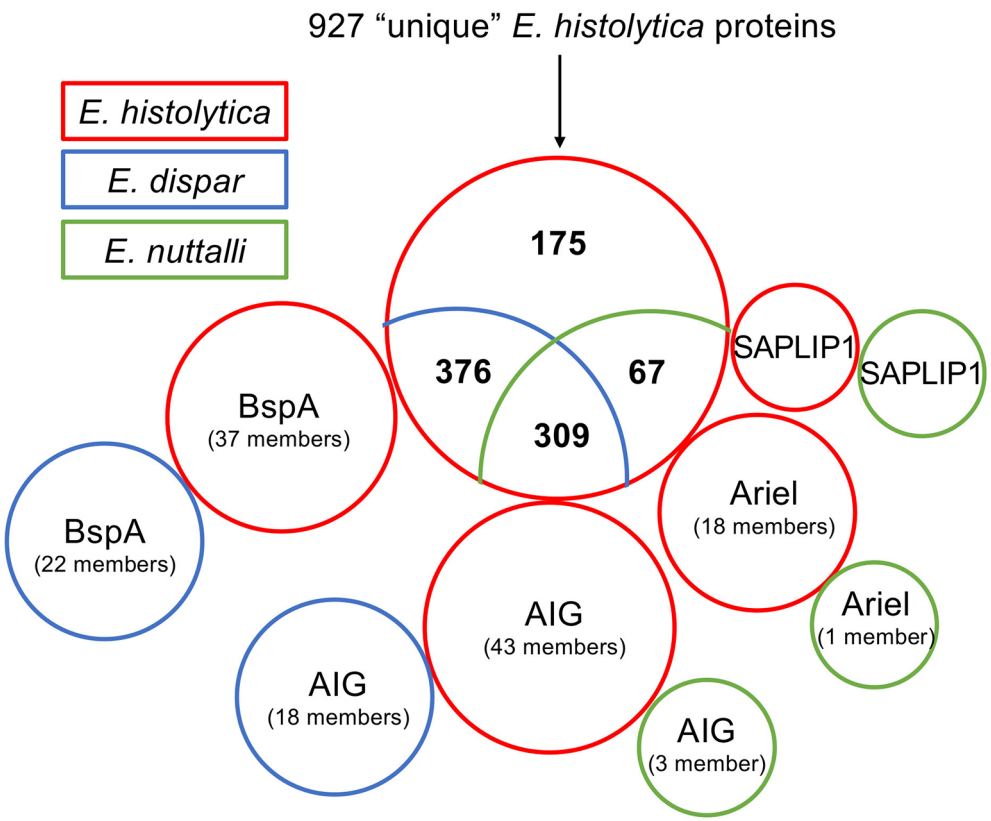

FIGURE 1 | Comparative blastp analysis of 927 proteins identified as unique for *E. histolytica* (Wilson et al., 2019). Shown is the comparison of *E. histolytica* with *E. nuttalli* and *E. dispar* as a venn diagram. The three protein families Ariel, AIG and BspA as well SAPLIP1 are shown separately. The proteins found in *E. histolytica* are boxed in red, those of *E. dispar* in blue and those of *E. nuttalli* in green.

from these organelles were integrated into the genome (Hannaert et al., 2003). Horizontal gene transfer has also been described for *Entamoeba*. However, almost all identified genes could be traced back to bacteria (mainly Bacterioidetes) so far (Romero et al., 2016). Comparison of the amino acid sequences revealed that *E. dispar* EDI_260680, contains a deletion of about 50 aa. Comparison of the sequence with those of plant aldo-keto reductases shows that this area contains amino acids that are important for the enzymes' activity (Sengupta et al., 2015). In plants aldo-keto reductases play a role in stress response and detoxification of toxic aldehydes among other things (Sengupta et al., 2015). Since nothing is known about the function of the protein in *E. histolytica*, no statement can be made as to whether the deletion present in *E. dispar* has an influence on its activity.

PROTEINS OCCURRING IN *E. HISTOLYTICA* AND *E. DISPAR*, BUT NOT IN *E. NUTTALLI*

In the gene set of *E. histolytica* "unique" genes underlying this work, 37 genes encoding members of the BspA family (leucine-rich repeat protein) were identified (Wilson et al., 2019). BlastP analysis of the proteins encoded by *bspa* showed that for all but two (EHI_098720/EHI_173850: 49% identity), homologous proteins (in total 22 members) with an identity of ≥ 50% could be detected in *E. dispar* (**Figure 1**, **Tables S3** and **S4**). However, no homologous proteins could be found in *E. nuttalli*. The length of *bspa*-encoded proteins listed here ranges from 101 to 447 aa. It is striking that almost all *bspa*s are found on very short contigs and then often on the edge of the contigs. Therefore, it is not clear whether all of these are really full-length proteins since, with a few exceptions, the BspA proteins characterized so far have an average length of about 550 amino acids (Davis et al., 2006b). For some members of the BspA family a surface localization was described, although neither signal sequences nor transmembrane domains were detected (Davis et al., 2006b, Silvestre et al., 2015). However, the phenomenon of a surface localization without the detection of protein domains that would allow membrane anchoring has been described for a number of other proteins (Biller et al., 2014). It has been shown that BspA-like proteins are involved in adhesion to extracellular membranes, epithelial cell invasion and fibronectin and fibrinogen binding (Mengaud et al., 1996; Sharma et al., 1998; Inagaki et al., 2006). A BspA-like gene family could also be

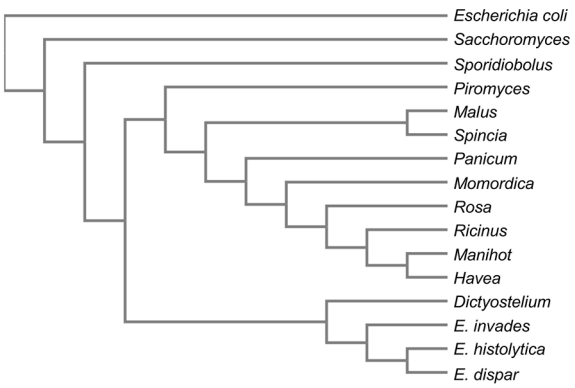

FIGURE 2 | Phylogram of aldose reductases. The aldose reductases sequences of *E. histolytica*, *E. dispar*, *E. nuttalli* were compared for homologous sequences using BlastP. The best scoring 10 proteins as well as three aldose reductase sequences used as outgroups were used to generate a phylogram using the online tool Clustal Omega (Sievers et al., 2011). Sequences used: *E. histolytica* aldose reductase (EHI_029620/EHI_039190), *E. invadens* aldo_ket_red domain-containing protein (EIN_497000), *E. dispar* NADPH-dependent alpha-keto amide reductase (EDI_260680), *Piromyces finnis*_aldehyde reductase_(ORX59359), *Ricinus communis*_NADPH-dependent aldo-keto reductase, chloroplastic_(XP_002529872), *Manihot esculenta*_NADPH-dependent aldo-keto reductase, chloroplastic-like_(XP_021619253), *Hevea brasiliensis*_NADPH-dependent aldo-keto reductase, chloroplastic-like (XP_021670007), *Momordica charantia*_NADPH-dependent aldo-keto reductase, chloroplastic-like_(XP_022143954), *Dictyostelium discoideum*_aldehyde reductase_(XP_628918), *Malus domestica* NADPH-dependent aldo-keto reductase, chloroplastic-like (XP_008369945), *Spinacia oleracea*_NADPH-dependent aldo-keto reductase, chloroplastic-like (XP_021845528), *Panicum hallii* aldo-keto reductase family 4 member C10-like isoform X2 (XP_025809823), *Rosa chinensis*_NADPH-dependent aldo-keto reductase, chloroplastic-like (XP_024188589), *Escherichia coli* aldo-keto reductase (E0IVZ7), *Saccharomyces cerevisiae* NADPH-dependent aldose reductase (P38715), *Sporidiobolus salmonicolor* aldehyde reductase (P27800).

identified in *Trichomonas vaginalis* (Hirt et al., 2002; Noel et al., 2010). The BspA proteins of *T. vaginalis* are believed to play various and important roles in the pathobiology of this parasite by contributing to invasion and long-term infections of the urogenital tract (Noel et al., 2010). However, the exact functions of the proteins in *T. vaginalis* as well as in *E. histolytica* and *E. dispar* are not yet deciphered. As these are large gene families, it can be assumed that they play an important role in the life cycle of the amoebae. It is therefore all the more surprising that homologous proteins are not detectable in the closest relative of *E. histolytica*, *E. nuttalli*.

Wilson and colleagues found 17 members of the AIG family that were "unique" to *E. histolytica* (Wilson et al., 2019). However, by BlastP analysis 8 AIG proteins were identified in *E. dispar*, showing 49 – 77% homology to the 17 different *E. histolytica* AIG1 proteins. Eight of the *E. histolytica* AIGs are most similar (53 – 63% identity) to *E. dispar* EDI_185310. For only one AIG1, EHI_126560, homologs were identified in both *E. dispar* (EDI_274460, 77% identity) and *E. nuttalli* (ENU1_158210, 97% identity)(**Tables S3**, **S5**). In addition to the 17 genes, 26 more genes encoding AIG proteins can be identified in the *E. histolytica* genome. For all these proteins, homologs with a sequence identity between 53 and 83% can be detected in *E. dispar*. However, seven *E. histolytica* AIGs each show the highest homology to only two *E. dispar* AIGs (EDI_036000 and EDI_243490). Furthermore, two more AIGs, ENU1_161270 and ENU1_207600, are found in *E. nuttalli*, which are homologous to AIGs from *E. histolytica* (EHI_180390, 95% identity, and EHI_191790, 98% identity, respectively) (**Figure 1**, **Table S5**).

AIGs belong to the GTPases, were originally isolated from *Arabidopsis thaliana* and are thought to confer resistance to bacterial infections. It is also believed that AIG proteins are involved in the development of *A. thaliana* and its response to environmental stimuli (Reuber and Ausubel, 1996). Orthologous proteins are also found in mammals and play a role in B-cell and T-cell development *via* interaction with proteins of the Bcl2 family (Nitta et al., 2006; Nitta and Takahama, 2007). However, very little is known about the function of AIG proteins in *E. histolytica*. In a comparative genome analysis of *E. histolytica* isolated from a patient presenting with diarrhea and an asymptomatic patient, it was shown that one *aig* gene from a tandem array of three *aig* genes was deleted by homologous recombination in the isolate from the asymptomatic patient. Overexpression of this *aig* (EHI_176590) resulted in increased formation of cell surface protrusions and increased adhesion to human erythrocytes. Furthermore, the gene EHI_176590 was detected in approximately 60% of stool samples from symptomatic patients, but only in 15% of stool samples from asymptomatic individuals infected with *E. histolytica*. It is therefore postulated that the AIG protein plays a central role in the virulence of *E. histolytica* by regulating host cell adhesion (Nakada-Tsukui et al., 2018). In addition, a quantitative real-time PCR approach showed that 18 of 34 investigated *aig* genes are increasingly expressed in pathogenic compared to non-pathogenic HM-1:IMSS cell lines (Biller et al., 2010). But as with the Ariel family and the BspA family, the function of the AIG family is not yet clear.

PROTEINS OCCURRING IN *E. HISTOLYTICA* AND *E. NUTTALLI*, BUT NOT IN *E. DISPAR*

In the set of "unique" *E. histolytica* genes on which this work is based, 67 had homologs in *E. nuttalli* but not in *E. dispar* (**Figure 1**, **Table S6**) (Wilson et al., 2019). As already described for the other comparisons, these are mostly hypothetical proteins. However, the list also contains the non-pathogenic pore-forming peptide EHI_169350. EHI_169350 (entbd24tf or SAPLIP1) belongs to a family of 15 saposin-like proteins (SAPLIPs) which were first identified by Bruhn and Leippe (Bruhn and Leippe, 2001; Winkelmann et al., 2006). Like amoebapore A, B and C the SAPLIPs are characterized by a conserved sequence motif consisting of six cysteine residues.

Interestingly, of the entire SAPLIP family, only EHI_169350 can be clearly assigned to the amoebapore subfamily, with the largest sequence identity of about 65% shared with amoebapore A (Bruhn and Leippe, 2001). Only two members of the SAPLIP family including EHI_169350 possess a typical signal peptide and only EHI_169350 resembles amoebapores in its net charge and dispersed charge distribution, with the greatest similarity being to amoebapore C. However, EHI_169350 lacks the typical C-terminal histidine residue of amoebapores, which is essential for oligomerization during channel formation (Andra and Leippe, 1994). EHI_169350 has an asparagine residue at position 90 instead. It can therefore be assumed that EHI_169350 has no pore-forming activity (Bruhn and Leippe, 2001). No statement can therefore be made about the function of EHI_169350 especially concerning its role in pathogenicity.

THE PEPTIDASES OF E. HISTOLYTICA, E. DISPAR AND E. NUTTALLI

As described above, CPs play an important role in the destruction and invasion of human tissue. This has been demonstrated in a variety of *in vitro* and *in vivo* studies (Bruchhhaus and J, 2015; Gilmartin et al., 2020). A new *in silico* analysis of the genome of *E. histolytica* (AmoebaDB, release 48 beta, 27 Aug 2020) revealed a total of 33 genes encoding CPs of clan CA, C1 (papain-like) family (**Table S9**). Twelve of the CPs could be assigned to the CP-A family, ten to the CP-B family and eleven to the CP-C family. In *E. dispar* there are only nine members of the CP-A family. Genes coding for EdCP-A1, -A5, -A7 are not present or are present as pseudogenes. The CP-B family consists of seven members; here the genes coding for EdCP-B1, -B8 and -B9 are missing. The EdCP-C family has eleven members, like the EhCP-C family. In *E. nuttalli* there are no homologous proteins to EhCP-A1, -A6, -A7, and -A8; thus the EnCP-A family comprises eight members. The EnCP-B family consists of nine members (EnCP-B2 is missing here), and EnCP-C5 is missing in the CP-C family, so there are ten members in total (**Figure 3**, **Table S9**).

Only for four of the CP encoding genes (*ehcp-a1, -a2, -a5* and *-a7*) could it be shown that they are highly expressed in *E. histolytica* under the standard axenic culture conditions (Bruchhaus et al., 2003; Clark et al., 2007; Tillack et al., 2007). Interestingly, the ability to disrupt a cell monolayer (cytopathic activity) was dramatically increased for amoebae overexpressing *ehcp-a5*, whereas it showed only a moderate increase in transfectants overexpressing *ehcp-a1* or *ehcp-a2*. Furthermore, the overexpression of *ehcp-a1* and *ehcp-a2* in *E. histolytica* trophozoites did not increase ALA formation in gerbils, whereas overexpression of *ehcp-a5* resulted in significantly larger ALAs compared to controls. If the *ehcp5* is overexpressed in the HM-1:IMSS derived G3 isolate, in which the *amoebapore a* gene is silenced and therefore has only low virulence, this is sufficient to compensate for the reduction in virulence (Hellberg et al., 2001; Tillack et al., 2006). In previous studies EhCP-A5 was also believed to play an important role in intestinal invasion. EhCP-A5 has been shown to interact directly with the integrins on the surface of human colon epithelial cells and induce the secretion of pro-inflammatory cytokines (Hou et al., 2010). Thus, amoebae silenced for *ehcp-a5* expression do not trigger an inflammatory response of the host and do not induce the collagen remodeling required for invasion. Further investigations showed that CP-A5 can convert the pro-matrix metalloproteinase (MMP)-3 into its active form, which in turn activates pro-MMP-1 (Bansal et al., 2009; Thibeaux et al., 2012; Thibeaux et al., 2014). The

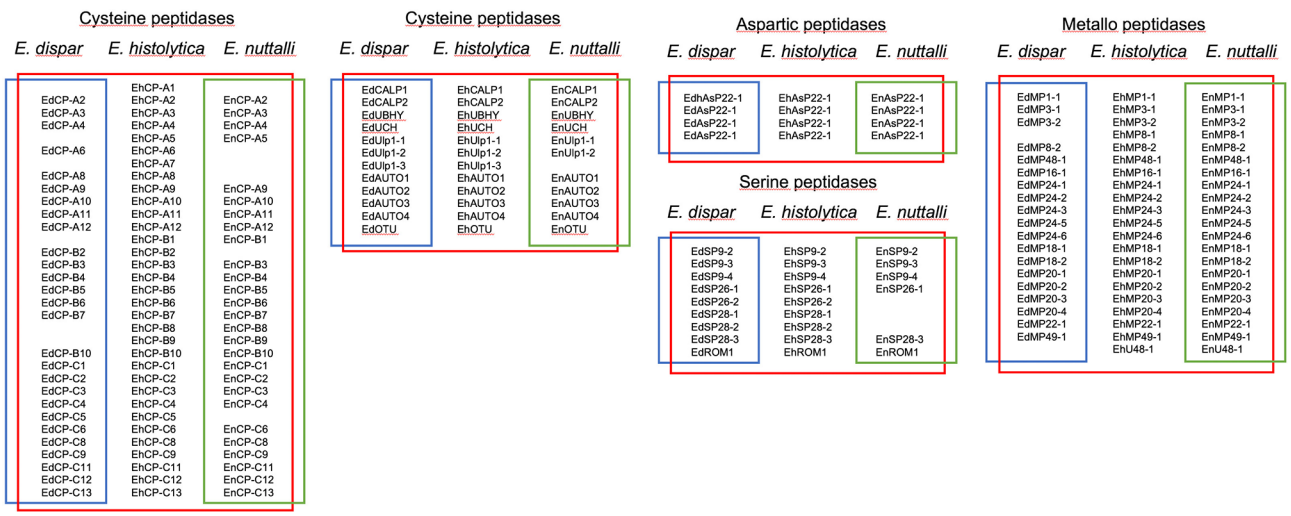

FIGURE 3 | Cysteine, asparagine, serine and metallopeptidases of *E. histolytica*, *E. dispar* and *E. nuttalli* (for more details see **Table S9**).

observation that CP-A5 occurs in the two pathogenic amoeba species *E. histolytica* and *E. nutalli*, but not in the non-pathogenic species *E. dispar*, supports the significance of this peptidase as an important pathogenicity factor (**Figure 3**, **Table S9**). However, a CP-A5 homologue is not detectable in the genome of the reptile pathogen *E. invadens*.

In the genome of *E. histolytica* another twelve genes encoding CPs can be identified and assigned to five different families (C2, C19, C48, C54, C65; all clan CA) (**Figure 3**, **Table S9**). Except for the Ulp1 protease Ulp1-3 (C48 family) in *E. nuttalli*, homologs for all CPs in *E. dispar* and *E. nuttalli* could be found.

Homologs for the four members of the aspartic peptidase family (clan AD, family A22, A) of *E. histolytica* are found in both *E. dispar* and *E. nuttalli*. However, nothing is known about the function of these peptidases (**Figure 3**, **Table S9**).

Nine serine peptidases can be detected, which can be assigned to four families (clan SC, family S9, C; Clan SF, family S26, B; Clan SC, S28; clan ST, family S54) (**Figure 3**, **Table S9**). For the serine peptidases EhSP26-2, EhSP28-1 and EhSP28-2 no homologs can be detected in *E. nutalli*. Functional analysis has only been performed for the S28 and rhomboid proteases. Both are found associated with the amoeba membrane and the rhomboid protease probably plays an important role in mobility and adhesion of amoebae to the host tissue (Barrios-Ceballos et al., 2005; Baxt et al., 2008; Rastew et al., 2015; Welter et al., 2020).

A total of 21 metallopeptidases belonging to eleven different families were identified in the genome of *E. histolytica* (**Figure 3**, **Table S9**). Only for 2 of them no homologous proteins can be identified in *E. dispar*. These are MP8-1 (cell surface protease gp63) and U48-1, a CAAX prenyl protease. In *E. nuttalli* the two peptidases can be detected, just like in *E. histolytica*. For EhMP8-1, Teixeira and colleagues showed that it is a functional metallopeptidase localized on the surface of E. histolytica trophozoites. By silencing *ehmp8-1* expression, the adherence of trophozoites to cells was increased, while the surface staining of several antigens, including the Gal/GalNAc lectin, remained unchanged. Amoebae which were silenced for *ehmp8-1* expression also showed decreased cytopathic activity and reduced mobility, but phagocytic activity was increased (Teixeira et al., 2012). In contrast to the EhMP8-1 there is unfortunately very little information about the EhMP8-2. The *ehmp8-2* gene is approximately 150 times more highly expressed in non-pathogenic amoebae than in pathogenic amoebae. Furthermore, it was shown that overexpression of *ehmp8-2* in pathogenic amoebae significantly reduced ALA formation in the mouse model. Thus, the presence of EhMP8-2 leads to a non-pathogenic phenotype of the amoebae (Meyer et al., 2016). Another peptidase, which is found only in *E. histolytica* and *E. nuttalli*, but not in *E. dispar*, is a CAAX prenyl protease of unknown function in the U48 family. The question arises for EhMP8-2 as well as for EhU48-1 whether the absence of these molecules has an influence on the virulence of amoebae.

CONCLUSION

Unfortunately, the majority of proteins found exclusively in pathogenic *E. histolytica*, or in *E. histolytica* and *E. nuttalli* but not non-pathogenic *E. dispar*, lack functional annotations. With Ariel, BspA and AIG, *E. histolytica* has three large protein families whose members are repeatedly discussed as virulence factors. The Ariel family, consisting of 18 members, is almost exclusively found in *E. histolytica*. Only one member was detected in *E. nuttalli*. In contrast, proteins of the BspA family are found in *E. histolytica* and *E. dispar*, and members of the AIG family in all three organisms. However, it is noticeable that *E. histolytica* contributes the most members to each of these protein families. One reason for this observation could be that, in analyses like these, incomplete or fragmented genome assemblies may contain gaps and genes may be missing in the annotated gene set rather because of this than because of the actual absence of these genes. For example, the lysine and glutamic acid-rich protein KERP1 is a virulence factor that is active in the development of amoebic liver abscesses (Seigneur et al., 2005; Santi-Rocca et al., 2008). Originally, it was assumed to be absent in the genome of *E. dispar*, but in fact it is present but only partially represented in the genome assembly (Weedall, 2020). Such a situation is probably a factor in defining genes as lineage-specific. It is probably also a wide-ranging confounding factor for *Entamoeba* genomes, which are particularly difficult to assemble for a number of reasons (Weedall and Hall, 2011), and is likely to have a greater impact on genome assemblies with low coverage such as *E. dispar*. Therefore, the correctness of the genome assembly should first be verified before further investigations on the influence of the *E. histolytica*-unique proteins on virulence are performed.

AUTHOR CONTRIBUTIONS

All authors (JK, BH, IW, GDW, NH, TR, NM, and IB) wrote, edited, and reviewed the drafts of the manuscript, and agree to be accountable for the content of the work. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.641472/full#supplementary-material

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REVIEW
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How Malaria Parasites Acquire Nutrients From Their Host

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Plasmodium parasites responsible for the disease malaria reside within erythrocytes. Inside this niche host cell, parasites internalize and digest host hemoglobin to source amino acids required for protein production. However, hemoglobin does not contain isoleucine, an amino acid essential for *Plasmodium* growth, and the parasite cannot synthesize it *de novo*. The parasite is also more metabolically active than its host cell, and the rate at which some nutrients are consumed exceeds the rate at which they can be taken up by erythrocyte transporters. To overcome these constraints, *Plasmodium* parasites increase the permeability of the erythrocyte membrane to isoleucine and other low-molecular-weight solutes it requires for growth by forming new permeation pathways (NPPs). In addition to the erythrocyte membrane, host nutrients also need to cross the encasing parasitophorous vacuole membrane (PVM) and the parasite plasma membrane to access the parasite. This review outlines recent advances that have been made in identifying the molecular constituents of the NPPs, the PVM nutrient channel, and the endocytic apparatus that transports host hemoglobin and identifies key knowledge gaps that remain. Importantly, blocking the ability of *Plasmodium* to source essential nutrients is lethal to the parasite, and thus, components of these key pathways represent potential antimalaria drug targets.

Keywords: *Plasmodium*, malaria, nutrients, new permeation pathway, transporters

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INTRODUCTION

Malaria is a devastating infectious disease caused by protozoan parasites belonging to the species *Plasmodium,* with an estimated 228 million cases in 2018 alone (World Health Organization, 2019). Despite a modest reduction in the burden of malaria in the last 20 years, it remains a global health problem because of the absence of an effective vaccine and limited chemotherapeutic options as a result of drug resistance. The most severe form of malaria is caused by *Plasmodium falciparum* that is transmitted between people via a mosquito vector (**Figure 1a**). After infection, the parasite has a complex lifecycle that includes an asymptomatic liver stage (**Figure 1b**). Following replication inside hepatocytes, merozoites are released into the blood where they invade erythrocytes to initiate the blood stage of the parasite lifecycle; here, rapid asexual replication occurs, and the clinical symptoms of malaria become evident (**Figure 1c**).

Plasmodium falciparum is an obligate intracellular parasite and is dependent on its host to supply the nutrients required to support its development. However, *P. falciparum* faces some challenges by selecting to reside in mature, metabolically inactive erythrocytes. While the parasites are able to take up hemoglobin from the host cell cytoplasm and rapidly metabolize it, human hemoglobin does not contain all the amino acids necessary for *P. falciparum* growth; isoleucine is absent, and

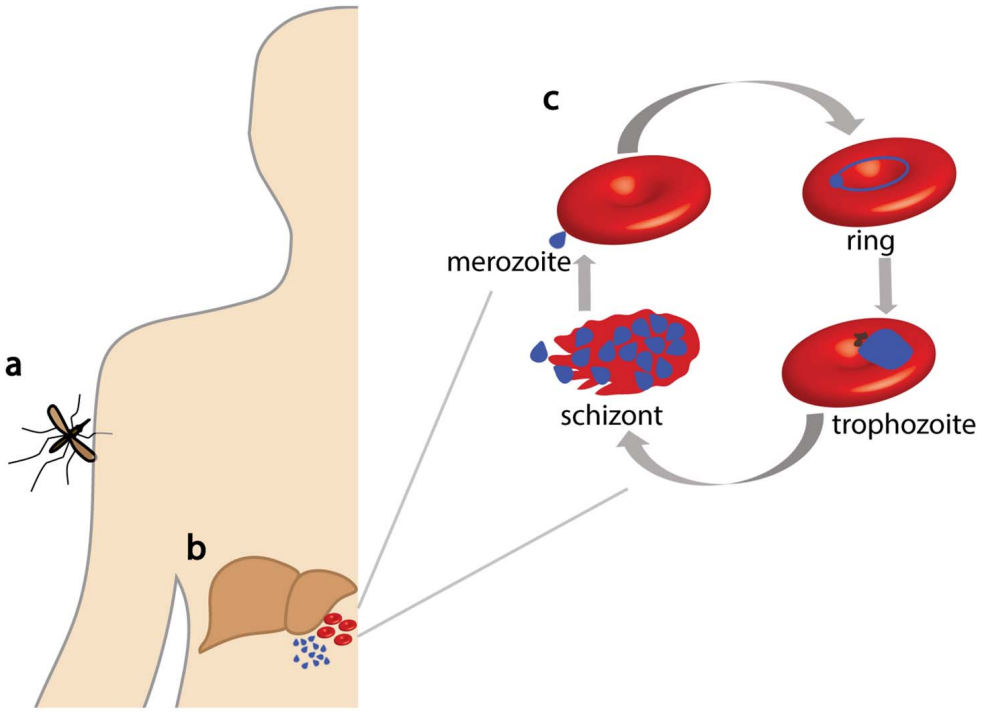

FIGURE 1 | Lifecycle of *Plasmodium falciparum*. *P. falciparum* parasites are injected into a human host via mosquito bites **(a)** where they then travel to the liver **(b)**. After parasite replication, merozoites are released into the blood where they invade erythrocytes to commence the asexual replication cycle **(c)**. Parasites develop within the erythrocyte in distinct forms (ring, trophozoite, and schizont) until erythrocyte lysis occurs, releasing merozoites for reinvasion.

methionine, for example, is poorly represented, and thus these amino acids must be acquired from the human serum (Sherman, 1977; Divo et al., 1985; Spry et al., 2010). So too does pantothenate (vitamin B_5), the precursor to coenzyme A, as it cannot be synthesized by the parasite *de novo*, and it is also essential for parasite growth (Saliba et al., 1998). Although *P. falciparum* can synthesize thiamine (vitamin B_1), it cannot do so in sufficient quantities (Wrenger et al., 2006). Also, rate-limiting to parasite growth are the diminishing intracellular pools of other nutrients including folate and purines (Liu et al., 2006; Wang et al., 2007), and as *P. falciparum* is unable to synthesize purines, it is dependent upon the salvage of exogenous purines via the purine salvage pathway (Reyes et al., 1982; Downie et al., 2008). While *P. falciparum* has some capacity to synthesize its own lipids (reviewed in O'Neal et al., 2020), several classes of lipids are rate-limiting and must also be obtained from an extracellular source (Gulati et al., 2015; O'Neal et al., 2020).

As *P. falciparum* resides within a parasitophorous vacuole (PV) in the host erythrocyte (**Figure 2**), nutrients acquired from the extracellular milieu must traverse otherwise impermeable membranes, namely, the erythrocyte plasma membrane (EPM), the PV membrane (PVM), and the parasite plasma membrane (PPM). The erythrocyte plasma membrane harbors a range of specialized transporters that form channels, carriers, or pumps to facilitate the passage of substrates. However, not all substrates required by *Plasmodium* (e.g., isoleucine and pantothenate) can be obtained via the endogenous transporters, or they are transported in insufficient quantity (Saliba et al., 1998; Martin and Kirk, 2007). Therefore, additional mechanisms must be employed to enable *P. falciparum* to acquire essential nutrients from the host serum. This is achieved through the creation of new permeation pathways (NPPs) that facilitate the transport of a broad range of substrates across the erythrocyte membrane (Ginsburg et al., 1985; Desai et al., 1993). NPPs result from modifications to the host erythrocyte membrane (**Figure 2**); they behave as channels and display a preference for anions over cations (Kirk et al., 1994), but also likely act to dispel waste products generated from hemoglobin digestion and metabolic processes and to maintain ion gradients essential for cell function. NPPs are established at the erythrocyte membrane from 15 h after invasion and reach a plateau after 36 h (Staines et al., 2001). Despite the importance of this channel to parasite survival and its potential as a drug target, until recently, very little was known about its molecular composition, especially compared with our current knowledge of other *P. falciparum* transporters (reviewed in Martin, 2020). The molecular makeup of the "sieve" that allows host nutrients to then cross the encasing PVM so that they can then be transported by the many transporters that decorate the PPM has also only just been revealed. So too have components of the endocytic apparatus that traffics hemoglobin to the digestive food vacuole. This review discusses the strategies the parasite employs to obtain nutrients from the host serum and host cell cytoplasm and the recent advances that have been made in identifying the molecular constituents of these pathways.

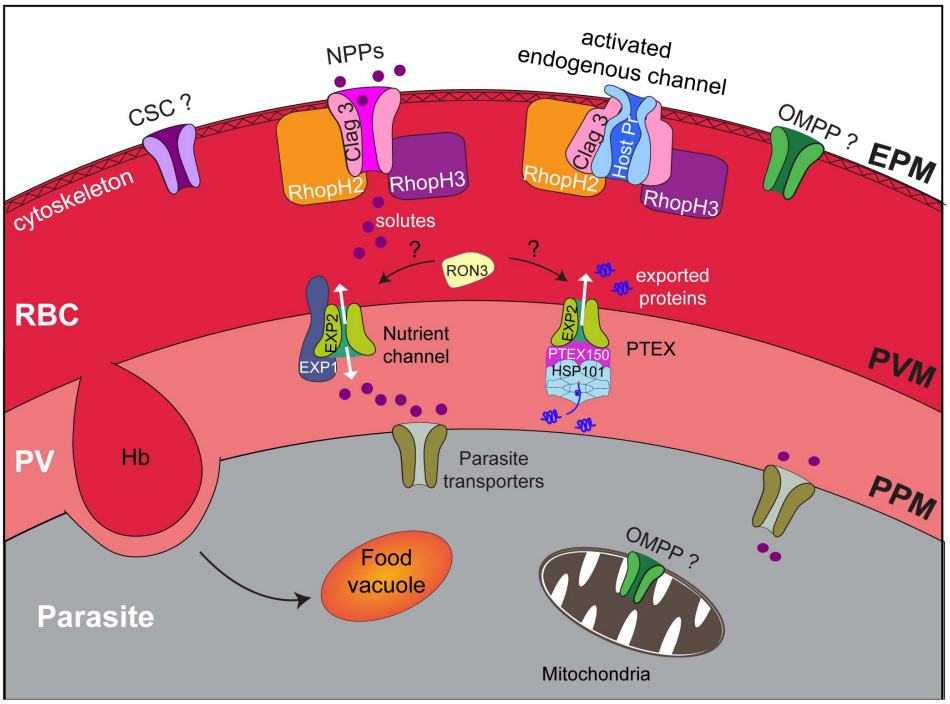

FIGURE 2 | Nutrient acquisition by *P. falciparum* relies on membrane transporters and the cytostome. Upon infection by *Plasmodium* parasites, erythrocytes are extensively remodeled to accommodate the growing parasite. The parasite, contained by its own PPM, is encased within a parasitophorous vacuole (PV) and a PV membrane (PVM). Each membrane in the infected erythrocyte, including the erythrocyte plasma membrane (EPM), is modified to include transporters that enable the parasite to access nutrients from its host. These include the NPPs at the EPM, the nutrient channel at the PVM, and transporters at the PPM, whereas hemoglobin uptake and transport to the food vacuole occur via the cytostome. The NPP channel is proposed to be a CLAG3 dimer/oligomer that associates with RhopH2 and RhopH3. Alternatively, CLAG3, RhopH2, and RhopH3 may activate an endogenous host channel. The contribution of other parasite-encoded channels to the NPPs such as the calcium-dependent, stress-gated ion channel (CSC), and OMPP is unknown; the localization of both of these proteins is yet to be ascertained. The nutrient channel at the PVM comprises EXP2, with EXP1 being critical for proper distribution of the EXP2 nutrient channel. EXP2 also forms the pore of the translocon of exported proteins (PTEX). RON3 is implicated in both nutrient uptake across the PVM and protein export with an unknown function. The PPM is also decorated with a raft of transporters that facilitate the uptake of diverse substrates to satisfy the metabolic requirements of the growing parasite.

CROSSING THE ERYTHROCYTE MEMBRANE

The human erythrocyte membrane contains more than 50 types of transmembrane proteins in its lipid bilayer that are responsible for a range of functions including substrate transport, cell adhesion, and structural integrity (reviewed in Pretini et al., 2019). Substrate transport is facilitated by ATP-powered transporters, organic cation and anion channels, glucose transporters, *P*-type ATPases, urea transporters, and aquaporins, as well as monocarboxylate transporter 1 (MCP1), anion-exchanger 1, and equilibrative nucleoside transporter 1 (Pretini et al., 2019). These transporters serve to maintain the selective permeability of the membrane. It has long been recognized that *P. falciparum* infection induces a measurable increase in the permeability of host erythrocytes (Overman, 1947) to a diverse range of structurally unrelated low molecular mass solutes including sugars, amino acids, vitamins, purines, and ions (reviewed in Martin, 2020), and this change is attributed to NPPs (Ginsburg et al., 1985; Kirk et al., 1994). As the NPPs have the characteristics of an anion-selective channel, it is referred to as the *Plasmodium* surface anion channel (PSAC) (reviewed in Desai, 2012). Despite the recognition of the existence of NPPs for several decades, its biochemical composition has not been well defined, and indeed, it is not known whether PSAC represents the only NPP and whether the NPPs are solely parasite-derived or comprise host cell constituents.

Contribution of CLAG Proteins to NPPs

The *P. falciparum* gene products encoded by *clag3.1* (PF3D7_0302500) and *clag3.2* (PF3D7_0302200) genes were the first parasite-encoded proteins implicated in NPPs and were identified using nutrient restriction, NPP inhibitors, and genetic approaches (Nguitragool et al., 2011; Pillai et al., 2012; **Figure 2**). Parasites in which CLAG3 expression has been depleted through either epigenetic silencing or *in vitro* selection exhibit delayed *in vitro* growth (Comeaux et al., 2011) and altered PSAC activity (Mira-Martinez et al., 2013; Sharma et al., 2013, 2015); yet, interestingly, parasites are still able to survive. Even complete knockout of the *clag3* genes is not lethal, and transport activity is not completely lost in these parasites (Gupta et al., 2020). In the latter case, parasites only displayed a fitness cost when grown in a modified medium that more closely resembles human plasma.

That the CLAG3 proteins were implicated in NPPs was surprising as they do not resemble known transporters, and moreover, it was expected that proteins comprising the NPP channel would be essential for parasite survival given the vital nature of the substrates it transports. However, *clag3.1* and *clag3.2* belong to a unique *Plasmodium* multigene family referred to as RhopH1, which also comprises *clag2*, *clag8*, and *clag9* in *P. falciparum* (Kaneko et al., 2001; Ling et al., 2004; Kaneko et al., 2005). As all five *clag* genes have the same intron–exon structure and are highly conserved, it is conceivable that the loss of the *clag3* genes can be functionally complemented by the other paralogs. Unfortunately, this is very difficult to assess in *P. falciparum* given the technical limitations of knocking out multiple *clag* paralogs in the one parasite line. Alternatively, it is possible that the different *clag* paralogs form distinct channels that facilitate the uptake of different solutes. To date, gene knockouts of *clag2* and *clag8* have not been reported in the literature to assess this. However, *clag2* expression was found to be silenced in blasticidin-resistant parasites in which silencing of both *clag3* genes led to reduced uptake of blasticidin (Sharma et al., 2013, 2015). This is despite *clag2* expression being independent of the epigenetically regulated *clag3* switching program (Cortés et al., 2007). This finding suggests that *clag2* may also contribute to NPP function. For *clag9*, several studies have shown that this gene is not essential for *P. falciparum* growth *in vitro*. Interestingly, CLAG9 is the most distinct of all the CLAG paralogs and lacks the small hypervariable region that is present in its paralogs and which has been shown to be exposed at the erythrocyte surface (Nguitragool et al., 2011, 2014). However, to date, no studies have directly examined the impact of the loss of *clag9* on solute uptake. Prior studies have instead linked CLAG9 to cytoadherence of infected erythrocytes to CS32 melanoma cells and the endothelial receptor CD36, potentially via assisting with the trafficking of the major virulence factor PfEMP1 onto the erythrocyte surface (Trenholme et al., 2000; Goel et al., 2010). However, a subsequent study observed that the loss of binding of infected erythrocytes to CD36 was a result of expression of a non-functional PfEMP1 rather than lack of *clag9* (Nacer et al., 2011). CLAG9 has also been linked to merozoite binding to erythrocytes by interacting with the glycophorin A–band3 receptor–co-receptor complex (Chourasia et al., 2020). Although it is not uncommon for protein and protein fragments to bind non-specifically to erythrocyte membranes, RhopH3 that forms a complex with *clag9* (discussed below) has also been shown to be involved in erythrocyte invasion. Nevertheless, at this stage, it still remains to be formally examined whether CLAG9 also plays a role in NPP activity.

Contribution of RhopH2 and RhopH3 to NPP

The CLAG3 proteins are synthesized toward the end of the parasite asexual cell cycle, where they are stored in the rhoptry organelle in a complex, termed the RhopH complex, with two unrelated proteins named RhopH2 and RhopH3, which are encoded by single copy genes [(Cooper et al., 1988) and reviewed in Counihan et al. (2013)]. The RhopH protein complex is highly conserved among *Plasmodium* species, although the number of *Clag* genes varies among *Plasmodium* species. Upon merozoite invasion, all three proteins are transferred to the newly infected erythrocyte (Vincensini et al., 2008) and are transported to the host cell membrane via undefined trafficking routes. At the erythrocyte membrane, RhopH1, RhopH2, and RhopH3 exist as a complex (**Figure 2**; Sam-Yellowe et al., 1988) in a stoichiometry yet to be resolved, where RhopH2 has been shown to associate with host skeletal proteins (Counihan et al., 2017). Both the *rhopH2* and *rhopH3* genes are refractory to deletion and essential for *Plasmodium* survival (Cowman et al., 2000; Janse et al., 2011; Zhang et al., 2018). Using inducible knockdown/knockout approaches, depletion of RhopH2 and RhopH3 caused a significant defect in parasite growth, and NPP activity was limited (Counihan et al., 2017; Ito et al., 2017; Sherling et al., 2017). Interestingly, depletion of RhopH2 resulted in a reduction of essential vitamins and cofactors including folate and decreased *de novo* synthesis of pyrimidines, which likely contributed to the delayed growth phenotype observed (Counihan et al., 2017). Normally, *P. falciparum* requires ∼48 h to complete a lifecycle, and rapid growth and maturation are observed in the second half of the cycle (**Figure 1**); reduced levels of RhopH2 and RhopH3 resulted in parasites stalling about midway through their lifecycle, which is consistent with when NPPs are active (15–36 h after invasion).

What Is the Channel-Forming Component of the NPP?

There is compelling evidence that the RhopH proteins participate in NPP formation, but there are still fundamental questions concerning the makeup of NPP that need to be determined, particularly the component(s) that form the actual channel through the erythrocyte membrane. The CLAG proteins do not resemble any known proteins, including known transporters, although some of the top hits to the CLAG3 proteins using the protein prediction and structure program iTasser (Zhang, 2008) are channel proteins. The CLAGs are predicted to harbor a region that may form a transmembrane domain that is lined with polar residues on one side and non-polar on the other, reminiscent of the domains that form water-filled pores (Sharma et al., 2015), and a pool of CLAG3 is integral to the membrane (Nguitragool et al., 2011).

A soluble form of RhopH protein complex structure has recently been solved via cryoelectron microscopy (Schureck et al., 2021). Structural analysis reveals a heterotrimeric complex of CLAG3, RhopH2, and RhopH3 with a 1:1:1 stoichiometry. CLAG3 is situated in the middle of the complex, where it interacts tightly with RhopH2 and RhopH3. However, the predicted transmembrane domain of CLAG3, which does indeed form an a-helix near the CLAG3 *C*-terminus, is buried in the core of the RhopH complex and is not exposed to solvent. So too is the hypervariable region that was previously shown to be exposed on the surface of infected erythrocytes (Nguitragool et al., 2011). Thus, in order for the complex to be incorporated into the membrane, large-scale conformational changes would be required. The process(es) that may drive this are unknown

but could potentially involve posttranslational modifications such as phosphorylation or regulation of allosteric disulfides or may require interactions with proteins or lipids at the erythrocyte plasma membrane.

RhopH2 and RhopH3 are even less convincing as potential channel proteins, showing no structural homology to known channel proteins. Moreover, protease protection assays indicate that RhopH2 and RhopH3 are not exposed on the erythrocyte surface (Ito et al., 2017). Some protein prediction programs such as Phobius suggest the presence of at least 1 transmembrane domain in RhopH2 and RhopH3, and thus, there is the possibility that CLAG forms a functional channel with RhopH2 and RhopH3 or indeed forms homo-oligomers or oligomerizes with other proteins to form a channel (see **Figure 2** for a model). However, the predicted transmembrane domains of RhopH2 and RhopH3 are also buried in the RhopH soluble structure, 46–101 Å apart from the CLAG3 predicted transmembrane domain. Thus, massive rearrangement of the complex structure would be required if the RhopH2, RhopH3, and CLAG3 transmembrane domains come together to form a channel in the erythrocyte plasma membrane (Schureck et al., 2021). Given the contribution of RhopH2 and RhopH3 to NPP function, it is likely, therefore, that these two proteins play an essential accessory role.

There is also the possibility that CLAG3 is not the NPP channel and that other parasite or host proteins serve this function (**Figure 2**). Parasite-encoded proteins that exhibit homology to anion-selective channels have been identified among the *Plasmodium* transportome, including the outer membrane pore-forming protein (OMPP) found in bacteria and eukaryotic organelles such as the mitochondria and chloroplasts (Reddy and Saier, 2016) and the calcium-dependent, stress-gated ion channel (CSC) that belongs to the calcium-dependent chloride channel (Ca-CIC) family (Martin, 2020). The localization of both of these proteins in *Plasmodium* has yet to be assessed; only CSC harbors a signal sequence, and neither protein is predicted to contain export motifs to facilitate trafficking into the erythrocyte after synthesis. Neither of these proteins interacts with members of the RhopH complex; thus, if they are involved in NPPs, they must function independently of the RhopH complex (**Figure 2**).

Immunoprecipitation experiments have revealed that ∼30 parasite proteins affinity purify with RhopH2 (Counihan et al., 2017). While these proteins are exported or predicted to be exported into the host cytoplasm, none resemble channel proteins, and therefore, unlikely to serve as the NPP channel. Interestingly, erythrocyte anion exchanger 1 (AE1) affinity is purified with the RhopH complex, but as this endogenous protein is highly abundant, its binding may be non-specific. Moreover, if AE1 served as the NPP channel, it would have to be modified by the parasite during infection given that NPPs are not established until 15 h postinfection. While AE1 is progressively phosphorylated in response to oxidative stress when parasites transition from the ring to schizont stage (Pantaleo et al., 2010), the resulting reduction in AE1 affinity for the cytoskeleton and consequential membrane destabilization occur at the end of the cell cycle (Ferru et al., 2011), which is more consistent with a role in merozoite egress rather than NPP function.

Another erythrocyte voltage-dependent anion channel (VDAC), which is either expressed alone or as a component of the peripheral-type benzodiazepine receptor (PBR) complex, is the VDAC. In uninfected erythrocytes, PBR mediates the AE1-independent anion conductance and becomes upregulated when erythrocytes are infected by *P. falciparum* (Bouyer et al., 2011). The properties of VDAC resemble those of the NPP, and PBR ligands reduce membrane transport and conductance in *P. falciparum*-infected erythrocytes and block parasite growth *in vitro*. However, VDAC/PBR does not immunoprecipitate with the RhopH complex (Counihan et al., 2017), indicating that this transporter exists as an independent entity at the erythrocyte membrane and is not regulated by the RhopH components, but whether it associates with other parasite proteins is unknown. It also remains unknown what substrates VDAC/PBR transports in *Plasmodium*-infected erythrocytes. Thus, despite the importance of NPPs to parasite growth, the molecular composition of this channel still remains elusive.

NUTRIENT TRANSFER ACROSS THE PVM

The erythrocyte membrane is not the only barrier to host nutrient uptake by the parasite. Nutrients must also be able to cross the PVM. Soluble macromolecules such as amino acids and monosaccharides up to 1,400 Da in size are able to readily cross this membrane via a channel that is permeable to both cations and anions (Desai and Rosenberg, 1997). Cell-attached patch clamping on parasites isolated from infected erythrocytes has revealed that this channel is present at the PVM in high density and is open most of the time (Desai et al., 1993). Permeation through this channel does not involve the binding of substrates; rather, the channel behaves like a sieve. Thus, transfer across the PVM is constrained by size of the solute and its diffusion coefficient in the cytosol (Desai et al., 1993; Desai and Rosenberg, 1997).

The PVM in the related apicomplexan parasite, *Toxoplasma gondii*, has a similar exclusion limit for macromolecules to that of *Plasmodium* (Schwab et al., 1994), suggesting that related channels may exist to transport soluble macromolecules across their respective PVMs. Two *T. gondii* proteins, termed GRA17 and GRA23, which are secreted from the dense granule organelles following invasion, perform this role. These proteins were shown to be required for the uptake of membrane-impermeable dyes lucifer yellow (522 Da) and the fluorescent form of 5-(and-6)-carboxy-20,70-dichlorofluorescein diacetate (CDCFDA; 445 Da) into the PV (Gold et al., 2015). In contrast, and consistent with the size constraint of this channel, dextran (3,000 Da) was excluded from entry. Moreover, injection of GRA17 and GRA23 cRNA into *Xenopus* oocytes altered their membrane conductance (Gold et al., 2015). Although the GRA proteins appear to act synergistically, it remains unclear if they have separate or overlapping functions. Studies in mice have revealed that GRA17-mediated PVM permeability is important for the growth of tachyzoites (the rapidly growing life stage of *T. gondii*) and virulence in mice, whereas both GRA17 and GRA23 are

important for the viability of the *T. gondii* life stage inside cysts (Gold et al., 2015; Paredes-Santos et al., 2019; Li et al., 2020).

Interestingly, GRA17 and GRA23 show predicted structural similarity to *Plasmodium* EXP2, the pore-forming component of the *Plasmodium* translocon of exported proteins (PTEX) that is also secreted from dense granules. The PTEX machinery is responsible for the export of *Plasmodium* proteins into the host cytosol (de Koning-Ward et al., 2009), and GRA17 loss-of-function phenotypes can be rescued by *Plasmodium* EXP2 (Gold et al., 2015). Yet, intriguingly, GRA17 or GRA23 does not play a role in protein export. However, recently, it was discovered that a PTEX-independent pool of EXP2 exists at the PVM in *P. falciparum* that facilitates the exchange of soluble macromolecules across this membrane (Garten et al., 2018). Indeed, there is a correlation between EXP2 expression levels and the frequency with which the channel can be detected. Removal of 53 amino acids from the *C*-terminus of EXP2, a region that is abundant in acidic residues, gives rise to a PVM channel with an altered voltage response. Combined, these studies suggest that EXP2 not only gates the PVM channel, but also forms the actual nutrient channel through the PVM. Interestingly, pores are also found in the PVM of the liver stage parasites (Bano et al., 2007). As EXP2 is also expressed in the liver stage parasites (Vaughan et al., 2012), it may be performing a similar role, pointing toward a conserved mechanism in both lifecycle stages.

If the nutrient pore of *P. falciparum* exists in an open confirmation, the incorporation of EXP2 into membranes would need to be regulated to permit nutrient exchange only at the PVM. One candidate regulatory protein is EXP1, a PV-resident protein previously proposed to function as a glutathione S-transferase (Lisewski et al., 2014). EXP1 coimmunoprecipitates with EXP2 (Mesén-Ramírez et al., 2016) and is critical for PVM nutrient channel activity (Mesén-Ramírez et al., 2019). Conditional knockout of EXP1 using the dimerizable Cre system (Collins et al., 2013) led to slow growth phenotypes comparable to those resulting from amino acid starvation (Babbitt et al., 2012) or after conditional depletion of members of the RhopH complex (Counihan et al., 2017; Ito et al., 2017; Sherling et al., 2017). Compared with wild-type parasites, EXP1 knockout parasites showed a reduced frequency of channel detection by patch clamping, and transgenic parasites expressing limiting levels of EXP1 were hypersensitive to low levels of amino acids. These phenotypes may be attributable to altered PVM morphology and distribution of EXP2. EXP2 membrane association and protein export via PTEX did not appear to be affected (Mesén-Ramírez et al., 2019), indicating that EXP1 does not regulate the insertion of EXP2 into the membrane. Nessel et al. (2020) have also generated EXP1 knockdown parasites using the TetR-DOZI aptamer system (Ganesan et al., 2016) and similarly concluded that EXP1 is responsible for proper distribution of EXP2 at the PVM. However, as their knockout EXP1 parasites progressed further through the cell cycle than the EXP2 knockdown parasites, this led the authors to conclude that EXP1 does not contribute to EXP2-dependent transport activities. The differences between the findings of the two EXP1 studies may stem from the timing of EXP1 knockdown and when this impacts on PVM morphology. It is likely that disturbances to the PVM from EXP1 knockdown would also affect the localization/function of other PVM proteins that are critical for the parasite to complete the cell cycle, although this was not investigated.

RON3, a protein secreted from the rhoptry bulb and that localizes to the parasite periphery following invasion, has also been linked to PVM nutrient uptake. Parasites deficient in RON3 exhibit a similar phenotype to parasites deficient in EXP2 and fail to progress beyond the ring stages (Low et al., 2019). They are not only affected in their ability to import glucose, as they also fail to export proteins into the erythrocyte cytosol. However, RON3 is unlikely to be a direct interacting partner of nutrient pore EXP2 or PTEX EXP2; the BioID2 proximity ligase system fused to EXP2 did not reveal an interaction with RON3 (Mesén-Ramírez et al., 2016), and only few peptides were identified in a PTEX immunoprecipitation (de Koning-Ward et al., 2009), despite the fact that RON3 is a 263-kDa protein. Thus, it remains unclear how RON3 contributes to both nutrient uptake and protein export, and further studies are required to assess whether EXP2 localization or assembly is affected in RON3-deficient parasites.

TRANSPORT OF NUTRIENTS ACROSS THE PPM

Once soluble macromolecules from the host have gained access to the PV, they can be then be transported across the PPM via an array of parasite transporters. A recent review by Martin (2020) has documented parasite transporters present at the different membranes in a parasitized erythrocyte, including the PPM, and so here we highlight only a subset for which there is experimental support for localization at the PPM. These include aquaglyceroporin (AQP), formate-lactate channel (FNT), putative copper channel, cationic amino acid transporter (NTP1), hexose transporter (HT1), putative amino acid transporter (AAT1, AAAP3), putative Mg^{2+} transporter, ATP: ADP antiporter (AAC1, AAC2), putative MCP1, folate–biopterin transporter (FT1, FT2), equilibrative nucleoside transporter (ENT, ENT4), Zn^{2+} and Fe^{2+} transporter (ZIPCO), the ABC transporters known as multidrug-resistant proteins (MDR1, MDR2, and MDR5) and multidrug-resistant associated proteins (MRP1 and MRP2), putative inorganic anion exchanger (SulP), Pi: Na^{+} symporter (PiT), putative lipid/sterol: H^{+} antiporter (NPC1R), V type H^{+} ATPase, and a variety of P-ATPases (ATP1, ATP2, ATP4, ATP8, CuTP, and GCa).

Not all of the transporters at the PPM are essential for parasite survival. Gene disruption studies (reviewed in Martin, 2020) and a large-scale mutagenesis screen (Zhang et al., 2018) have provided insight into which of these many transporters are essential and therefore could constitute potential antimalaria drug targets. Although the identity of the PPM transporter for pantothenate remains unknown, it is also a candidate drug target given the reliance on pantothenate uptake for parasite survival. The putative pantothenate transporter (PPT) was suggested to serve this role as it could complement the growth phenotype of a yeast mutant deficient in pantothenate transport (Augagneur et al., 2013). However, a study using rodent malaria parasites

revealed that this transporter, which is also referred to as the putative metabolite transporter TFP1, is not expressed at the PPM in asexual blood stages and instead localizes to osmiophilic bodies in gametocytes (Kehrer et al., 2016). Moreover, the *tfp1* gene is dispensable (Hart et al., 2014; Kehrer et al., 2016; Zhang et al., 2018). Alternate pantothenate transporter candidates have been proposed, including the putative metabolite/drug transporter *umf* and monocarboxylate transporter *mcp1* or one of the parasite's mitochondrial carrier superfamily proteins (Martin, 2020), but experimental evidence is lacking for any of these. Interestingly, the identity of the isoleucine PPM transporter, another potential antimalaria drug target due to the parasite's requirement to take up isoleucine, is also unknown. Proposed candidates include the putative amino acid transporters AAT1 and AAAP3 (Martin, 2020), both of which were not mutable in *P. falciparum* (Zhang et al., 2018).

INGESTION OF HOST HEMOGLOBIN VIA FORMATION OF THE CYTOSTOME

Unlike other microbes that source heme iron from hemoglobin through extraction from soluble and cell surface/wall receptors and subsequent transfer to cell wall and ABC transporters (Pishchany and Skaar, 2012), *P. falciparum* utilizes hemoglobin as a source of amino acids, which it instead acquires by endocytosing a large proportion of the host cell cytosol. This endocytosis process is non-selective, involving double-membrane invagination of both the PVM and the PPM, resulting in formation of a cytostome and potentially a larger structure known as a phagotroph (Aikawa et al., 1966; Slomianny et al., 1985; Slomianny, 1990; Elliott et al., 2008). The content of cytostomes has been assumed to be subsequently transported to the parasite's digestive vacuole (DV); however, it is important to note that this has yet to be experimentally shown; consequently, the mechanism(s) by which hemoglobin and the host cell cytosol are delivered from endocytic structures to the DVs is yet to be resolved. We refer readers to a review by Spielmann et al. (2020) for proposed endocytic routes to the DV, which involve either the entire cytostome or only a portion pinching off and the subsequent transport to the DV, with potential involvement of intermediatory compartments. Digestion of hemoglobin occurs *en route* and within the DV (Abu Bakar et al., 2010), with the globin chains first cleaved from hemoglobin by aspartic proteases and cysteine endoproteases, and the liberated polypeptides then further digested by metalloproteases and series of aminopeptidases into oligopeptides and dipeptides to provide a source of amino acids for the parasite (Goldberg, 2005).

The molecular constituents that govern the uptake of the host cytosol by *P. falciparum* are also not resolved, largely because findings have either been conflicting or direct functional data have been lacking. We refer readers to two reviews that summarize the large body of work that has been undertaken to tease out potential molecular players in endocytosis of the host cell cytosol, with candidates including adapter protein-2 (AP-2), phosphatidylinositol-3-kinase, coronin, actin, dynamin, epidermal growth factor receptor substrate-15 (Eps15) homology domain (EHD) protein, soluble *N*-ethylmaleimide sensitive factor attachment protein (SNAP) receptor proteins (SNAREs), and Rab proteins (Spielmann et al., 2020; Xie et al., 2020). More recently, two studies from the Spielmann laboratory using genetic and proximity ligation approaches have shed further light on this process, revealing that components of a non-canonical endocytic apparatus are involved. In the first study, vacuolar protein sorting associated protein 4, which serves as an endolysosomal transport protein in other eukaryotes (Cowles et al., 1994; Piper et al., 1994), was shown to be required for host cell cytosol uptake (Jonscher et al., 2019). Then, a follow-up study examining the cellular function of Kelch 13 and its contribution to artemisinin resistance demonstrated that a Kelch-13 protein complex is also required for endocytosis of hemoglobin from the host cell (Birnbaum et al., 2020). This complex comprises Kelch 13, epidermal growth factor receptor substrate-15 (Eps15), ubiquitin carboxyl-terminal hydrolase (UBP1), AP-2, and a handful of other Kelch interacting proteins (Birnbaum et al., 2020). Unlike Kelch 13, which is only critical for the endocytosis of the host cell cytosol at ring stages, Eps15, UBP1, and AP-2 are required for endocytosis at both ring and trophozoite stages, such that inactivation of these proteins leads to a reduced transport of hemoglobin to the DV (Birnbaum et al., 2020). The identification of some of the molecular components of this endocytic pathway will now enable detailed mechanistic dissection of host cell cytosol uptake by the parasite.

CONCLUDING REMARKS

Until recently, many of the molecular players involved in nutrient uptake by *Plasmodium* parasites have remained elusive, largely due to the difficulties associated with genetic manipulation of the *Plasmodium* genome and studying the function of essential genes (de Koning-Ward et al., 2015). However, the use of advanced molecular approaches to conditionally knock out or knock down genes and their products has enabled a better understanding of this process. Many of the genes identified in the pathways discussed in this review are essential to parasite survival, which highlights that blocking parasite nutrient acquisition from the host with antimalaria drugs is a worthwhile strategy to pursue. For drugs such as artemisinin that are activated by hemoglobin-derived heme and thus are dependent on the adequate uptake of hemoglobin by the parasite, understanding how this is achieved could inform approaches that could be used to antagonize drug resistance. Conversely, the molecular constituents of the hemoglobin uptake pathway could also be targeted with drugs, albeit not in combination with artemisinin or other heme-activated compounds. However, there are still some key knowledge gaps remaining. Functional evidence for CLAG3 serving as the channel-forming component of the NPP is lacking; if it does form the channel, what processes occur to drive insertion of CLAG3 into the membrane. If CLAG3 does not perform this role, what protein does, and is it parasite-derived? What function is served by the other CLAG proteins; are they required for the uptake of specific substrates? Why is the RhopH

complex synthesized at the end of the cell cycle and secreted during invasion when the NPPs are not established for 15 h later, and what essential role do RhopH2 and RhopH3 play in NPP formation? What process ensures that EXP2 can form both a protein export channel and a nutrient channel, and what regulates its assembly into the PVM so that it does not prematurely form pores in membranes prior to this time? And is EXP2 the only nutrient channel at the PVM? What is the identity of the PPM transporters that are responsible for the uptake of isoleucine and pantothenate that are known to be essential to the parasite? These are just some of the questions that remain unanswered and are key to fully understanding the essential process of nutrient acquisition by *Plasmodium* parasites.

AUTHOR CONTRIBUTIONS

NC, JM, and TdK-W were responsible for writing and editing manuscript drafts and the final document. All authors contributed to the article and approved the submitted version.

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REVIEW
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Theileria's Strategies and Effector Mechanisms for Host Cell Transformation: From Invasion to Immortalization

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One of the first events that follows invasion of leukocytes by *Theileria* sporozoites is the destruction of the surrounding host cell membrane and the rapid association of the intracellular parasite with host microtubules. This is essential for the parasite to establish its niche within the cytoplasm of the invaded leukocyte and sets *Theileria* spp. apart from other members of the apicomplexan phylum such as *Toxoplasma gondii* and *Plasmodium* spp., which reside within the confines of a host-derived parasitophorous vacuole. After establishing infection, transforming *Theileria* species (*T. annulata*, *T. parva*) significantly rewire the signaling pathways of their bovine host cell, causing continual proliferation and resistance to ligand-induced apoptosis, and conferring invasive properties on the parasitized cell. Having transformed its target cell, *Theileria* hijacks the mitotic machinery to ensure its persistence in the cytoplasm of the dividing cell. Some of the parasite and bovine proteins involved in parasite-microtubule interactions have been fairly well characterized, and the schizont expresses at least two proteins on its membrane that contain conserved microtubule binding motifs. *Theileria*-encoded proteins have been shown to be translocated to the host cell cytoplasm and nucleus where they have the potential to directly modify signaling pathways and host gene expression. However, little is known about their mode of action, and even less about how these proteins are secreted by the parasite and trafficked to their target location. In this review we explore the strategies employed by *Theileria* to transform leukocytes, from sporozoite invasion until immortalization of the host cell has been established. We discuss the recent description of nuclear pore-like complexes that accumulate on membranes close to the schizont surface. Finally, we consider putative mechanisms of protein and nutrient exchange that might occur between the parasite and the host. We focus in particular on differences and similarities with recent discoveries in *T. gondii* and *Plasmodium* species.

Keywords: *Theileria*, Apicomplexa, invasion, annulate lamellae, microtubule, transformation, *Toxoplasma*, *Plasmodium*

INTRODUCTION

Theileria is a genus of tick-borne parasites and, together with *Babesia* spp., forms the order of Piroplasmida within the phylum of Apicomplexa. Apicomplexan parasites are important zoonotic and human pathogens and include members such as *Plasmodium* spp., the causative agents of malaria and responsible for more than 400,000 deaths per year (World Malaria Report, 2020); *Toxoplasma gondii*, arguably the most successful parasite worldwide capable of infecting all warm blooded animals and estimated to infect up to 30% of the human population (Aguirre et al., 2019); *Babesia* spp., for which humans are accidental hosts (Yabsley and Shock, 2013); and *Theileria* spp. that cause devastating diseases of cattle and result in huge economic losses in several countries of the Global South (Morrison, 2015). Due to their large impact on human and animal health, apicomplexan parasites have been the subject of extensive research efforts which have uncovered fascinating mechanisms of host cell manipulation, ranging from metabolic reprogramming to hijacking of host gene expression (recently reviewed by Villares et al., 2020). Among the most striking alterations of host cell phenotype are induced by the pathogenic *Theileria* species *T. annulata* and *T. parva*. Upon infection of bovine leukocytes, these parasites re-program cell signaling pathways to such an extent that they induce cellular transformation, conferring a proliferative phenotype and immortality on infected leukocytes (Dobbelaere and Rottenberg, 2003; Tretina et al., 2015). *Theileria*-infected leukocytes thus share many key hallmarks with cancer, and the clonal expansion of infected leukocytes and the associated cytokine storm have been implicated in pathogenesis (Glass et al., 2012). In infected cattle this causes East Coast Fever (*T. parva*) and Tropical Theileriosis (*T. annulata*), comprising symptoms including fever, anemia and enlarged lymph nodes. In many cases infection results in the death of the animal. The *Theileria* genus also comprises the so-called "non-transforming" species *T. orientalis*, which does not induce proliferation in the invaded blood cells and consequently does not cause lymphoproliferative diseases in livestock, although infection can still be lethal due to severe anemia (Watts et al., 2016).

Infective sporozoites are transmitted by ticks and invade white blood cells of cattle and other small ruminants, with *T. annulata* infecting bovine macrophages and B cells, and *T. parva* infecting bovine T cells and B cells (Spooner et al., 1989). Following invasion, the sporozoite develops into a multinucleated syncytium called a macroschizont, and proliferation of the parasitized leukocyte is triggered within three days of infection. Rather than undergoing multiple rounds of egress and reinvasion like *T. gondii*, *Theileria* schizonts interact closely with the mitotic machinery of the host cell and are divided to both daughter cells following cytokinesis, thus leading to clonal expansion of the schizonts together with the infected leukocytes. *Theileria* infection is associated with extensive changes in host cell kinase activity, and a significant increase in total protein phosphorylation in infected cells has been reported (ole-MoiYoi et al., 1993). *Theileria* schizonts take control of multiple signaling pathways in the host, leading to changes in gene expression that favor parasite survival and spread of the infected cell. One important example is the constitutive activation of the NF-κB pathway that is essential for protecting the *Theileria*-transformed leukocyte from apoptosis (Heussler et al., 2002). Another example is the AP-1-driven expression of metalloprotease-9 (MMP9) that confers an invasive phenotype on the infected host (Baylis et al., 1995). Intriguingly, survival and the maintenance of the transformed phenotype in *Theileria*-infected cells depends on the presence of a viable parasite, suggesting that transformation is driven by changes in gene expression and cell signaling pathways rather than mutations or alterations at the DNA level (Dobbelaere and Rottenberg, 2003). Although the extensive changes in host cell signaling pathways induced by *Theileria* infection have been relatively well studied (see reviews Woods K. et al., 2013; Cheeseman and Weitzman, 2015; Tretina et al., 2015), few parasite effector proteins have been characterized in detail, and even fewer have been linked convincingly to regulation of the transformed host cell phenotype.

In this review, we focus on host-parasite interactions in *Theileria*-infected leukocytes, in particular *Theileria* proteins that interact with host cell proteins at the schizont membrane, and those that are secreted into the host cytoplasm or nucleus where they have the potential to modify the host phenotype. We discuss the recent description of host-derived pore-containing membranes that align close to the schizont membrane, and we speculate on some of the shared and unique elements of *Theileria* protein export as compared to other apicomplexans. In addition to reviewing published work that addresses important elements of the *Theileria*-leukocyte interaction, we sought to identify some key open questions that will hopefully motivate further study into *Theileria* biology.

INVASION IN ANY ORIENTATION

Like most other apicomplexan parasites, *Theileria* have a complex life cycle (reviewed in Jalovecka et al., 2018). The invasion process of tick-borne *Theileria* sporozoites into their mammalian target cells differs strikingly from that of other apicomplexans such as *Toxoplasma* and *Plasmodium* and these differences are reflected in the morphology of the invasive sporozoites. *Theileria* sporozoites are approximately spherical, 0.75 – 1.5 μm in diameter, have a single nucleus occupying the basal region of the cell, and are covered by a 20 – 25 nm surface coat which is trypsin-sensitive (Fawcett et al., 1982; Shaw, 1991). Their cytoplasmic hemisphere contains a single mitochondrion without cristae, numerous free ribosomes, and dispersed secretory organelles named microspheres (equivalent to dense granules in *T. gondii* and *Plasmodium* spp.). The apical tip of the sporozoite is defined by few rhoptries attaching to the cell membrane via a peg-like structure similar to a polar ring, but otherwise lacks typical structures of a large apical complex such as a conoid (as do all piroplasms; class Aconoida), a subpellicular microtubule basket, and apical micronemes. Electron microscopy studies indicate that *Theileria* sporozoites are surrounded only by a plasma membrane and do not possess an inner membrane complex (Stagg et al., 1981; Jura et al., 1983; Shaw, 1991). Thus,

Theileria's sporozoite structure differs greatly from the larger (4 – 14 μm) and elongated sporozoites of *Toxoplasma* and *Plasmodium* (Speer et al., 1995; Frischknecht and Matuschewski, 2017). The lack of specialized secretory micronemes in the apical region of sporozoites, which in other apicomplexans release proteins essential for parasite gliding motility and are required for host cell attachment and invasion (Frénal et al., 2017), further reflects differences in the invasion process.

In contrast to all other apicomplexans, including the closely related *Babesia* spp. (Jalovecka et al., 2018), *Theileria* sporozoites are non-motile and their contact with a host cell happens by chance (Shaw, 1991). This initial attachment is irreversible and of an essentially passive nature, though inhibitable by proteases, suggesting the involvement of specific host-parasite surface molecule interactions. Invasion occurs within three minutes after attachment and the parasite is established inside the host cell cytosol within 15 min. Unlike other apicomplexan zoites, *Theileria* sporozoites do not reorientate for invasion and can enter the host cell in any direction. During this process parasite and host cell membranes are in close contact, and entry seems to occur rather passively by a "circumferential zippering" of the two membranes. As no involvement of the actin cytoskeleton of the parasite has been shown and no actin accumulation or pseudopodia formation occurs at the leukocyte surface, it is supposed that this process of zippering might be sufficient in itself to lead to internalization of the sporozoite (Fawcett et al., 1982). Invasion can be blocked by pre-treating the host lymphocytes with cytoskeleton-disrupting reagents (Shaw, 1991), though it is not known whether this inhibition is due to a reduction in sporozoite binding caused by altered surface receptor distribution on the host cell, or whether the invasion itself cannot take place. Though not fully understood, the invasion process of *Theileria* sporozoites is starkly different to, for example, the invasion of *Toxoplasma* tachyzoites, which is actively driven by parasite motility and involves the formation of moving junctions, complexes of parasite proteins interacting with the host cell surface to support penetration of the host cell (Frénal et al., 2017). During the internalization and zippering process, parts of the *Theileria* sporozoite surface coat are shed (Fawcett et al., 1984; Webster et al., 1985) including the *T. parva* surface protein p67 (TP03_0287) (Shaw, 2002). This suggests that successful invasion involves proteolytic processing of parasite or host surface molecules, which is supported by the fact that entry can be blocked by protease inhibitors. In *Theileria* sporozoites, microspheres and rhoptries are discharged approximately 15 min post-invasion and seem to be controlled by a timed mechanism starting at the moment of attachment, as discharge occurs still during invasion if the invasion process is slowed down with cytochalasin D (Shaw, 1991).

Molecularly, little is known about the invasion process and the host and parasite molecules that are involved. For *T. parva*, two sporozoite surface molecules have been identified which seem to play a role in invasion, namely p67 (Dobbelaere et al., 1985; Iams et al., 1990a) and PIM (Polymorphic immunodominant molecule; TP04_0051; the orthologs protein is named TaSP in *T. annulata*; TA17315) (Toye et al., 1991). For *T. annulata,* the major sporozoite surface protein is SPAG-1 (TA03755), which shares considerable sequence homology with p67. Antibodies against p67 and SPAG-1 have been shown to block sporozoite entry into the host cell (Williamson et al., 1989; Musoke, 1992) and immunization with either antigen provides some cross-species protection (Hall et al., 2000). Native and recombinant p67 competitively and reversibly blocks sporozoite binding, indicating that p67 interacts with the host cell, though in a rather weak manner (Shaw et al., 1995). Because attachment of the sporozoite to the host cell is quite strong and most likely irreversible, it is supposed that this attachment is mediated via numerous interactions resulting from the high density of p67 distributed on the sporozoite surface (Shaw, 2002). In contrast to *Theileria*, the molecular components of the invasion process of *Toxoplasma* and *Plasmodium* zoites have been well studied. Apical membrane antigen 1 (AMA1) is a secreted microneme protein conserved across the Apicomplexa that, by interacting with RON2, plays a critical role in the formation of the moving junction and host cell invasion (Hehl et al., 2000; Bargieri et al., 2013). Other well-characterized proteins required for host invasion are the *Plasmodium* thrombospondin-related anonymous protein (TRAP) and the *T. gondii* homolog microneme protein 2 (MIC2) (Sultan et al., 1997; Huynh and Carruthers, 2006). Although *Theileria* sporozoites are non-motile and their invasion process is considered rather passive, *Theileria* parasites do possess homologs of TRAP (TA07755) and AMA1 (TA02980), neither of which have been studied in *Theileria*.

While some hepatocyte receptors involved in *Plasmodium* sporozoite invasion are relatively well known (for example CD81 and SR-B1, reviewed in Dundas et al., 2019), it is not known which host cell molecules *Theileria* sporozoites interact with. It is likely that sporozoite surface markers recognize very specific host molecules, allowing them to selectively invade only a restricted population of host cells. It has been shown that antibodies against MHC class l and β-2 microglobulin block *T. parva* sporozoite invasion and that these molecules are an essential component of the host receptor involved in sporozoite-host binding (Shaw, 1991, Shaw et al., 1995). *T. annulata,* on the other hand, invades cells expressing MHC class ll molecules (Spooner et al., 1989), though no studies have been performed to show that MHC class ll molecules are directly involved in invasion. It is likely that the attachment and invasion process includes interactions with other surface molecules as well, as interaction with MHC class l or ll molecules would not explain the high selectivity in invasion.

ESTABLISHING A NICHE IN THE HOST CYTOPLASM

After invasion, the sporozoite is first encapsulated in a membrane of host cell origin, called the parasitophorous vacuole (PV). However, in contrast to many other apicomplexan parasites (e.g., *Toxoplasma* and *Plasmodium*), *Theileria,* as well as *Babesia,* rapidly escape this host membrane after invasion and reside directly in the host cell cytosol (Shaw, 1991; Repnik et al., 2015). The separation of host and parasite membranes in *Theileria* occurs simultaneously with rhoptry and microsphere discharge and starts at the regions where rhoptries are located (Shaw, 1991).

Unlike in other apicomplexans this seems not to be calcium-dependent (Shaw, 1991, 1997), and the escape from the host cell membrane is not dependent on the acidification of the parasite-containing vacuole as is the case for other intracytoplasmic pathogens such as *Listeria* (Li et al., 2017) and *Trypanosoma* (Batista et al., 2020). The first visible reaction of the host cell to the presence of the parasite is the arrangement of orderly microtubules (MTs) which associate with the parasite surface shortly after PV membrane (PVM) lysis and converge toward the centriole-Golgi region (Shaw, 1991).

CLASPING MICROTUBULES AT THE SCHIZONT SURFACE

Theileria schizonts have evolved a unique mechanism to facilitate their expansion in an infected cow. Upon development into a schizont, *Theileria* triggers dramatic changes in the phenotype of the infected cell, inducing uncontrolled proliferation that leads to the clonal expansion of infected leukocytes. Rather than egress and reinvade new host cells, *Theileria* hijacks the mitotic machinery of the host cell and aligns itself with the mitotic spindle to ensure its segregation to both daughter cells following cytokinesis of the transformed cell (von Schubert et al., 2010). The close association of *Theileria* with host MTs is observed very soon following PVM rupture and is maintained throughout the schizont stage. Division of the host cell into two daughter cells with the parasite being distributed equally between them happens as early as three days after infection (Hulliger et al., 1964; Stagg et al., 1981). MTs bind closely to the surface of the schizont and the parasite "incorporates" itself within the host central spindle during cytokinesis, ensuring its distribution to both throughout the cell cycle (**Figure 1**; Huber et al., 2017).

Live imaging of *Theileria*-infected cells stably expressing RFP-tubulin revealed that mitotic MTs aligned with the schizont surface are stable, unlike cytoplasmic MTs which are highly dynamic (von Schubert et al., 2010). Over the last decade a number of host proteins, including kinases and MT-associated proteins (MAPs) involved in regulating MT dynamics, have been found to localize close to the schizont membrane where they likely promote the stabilization of MTs. Von Schubert and colleagues described the biphasic association of the host mitotic kinase polo-like kinase 1 (Plk1) with the schizont surface (von Schubert et al., 2010). Plk1 is a mitotic kinase, expressed at high levels during G2 phase and mitosis, and degraded at the end of mitosis. Plk1 has a large array of interaction partners and phosphorylation targets, and by phosphorylating different targets, it controls numerous elements of the cytokinesis process including contractile ring formation and cleavage furrow ingression (Petronczki et al., 2008). Plk1 binding to the schizont is mediated via its Polo-box domain, is most prominent during G2 phase and anaphase, and is conspicuously absent during prometaphase and metaphase when Cdk1 activity is highest. Most importantly, inhibition of Plk1 activity with potent anti-Plk1 inhibitors prevented the binding of the schizont with the stable MTs of the central spindle. When the schizont-central spindle interaction was prevented by Plk1 inhibition at the onset of anaphase (thus still allowing spindle formation and cleavage furrow ingression), and at the same time the association of astral MTs with the parasite was blocked by depolymerization of MTs with nocodazole, the parasite was no longer properly segregated during cell division, often resulting in parasites being sequestered on one side of the cleavage furrow (von Schubert et al., 2010). This clearly demonstrates that the proper interaction between the parasite and MTs is crucial for the persistence of the schizont in the cytoplasm of the host, and that this interaction is at least in part mediated by Plk1 activity.

One parasite-encoded molecule that is involved in the parasite-MT interaction is p104 (TA08425), an immunodominant molecule that is expressed at the surface of the *T. annulata* schizont (Woods K. L. et al., 2013). p104 possesses a predicted signal peptide and cleavage site as well as a predicted GPI anchor sequence, although the functionality of the GPI anchor sequence has not been confirmed. While the N-terminal portion of p104 is structured and contains repeated FAINT (frequently associated in *Theileria*) domains that are unique to *Theileria*, the C-terminal half of the protein is highly disordered and contains a conserved End Binding Protein 1 (EB1)-binding SxIP motif. EB1 is a host cell MT-plus end binding protein that localizes to the plus ends of growing MTs where, by interacting transiently and dynamically with hundreds of different proteins, it plays a key role in regulating the dynamics of MTs (Gouveia and Akhmanova, 2010). *T. annulata* taps into the MT network by hijacking host EB1, a key regulator of MT dynamics, and EB1 associates with the schizont surface by a SxIP-motif dependent interaction with p104. Thus it seems that MTs are directed to the parasite surface via the p104-EB1 interaction (Woods K. L. et al., 2013).

The presence of EB1-binding motifs at the schizont membrane are not alone sufficient to explain the capture and subsequent stabilization of MTs along the surface of the parasite, and in this context the host MT-stabilizing protein CLASP1 was found to coat the entire schizont throughout the cell cycle (**Figure 1**; Huber et al., 2017). Even following isolation of the schizont with a method that requires depolymerization of MTs, bovine CLASP1 remains firmly bound to the schizont surface. CLASP1 functions to stabilize MTs by facilitating MT rescue and limiting catastrophe by recruiting tubulin dimers (Al-Bassam et al., 2010; Leano and Slep, 2019). It was previously shown that isolated schizonts can facilitate MT binding and polymerization at their surface (Kuehni-Boghenbor et al., 2012), and it is likely that CLASP1 plays a role in this. CLASP1, a known EB1-binding protein, contains two SxIP motifs which presumably also contribute to the interaction of host EB1 with the parasite. The CLASP1 SxIP-motifs are not required for parasite localization, because the short C-terminal kinetochore-binding domain was found to be sufficient and necessary for the interaction with the schizont membrane (Huber et al., 2017). Co-immunoprecipitation and proximity ligation experiments confirmed that CLASP1 interacts with both p104 and EB1 at the schizont surface. Western blotting and mass spectrometry analysis revealed that p104 is heavily phosphorylated in a cell cycle dependent manner, with both endogenous and ectopically expressed p104 being hyper-phosphorylated during mitosis in

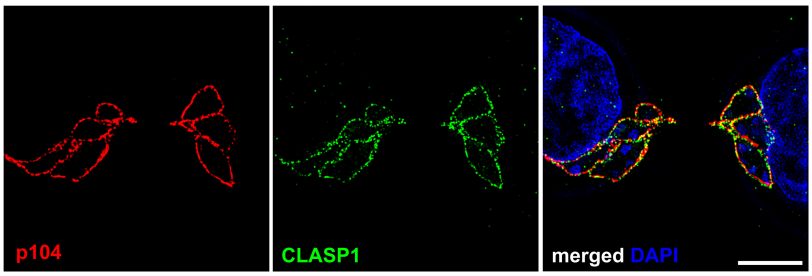

FIGURE 1 | Punctate localization of *Theileria annulata* p104 and bovine CLASP1 on the schizont membrane. Structured illumination microscopy (SIM) was performed on *T. annulata* infected macrophages (TaC12) with anti-p104 (red) and anti-CLASP1 (green) antibodies. *Z*-stack images were taken at 12.5 nm intervals, one *z*-stack is shown. Scale bar is 5 μm.

a manner at least in part regulated by host Cdk1 activity (Woods K. L. et al., 2013; Wiens et al., 2014). EB1 binding to the schizont, like that of Plk1, correlates inversely to host Cdk1 activity and the hyper-phoshorylation of *T. annulata* p104. Interestingly, host Cdkl1 has recently been found to partially co-localize with and phosphorylate the parasite surface molecule TaSP (*T. annulata* surface protein, TA17315), and it was postulated that phosphorylated TaSP might act as a docking protein for other cell cycle regulatory proteins (Mackiewicz et al., 2020).

CLASP2 is a close homolog of CLASP1 and both proteins share largely overlapping functions and localization in mammalian cells (Mimori-Kiyosue et al., 2006). CLASP2 is also enriched at the schizont surface, but in a MT-dependent manner with a localization resembling that of tubulin (Huber et al., 2017). Knockdown of CLASP1 expression in *Theileria*-infected cells had no effect on schizont position, morphology or distribution during host cytokinesis, presumably due to the continued presence of CLASP2. Over-expression of the CLASP1 MT-binding domain did however have a striking dominant negative effect on infected cells with large, round parasites residing in enlarged host cells containing bundled MTs. While this experiment emphasized the importance of a properly functioning host MT network on *Theileria* morphology and structure, the MT-binding domain of CLASP1 also has a dominant negative effect on non-infected cells (Maiato et al., 2003), making it difficult to separate the dependencies of host and parasite on a functional MT network.

End Binding Protein 1, Plk1 and CLASP1 are not the only host cell proteins to be sequestered at the schizont. The striking localization of CLASP1 at the schizont surface meant that the kinetochore binding fragment of CLASP1 served as an effective bait for proximity ligation pull-down experiments to identify further host-parasite interactions. These experiments led to the identification of a number of host adaptor proteins that coat the schizont surface including CD2 associated protein (CD2AP), c-Cbl-interacting protein of 85 kDa (CIN85) and Arf-GAP with SHD domain ANK repeat and PH domain-containing protein 1 (ASAP1) (Huber et al., 2018). Adaptor proteins are proteins that contain several protein-binding domains that facilitate the formation of large signaling complexes (Flynn, 2001). CD2AP and CIN85 interact with numerous binding partners and have been implicated in diverse biological processes including vesicle trafficking, cytokinesis and cytoskeleton dynamics (reviewed in Dikic, 2002). It is tempting to speculate that some of these adaptor proteins may contribute to the generation of signaling complexes at the parasite surface, and thus contribute to the parasite-driven remodeling of the host. CD2AP and CIN85 are also recruited by *Toxoplasma* to the moving junction during invasion, and invasion-deficient *T. gondii* mutants with depleted rhoptry proteins RON2, RON4, and RON5 fail to recruit CD2AP/CIN85 (Guérin et al., 2017). However, it is unlikely that CD2AP and CIN85 play any role in *Theileria* invasion because these proteins were not detected at the parasite surface until development into a schizont had started (Huber et al., 2018).

Host CD2AP, CIN85 and ASAP1 were found to form a complex at the parasite surface together with host CLASP1 and EB1, *T. annulata* p104 and a novel *T. annulata* encoded protein named *T. annulata* proline rich microtubule and SH-domain interaction protein (TaMISHIP, TA20980, Huber et al., 2018). TaMISHIP is a protein that contains a signal peptide and predicted cleavage site, is highly disordered, and is unique to transforming species of *Theileria*. TaMISHIP partially co-localizes with p104 in sporozoites, presumably in rhoptry organelles, and upon schizont development TaMISHIP is found in a punctate pattern in close proximity to p104 on the parasite membrane (Huber et al., 2018). A *T. annulata* GPI anchored protein named GPI anchored schizont protein 34 (gp34; TA06510) was also found to be present in the TaMISHIP-protein complex at the schizont surface (Huber et al., 2018). Gp34, like TaMISHIP, covers the schizont surface in punctate foci (Xue et al., 2010; Huber et al., 2018). Upon over-expression in infected or non-infected cells, both TaMISHIP and gp34 translocate to the nucleus and induce a small but significant increase in binucleation. Although it is unlikely that the nuclear localization of over-expressed TaMISHIP or gp34 reflect the localization of the endogenous proteins, these experiments, combined with the fact that both TaMISHIP and gp34 are part of a complex including MT-regulators CLASP1 and EB1, indicate that both proteins might contribute to host-parasite interactions during host cell division (Xue et al., 2010). TaMISHIP encompasses two EB1-binding SxIP motifs, and live cell imaging with uninfected cells over-expressing GFP-TaMISHIP revealed that TaMISHIP tracks the plus ends of

growing MTs (Huber et al., 2018). Mutation of the SxIP motif abolished plus end tracking, confirming that the interaction between TaMISHIP and EB1 is, like p104, mediated via the SxIP motif. TaMISHIP also contains three putative SH3-binding Px(P/A)xPR motifs which are predicted to mediate interactions with SH3 domains. Mutational studies combined with co-immunoprecipitation showed that the interaction between TaMISHIP and CD2AP, which contains three SH3 domains, is mediated via the SH3 domains of TaMISHIP (Huber et al., 2018).

While a number of host and parasite molecules that contribute to maintaining the tight association between the schizont and host MTs have been identified, many questions remain open. How the final stages of cytokinesis and abscission are regulated in infected cells is worthy of further investigation. *Theileria* synchronizes its cell cycle to that of its host by initiating DNA synthesis during host mitosis (Irvin et al., 1982; Wiens et al., 2014), but how these processes are controlled and coordinated – does host cell cycle progression drive parasite DNA synthesis, or vice versa? – remains unknown.

The host MT network is also rearranged and recruited to the *T. gondii* PVM. Differently to *Theileria*-infected cells, *T. gondii* infected cells rarely complete cytokinesis, and it has been proposed that the interactions between *T. gondii* and host MTs might suppress cell division and therefore provide a larger space in which the parasite can replicate (Walker et al., 2008). The rearrangement of host organelles in *T. gondii* infected cells is well documented. For example, the mitochondria and rough ER physically associate with the PV (Sinai et al., 1997), and host endolysosomes and Rab vesicles cluster round the PV and have been implicated in nutrient uptake (Coppens et al., 2006; Coppens and Romano, 2018). It seems likely that *T. gondii* rearranges host MT networks in order to facilitate the rearrangement of intracellular organelles to the benefit of the parasite. Although it has not been shown in *Theileria*-infected cells, a similar recruitment of host organelles such as the ER, golgi, mitochondria or endolysosomal vesicles is possible (Stagg et al., 1981).

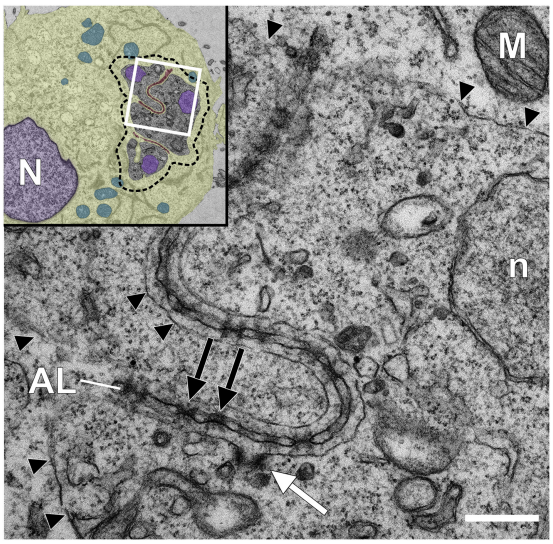

FIGURE 2 | Transmission electron microscopy analysis of a *Theileria annulata* infected macrophage reveals annulate lamellae pore complexes close to the schizont surface. Glutaraldehyde-fixed and Epon-embedded TaC12 cells were analyzed using TEM. Annulate lamellae (AL) and AL pore complexes (ALPC, black arrows) are indicated. The parasite plasma membrane (PPM) is indicated with black arrow heads. The white arrow indicates a putative cytostome. Scale bar is 400 nm. The white box in the inset indicates the magnified area. Inset: overview of the infected cell. Host (N) and parasite (n) nuclei have been colored purple, the host cytoplasm has been colored yellow, host mitochondria are blue, and host derived AL are highlighted brown. The outline of the schizont is indicated with a dashed line.

THE ELUSIVE ROLE OF POROUS ANNULATE LAMELLAE AT THE HOST-PARASITE INTERFACE

Recent transmission electron microscopy (TEM) combined with high resolution fluorescent imaging revealed that *Theileria* schizonts are surrounded by porous, cytomembranous structures called annulate lamellae (AL) during host interphase (**Figure 2**; Huber et al., 2020). AL are poorly characterized cytoplasmic organelles composed of stacked membrane cisternae which often occur in rapidly growing cells such as embryonic cells, oocytes and tumor cells (Kessel, 1992). AL contain pores that are both structurally and biochemically similar to nuclear pore complexes (NPCs). In *Theileria*-infected leukocytes, these host-derived porous membranes align close to, but not touching, the schizont membrane, and were often detected in between the lobes of the schizont (**Figure 2**; Huber et al., 2020). The AL disperse as the cell enters mitosis with the same dynamics as the host nuclear envelope, reforming and realigning close to the schizont membrane upon completion of cytokinesis. Almost all known structural components of NPCs associate with AL pore complexes (ALPCs) at the parasite plasma membrane (PPM), along with components of nuclear trafficking machinery. Host cell proteins that accumulate at AL close to the parasite membrane include the small GTPase Ran, RanGTPase activating protein (RanGAP1), Ran binding protein (RanBP2) and importin (Huber et al., 2020). NLS-domain containing proteins destined for nuclear import bind to karyopherins such as importin and are trafficked into the nucleus upon the formation of a Ran GTPase gradient between the nucleus and the cytoplasm (D'Angelo and Hetzer, 2008). Although experimental evidence is lacking, a model by which secreted nuclear localization signal (NLS)-containing *Theileria* proteins are "picked up" at the PPM by host importin and trafficked into the nucleus of the host is very attractive and would be interesting to investigate further.

Whether *Theileria* schizonts depend on host cell nutrients or metabolites remains unexplored, although genomic analyses have suggested that, like other Apicomplexa, *Theileria* may have reduced metabolic capacity that could indicate a reliance on host cell metabolite-scavenging (Hayashida et al., 2012). If this is the case, the potential mechanism by which nutrients could be imported over the PPM is not known. The schizont membrane has a lobular shape and possesses a rather large membrane surface that is exposed to the host cell. Membrane

contact sites between intracellular organelles have emerged in recent years as important hot spots for cellular signaling, lipid exchange and communication (Jain and Holthuis, 2017; Bohnert, 2020). While regions of direct contact between the schizont PPM and porous AL or other intracellular organelles have not been detected, cytostome-like structures are frequently observed embedded in the schizont membrane at points of close proximity to AL, raising the possibility of the direct transfer of lipids or metabolites between the parasite and host via these structures (**Figure 2**; Huber et al., 2020). Filamentous protrusions resembling nanotubes described from *Plasmodium* gametocytes (Rupp et al., 2011) can be readily detected at the *Theileria* schizont surface membrane. Driven by parasite actin polymerization, they extend toward the host cell nucleus or cell periphery (Kuehni-Boghenbor et al., 2012). It is possible that these protrusions could also function to interact with host cell components. The autophagy protein LC3 was detected within and surrounding *Theileria* schizonts in transformed cells, raising the possibility that host and/or *Theileria* autophagy might play a role in parasite survival, although the significance of this observation has not been explored (Latré De Laté et al., 2017). In *Plasmodium*-infected hepatocytes, functional host cell autophagy is required for *Plasmodium* growth and differentiation, perhaps as a source of nutrients for the developing parasite (Thieleke-Matos et al., 2016). On the other hand, recognition of the PVM by autophagic machinery leads to the elimination of the parasite. Live imaging revealed that in the case of successful infection, host autophagy proteins are progressively shed from the *Plasmodium* PVM and accumulate in the tubovesicular network of the PVM (TVN), thus preventing autophagic destruction of the developing parasite (Prado et al., 2015; Agop-Nersesian et al., 2017). How, or if, *Theileria* parasites interact with the host autophagy pathway remains to be seen.

HOW ARE *THEILERIA* PROTEINS EXPORTED ACROSS THE PARASITE PLASMA MEMBRANE?

Although several *Theileria* proteins are known to localize to the host cell cytosol or nucleus in infected cells, the mechanism by which *Theileria* exports proteins into its host cell are not known. Lacking a PV, it is not surprising that *T. annulata* and *T. parva* do not share many orthologs with *T. gondii* and *Plasmodium* spp. protein complexes situated in the PVM and that are involved in the export of effector proteins from the tachyzoite (MYR1 complex; Franco et al., 2016; Marino et al., 2018; Cygan et al., 2020) and red blood cell stage (PTEX complex; de Koning-Ward et al., 2009). The only *Theileria* protein with a homologous sequence to known translocon proteins of other parasites is the putative chaperon protein ClbP (TA07095) which shares sequence identities to HSP101, a member of the PTEX translocon of *Plasmodium* (Beck et al., 2014). TaClbP contains a signal peptide but no transmembrane domain and its localization has not been determined. As HSP101 is expressed in *Plasmodium* liver stage forms (Matthews et al., 2013), its involvement in protein export into the hepatocyte appears likely, although it has not yet been proven. In *Plasmodium* trophozoites, areas of close contact between the PPM and the PVM can be observed, and domains of protein export can be observed on the PVM in these regions (Garten et al., 2020). It is not known whether the PPM of the *Theileria* schizont is similarly structured in patches with distinct functional regions, but the visualization of evenly distributed "knobs" on the PPM by high resolution scanning electron microscopy (SEM) could indicate the accumulation of specialized protein clusters on the parasite surface (Kuehni-Boghenbor et al., 2012). Intriguingly, *Theileria* proteins can form rather large complexes in punctate foci on the membrane surface with multiple host and parasite proteins involved (**Figure 1**; Xue et al., 2010; Huber et al., 2017, 2018). The most well-studied protein complex present at the schizont surface is the CLASP1/CD2AP/EB1-complex which interacts with the MT system of the host and involves a total of at least seven host and four parasite proteins, including p104 (**Figure 3**; Huber et al., 2018). The EB1 binding *T. annulata* protein p104 is equipped with a predicted c-terminal GPI-anchor signal sequence and resides on the entire parasite membrane and interacts (directly or indirectly) with TaMISHIP and TA03615, neither of which contain a transmembrane domain but are both found to interact with host proteins on the surface. Several other parasite proteins such as gp34, TaSP and TaSE (TA20205) have been described to also reside on or within the PPM. It is tempting to speculate that parasite proteins incorporated in larger protein complexes are involved in protein, nutrient or small molecule trafficking between the parasite and the host cell cytoplasm. Given the absence of a transport machinery orthologous to those defined in *Plasmodium* or *Toxoplasma*, we consider two attractive possibilities for the export of effector proteins. First, it is possible that no export machinery exists but proteins are exported either passively or via exosome vesicles outside of the schizont confinement into the bovine cell. A role for schizont-derived vesicles being exported into the host cell compartment has not yet been explored. A second possibility is that a *Theileria*-specific translocon apparatus exists, although it has not been identified.

Another question that arises is whether families of effector proteins are actively stored in distinct secretory organelles or if they are continuously synthesized directly before export. In tachyzoites of *T. gondii*, micronemes, rhoptries and dense granules comprise different families of effector proteins. The distinct proteome of these organelles has recently been identified (Barylyuk et al., 2020), though only a limited fraction has been fully characterized. Many *Toxoplasma* and *Plasmodium* proteins targeted for export contain a Texel or HT/Pexel recognition motif, respectively, and these are cleaved by an aspartyl protease (ASP) prior to export (Hiller et al., 2004; Marti et al., 2004; Coffey et al., 2015). A PEXEL-like motif (PLM) has also been identified in *Babesia*, and experiments with recombinant reporters consisting of N-terminal portions of *Babesia* secreted proteins and a fluorescent tag revealed the accumulation of proteins destined for secretion in large vesicular organelles termed "spherical bodies" prior to export into the erythrocyte (Pellé et al., 2015). Although a Pexel or Texel-like motif has not been identified in the *Theileria* genome, it is possible that *Theileria* proteins are processed and cleaved by an ASP prior

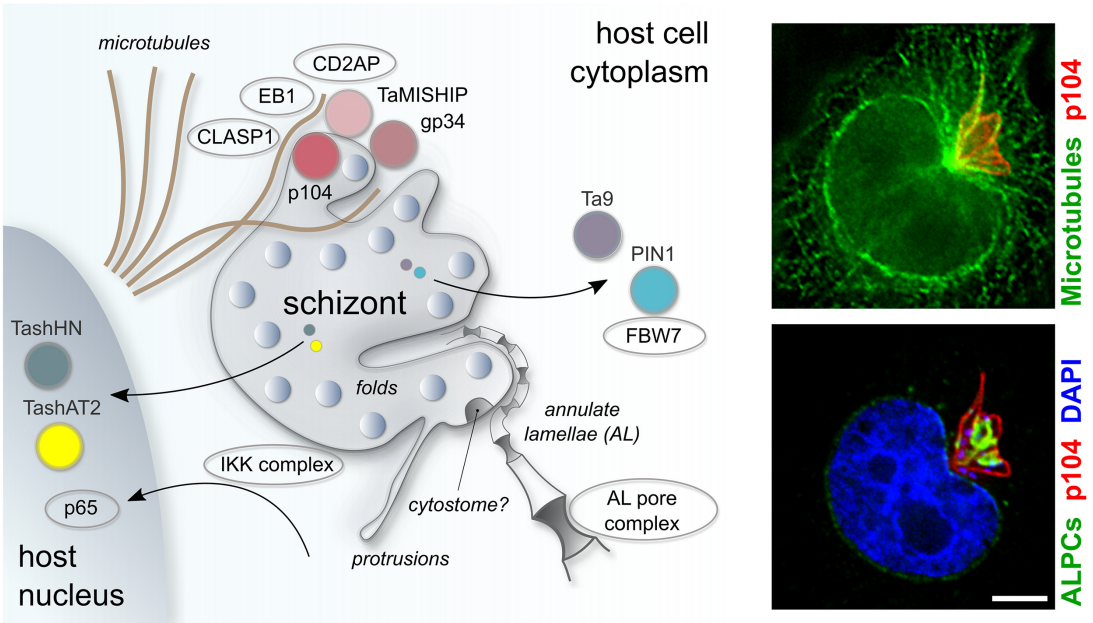

FIGURE 3 | Schematic representation of key host-parasite interactions in *Theileria annulata* infected leukocytes. *Theileria* schizonts export proteins into the host nucleus (TashAT, TashHN) or cytoplasm (PIN1, Ta9), where they have the potential to interact with host proteins (FBW7) to modify the host phenotype. *Theileria* interacts closely with host MTs, mediated in part by a protein complex on the schizont surface comprising bovine CLASP1, CD2AP, EB1 and the schizont proteins p104 and MISHIP. The IKK complex is recruited to the parasite surface, enabling the nuclear localization of NF-kB subunits (p65) to the host nucleus. Porous annulate lamellae that align closely to the schizont membrane are depicted, as are structural elements of the parasite plasma membrane such as dynamic protrusions and a potential cytostome. Fluorescent images show host MTs, labeled with anti-CLASP2 antibodies (green, top panel) and host ALPCs, labeled with anti-RanGAP1 (green, bottom panel) in relation to the schizont membrane, which is labeled with anti-p104 (red). The scale bar is 5 μm.

to storage in distinct organelles and export. *T. annulata* shares an orthologous ASP (TA17685; in *T. parva* TP03_0676) with plasmepsin V (PMV) of *Plasmodium* spp. (Dogga et al., 2017), and it will be interesting to identify the protein targets of this peptidase. *Theileria* sporozoites only harbor rhoptries and microspheres. Though present in some tick stages of *Theileria* (Mehlhorn et al., 1975), apical micronemes are absent from invasive zoites, consistent with the lack of an actin-myosin-based motor complex for active invasion and gliding motility. So far the only described rhoptry protein is p104, first identified as a strongly antigenic protein of 104 kDa in *T. parva,* and detected by immunogold labeling in the rhoptries of sporozoites (Iams et al., 1990b). While *T. annulata* p104 is localized on the PPM of schizonts, immunofluorescence analysis suggests that p104 is, as in *T. parva*, localized in the rhoptries of sporozoites (Huber et al., 2018). Immunogold labeling of *T. parva* sporozoites identified p150 and PIM (named TaSP in *T. annulata*) to be stored in microspheres, which are secreted after invasion (Skilton et al., 1998; Toye et al., 2014). In schizonts, TaSP is expressed on the PPM surface and seems to contribute to the interaction of the schizont with MTs (Seitzer et al., 2010). Intracellular organelles, and in particular storage or secretory vesicles, have not been well characterized in *Theileria* schizonts, although a recent analysis of two HSP90 variants allowed the visualization of the endoplasmic reticulum (TA06470) and the apicoplast (TA10720) of the schizont for the first time (Kinnaird et al., 2017). TEM studies do indicate the presence of electron dense structures within the schizont that resemble rhoptries or dense granules (**Figure 2**; Shaw and Tilney, 1995). Whether exported protein storage units exist in *Theileria* schizonts, what their contents are, and to what extent the secretion of *Theileria* effector proteins drives cellular transformation, remains to be unraveled.

THE SCHIZONT SURFACE IS A SIGNAL TRANSDUCTION PLATFORM

One key signaling pathway that *Theileria* subverts is the NF-κB pathway, and this is achieved by physically recruiting signaling molecules to the schizont surface (Heussler et al., 2002). In *Theileria*-transformed cells, NF-κB is constitutively activated, conferring resistance to apoptosis upon the infected cell. In non-infected cells, NF-κB is retained in the cytoplasm by binding to its cellular inhibitor IκB (inhibitor of κB). The turnover of IkB is regulated by the activity of the IκB kinase complex, IKK (Ghosh and Karin, 2002). In *Theileria* infected cells, IKK complexes, termed signalosomes, are recruited to the schizont surface where they are continually phosphorylated and activated. This leads to the phosphorylation and subsequent degradation of IκB, thus freeing NF-κB subunits for translocation into the nucleus (Heussler et al., 2002). Exactly how IKK is recruited to the parasite surface remains unclear. There is some evidence that the architecture of the actin cytoskeleton in infected cells plays a role in NF-κB activation, and Schmuckli-Maurer and

colleagues noticed that in some *T. annulata* infected clones, high NF-κB activity tended to correlate with a looser actin cytoskeleton (Schmuckli-Maurer et al., 2010). In line with this, disruption of the actin cytoskeleton with cytochalasin D or jasplakinolide led to an increase in NF-κB activity. A *T. parva* schizont surface protein termed TpSCOP, absent in the non-transforming *T. orientalis*, was postulated to contribute to NF-κB activation in infected T-cells (Hayashida et al., 2010). TpSCOP binds to host cell F actin via its N-terminal region and partially colocalizes with actin filaments at the schizont surface. Interestingly, over-expression of TpSCOP in mouse T-cells conferred a marked resistance to Fas-mediated apoptosis and led to an increase in phosphorylation of IκB as well as upregulation of NF-κB activity and elevated expression of the anti-apoptotic protein A1/Bfl-1. To date, TpSCOP is the only *Theileria*-encoded protein that has been linked to *Theileria*-dependent transformation via stimulation of NF-κB activity. Infection with *T. gondii* type II strains also leads to an increase in host NF-κB signaling, and recent work showed that activation of the NF-κB pathway is achieved via the interaction between the effector protein GRA15, which is secreted into the PV and localized to the PVM, and TNF receptor-associated factors (TRAFs), adaptor proteins that function upstream of the NF-(κB transcription factor (Sangaré et al., 2019). Simultaneously, *T. gondii* antagonizes the NF-κB signaling pathway by the secreted dense granule protein HCE1/TEEGR (Braun et al., 2019).

Constitutive c-Jun N-terminal kinase (JNK) activity, phosphorylation of c-Jun and activation of the transcription factors ATF-2 and AP-1 has been reported for *T. annulata* and *T. parva* infected lymphocytes, and inhibition of JNK activity or overexpression of JNK mutants result in apoptosis of the infected cell (Baylis et al., 1995; Chaussepied et al., 1998; Adamson et al., 2000; Lizundia et al., 2006). The two major isoforms of JNK, JNK1 and JNK2, exert opposing effects on cell survival. While JNK1 activity increases the stability of c-Jun, JNK2 activity promotes the degradation of c-Jun. Loss of *jnk2* in mouse cells leads to an increase in cell proliferation, while loss of *jnk1* leads to decreased proliferation (Bode and Dong, 2007; Latré De Laté et al., 2019). In *T. annulata* infected macrophages, bovine JNK1 was detected in both the nucleus and cytoplasm of the host, while JNK2 is localized mainly in the cytoplasm and accumulates at the membrane of the schizont (Latré De Laté et al., 2019). *T. annulata* p104 contains three putative JNK-binding motifs and co-precipitates with JNK following immunoprecipitation with anti-pan JNK antibodies. The interaction between p104 and bovine JNK was ablated following treatment with cell-penetrating peptides containing the wild type p104 JNK-binding motif. Importantly, an S > A mutant peptide failed to block the interaction between p104 and JNK, suggesting that the interaction between p104 and JNK2 is promoted by phosphorylation of the S806 and/or S808 residues. Blocking the p104-JNK2 interaction with a penetrating peptide was accompanied by a reduction in JNK1 expression in the nucleus and a reduction in nuclear c-Jun phosphorylation. AP-1 is a transcription factor composed of proteins belonging to the c-Fos, c-Jun, ATF and JDP families. In *Theileria*-infected macrophages, constitutive AP-1 activity drives the transcription of *mmp9*, leading to an increase in matrix metallopeptidase 9 (MMP9) activity. This in turn promotes invasion and dissemination, as measured by matrigel traversal (Adamson et al., 2000; Cock-Rada et al., 2012; Echebli et al., 2014). Following disruption of the p104-JNK2 interaction with the wild type JNK-binding motif peptide, MMP9 activity, as revealed by gelatin gel assay, was reduced and matrigel traversal was significantly decreased. Treatment with the (S > A) mutant peptide as well as irrelevant peptides served as an important control and exerted no effect on matrigel traversal. These data point to a role for p104 in sequestering JNK2 at the parasite surface which might contribute to promoting the survival of the transformed cell by stabilizing c-Jun in the nucleus (Latré De Laté et al., 2019). Secretion of a parasite prolyl isomerase, TaPIN1 (TA18945), has also been implicated in stabilizing c-Jun levels and thus promoting *Theileria*-induced transformation (Marsolier et al., 2015), and will be discussed in the next section.

P53 is a nuclear transcription factor that functions to drive apoptosis in response to stress or DNA damage, thus protecting cells from accumulating genomic aberrations that can lead to cancer. In many tumors, the tumor suppressor protein p53 is mutated, leading to a loss of function (Ozaki and Nakagawara, 2011). In *Theileria*-transformed cells p53 activity has also been reported to be low, and two different mechanisms by which the parasite achieves this have been proposed (Haller et al., 2010). Haller and colleagues detected p53 decorating the surface of the schizont in *T. annulata* infected macrophages. They proposed that the sequestration of p53 at the schizont surface prevents its translocation to the nucleus, and in line with this observation, p53-transcribed apoptotic genes were found to be regulated in a parasite-dependent manner. The expression of the anti-apoptotic protein Bcl-2 increases upon buparvaquone treatment, while the expression of pro-apoptotic proteins Apaf-1 and Bax decreases upon killing of the parasite with buparvaquone treatment (Haller et al., 2010). In another study, no schizont surface localization of p53 could be detected in *T. parva* infected T cells (Hayashida et al., 2013). Instead, those authors found that MDM2, a well-characterized oncoprotein, is expressed at high levels in *T. parva* infected lymphocytes. MDM2 protein is a negative regulator of p53 activity that controls p53 expression at both the transcriptional and translational level as well as by regulating proteasomal degradation of p53. *T. parva* infected lymphocytes are sensitive to MDM2 inhibitors such as TIBC and nutlin-3, whereas non-infected ConA-stimulated lymphocytes were insensitive. In *T. parva* infected lymphocytes, protein levels of p53 are low and do not increase in response to DNA damaging agents such as cisplatin, even as transcript levels of p53 increase. Treatment of infected cells with the MDM2 inhibitor TIBC led to nuclear accumulation of p53, and restoration of p53-targeted pro-apoptotic molecules such as Bax. Together these data led to the conclusion that highly expressed MDM2 in *T. parva* infected cells contributes to suppression of p53 protein expression by targeting p53 for proteasomal degradation. Variable expression levels of MDM2 observed in different *Theileria*-infected clones (Hayashida et al., 2013) might lead to varied p53 expression and could explain the difficulty in detecting p53 at the schizont surface in some *T. parva* and *T. annulata* infected cell lines

(Hayashida et al., 2013). While it is clear that several changes observed in Theileria-infected cells, including alterations to p53 and NF-κB pathways, resemble those seen with cellular transformation in cancer, it has not known how soon following transformation these alterations occur. A transcriptomic or proteomic analysis of freshly infected and transformed cell lines will help to unravel whether these alterations drive *Theileria*-dependent transformation, or are in fact acquired subsequently to parasite-induced transformation, as occurs with secondary mutations in many cancers (Brown et al., 2019).

SECRETION OF *THEILERIA*-ENCODED EFFECTOR PROTEINS INTO HOST CELL COMPARTMENTS

Several *Theileria* effector proteins, defined as proteins of transformative *Theileria* species which are translocated into the host cell or integrated into the schizont membrane and interact with host proteins, have been described in recent years (reviewed in Tajeri and Langsley, 2021). Some proteins have been shown to translocate to the host cell nucleus, and key examples are three Tash family proteins TashAT2 (TA20095), TashHN (TA20090) and SuAT1 (TA03135) (Swan et al., 1999; Swan et al., 2001; Swan et al., 2003; Shiels et al., 2004). Others reside in the host cell cytoplasm, as it is the case for Ta9 (TA15705) and TaPIN1 (TA18945), both of which have been implicated in the proliferative phenotype of the host cell (Marsolier et al., 2015, 2019; Unlu et al., 2018). *T. annulata* and *T. parva* genomes contain multiple expanded protein families, which are absent in non-transformative species such as *T. orientalis*. The Tash and Ta9 protein families are both examples of such gene families. The Tash family encompasses 17 genes in *T. annulata* and 20 genes in *T. parva* (Pain et al., 2005). All Tash family proteins contain an N-terminal signal sequence and some contain an AT hook DNA-binding domain (Swan et al., 1999; Swan et al., 2001). Eight direct orthologous gene pairs can be found located at either end of the gene cluster of the Tash genes between *T. annulata* and *T. parva*. However, in the middle of the cluster a significant species-specific diversification is visible (Pain et al., 2005; Shiels et al., 2006; Weir et al., 2009). Following overexpression of *TashAT2* in non-infected bovine cells (BoMac), changes in morphology became apparent although no difference in proliferation was observed. In *TashAT2*-transfected BoMac cells transcripts for the mitochondrial genes cytochrome oxidase and NADH dehydrogenase were upregulated compared to cells expressing a control plasmid, while expression of the ubiquitin-like protease UBP43 and its ubiquitin-like substrate, bISG15, were significantly reduced in TashAT2-expressing cells (Oura et al., 2006). The ISGylation system, comprising of UBP43 and ISG15 together with UBE1L and Ubc8, plays a role in cellular defense against viral and bacterial infection (Ritchie et al., 2004; Kim et al., 2005), and is strongly induced by type 1 interferons (IFNs) and lipopolysaccharide (LPS). Although UBP43 and ISG15 are expressed more strongly in *Theileria*-infected cells compared to their uninfected counterparts, infected cells were found to be refractory to LPS- and IFN-α stimulated induction of very high levels of ISG15 and UBP43. This led to a tentative suggestion that the parasite dependent repression of elevated ISG15 levels might allow *Theileria* to escape from a protective immune response (Oura et al., 2006). Overexpression of SuAT1 in BoMac cells induced a change in gene expression for cytoskeletal polypeptides and caused a similar change in morphology to that seen upon overexpression of TashAT2, with cells becoming larger and more spread out (Shiels et al., 2004).

The Ta9 family protein cluster is located on chromosome 2 with five members in *T. annulata* and six members in *T. parva*. In the non-transforming *Theileria* species *T. orientalis,* only one protein with weak homology to Ta9 has been identified in the syntenic region on chromosome 2 (Hayashida et al., 2012). Ta9 has been shown to localize to the host cell cytoplasm by immunofluorescence analysis in *T. annulata* (Unlu et al., 2018) and is one of the main CD8 (T cell antigens in both T. annulata and T. parva infection (MacHugh et al., 2011). Expression of Ta9 increases during schizont development and is reduced again at the onset of merogony (Hayashida et al., 2012), suggesting a role in the manipulation of the host cell during the schizont stage. Luciferase reporter assays in Ta9-expressing HEK293T cells indicate that Ta9 expression might activate AP-1 driven transcription (Unlu et al., 2018). However, this has not been shown in bovine cell lines or parasitized cells and, although the relevant polypeptide sequence has been narrowed down to the C-terminal region of the Ta9 protein, the mechanism by which AP-1 activation occurs is still unknown. A second protein which has been detected in the host cell cytoplasm is the *T. annulata* peptidyl-prolyl isomerase 1 (TaPin1). TaPin1 was identified by a comparative genomics approach and subsequent characterization showed that it interacts with the host ubiquitin ligase FBW7 and might influence the host c-Jun signaling pathway (Marsolier et al., 2015). C-Jun signaling is involved in oncogenic transformation and has been previously shown to be modulated by *Theileria* schizonts (Lizundia et al., 2006). Further analysis of TaPin1 revealed that the *T. annulata* protein also interacts with the host pyruvate kinase isoform M2 (PKM2), and elevated levels of PKM2 were shown in parasitized lymphocytes compared to non-infected cells (Marsolier et al., 2019). As a cofactor of the transcription factor hypoxia-inducible factor 1 alpha (HIF1α), PKM2 plays an important role in the transcriptional control of glycolytic enzymes in cancer cells (Wang et al., 2014). These enzymes are responsible for the Warburg effect, a metabolic alteration toward aerobic glycolysis which is also observed in *Theileria*-infected lymphocytes (Medjkane and Weitzman, 2013; Medjkane et al., 2014; Metheni et al., 2015). Interestingly, transcriptional activity of HIF1α and expression of HIF1α target genes is reduced in parasitized cells upon treatment with the theilericidal drug buparvaquone. This loss of activity was ascribed to the prolyl isomerase activity of TaPin1 and can be partially rescued by expressing exogenous TaPin1 in the parasitized cells. It is likely that only a small fraction of the potential arsenal of *Theileria*-encoded secreted effector proteins have been identified to date. Whether *Theileria* encodes a single "master regulator" protein that drives host transformation, or whether multiple secreted schizont-encoded proteins work together to modulate the host phenotype, remains to be seen.

CONCLUDING REMARKS

Theileria schizonts have the unique ability to induce the transformation of their host cell, leading to a significant remodeling of the host phenotype and the clonal expansion of parasitized leukocytes. A striking aspect of *Theileria* biology is the close interaction the schizont maintains with host MTs during its intracellular life. The mimicking of particular protein binding motifs, such as the EB1-binding SxIP motif in the schizont surface protein p104, demonstrates the elegant mechanism used by the parasite to ensure its distribution to daughter cells during host mitosis and cytokinesis. A remarkable degree of co-dependency exists between the schizont and the transformed leukocyte. Gene expression studies have revealed that the parasite irreversibly rewires gene expression patterns in the host (Kinnaird et al., 2013), and killing the parasite with the potent and specific anti-*theilerial* drug buparvaquone leads to a halt in proliferation and the onset of apoptotic cell death. The close relationship between the intracellular schizont and the leukocyte brings with it a number of challenges and raises some intriguing questions on how *Theileria* induces such fundamental changes.

First, how does the schizont drive cellular transformation, and what effector proteins are involved? To date, only few *Theileria*-encoded secreted proteins that can interact with the host have been characterized, key examples being Tash family proteins (Swan et al., 1999, 2001, 2003; Shiels et al., 2004), a parasite-encoded prolyl-isomerase Pin1 (Marsolier et al., 2015, 2019), and Ta9, a secreted protein that is unique to transforming *Theileria* species (Unlu et al., 2018). Unfortunately, with the lack of an efficient method to genetically manipulate *Theileria*, functional studies into the mode of action of potential *Theileria* effectors are limited. A great hope for the future is that with the availability of efficient CRISPR-Cas9 gene editing tools, a robust transfection method will be established and will open the field for mechanistic investigations into *Theileria*-host interactions. *Theileria* modulates the host phenotype not only by secreting proteins to the host cytoplasm or nucleus, but also by recruiting host signaling molecules to its surface. Some interesting examples are the recruitment and activation of IKK signalosomes at the PPM, leading to anti-apoptotic NF-kB signaling (Heussler et al., 2002), or the p104-mediated binding of JNK2 to the parasite surface that might contribute to c-Jun activation (Latré De Laté et al., 2019). However, the details of the mechanisms by which JNK2 and IKK are sequestered at the PPM have not been fully uncovered.

Further, how parasite effector proteins are stored within the schizont and exported into the host remains unknown. Does *Theileria* possess a translocon-like complex on its membrane that facilitates protein export? In this context the presence of knob-like structures on the schizont PPM, as well as the identification of the CLASP1-CD2AP-EB1-p104-TaMISHIP protein complex in punctate foci on the membrane is interesting, although further investigation into protein complexes at the PPM is required. Another question that remains unexplored is if and how *Theileria* schizonts scavenge nutrients from the host. In particular, the frequent observation of a cytostome-like structure in close proximity to AL hints at the mechanism for uptake of lipids and other molecules. Little is known about the potential recruitment of host organelles to the schizont surface, although the close alignment of porous AL and associated nuclear transport machinery with the schizont membrane is intriguing. Ongoing studies investigating the host-pathogen interactions in *Theileria*-transformed leukocytes will hopefully yield fascinating insights into the survival strategies of this remarkable pathogen.

AUTHOR CONTRIBUTIONS

KW and PO developed the ideas for the manuscript. KW, CP, FB, and PO all contributed to writing and editing. All authors contributed to the article and approved the submitted version.

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Staging Encystation Progression in *Giardia lamblia* Using Encystation-Specific Vesicle Morphology and Associating Molecular Markers

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Differentiation into environmentally resistant cysts is required for transmission of the ubiquitous intestinal parasite *Giardia lamblia*. Encystation in *Giardia* requires the production, processing and transport of Cyst Wall Proteins (CWPs) in developmentally induced, Golgi-like, Encystation Specific Vesicles (ESVs). Progress through this trafficking pathway can be followed by tracking CWP localization over time. However, there is no recognized system to distinguish the advancing stages of this process which can complete at variable rates depending on how encystation is induced. Here, we propose a staging system for encysting *Giardia* based on the morphology of CWP1-stained ESVs. We demonstrate the molecular distinctiveness of maturing ESVs at these stages by following *Gl*Rab GTPases through encystation. Previously, we established that *Giardia*'s sole Rho family GTPase, *Gl*Rac, associates with ESVs and has a role in regulating their maturation and the secretion of their cargo. As a proof of principle, we delineate the relationship between *Gl*Rac and ESV stages. Through proteomic studies, we identify putative interactors of *Gl*Rac that could be used as additional ESV stage markers. This staging system provides a common descriptor of ESV maturation regardless of the source of encysting cells. Furthermore, the identified set of molecular markers for ESV stages will be a powerful tool for characterizing trafficking mutants that impair ESV maturation and morphology.

Keywords: *Giardia*, encystation, Rho GTPase, Rac, ESV, membrane trafficking

INTRODUCTION

Giardia lamblia (syn. *Giardia intestinalis* and *Giardia duodenalis*) is a major intestinal parasite which infects more than 280 million people every year (Lane and Lloyd, 2002). The lifecycle of this diplomonad protozoan is simple, featuring only two stages – the binucleate, double-diploid, proliferative trophozoites which non-invasively colonize host intestines and the environmentally resistant, infectious, non-motile cysts that are shed in host's feces. Regulation of encystation ensures the production of viable cysts and promotes transmission of this ubiquitous parasite. Being a popular life-cycle strategy also adopted by other protozoan parasites, studying this differentiation process is important and *Giardia* is the best-developed model available (Eichinger, 2001).

Giardia encystation requires the construction of its protective cyst wall, an extracellular matrix composed of Cyst Wall Material (CWM). CWM contains three paralogous Cyst Wall Proteins (CWP1-3) and a unique β-1,3-linked N-acetylgalactosamine (GalNAc) homopolymer (Lujan et al., 1995; Gerwig et al., 2002; Sun et al., 2003). When induced, large quantities of CWPs are synthesized and transported from the endoplasmic reticulum (ER) to the cell surface in membrane-bound organelles called Encystation-Specific Vesicles (ESVs). *Giardia* lacks classical Golgi apparatus. However, since nascent ESVs arise from ER-exit sites (ERES; Faso et al., 2013) and are marked by several Golgi markers they are thought to be developmentally induced Golgi (Marti et al., 2003). This view is supported by the roles ESVs play as the only recognizable post-ER delay compartments. ESVs feature machinery needed for the post-translational processing and subsequent partitioning of CWPs into distinct phases (Reiner et al., 2001; Slavin et al., 2002; Davids et al., 2004). After proteolytic cleavage of CWP2, CWP1 and the N-terminal end of cleaved CWP2 are sorted into the outer fluid phase while CWP3 and C-terminal end of cleaved CWP2 remain as the inner condensed core (Touz et al., 2002; DuBois et al., 2008; Konrad et al., 2010). Additionally, ESVs coordinate secretion of CWM to the cell surface, likely mediated by a higher order networking structure (Štefanić et al., 2009). Processed CWPs are deposited sequentially; the fluid phase first at a rapid rate where binding of CWP1 to the cell surface is mediated by its lectin binding domain that recognizes GalNac fibrils on the surface of the encysting cell (Chatterjee et al., 2010). This is followed by slower secretion of the condensed phase (Štefanić et al., 2009; Konrad et al., 2010). These events are trackable by following ESV morphology and CWP localization.

As a lab-inducible and -tractable secretory pathway of a minimalistic organism, *Giardia*'s encystation process provides a unique opportunity to uncover the constraining principles of membrane trafficking. Despite fundamental differences in compartment organization, canonical membrane trafficking players continue to perform conserved roles in *Giardia* (Touz and Zamponi, 2017). The accumulation of CWP and *de novo* ESV biogenesis at the ERES is dependent on COPII and the small GTPase, SarI – vesicle coat proteins that transport cargo from the rough ER to the Golgi apparatus in higher eukaryotes (Štefanić et al., 2009). Additionally, Arf1, a small GTPase that canonically plays a central role in intra-Golgi transport by regulating COPI and clathrin membrane coats, is required for ESV maturation; inhibiting Arf1 activity interfered with the transport and secretion of CWM to the cell surface (Štefanić et al., 2009). The sole Rho GTPase in *Giardia*, *Gl*Rac, whose homologs are known to coordinate vesicle trafficking and the cytoskeleton in plants and animals, was found to regulate CWP1 trafficking and secretion (Krtková et al., 2016).

Similar to its homologs, *Gl*Rac is thought to regulate *Giardia* encystation by acting as a molecular switch for the recruitment and regulation of effector proteins which drive the progress of encystation. We previously demonstrated that *Gl*Rac is required for the temporal coordination of CWP1 production, ESV maturation, and CWP1 secretion (Krtková et al., 2016). The relationship between *Gl*Rac and its roles in CWP trafficking are complicated by the dynamic association of *Gl*Rac with ESVs (Krtková et al., 2016). Our efforts to specify the molecular events that coincide with *Gl*Rac activity at ESVs, highlighted the need for a standardized system to distinguish the advancing stages of encystation. While it is recognized that ESVs go through stages of maturation (Konrad et al., 2010), there is no established criteria for specifically identifying these stages. Previous studies used timing post induction of encystation as a convenient means to stage ESVs (Hehl et al., 2000; Faso et al., 2013; Merino et al., 2014; Vranych et al., 2014; Frontera et al., 2018). However, the amount of time it takes to encyst varies by the method used to promote encystation and the process is only semi-synchronous due to a requirement for cells to be in G2 before proceeding into the encystation pathway (Luján et al., 1996; Reiner et al., 2008; Konrad et al., 2010; Einarsson et al., 2016). The variability between encystation experiments limits the meaningfulness of noting hours post induction of encystation (h p.i.e.) as the criteria for staging encysting cells. To illustrate the differences between the most common methods for inducing encystation, we monitored CWP1 and CWP2 induction using luciferase reporters and observed variation in how each method induces CWP levels (**Supplementary Figures 1A,B**). Additionally, we used the ESV staging system described below to compare the distribution of stages observed for encystation method at 8 and 24 h p.i.e. which identifies marked differences in encystation dynamics (**Supplementary Figures 1C–E**). Here, we develop a system for staging encysting *Giardia* cells that is based on our current understanding of molecular events during encystation that is observed by changes in ESV morphology (Konrad et al., 2010). Furthermore, this would allow us to navigate around ambiguities introduced by variation in the timing and efficiency of encystation when different protocols for induction are used. Our survey of *Giardia* Rabs, small GTPases that are known to control the specificity and directionality of membrane trafficking pathways as well as mark specific organelles, highlight the molecular distinctiveness between the stages we propose. As a proof of principle, we characterized putative interactors of *Gl*Rac as identified by proteomics and confirm their association with ESVs. This work will facilitate future studies where the functions of *Gl*Rac effectors can be precisely mapped in this crucial trafficking pathway.

MATERIALS AND METHODS

Strain and Culture Conditions

Giardia lamblia strain WB clone C6 (ATCC 50803; American Type Culture Collection) was cultured in TYDK medium (per 100 ml; 2 g casein digest, 1 g yeast extract, 1 g glucose, 0.2 g NaCl, 0.06 g KH_2PO_4, 0.095 g K_2HPO_4, 0.2 g L-cysteine, 0.02 g L-ascorbic acid, 1.2 mg ferric ammonium citrate) supplemented with 10% adult bovine serum with pH adjusted to 7.1. To induce encystation in trophozoites, the two-step encystation protocol was followed. Trophozoite cultures were first grown to confluency ($\sim 1 \times 10^6$ cells/ml) for 36 h in pre-encystation medium (same as growth medium above but without bile and pH at 6.8). Encystation was induced by switching culture medium to

encystation medium (same as growth medium but pH 7.8 and supplemented with 10 g/l bovine/ovine bile instead of bovine bile) and incubating the cells further for either 8 or 24 h as indicated.

Vector Construction

All constructs were made using traditional cloning, Gibson assembly (Gibson et al., 2009) or In-Fusion kit (Takara Bio); for full details and Gene Accession numbers, consult **Supplementary Table 1**. Note, constructs made to tag *Giardia* Rabs and putative *Gl*Rac-interactors were designed for stable integration by homologous recombination into the *Giardia* genome for endogenous expression. Generation of the native promoter morpholino-sensitive (ms) HALO_C18_*Gl*Rac_PuroR (designed for endogenous expression) and PAK promoter_CRIB_N11_mNG_3HA_NeoR (designed for episomal expression) constructs have been described previously (Hardin et al., 2021). Plasmid backbones used to build constructs in this paper were sourced from Gourguechon and Cande (2011); Krtková et al. (2016), Michaels et al. (2020), and Paredez et al. (2014).

For transfection, 5 to 50 μg of DNA was electroporated (375 V, 1,000 μF, 750 Ω; Gene Pulser Xcell; Bio-Rad, Hercules, CA, United States) into trophozoites. Following electroporation, the cells were added to 13 ml pre-warmed, fresh TYDK medium and allowed to recover at 37°C overnight before beginning selection with G418 or Puromycin for 4–7 days. Strains were maintained at a final concentration of 30 μg/ml G418 or 0.3 μg/ml Puromycin.

Luciferase Assay

Confluent cell cultures were incubated with the three different encystation media (two-step, Uppsala, and lipoprotein-deficient) for 8 and 20 h, then pelleted and resuspended with HEPES-Buffered Saline. 200 μl of diluted cells (2×10^4 cells) and 50 μl (8 mg/ml) D-Luciferin (GoldBio, United States) were loaded into each well. Plates were incubated for time increment from 5 to 30 min at 37°C. Luciferase activity was determined with an Envision MultiLabel Plate Reader (Perkin Elmer, United States).

Immunofluorescence Microscopy

Immunofluorescence assays were performed as described previously (Krtková et al., 2016), To detect triple hemagglutinin (3HA) tag, anti-HA rat monoclonal antibodies 3F10 (Roche) diluted to 1:125 followed by Alexa 488-conjugated anti-rat antibody (Molecular Probes) diluted to 1:250 were used. To detect HALO tag 0.5 μM Janelia Fluor 549 (Promega) dye or HaloTag® TMR Ligand (Promega) were used. CWP1 was detected with Alexa 647-conjugated anti-CWP1 antibody (Waterborne, New Orleans, LA, United States).

Fluorescent images were acquired on a DeltaVision Elite microscope using a 100×, 1.4-numerical aperture objective and a PCO Edge sCMOS camera. Deconvolution was performed with SoftWorx (API, Issaquah, WA, United States) and images were analyzed using Fiji, ImageJ (Schindelin et al., 2012). Pearson Coefficient, Manders Correlation Coefficient and Costes' automatic thresholding analyses were obtained using the JACoP plugin for ImageJ (Bolte and Cordelières, 2006). 3D viewing and manual scoring of cells were performed using Imaris (Bitplane, version 8.9). Figures were assembled using either Adobe Photoshop or Adobe Illustrator. A minimum of 120 cells were imaged for each cell line and timepoint post induction of encystation which corresponded to between 15 and 20 cells at each of our defined stages.

Affinity Purification

Affinity purification of OneSTrEP-*Gl*Rac (OS-*Gl*Rac) was done according to a previously used protocol (Paredez et al., 2014). Note that two-step encystation protocol was followed during culturing for the 8 h p.i.e. experiments. The resulting elutes were analyzed using liquid chromatography with tandem mass spectrometry.

Mass Spectrometry

Samples were prepared using the FASP method (Wiśniewski et al., 2009). Briefly, the samples were concentrated in an Amicon Ultra 10K filter, washed twice with 25 mM ammonium bicarbonate (ABC), and the disulfides reduced using 10 mM Tris 2-carboxyethyl phosphine (TCEP) for 1 h at 37°C. The resulting thiols were alkylated with 12 mM iodoacetamide for 20 min prior to spinning out the liquid. The proteins were washed twice with 100 μl 50 mM ABC. Digestion occurred after addition of 1 μg trypsin (Promega sequencing grade) in 200 μl 50 mM ABC, and overnight incubation at 37°C. The peptides were spun out of the filter and dried using a vacuum centrifuge.

All mass spectrometry was performed on a Velos Pro (Thermo) with an EasyLC HPLC and autosampler (Thermo). The dried pulldowns were solubilized in 25 μl of loading buffer (0.1% trifluoroacetic acid and 2% acetonitrile in water), and 6 μl was injected via the autosampler onto a 150-μm Kasil fritted trap packed with ReproSil-Pur C18-AQ (3-μm bead diameter, Dr. Maisch) to a bed length of 2 cm at a flow rate of 2 μl/min. After loading and desalting using a total volume of 8 μl of loading buffer, the trap was brought on-line with a pulled fused-silica capillary tip (75-μm i.d.) packed to a length of 25 cm with the same Dr. Maisch beads. Peptides were eluted off the column using a gradient of 2–35% acetonitrile in 0.1% formic acid over 120 min, followed by 35–60% acetonitrile over 5 min at a flow rate of 250 nl/min. The mass spectrometer was operated using electrospray ionization (2 kV) with the heated transfer tube at 25°C using data dependent acquisition (DDA), whereby a mass spectrum (m/z 400–1,600, normal scan rate) was acquired with up to 15 MS/MS spectra (rapid scan rate) of the most intense precursors found in the MS1 scan.

Tandem mass spectra were searched against the protein sequence database that was downloaded from GiardiaDB, using the computer program Comet (Eng et al., 2013). Iodoacetamide was a fixed modification of cysteine, and oxidized methionine was treated as a variable modification. Precursor mass tolerance was 2 Da, and fragment ion tolerance was ± 0.5 Da. Discrimination of correct and decoy spectra was performed using Percolator (Käll et al., 2007) with a 1% *q*-value cutoff. Proteins that had more than one unique peptide and significantly higher values for normalized total spectral counts (as determined by Fisher Exact Test with a Bonferroni multiple testing correction with an alpha

of 0.01) in the OneSTrEP-*Gl*Rac pulldown sample compared to its counterpart WT control sample, were noted to be hits.

RESULTS

Encystation Staging

Encystation was induced with a two-step protocol where *Giardia* trophozoites were initially cultured in no-bile, low-pH media (pH 6.8) and then moved into high-bile, high pH media (pH 7.8) (Boucher and Gillin, 1990). The rate at which encystation proceeds through this method lands between encystation rates induced by the lipoprotein depletion and the Uppsala high bile methods in terms of cyst production (**Supplementary Figure 1**). Also, the two-step protocol is thought to maximally synchronize the encystation process (Konrad et al., 2010). In our hands, while some level of synchronization could be achieved from this method, cells remained in various stages within the process of cyst development, as judged by localizing CWP1 and visualizing ESV morphology (**Figure 1**). We, therefore, sought to increase the resolution of staging encysting cells by going beyond noting h p.i.e. and categorizing each cell based on the morphology of CWP1-stained ESVs instead (**Figure 1**).

Based on our current understanding of the sequence of events involved in encystation, we propose the following key for staging encysting cells (**Figure 1**): Stage I – CWP1 localizes to the ER; Stage II – CWP1 localizes in the ER and also in ER-associated punctate structures thought to be ER-exit sites (Faso et al., 2013); Stage III – CWP1 localizes homogenously in small ESVs; Stage IV – CWP1 localizes to doughnut-shaped structures as a result of CWP2 being proteolytically processed to drive core condensation which pushes fluid phase CWP1 to the vesicle periphery. Stage V – found in cultures induced to encyst longer than 8 h (24 h p.i.e. in this study); CWP1 now localizes to large doughnut-shaped structures of matured ESVs about to be partitioned from condensed-phase cyst wall material (CWM) into separate vesicles. Stage VI – CWP1 is present in smaller separate vesicles that are close to the surface of the cells and ready to be secreted out to form the cyst wall. Our focus here is on ESV maturation; therefore, we did not analyze partially to fully formed cysts. Nonetheless, these staging parameters can be expanded to include them in the future.

Giardia Rab GTPases Associate to ESVs in Stage-Specific Manner

It is well established that different compartments of the membrane trafficking pathway feature unique molecular identities. Rab GTPases, through the recruitment of specific effectors, regulate the trafficking of each other through the endocytic/sorting/secretory pathway (Pfeffer, 2017). The resulting spatio-temporal specific recruitment of Rabs help direct cargo traffic. Rab GTPases can, therefore, be used as identity markers for differentiating trafficking compartments. We hypothesized that a subset of the nine Rab GTPases encoded in the *Giardia* genome would also demarcate ESVs as they mature, providing molecular markers for different stages of these compartments as they sort and secrete CWPs. Each *Gl*Rab was tagged on the N-terminus with mNeonGreen (mNG) to visualize their localization and the cells were subjected to the two-step encystation process before being processed for immunofluorescence assays.

Seven of the analyzed Rab proteins associated with ESVs with peak association at specific stages (**Figure 2**). To confirm that these Rab proteins co-localized with ESVs we analyzed co-localization by calculating Pearson and Manders' Coefficients for three cells per stage following Costes' automatic thresholding (**Supplementary Figure 2**). Note that these global statistical measurements are not effective for highlighting qualitative differences when proteins display complex localization patterns (Bolte and Cordelières, 2006; **Supplementary Figure 2**). In the case of ESV maturation and Rab proteins, their levels change during ESV maturation (Einarsson et al., 2016). The change in levels prevents the use of a standard set of acquisition parameters with matched signal to noise that is further complicated by changes in the organization of CWP1 as it proceeds from the ER and into ESVs that vary in size and shape as encystation proceeds. These changes preclude the use of standard correlation coefficients to uncover the dynamic relationship between CWP1 and Rab proteins. Therefore, we devised a qualitative scoring system to capture the dynamic relationship between Rab proteins and CWP1. After imaging a minimum of 120 cells per cell line, which corresponded to 15–20 cells per stage, we viewed the cells in 3D using Imaris and then assigned scores between 0 and 5, with higher scores being granted to cells featuring more robust recruitment of tagged-Rab proteins colocalizing with CWP1-stained vesicles (**Supplementary Figure 3**). Consistency between scores assigned by two team members for a sample subset of cells gave us confidence that this approach is reproducible. Of interest were Rabs with CWP1 colocalization scores that peaked at the different encystation stages – *Gl*Rab2a at Stages I and II, *Gl*RabA at Stage III, *Gl*Rab D at Stage IV and *Gl*Rab1a at Stages V and VI (**Figure 2**). *Gl*Rab32 did not localize to ESVs at any of the stages we looked at (**Supplementary Figure 3H**). Multiple attempts at tagging *Gl*Rab2b were unsuccessful and therefore this Rab GTPase was not included in our analysis. Our data is consistent with the cisternal maturation model where ESVs cargo remains in place and the molecular identity of the compartment changes as CWP is processed and sorted (Mani and Thattai, 2016). Here, we have shown that the staging of ESVs via their morphologies demarcates ESVs undergoing unique molecular events and provides a better framework for dissecting ESV molecular biology and the developmental state of encysting cells.

*Gl*Rac Activity During Encystation Is Stage-Specific

Rho family GTPase proteins, by cycling between an active GTP-loaded conformation and an inactive GDP-bound conformation, act as molecular switches that spatially and temporally regulate the recruitment of effector proteins. We previously noted that *Giardia's* sole Rho GTPase, *Gl*Rac, has variable association with ESVs (Krtková et al., 2016), but we did not determine the encystation stage-specificity of it. *Gl*Rac was endogenously tagged

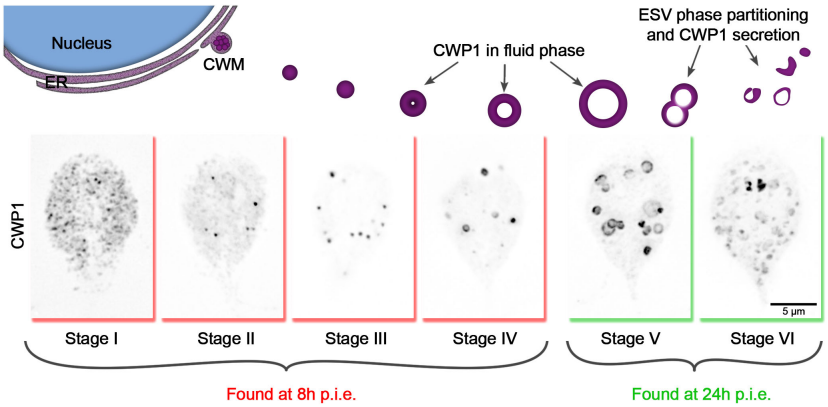

FIGURE 1 | Cyst wall protein trafficking in ESVs during encystation can be divided into stages. Graphic showing trafficking of CWP1-containing ESVs. Once *Giardia* cells sense the signal to encyst, large amounts of CWM are produced in the ER (Stage I), which then accumulate at ER exit sites (Stage II) and are secreted out into the cytoplasm compartmentalized in ESVs (Stage III). As CWP cargo are sorted and processed, they are separated into phases with CWP1 now being confined to the outer fluid-phase of the ESVs (Stage IV) which grow larger in size as they continue to mature (Stage V). The distinct phases of ESVs are then partitioned into separate vesicles (Stage VI) which are subsequently sequentially secreted to form *Giardia's* cyst wall. These stages can be tracked by visualizing CWP1 and tracking ESV morphology. Cells shown here were harvested and fixed at 8 and 24 h p.i.e. then stained with CWP1 antibody.

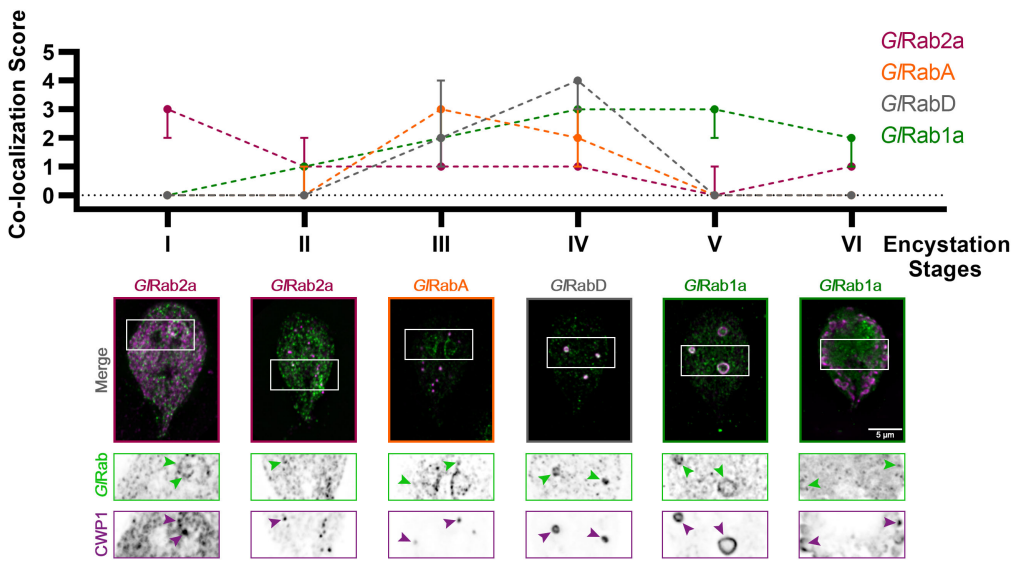

FIGURE 2 | *Giardia* Rabs associate with ESVs during encystation in a stage-specific manner. Summary of findings from colocalization analysis of *Giardia* Rabs and CWP1 through all the encystation stages described above. Cells expressing endogenously tagged mNG-*Gl*Rabs were subjected to the two-step encystation process. They were then harvested at 8 and 24 h p.i.e. to be fixed and stained for CWP1. 15–20 cells per encystation stage were then imaged to visualize mNG-tagged *Gl*Rabs (green) and CWP1 (magenta) and scored for the level of colocalization between the tagged *Gl*Rabs and CWP1 stained structures. Plot shows median scores with 95% confidence interval. Arrowheads indicate mNG-*Gl*Rabs colocalizing with CWP1-stained ESVs.

with HALO on its N-terminus and the cells were subjected to the same ESV colocalization assay as described above. HALO-*Gl*Rac strongly colocalizes with CWP1 during Stage I, an association which wanes through the mid-stages while maintaining some association, and then increases at Stages V and VI of encystation (**Figure 3A**). This is consistent with the proposed activities of *Gl*Rac during encystation. A large proportion of *Gl*Rac is thought to be sequestered in its inactive form at the ER while *Gl*Rac association with mid-stage and late-stage ESVs promotes ESV maturation and CWP secretion, respectively (Krtková et al., 2016). We then used a CRIB domain-based Rho GTPase biosensor (Manser et al., 1994; Srinivasan et al., 2003), CRIB-mNG, to confirm our functional analyses. CRIB-mNG, which marks active GTP-loaded *Gl*Rac, colocalized with ESVs as their CWP cargo were being first sorted into condensed and fluid phases i.e., Stages III and IV, and also at Stage VI of encystation when ESVs have completed undergoing the second sorting step and were beginning to secrete their cargo to form the cyst (**Figure 3B**). This pattern of localization by CRIB-mNG indicates that the ER associated population of

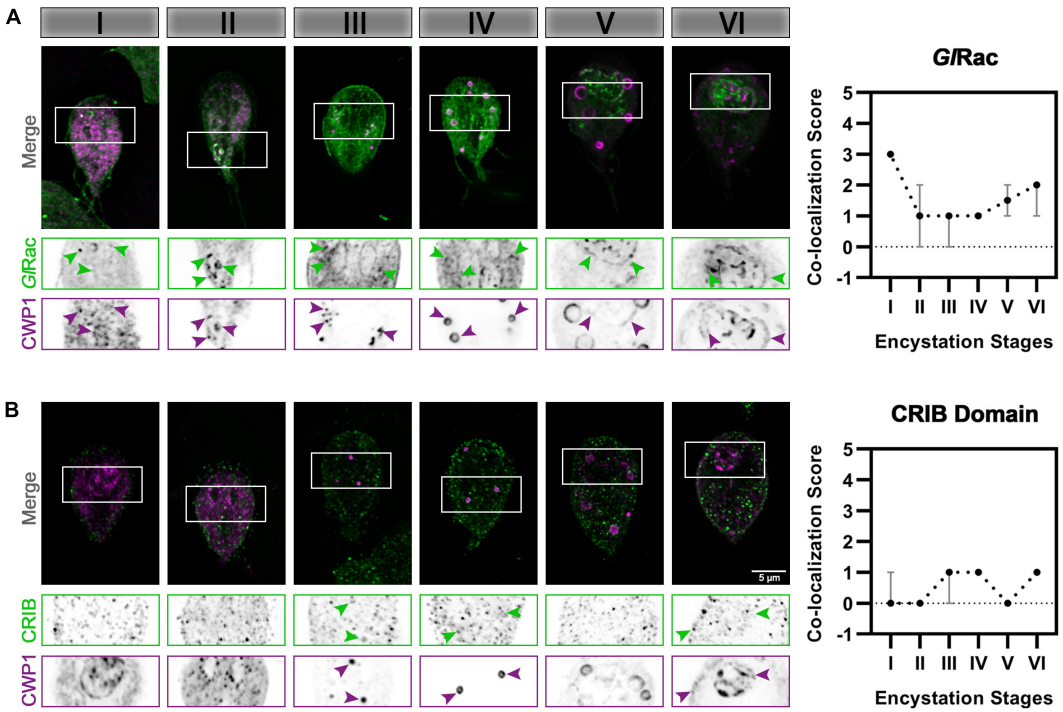

FIGURE 3 | *Gl*Rac activity during encystation is stage-specific. Cells expressing **(A)** HALO-*Gl*Rac (endogenous tag) or **(B)** CRIB-HA-mNG (exogenous expression) were subjected to the two-step encystation process. They were then harvested at 8 and 24 h p.i.e. to be fixed and stained for HALO-*Gl*Rac or CRIB-HA-mNG and CWP1. 15–20 cells per encystation stage were then imaged to visualize HALO-*Gl*Rac (green) or CRIB-HA-mNG (green) and CWP1 (magenta) and scored for the level of colocalization between HALO-*Gl*Rac or CRIB-HA-mNG and CWP1 stained structures. Plot shows median scores with 95% confidence interval. Arrowheads indicate HALO-*Gl*Rac or CRIB-HA-mNG colocalizing with CWP1-stained ESVs.

*Gl*Rac is GDP-loaded while the *Gl*Rac associated with ESVs is GTP-loaded. The specific association of CRIB with Stage III, IV, and VI ESVs is in agreement with our previous functional analysis that demonstrated a role for *Gl*Rac in promoting the development of Stage IV (doughnut shaped) ESVs and a later role in regulating secretion of CWP1 (Krtková et al., 2016).

Putative Effectors of *Gl*Rac During *Giardia* Encystation

Next, we sought to identify the effectors regulated by *Gl*Rac during encystation – in this study we specifically focused on mid-stage encysting cells featuring ESVs that associate with active *Gl*Rac. *Giardia* cells exogenously expressing N-terminally OneSTrEP (OS-) tagged *Gl*Rac were induced to encyst using the two-step method. We then performed an affinity purification with OS-*Gl*Rac lysates at 8 h p.i.e. Purifications were completed in triplicate, and wild type (WT) trophozoites were used as a control to identify non-specific binding to the Strep-Tactin beads. The eluted proteins were then analyzed via mass spectrometry to identify putative interactors of *Gl*Rac. We identified 57 proteins, statistically significantly associating with *Gl*Rac in at least one replicate, in both trophozoites and encysting cells. Similarly, 33 proteins associated with *Gl*Rac exclusively in non-encysting trophozoites while 28 proteins were associated with encysting cells. The complete list, including low-abundance hits and proteins also identified in our mock control, is given in **Supplementary Table 2**. Altogether, we identified 18 proteins predicted to have a role in membrane trafficking that were enriched in our encysting population (**Supplementary Table 3**). Out of these, four were previously known to associate with ESVs supporting the idea that our list could have additional ESV components. We focused on 11 proteins that were homologs of known players of membrane trafficking in other eukaryotes (**Table 1**). *Gl*Rab1a and *Gl*Rab2a had already been visualized earlier (**Supplementary Figure 3**). Each of the rest of the candidate genes were endogenously expressed with a dual tag of 3HA fused to mNG in cells already endogenously expressing Halo-*Gl*Rac. The candidates were then visualized via immunofluorescence assays along with *Gl*Rac and CWP1 (**Supplementary Figure 4**). Six of the eight candidates listed showed patterns of colocalization with CWP1 similar to that of *Gl*Rac – *Gl*Rab2a (**Figure 2**), *Gl*Sec61-α, *Gl*Coatomer α subunit, *Gl*Coatomer β′ subunit, and *Gl*v-SNARE (**Figure 4**) suggesting that they might be involved in the same pathway regulating encystation as *Gl*Rac.

DISCUSSION

Here, we have developed a staging system for encysting cells and have identified novel molecular markers for ESV stages that correlate with the progression of encystation. We

TABLE 1 | List of candidate genes selected for this study and identified as hits in OS-*Gl*Rac pulldown experiment in trophozoites and encysting cells (8 h p.i.e.) that are homologs of known membrane trafficking players.

Gene ID	Gene Name	Trophozoites			8 h encyst		
		Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
GL50803_8496	**Rac/Rho-like protein**	**x**	**x**	**x**	**x**	**x**	**x**
GL50803_5744	Sec61-α	x	x	x	x	x	x
GL50803_9558	Rab1a		x	x	x	x	x
GL50803_15567	Rab2a		x	x	x	x	x
GL50803_11953	Coatomer α subunit		x	x		x	x
GL50803_9593	Coatomer β′ subunit		x	x		x	x
GL50803_17304	α-adaptin					x	
GL50803_114776	NSF					x	
GL50803_9489	v-SNARE					x	

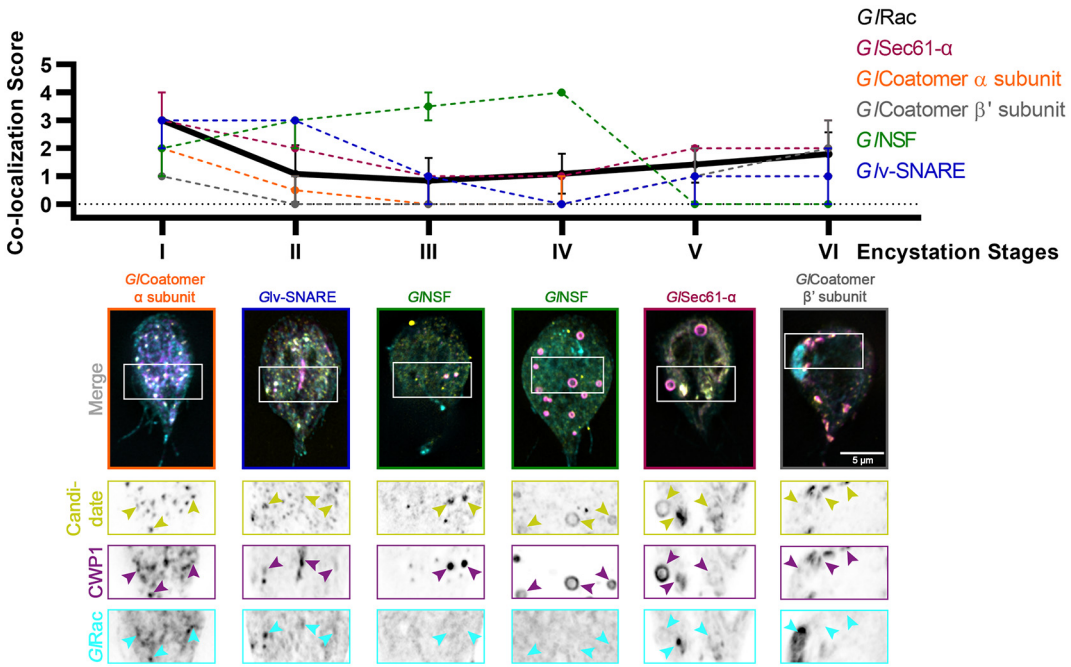

FIGURE 4 | Putative effectors of *Gl*Rac colocalize with CWP1 in a stage-specific manner in a pattern that is similar to *Gl*Rac. Summary of findings from colocalization analysis of putative *Gl*Rac interactors and CWP1 through all the encystation stages described above. Cells expressing endogenously tagged HALO-*Gl*Rac and mNG-HA tagged candidates were subjected to the two-step encystation process. They were then harvested at 8 and 24 h p.i.e. to be fixed and stained for CWP1. 15–20 cells per encystation stage were then imaged to visualize HALO-*Gl*Rac (cyan) or HA-mNG-candidate/candidate-HA-mNG (yellow) and CWP1 (magenta) and scored for the level of colocalization between the tagged candidate and CWP1 stained structures. Plot shows median scores with 95% confidence interval. Arrowheads indicate candidate proteins colocalizing with CWP1-stained ESVs.

previously noted that *Gl*Rac associates with ESVs at defined points of their maturation process (Krtková et al., 2016). The lack of an established method to unambiguously pinpoint progression of encystation in individual cells, along with the variability of encystation rates that result from different *in vitro* encystation protocols, prompted us to develop a staging system that is based on ESV morphologies and independent of the method used to induce encystation. The encystation stage-specific recruitment of these molecular markers is consistent with the cisternal maturation model (Mani and Thattai, 2016) and the observations of Štefanić et al. (2009). Briefly, ESVs are thought to act as developmentally induced Golgi (Marti et al., 2003). CWPs are first detected approximately 2 h after the induction of encystation (two-step protocol); the export of CWPs from the ER, maturation of ESVs and secretion of processed CWPs to the plasma membrane together represent a simplified version of the Golgi cisternal maturation model. CWP export from the ER was found to require functional Arf1, Sar1, and Rab1 (Štefanić et al., 2009). ESVs generated *de novo* from this export process at ERESs contain pre-sorted material that is simultaneously transported and processed.

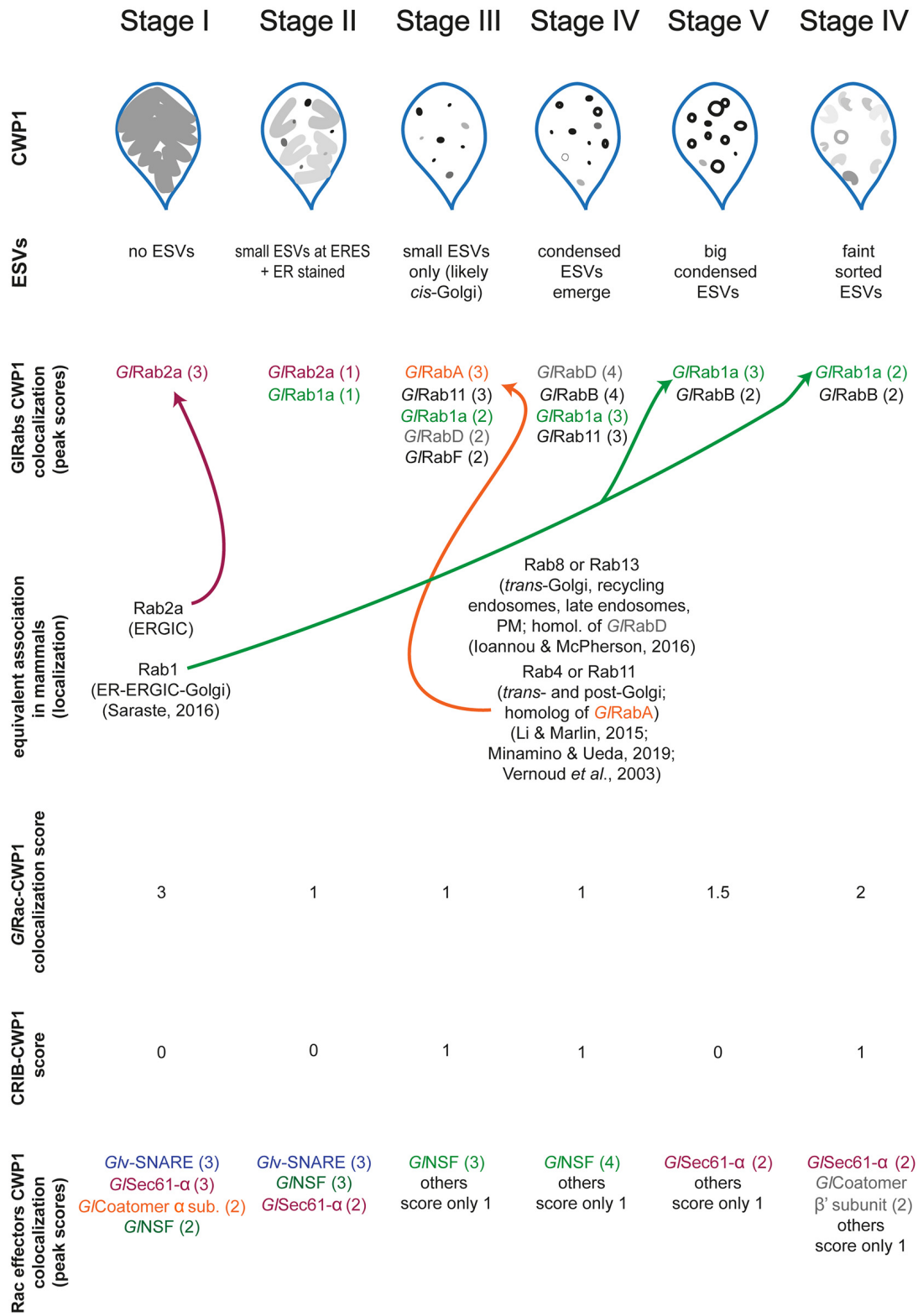

FIGURE 5 | Graphical summary showing the relationship between all proteins studied here relative to ESV stages. Values in parenthesis indicate our qualitative colocalization scores.

Staging ESV maturity previously relied on timing p.i.e. or simply referring to encysting cells as "early" or "late" without a standardized criteria for making these distinctions (Luján et al., 1996; Konrad et al., 2010; Einarsson et al., 2016). Timing p.i.e. is a practical approach to experimentation and can even be meaningful when comparing cells exposed to the same encystation medium when broad trends are being studied. Yet, this method is imprecise due to *in vitro* encystation protocols being only semi-synchronous. It is thought that there is a restriction point that prevents cells outside of G2 from entering the encystation response (Reiner et al., 2008). Given an approximately 8 h cell cycle, it is not surprising that we find cells at differing encystation stages when examining induced populations. Additionally, the timing and efficiency of inducing encystation varies between the three main *in vitro* encystation methods – cholesterol starvation (Luján et al., 1996), the Uppsala method (Einarsson et al., 2016), and the two-step encystation protocol [(Boucher and Gillin, 1990), used in this study]. To a smaller extent lot-to-lot variation of serum and bile components of growth medium and encystation medium impact doubling times and, therefore, the efficiency of inducing encystation. Most importantly, *in vivo* studies employing animal models of infection currently cannot be synchronized and timing h p.i.e. is not meaningful. Recent efforts to study the encystation response *in vivo* has raised questions about whether *in vitro* studies can recapitulate *in vivo* encystation dynamics (Pham et al., 2017). Thus, the emerging view is that *in vitro* observations should be verified *in vivo* and this encystation staging system would also be a practical way to characterize the distribution of encysting cells within host intestines.

Our universally applicable staging system will allow the field to standardize encystation staging regardless of the method of induction used. Each ESV morphology change is thought to correspond with sequential molecular events, including secretion from the ER, CWP processing, sorting, and pulsed cellular secretion of processed CWP. ESV morphology changes, therefore, provide landmarks for encystation stages. Observing and categorizing ESV morphologies are obvious and easy to follow, therefore making it accessible for adoption by the wider *Giardia* research community. This would be advantageous since it would reduce ambiguity when the timeline of molecular pathways directing CWP traffic are being specified. Finally, as the resolution of these events increase, encystation stages could be further subdivided to accommodate new discoveries.

Beyond morphological categorization we have identified several *Giardia* Rab GTPases that have peak association with specific encystation stages, indicating that our staging system corresponds to unique molecular identities. The association of Rab proteins before and after peak association is not surprising; in model eukaryotes it has been shown that Rab recruitment proceeds through cascades where handoff steps must occur (Rivera-Molina and Novick, 2009). Some *Giardia* Rab GTPases were previously known to be associated with ESVs and overall this group of proteins is upregulated during encystation (Marti et al., 2003; Einarsson et al., 2016). Here, we tagged and followed eight out of nine *Giardia* Rab GTPases over the course of encystation. Seven of the eight Rab GTPases analyzed here associated with ESVs. By scoring the degree of colocalization with CWP1 during the different encystation stages, we were able to identify their distinct patterns of ESV association thus potentially indicating the point at which their functions were required as encystation progressed. Functional equivalence between some of the *Gl*Rabs and their homologs can be inferred. *Gl*Rab2a and CWP1 colocalization peaked at Stages I and II, paralleling its mammalian homologs which reside in the ER-to-Golgi Intermediate Compartments (ERGIC) and regulate Golgi biogenesis, and bidirectional transport between the two compartments (Saraste, 2016). *Gl*RabD shows the closest homology to Rab8 or Rab13 known to localize at the *trans* Golgi network, recycling endosomes, late endosomes and the plasma membrane. They have especially been implicated in biosynthetic and recycling endosomal pathways (Ioannou and McPherson, 2016). In *G. lamblia*, RabD colocalization with ESVs peaks at Stage IV and equivalence could be argued.

While the similarities between *Giardia* Rabs and their eukaryotic homologs allow for some inferred function, not all *Giardia* Rabs have clear orthologs. The closest homologs of *Gl*RabA (RabA in plants/Rab4 or Rab11 in mammals) are known to associate with the *trans* Golgi and post-Golgi networks, specifically, the recycling endosomes (Vernoud et al., 2003; Li and Marlin, 2015; Minamino and Ueda, 2019) and *Gl*RabA was shown to associate with ESVs the most at Stage III, which is likely equivalent to *cis* Golgi earlier in the canonical secretory pathway. Additionally, *Gl*Rab1a colocalization with ESVs peaks at Stages V and VI which is when the ESVs undergo further sorting and the fluid phase of ESVs are beginning to disassociate for secretion. This is different from canonical Rab1 isoforms that have been established to regulate transport between the ER-ERGIC-Golgi interface (Saraste, 2016).

The importance of Rabs in regulating *Giardia* encystation is yet to be fully understood. Previously, *Gl*Rab1a was shown to be necessary for ESV development and cyst wall formation (Štefanić et al., 2009) complementing the observations we have made here. The specific functions of *Gl*Rabs would make an interesting topic for future studies.

As a proof of principle for our staging system, we turned to *Gl*Rac which has a complex relationship to ESVs. In agreement with previously published data, *Gl*Rac colocalization with CWP1 peaked at stages I and VI with low level colocalization being detectable throughout the rest of the stages. *Gl*Rac was hypothesized to be sequestered in the ER in an inactive state and then have a role in promoting ESV maturation and secretion of CWP1 (Krtková et al., 2016). Based on our hypothesis, *Gl*Rac was expected to be active at Stages III to promote maturation to Stage IV and at Stage VI to promote CWP1 secretion, which we confirmed using CRIB-mNG as a *Gl*Rac signaling biosensor. A surprising finding is that we found bright accumulations of *Gl*Rac during Stage VI which we overlooked in our previous analysis. Since we first sorted cells into bins by stage, it became apparent that the accumulation of *Gl*Rac was not a one-off occurrence (**Supplementary Figure 4**). The function of these *Gl*Rac accumulations are unknown, but since Rac homologs can direct secretion, we speculate that these regions could be involved in CWP1 secretion during encystation. CWP1 is thought to be rapidly secreted as cells seem to either have CWP1 in ESVs or on their surface and intermediates are rarely observed.

Our understanding of the encystation process and CWP1 trafficking would certainly benefit from live imaging analyses in order to study such transient processes.

As a molecular switch, *Gl*Rac is expected to recruit effectors that promote maturation of ESVs and secretion of CWP1. To identify *Gl*Rac effector proteins we affinity purified *Gl*Rac interactors from non-encysting and encysting populations. We were not surprised to find many statistically significant interacting proteins since *Gl*Rac, as the sole Rho GTPase in *Giardia*, is presumably responsible for many of the same roles the multitude of Rho GTPases carry out in other eukaryotes (Hodge and Ridley, 2016; Lawson and Ridley, 2018; Phuyal and Farhan, 2019). In addition to its role in membrane trafficking, we have shown that *Gl*Rac has important roles in regulating the cytoskeleton (Paredez et al., 2011; Krtková et al., 2017; Hardin et al., 2021); thus we expect many of the statistically significant interactors in non-encysting cells will prove to have roles in regulating the cytoskeleton. Here, we chose to focus on proteins predicted to have a role in trafficking as we had the potential to identify novel ESV components. Indeed, every protein selected from the proteomics hits associated with the ER and/or ESVs with *Gl*Rac. Our findings are summarized in **Figure 5**.

A number of candidates displayed patterns of colocalization that were similar to the patterns displayed by *Gl*Rac with CWP1, including *Gl*Sec61-α, *Gl*Coatomer-α subunit, *Gl*Coatomer-β′ subunit, and *Gl*v-SNARE. Each of these proteins were found to be in the ER at Stage I with high colocalization scores with CWP1 which reduced in subsequent stages to rise back up again in the final two stages. This would suggest that they localize in the same compartments during encystation. Note that while GTP-loaded GTPases are typically considered the active conformation, effector proteins can also bind the inactivated GDP-bound form of Rho GTPases. So it is not surprising that some of these candidates colocalize with *Gl*Rac at the ER where *Gl*Rac is largely GDP-loaded. All of these except *Gl*v-SNARE were also pulled down by *Gl*Rac in trophozoites indicating additional constitutive roles, while *Gl*NSF, *Gl*α-adaptin and *Gl*v-SNARE appears to have an encystation-specific association with *Gl*Rac, which will require further investigation. Here, we have set the scene for future studies to dissect the role of these newly identified components.

In summary we have devised a universally applicable ESV staging system based on ESV morphology using CWP1 as a marker. As CWP1 is an easily accessible encystation marker, our staging system can be readily adopted across the field. Beyond morphological differences in ESV stages we have identified molecular markers that can be used to distinguish different stages. As the *Giardia* toolkit grows, the field will have greater access to tools that allow for functional studies. We anticipate future studies of *Gl*Rabs and other ESV-associated proteins identified here that will impact ESV maturation and ESV morphology. Therefore, morphology alone may be insufficient for characterizing/staging the resulting ESVs. The newly identified molecular markers for different ESV stages will be a powerful tool for the purpose of characterizing the stages of abnormal ESVs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, and the unprocessed mass spec data are available via ProteomeXchange with identifier PXD024944. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

ET and AP designed the experiments and wrote the manuscript. ET and RS performed the colocalization with CWP1 analyses. ET and JK performed the affinity purifications. H-WS and GA performed expression analysis and staging of encysting cells induced with different encystation protocols. RJ performed mass spectrometry analysis and analyzed the proteomics data. MM and AP were responsible for fund acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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ORIGINAL RESEARCH
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Comparative Proteomics Analysis for Elucidating the Interaction Between Host Cells and *Toxoplasma gondii*

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Toxoplasma gondii, a representative model organism belonging to the phylum Apicomplexa, can infect almost all warm-blooded organisms, including humans. The invasion of host cells *via* host–parasite interaction is the key step for *T. gondii* to complete its life cycle. Herein we performed tandem mass tag analysis to investigate global proteomic changes in host cells (human foreskin fibroblasts, HFFs) [HFFs infected with *T. gondii* (HT) *vs*. HFFs (H)] and *T. gondii* [HT *vs*. *T. gondii* (T)] during intracellular infection. Overall, 3477 and 1434 proteins were quantified, of which 375 and 1099 proteins were differentially expressed (adjusted p-value < 0.05 and >1.5 or <0.67-fold change) in host cells and *T. gondii*, respectively. *T. gondii* invasion relies on the secretion of numerous secretory proteins, which originate from three secretory organelles: micronemes, rhoptries, and dense granules. In the HT *vs*. T group, few secretory proteins were upregulated, such as microneme proteins (MICs: MIC6, MIC10), rhoptry bulb proteins (ROPs: ROP5, ROP17), and dense granule proteins (GRAs: GRA4, GRA5, GRA12). In contrast, dozens of known secretory proteins were significantly downregulated in *T. gondii*-infected HFFs. In HFFs, gene ontology and Kyoto Encyclopedia of Genes and Genomes pathway analyses revealed a large number of differentially expressed proteins (DEPs) enriched in metabolic processes and immune-associated signaling pathways, such as NF-κB, cAMP, and Rap1 signaling pathways. Further, in case of *T. gondii*, DEPs were involved in ribosome biogenesis, citrate cycle, and galactose metabolism, indicating that cell biosynthesis and metabolism of *T. gondii* were altered after host cell invasion. These findings reveal novel modifications in the proteome of host cells as well as *T. gondii*, helping us better understand the mechanisms underlying host–parasite interaction.

Keywords: *Toxoplasma gondii*, host–parasite interaction, TMT, comparative proteomics, differentially expressed proteins

INTRODUCTION

Toxoplasmosis is a life-threatening opportunistic infection caused by the protozoan parasite *Toxoplasma gondii*, an obligate intracellular organism. Around one-third of the global population is considered to have a latent infection of *T. gondii* (Montoya and Liesenfeld, 2004). *T. gondii* is a representative model organism of the phylum Apicomplexa that contains many important parasites of humans and animals, including *Plasmodium* spp., *Eimeria* spp., *Neospora*, and *Cryptosporidium* (Zhang et al., 2012; Janouškovec et al., 2015). *T. gondii* can infect almost all warm-blooded organisms, including humans, and can exist in various tissues and organs of infected hosts. Moreover, toxoplasmosis can be fatal in immunocompromised patients, such as those with AIDS and those undergoing immunosuppressive chemotherapy (Kim and Weiss, 2008; Flegr, 2013; Torrey and Yolken, 2013). In addition, *T. gondii* can often cause adverse pregnancy outcomes, such as abortion and preterm labor, and even if pregnant women can successfully deliver, surviving offspring often show serious neurological and ocular sequelae (Robert-Gangneux et al., 2011; Jiao et al., 2017).

T. gondii actively invades host cells and can infect and replicate within almost any nucleated cell, which suggests that the pertinent mechanism is precise as well as effective. Host cell invasion is pivotal for *T. gondii* to complete its life cycle and is a consequence of host–parasite interaction (Hakimi et al., 2017). *T. gondii* participates in the signal transduction of host cells, making them prone to invasion. The invasion of host cells by *T. gondii* can be achieved in merely 15–20 s and relies on the actin–myosin motor complex and multiprotein complexes secreted by specialized organelles at its apical end, such as micronemes, rhoptries, and dense granules (Rabenau et al., 2001; Frénal et al., 2010; Swierzy et al., 2017). These secretory organelles have a crucial function in host cell invasion and parasitophorous vacuole establishment (Soldati et al., 2001). The microneme protein (MIC) plays an important role in the recognition, adhesion, and attachment of *T. gondii* to host cells during the early stage of invasion. Rhoptry proteins, comprising rhoptry neck proteins (RONs) and rhoptry bulb proteins (ROPs), are secreted during invasion and are believed to contribute to parasitophorous vacuole formation. Further, dense granule proteins (GRAs) participate in the modification of parasitophorous vacuole and its membrane, facilitating survival and replication of the organism.

The host–parasite response to an infection and the identification of host proteins that interact with *T. gondii* proteins should help in elucidating the mechanism of infection and virulence at the tachyzoite stage. Previous studies have majorly used two-dimensional electrophoresisto detect proteomic changes in human foreskin fibroblasts (HFFs) or *T. gondii* (Cohen et al., 2002; Nelson et al., 2008); however, this method has a relatively low throughput for protein identification or quantification. Herein we performed tandem mass tag (TMT) analysis to investigate global proteomic changes in both host cells and *T. gondii* during intracellular infection. We report novel insights into the proteomics landscape of *T. gondii* before and after infection of host cells and also report data pertaining to modifications in the host cell proteome during *T. gondii* infection, with the aim of elucidating the complex relationship between host cells and parasites.

MATERIAL AND METHODS

Parasite, Cell Culture, and Cell Invasion Experiments

T. gondii RH strain tachyzoites were maintained in HFFs at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (catalog no. SH30022.01B, Hyclone, China) supplemented with 10% fetal bovine serum (catalog no. 10099133C, Gibco, USA), 100 IU/mL penicillin, and 100 μg/mL streptomycin (catalog no. P1400, Solarbio, China). Tachyzoites were harvested from monolayer HFFs and purified using a 5-μm filter. They were then washed using phosphate-buffered saline and counted with trypan blue stain.

For cell invasion experiments, tachyzoites were added to HFF monolayers (approx. 85% confluency) at an infection ratio of 3:1. Tachyzoites that had not invaded the cells were washed away with phosphate-buffered saline after 2 h. The cells were harvested at 24 h when approximately 80% of them appeared infected. The control groups included an equal number of non-infected cells and an equal number of *T. gondii* tachyzoites. All cells were washed using cold phosphate-buffered saline, centrifuged at 400 g for 10 min, and stored at −80°C until needed. Each sample was replicated three times to yield nine samples (three groups × three biological replicates) and the three groups were designated H (HFFs), T (*T. gondii*), and HT (HFFs infected with *T. gondii*).

Protein Extraction and Quantification

The samples were suspended in a lysis buffer (8 M urea and 1% protease inhibitor) and disrupted three times on ice by sonication. The supernatant was then separated by centrifugation at 4°C and 12,000 *g* for 10 min, and the pellet was removed. Finally, protein concentration was determined using a BCA protein assay kit (catalog no. P0011-1, Beyotime, China), according to manufacturer instructions.

Trypsin Digestion and TMT Labeling

Dithiothreitol (5 mM, catalog no. D9163, Sigma-Aldrich, USA) was added to the protein solution, and reduction was allowed to proceed at 56°C for 30 min. The protein solution was then incubated with 11 mM iodoacetamide (catalog no. V900335, Sigma-Aldrich, USA) at room temperature in darkness for 15 min. The urea concentration of the solution was then diluted to <2 M using 100 mM tetraethylammonium bromide (catalog no. 17902, Sigma-Aldrich, USA). Finally, trypsin (catalog no. V5111, Promega, USA) was added at a trypsin-to-protein mass ratio of 1:50 for the first round of digestion for overnight and 1:100 for the second round of digestion for 4 h. Peptides were desalted on a Strata X C18 SPE column (Phenomenex, USA) and vacuum dried after trypsin digestion. They were then labeled using a TMT 10-plex isobaric

label kit (catalog no. 90111, Thermo, USA), according to manufacturer instructions.

High-Performance Liquid Chromatography Fractionation and LC-MS/MS

The labeled peptides were fractionated on a 300 Extend C18 column (4.6 mm × 250 mm, 5 μm, Agilent, USA) and separated with a gradient of 8%–32% acetonitrile (pH 9.0) over 60 min into 60 fractions. The peptides samples were then combined into 14 fractions and dried in a vacuum centrifuge. Subsequently, they were dissolved in 0.1% formic acid solution and loaded onto an EASY-nLC 1000 UPLC system (Thermo, USA). Solvent A was 0.1% formic acid in 90% acetonitrile solution. The gradient comprised an increase from 9% to 26% of solvent B (0.1% formic acid in 90% acetonitrile) over 40 min, a rise from 26% to 35% in 14 min, and then from 35% to 80% in 3 min, followed by holding at 80% for the last 4 min; the flow rate was kept constant at 350 nL/min at all times. The peptides were subjected to NSI source, followed by tandem mass spectrometry (MS/MS) on a Q Exactive Plus Orbitrap LC-MS/MS System (Thermo, USA). The electrospray voltage was 2.0 kV. The mass spectrometer scan range was 350–1800 *m/z* and the orbitrap resolution was set to 70,000. The MS/MS scan range and orbitrap resolution were 100 *m/z* and 35,000, respectively. Data-dependent acquisition was used for data collection. Automatic gain control was set to 5E4, and the dynamic exclusion duration was 30 s.

Database Search

The raw files from the same batch were processed together with MaxQuant against the SwissProt Homo sapiens and UniPort *T. gondii* ATCC_50861 databases, concatenated with the reverse decoy database. The cleavage enzyme was trypsin/P, with no more than two missing cleavages and the minimum peptide length was seven. For precursor ions, the mass tolerance of the first search and main search was set to 20 ppm and 5 ppm, respectively. The mass tolerance of fragment ions was 0.02 Da. Carbamidomethylation of cysteine residues and methionine oxidation were specified as a fixed and variable modification, respectively. The protein quantification method was set with TMT 10-plex and the reporter ion isotopic impurity distribution of TMT 10-plex was set as instructed by to the product manual. The results were filtered based on the peptide false discovery rate (FDR) of < 1%.

Data Analysis and Bioinformatics

We chose unique peptides for protein quantification; relative quantification of protein expression levels were calculated as the median of unique peptides. There biological replicates were assessed for each group. For each protein, the average ratio calculated using the three biological replicates indicated final protein expression. The protein quantification results were statistically analyzed using two-sample *t*-test and the p-value was corrected by the method of Benjamin and Hochberg FDR analysis. Proteins with a fold change > 1.5 or < 0.67 and adjusted p-value < 0.05 were considered to be differentially abundant. Differentially expressed proteins (DEPs) were selected for subcellular localization, clusters of orthologous groups of protein, gene ontology (GO) classification, and enrichment analyses. WoLF PSORT (v0.2 http://www.genscript.com/psort/wolf_psort.html) was used to predict the subcellular localization of DEPs. For GO analysis, all annotations were derived from the UniProt-GOA database (http://www.ebi.ac.uk/GOA/) and complemented with InterProScan soft (v5.14-53.0 http://www.ebi.ac.uk/InterProScan/). All DEPs were subjected to analysis of eukaryotic orthologous group (KOG) by aligning their sequences with the KOG protein sequence database (http://genome.jgi.doe.gov/help/kogbrowser.jsf). The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used for pathway enrichment analyses of DEPs. For GO and KEGG enrichment analyses, two-tailed Fisher's exact test was applied to test DEPs against all identified proteins. A corrected p value of <0.05 indicated statistical significance. MS data have been deposited in the ProteomeXchange Consortium *via* the PRIDE (Perez-Riverol et al., 2019) partner repository, with the dataset identifier PXD021736.

Parallel Reaction Monitoring (PRM) Validation

To confirm the authenticity and accuracy of results of TMT analysis, we performed PRM assay that included 14 peptides. Protein extraction and digestion were achieved using the aforementioned methods. The peptides were subjected to MS/MS on a Q Exactive Plus Orbitrap LC-MS/MS System (Thermo, USA). The electrospray voltage was 2.1 kV, MS scan range was 350–1050 *m/z*, and orbitrap resolution was 70,000. The MS/MS orbitrap resolution was 17,500. Data-independent acquisition was used for data collection. Automatic gain control of MS and MS/MS was set to 3E6 and 1E5, respectively. The maximum injection time was 50 ms for MS and 180 ms for MS/MS. The isolation window for MS/MS was 1.6 *m/z*. Three biological replicates were assessed.

Data Analysis of PRM Validation

The resulting MS data were processed using Skyline (v3.6). The peptide settings were as follows: the enzyme was set as trypsin [KR/P] and the max missed cleavage was set as 0. The peptide length was 7–25, and carbamidomethylation of cysteine residues was the fixed modification. The transition settings were as follows: precursor charges were set as 2, 3; ion charges were set as 1; and ion types were set as b, y. The product ions were set as from ion 3 to the last ion, and the ion match tolerance was 0.02 Da.

RESULTS

Protein Identification and Expression

From all nine samples, 240,537 spectra were generated, from which 26,251 peptides and 25,528 unique peptides were

obtained. A total of 4111 and 1708 proteins were identified as human and *T. gondii* proteins, of which 3477 and 1434 proteins were quantified, respectively (**Supplementary Table S1**). The standard criteria were adjusted p-value of <0.05 and > 1.5 or < 0.67 -fold change accordingly, 150 up- and 225 downregulated proteins were identified in the HT group in comparison with the H group (**Figure 1A** and **Supplementary Table S2A**). Further, 107 up- and 992 downregulated proteins were found in the HT group in comparison with the T group (**Figure 1B** and **Supplementary Table S2B**). All DEPs were subsequently subjected to comprehensive bioinformatics analysis.

Comparative Proteomics Analyses of Host Cells in the HT *vs*. H Group

In the HT *vs*. H group, the subcellular location of DEPs was as follows: the nucleus (25.33%), cytoplasm (24.26%), and extracellular (21.06%; **Figure 2A**). As evident from **Figure 3A**, DEPs were classified into 24 categories by cluster of orthologous group analysis. The top five categories with the most DEPs were signal transduction mechanisms [T]; general function prediction only [R]; posttranslational modification, protein turnover, chaperones [O]; cytoskeleton [Z]; and intracellular trafficking, secretion, and vesicular transport [U].

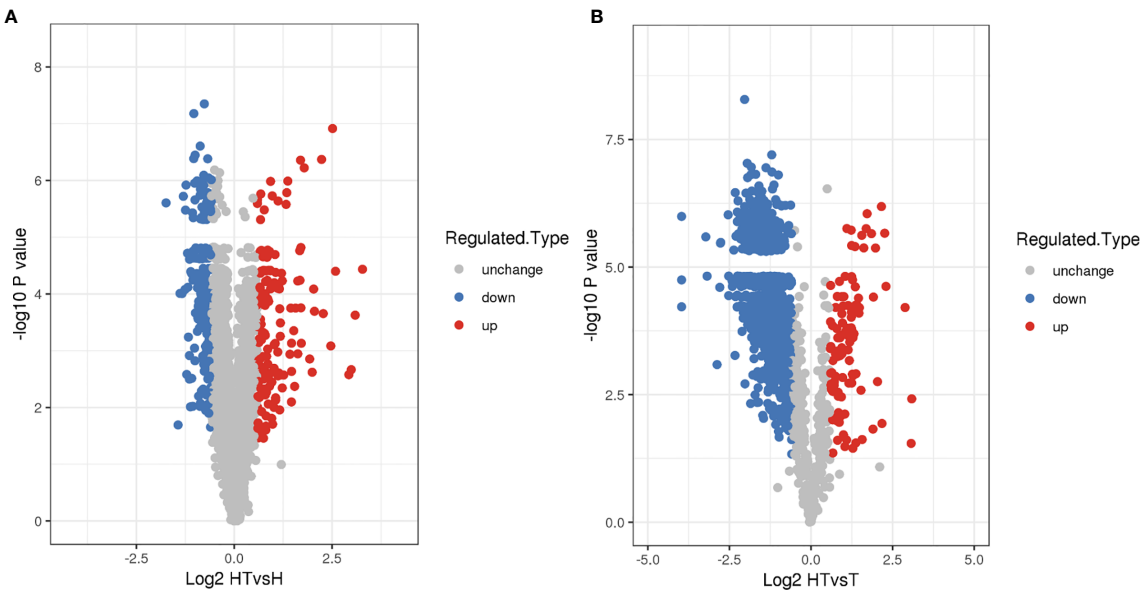

FIGURE 1 | Volcano map of DEPs in the **(A)** HT *vs*. H and **(B)** HT *vs*. T groups.

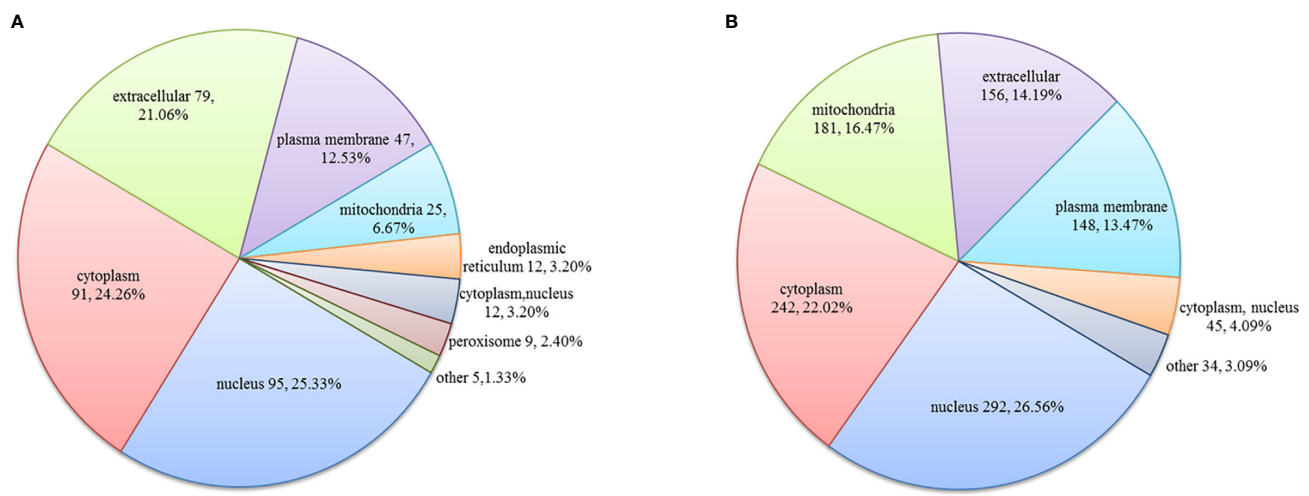

FIGURE 2 | Subcellular location of DEPs in the **(A)** HT *vs*. H and **(B)** HT *vs*. T groups. Numbers represent the number of DEPs.

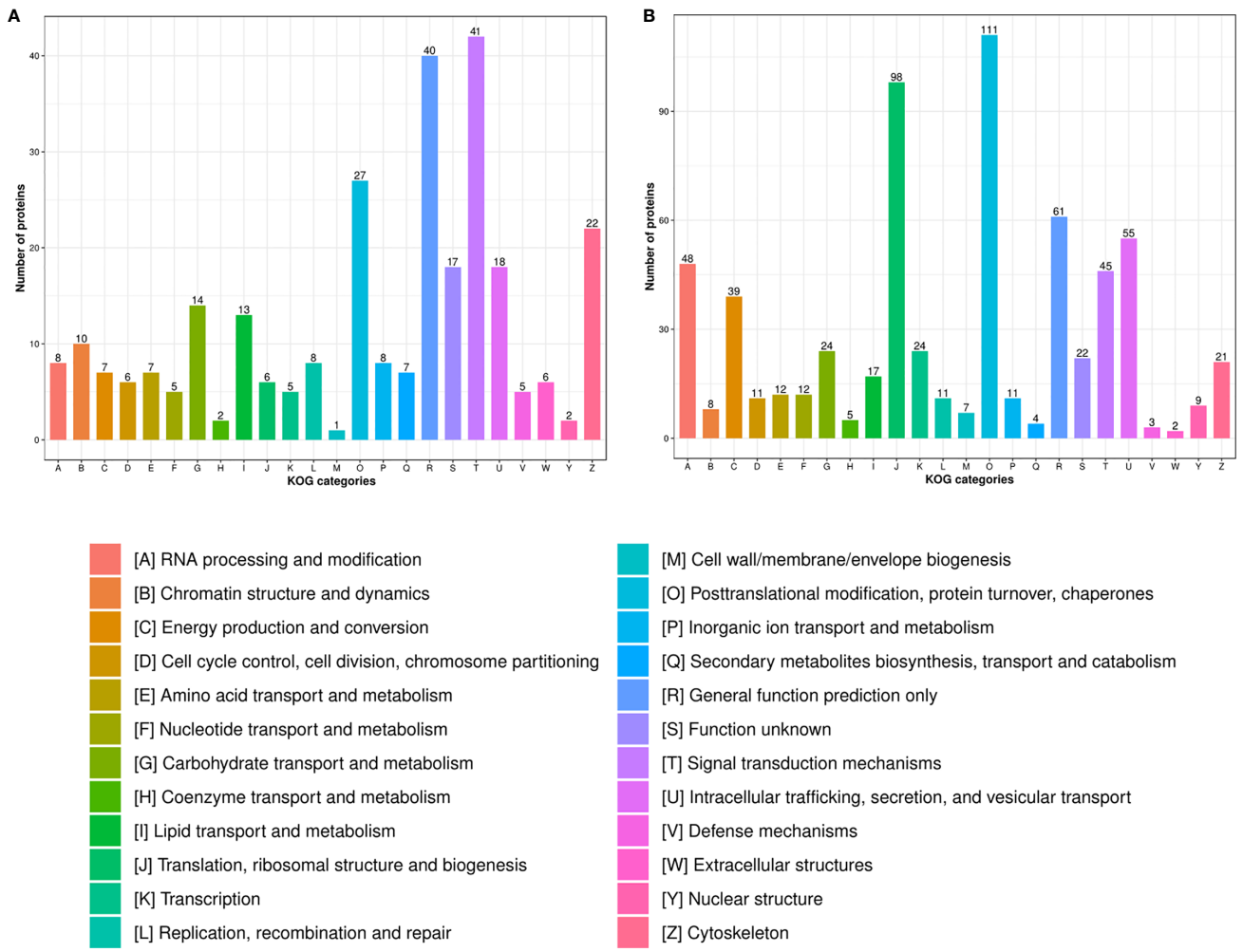

FIGURE 3 | COG analysis of DEPs in the **(A)** HT *vs*. H and **(B)** HT *vs*. T groups. X-axis represents COG/KOG categories. Y-axis represents the numbers of proteins.

We performed GO analysis to study biological changes in infected HFFs in the cellular component, biological process, and molecular function categories. Among the up- and downregulated proteins, 8, 14, and 8 terms in the cellular component, biological process, and molecular function categories were significantly enriched (**Figure 4** and **Supplementary Table S3**), respectively. In the biological process category, the enriched GO terms for immunity/defense-related functions were upregulated, such as regulation of lymphocyte-mediated immunity, regulation of leukocyte-mediated immunity, and regulation of adaptive immune response (**Figure 4A**), whereas those for cell metabolism-related functions were downregulated, such as oligosaccharide catabolic process, glycosaminoglycan catabolic process, and aminoglycan catabolic process (**Figure 4B**). According to KEGG pathway analysis, up- and downregulated proteins were significantly enriched in 19 and 25 pathways, respectively; some immune-related signaling pathways were present among them, such as the NF-κB signaling pathway, cAMP signaling pathway, cell adhesion molecules (CAMs), Rap1 signaling pathway, and calcium signaling pathway (**Figure 5A** and **Supplementary Table S4**).

Host Proteins Associated With the Immune Response Were Up- and Downregulated Upon Infection With *T. gondii*

T. gondii stimulated the host immune system. The expression of proteins associated with the immune response was upregulated, such as apoptotic proteins (Cytochrome c and TNF receptor-associated factor 1), adhesion proteins [intercellular adhesion molecule 1 (ICAM1), programmed cell death 1 ligand 1 (CD274), receptor-type tyrosine-protein phosphatase C (PTPRC), and integrin beta-2 (ITGB2)], and transcriptional regulatory factors (nuclear factor NF-kappa-B p100 subunit and prostaglandin G/H synthase 2). In contrast, the expression

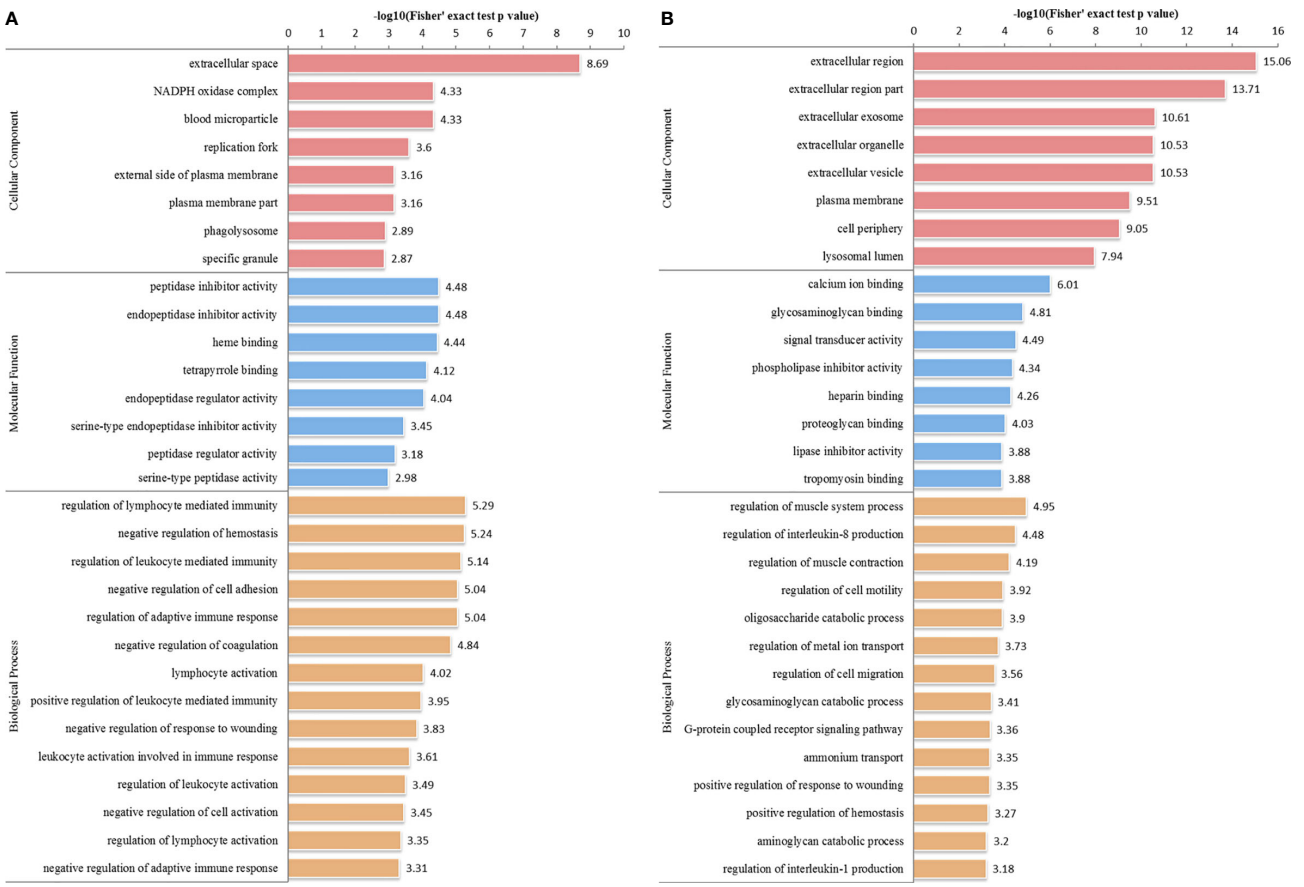

FIGURE 4 | GO distribution of DEPs in the HT *vs*. H group. The enriched GO terms under cellular component, biological process, and molecular function classifications for **(A)** upregulated and **(B)** downregulated proteins.

of ras-related proteins (rap-1b, m-ras, rab-32, and r-ras2) was downregulated (**Supplementary Table S2A**).

Signaling Pathways in Host Cells Were Altered During *T. gondii* Infection

In the HT *vs*. H group, all DEPs could be mapped to 91 pathways, of which 25 were significantly enriched (**Figure 6** and **Supplementary Table S4**). These significantly enriched pathways could be classified into cell metabolism and catabolism pathways (glycan degradation, phagosome and lysosome), immune-related signaling pathways (leukocyte transendothelial migration, calcium signaling pathway, Ras signaling pathway, cell adhesion molecules (CAMs), Rap1 signaling pathway, p53 signaling pathway, cAMP signaling pathway, complement and coagulation cascades) and signaling molecules and interaction pathways (cytokine-cytokine receptor interaction and ECM-receptor interaction).

Comparative Proteomics Analysis of *T. gondii* in the HT *vs*. T Group

In the HT *vs*. T group, the subcellular location of DEPs was as follows: the nucleus (26.56%), cytoplasm (22.02%), and mitochondria (16.47%; **Figure 2B**). All DEPs were divided into 24 categories by cluster of orthologous group analysis, and the top five categories with the most DEPs were posttranslational modification, protein turnover, chaperones [O]; translation, ribosomal structure, and biogenesis [J]; general function prediction only [R]; intracellular trafficking, secretion, and vesicular transport [U]; and RNA processing and modification [A] (**Figure 3B**).

In the HT *vs*. T group, 1099 DEPs were classified into eight terms of biological process, five terms of cellular component, and five terms of molecular function. Among the upregulated proteins, eight, six, and eight terms for cell component, biological process, and molecular function were significantly enriched, respectively, whereas the downregulated proteins were significantly enriched in one term of biological process and eight terms of molecular function (**Figure 7** and **Supplementary Table S5**). In the biological process category, cell biosynthesis and metabolism were upregulated on infection with *T. gondii*, including cellular protein metabolic, peptide metabolic, and peptide biosynthetic processes (**Figure 7A**). Further, KEGG pathway analysis of the HT *vs*. T group revealed that two significantly enriched pathways were

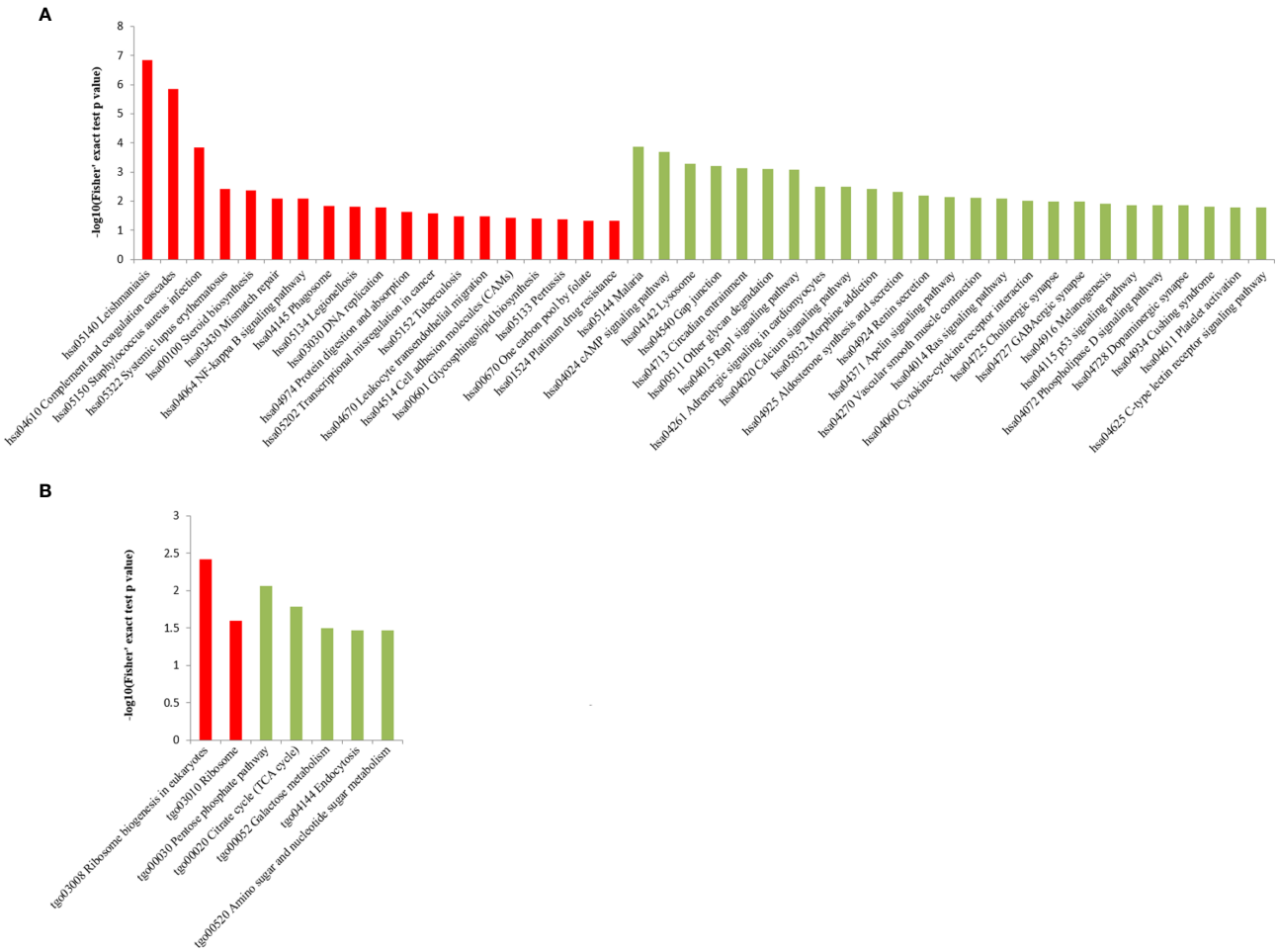

FIGURE 5 | KEGG pathway enrichment analysis of DEPs in the **(A)** HT *vs*. H and **(B)** HT *vs*. T groups. X-axis represents the significantly enriched KEGG pathways. Y-axis represents logarithmic corrected p values [–log10(p)].

upregulated, including ribosome and ribosome biogenesis in eukaryotes, and six enriched pathways were downregulated, including the pentose phosphate pathway, citrate cycle (TCA cycle), and galactose metabolism (**Figure 5B** and **Supplementary Table S6**).

T. gondii Infection Altered the Expression of Numerous Secretory Proteins

T. gondii invasion relies on the secretion of numerous secretory proteins, which mainly originate from three secretory organelles: micronemes, rhoptries, and dense granules. In the HT *vs*. T group, the expression of few secretory proteins was upregulated, such as MICs (MIC6, MIC10), ROPs (ROP5, ROP17), and GRAs (GRA4, GRA5, and GRA12). In contrast, the expression of dozens of known secretory proteins was significantly downregulated in *T. gondii*-infected HFFs (**Table 1**). As shown in **Table 1**, 10 SAG1-related sequence proteins (SRSs) exhibited a 0.67-fold or lower decrease in their expression levels in the HT *vs*. T group, whereas four SRSs exhibited a 1.5-fold or greater increase in their expression levels, including SRS29A (2.938 fold), SRS36D (1.919 fold), SRS57 (1.769 fold), and SRS20A (1.524 fold).

Validation of DEPs

We performed PRM assay for an independent comparison and validation of the results of TMT analysis. In the HT *vs*. H group, six proteins—desmoplakin protein (DSP), plexin-B2 (PLXNB2), annexin A1 (ANXA1), early endosome antigen 1 (EEA1), DNA topoisomerase 1 (TOP1), and probable ATP-dependent RNA helicase (DDX5)—were selected for analysis. As evident from **Table 2**, the results of PRM assay were consistent with those of TMT analysis. In case of the HT *vs*. T group, we selected MIC1, MIC2, ROP5, GRA12, and two heat shock proteins (HSP28 and HSP90) for PRM assay. The expression level of MIC1, MIC2, and HSP90 was downregulated and that of ROP5, GRA12, and HSP28 was upregulated, validating the consistency of results obtained using the two methods (**Table 2** and **Supplementary Table S7**).

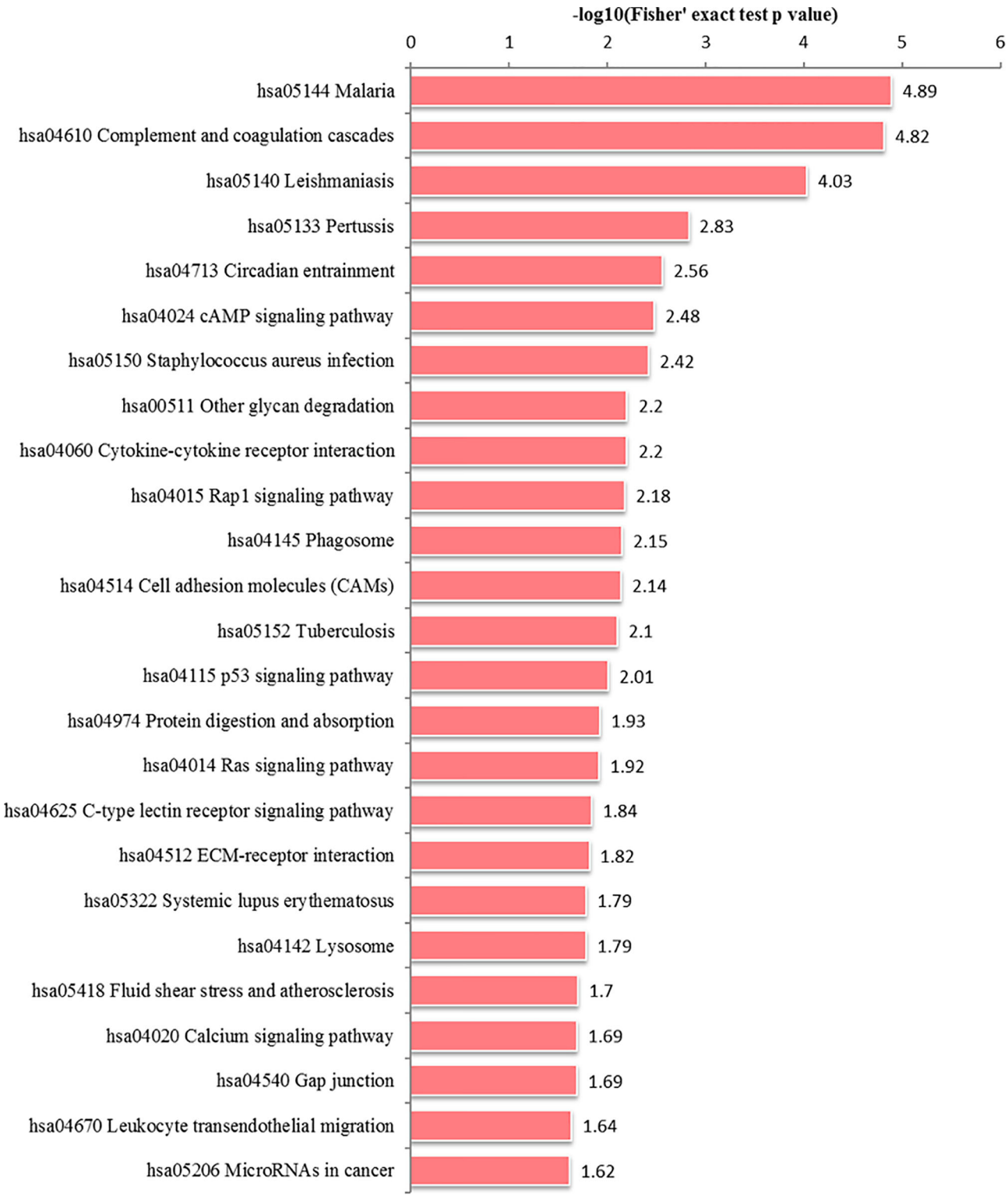

FIGURE 6 | Alteration of signaling pathways in host cells during *T. gondii* infection. X-axis represents significantly enriched KEGG pathways. Y-axis represents logarithmic corrected p values [–log10(p)].

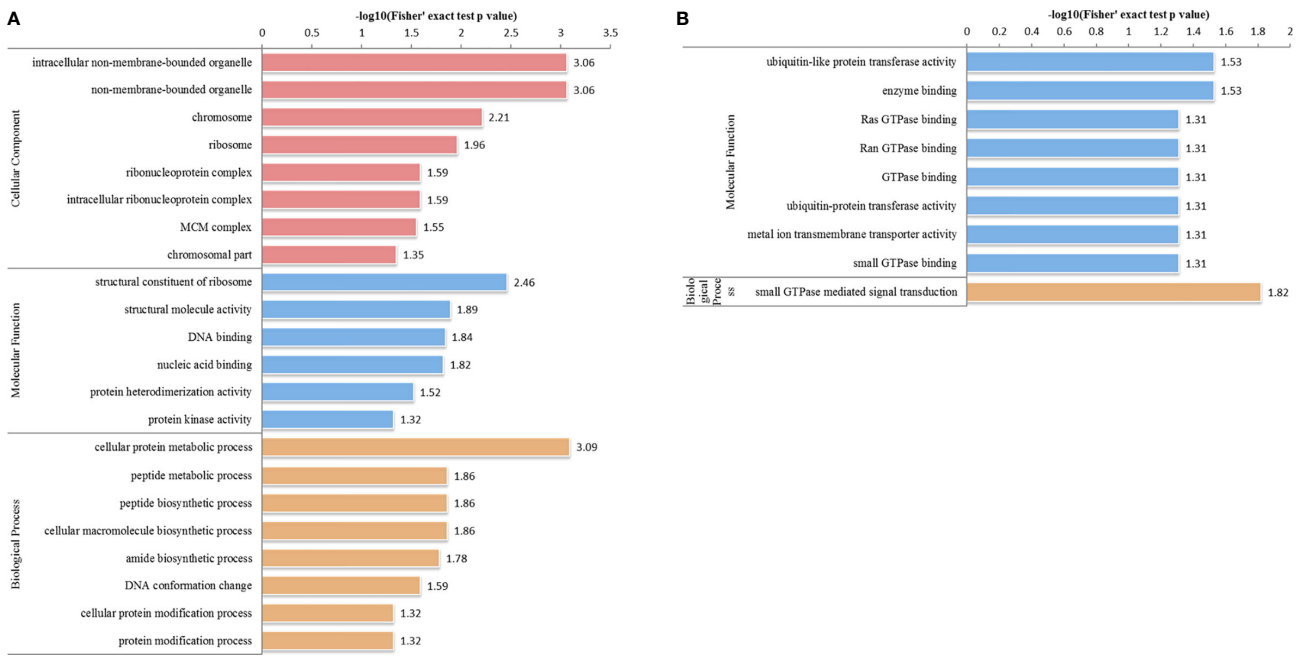

FIGURE 7 | GO analysis of DEPs in the HT *vs*. T group. The enriched GO terms under cellular component, biological process, and molecular function classifications for **(A)** upregulated and **(B)** downregulated proteins.

DISCUSSION

With technological advancements, mass spectrometry-based proteomic approaches have rapidly developed; compared to two-dimensional electrophoresis and difference gel electrophoresis, they are high throughput methods for protein identification and quantification. Nelson et al. (2008) identified 157 DEPs in HFFs before and after infection with *T. gondii* using two-dimensional electrophoresis and mass spectrometry, whereas we herein identified 1400 DEPs (adjusted p-value < 0.05 and >1.2 or <0.83 fold change, **Supplementary Table S1A**) in response to infection with *T. gondii* using TMT analysis. The host demonstrated global reprogramming of cell metabolism upon *T. gondii* invasion, as evident from the differential expression levels of proteins involved in key metabolic pathways (glycolysis, lipid and sterol metabolism, mitosis, apoptosis, and structural protein expression). In the present study, we focused on host cell signaling pathways related to cell survival, innate recognition of parasites, and immune response.

In the process of infecting hosts, parasites secrete various proteins to regulate the host signal transduction mechanism and modulate the immune response of host cells (Shapira et al., 2005; Muniz-Feliciano et al., 2013). In this study, immune-related signaling pathways were significantly enriched, such as the NF-κB, cAMP, Rap1, Ras, cytokine–cytokine receptor interaction, p53, platelet activation, and MAPK signaling pathways (**Supplementary Table S4**), suggesting that the innate and adaptive immunity of the host was majorly modulated in response to *T. gondii* infection.

As obligate intracellular parasites, *T. gondii* must traverse the host cell plasma membrane to initiate an infection and to ensure survival and replication. *T. gondii* invasion and infection establishment relies on secretory proteins, including MICs, ROPs, RONs, GRAs, and SRSs (Manger et al., 1998; Carey et al., 2000; Lekutis et al., 2001; Tomley and Soldati, 2001; Mercier et al., 2002; Bradley et al., 2005). The majority of MICs comprise multiple copies of a conserved adhesive domain, allowing them to bind to host cell surface receptors or carbohydrates during pathogen invasion. Carruthers and Tomley (2008) indicated that MIC secretion is rapidly upregulated when parasites make contact with host cells. We herein observed that the expression levels of MIC6 and MIC10 were upregulated, but those of most other MICs (MIC1–5, MIC7, MIC8, MIC11, MIC15, MIC17A, and M2AP) were downregulated; this could be because we analyzed the proteome at a single timepoint. In addition, the adhesion-related domains of MICs not only adhere to host cells but also promote complex formation between different MICs, making them more stable on the *T. gondii* surface and increasing its invasive capability. Multiple MICs have been previously reported, including MIC1–MIC4–MIC6, MIC2–M2AP, and MIC3–MIC8; all complex components were downregulated, except MIC6, in the current study. GRA6 can evidently activate the host transcription factor NFAT4, which promotes the synthesis of the chemokines Cxcl2 and Ccl2, and chemokines are known to control the spread of parasites (Ma et al., 2014; Hakimi et al., 2017). In this study, GRA6 expression level was downregulated after host cell invasion, consequently

TABLE 1 | Details pertaining to differentially expressed secretory proteins.

Protein accession	Protein description	Gene name	HT/T ratio	Regulated type	HT/T p value
B9Q4G2	SAG-related sequence SRS29A	BN1205_022090	2.938	Up	2.39E-06
B9PRE4	Microneme protein 10	BN1205_001390	2.008	Up	3.79E-05
V5BF07	SAG-related sequence SRS36D	TGVEG_292280	1.919	Up	3.80E-05
B6KB88	Rhoptry kinase family protein ROP17	BN1205_075890	1.781	Up	3.40E-03
A0A0F7V6G5	SAG-related sequence SRS57	BN1205_069910	1.769	Up	5.64E-04
B9QLZ0	Microneme protein MIC6	BN1205_071220	1.752	Up	8.44E-03
V4Z6U4	Rhoptry protein ROP5	TGVEG_308090	1.59	Up	4.40E-02
B9QGZ4	SAG-related sequence SRS20A	BN1205_033260	1.524	Up	3.61E-04
A0A125YSX9	Rhoptry neck protein RON5	TGVEG_311470	0.658	Down	2.22E-06
B9PUW8	Microneme protein MIC11	BN1205_044690	0.65	Down	5.41E-04
V5B190	Rhoptry kinase family protein ROP26	TGVEG_211260	0.636	Down	2.31E-05
A0A125YX19	Microneme protein MIC7	TGVEG_261780	0.629	Down	4.30E-05
A0A125YRV5	Rhoptry neck protein RON8	TGVEG_306060	0.585	Down	1.04E-04
V5BDH5	SAG-related sequence SRS38B	BN1205_085760	0.585	Down	1.93E-05
V4Z883	Microneme protein, putative	BN1205_055460	0.568	Down	1.24E-03
B9QHX6	Rhoptry protein ROP15	BN1205_009170	0.538	Down	5.86E-05
A0A0F7UVV5	Rhoptry kinase family protein ROP16	BN1205_071990	0.534	Down	5.79E-05
V4ZLN9	SAG-related sequence SRS30A	TGVEG_273130	0.528	Down	1.82E-04
A0A0F7UZH2	Microneme protein 2	BN1205_047640	0.522	Down	6.22E-07
B6KK34	SAG-related sequence SRS29C	BN1205_022060	0.516	Down	5.71E-05
A0A125YGR1	Rhoptry kinase family protein ROP40	TGVEG_291960	0.5	Down	2.28E-05
B6KB96	Rhoptry protein 6	BN1205_075810	0.494	Down	6.50E-05
B9QR15	Rhoptry kinase family protein ROP35	BN1205_041270	0.489	Down	3.14E-03
B9Q846	SAG-related sequence SRS67	BN1205_067870	0.48	Down	5.86E-07
V4YW47	Rhoptry neck protein RON3	BN1205_064400	0.477	Down	5.25E-04
B6KL08	Microneme protein MIC1	BN1205_084920	0.476	Down	5.59E-04
B9QFH2	Rhoptry protein ROP12	BN1205_045220	0.466	Down	2.01E-05
B9PXJ3	Microneme protein MIC4	BN1205_040160	0.453	Down	3.63E-05
A0A0F7V1Z5	SAG-related sequence SRS34A	BN1205_018330	0.421	Down	1.84E-05
V4Z9F7	Rhoptry kinase family protein ROP39	BN1205_072800	0.414	Down	3.19E-04
A0A125YXY8	Putative rhoptry protein	TGVEG_315210	0.403	Down	5.65E-04
V4Z8E3	Microneme protein MIC17A	TGVEG_200250	0.389	Down	1.66E-05
A0A125YHB4	Microneme protein MIC5	TGVEG_277080	0.381	Down	3.89E-05
V4YND8	Microneme protein MIC3	TGVEG_319560	0.379	Down	4.41E-06
B9Q6P5	SAG-related sequence SRS43	BN1205_062310	0.369	Down	1.54E-05
A0A0F7V8G6	Rhoptry protein ROP13	BN1205_096370	0.354	Down	1.89E-05
B6KDK1	SAG-related sequence SRS30C	BN1205_020430	0.326	Down	3.85E-05
B9QEX1	SAG-related sequence SRS44	BN1205_081610	0.314	Down	1.54E-05
B9PSP5	SAG-related sequence SRS25	BN1205_035070	0.302	Down	4.98E-07
V4ZDA0	Rhoptry protein ROP18	TGVEG_205250	0.301	Down	1.78E-04
B6K9I7	SAG-related sequence SRS52A	BN1205_100430	0.285	Down	1.01E-04
A0A0F7VCB9	Microneme protein 8	BN1205_006390	0.231	Down	1.64E-05
V5BFN0	Microneme protein MIC15	BN1205_004820	0.174	Down	2.93E-06

facilitating *T. gondii* survival. It has been reported that many SRSs are stage-specific; they play a key role in mediating the attachment to host cells and activate host immunity to regulate *T. gondii* virulence (Jung et al., 2004). For example, SRS57 serves as an adhesion protein by binding to the host cell surface, SRS29B is a virulence factor, and SRS34A and SRS16B play a crucial role in host cell immune modulation (Dzierszinski et al., 2000; Van et al., 2007; Tomita et al., 2018). In the HT group, the expression level of many SRS proteins, including, SRS29A, SRS36D, SRS57, and SRS20A, was upregulated and that of others (SRS38B, SRS30A, SRS29C, SRS67, SRS34A, SRS43, SRS30C, SRS44, SRS25, and SRS52A) was downregulated in comparison with the expression levels in the T group. So far, few studies exist on SRSs, and the understanding of their various biological functions needs to be enhanced in future.

Parasite invasion is an active process that relies on the cytoskeleton for motility and also on the host cytoskeleton (Dobrowolski and Sibley, 1996). Host microtubules have been observed to play a role in interactions with invading *T. gondii* tachyzoites, and the host cytoskeleton apparently contributes to the anchoring of the moving junction (Sweeney et al., 2010; Takemae et al., 2013). Microtubules are composed of heterodimers of alpha- and beta-tubulin. Takemae et al. (2013) reported that TUBB2C and TUBB5 interact with TgRON4. In our study, TUBB2C and TUBB5 were not identified as DEPs in the HT *vs.* H group, but other alpha- and beta-tubulin proteins were detected, including TUBA1C (0.625 fold), TUBA4A (0.595 fold), TUBB2A (0.801 fold), TUBB3 (0.688 fold), TUBB4B (0.646 fold), and TUBB6 (0.753 fold) (**Supplementary Table S1A**), indicating that infection with *T. gondii* causes the remodeling of the host cytoskeleton.

TABLE 2 | Comparison of the quantification results of TMT analysis and PRM assay of candidate DEPs in the HT *vs*. H and HT *vs*. T groups.

Protein accession	Protein gene	Protein description	Peptide sequence	PRM ratio	p value	TMT ratio	p value
O15031	PLXNB2	Plexin-B2	LVECGSLFK	0.50	2.48E-04	0.61	2.49E-06
P04083	ANXA1	Annexin A1	GVDEATIIDILTK	0.54	3.36E-05	0.62	1.89E-05
Q15075	EEA1	Early endosome antigen 1	EQALQDLQQQR	0.58	1.54E-04	0.67	1.72E-05
P11387	TOP1	DNA topoisomerase 1	ELTAPDENIPAK	1.49	4.80E-03	1.60	1.73E-06
P17844	DDX5	Probable ATP-dependent RNA helicase DDX5	NFYQEHPDLAR DWVLNEFK	1.80	2.66E-06	1.51	2.18E-06
P15924	DSP	Desmoplakin	LPVDIAYK	1.19	5.71E-02	1.59	1.78E-05
A0A0F7V6Y1	HSP90	Heat shock protein 90	EDQTEYLEDR	0.57	3.24E-04	0.64	9.44E-04
A0A0F7UZH2	MIC2	Microneme protein 2	NPWNEDQQHGGLSCEQQHPGGR	0.65	4.60E-03	0.52	2.28E-05
B6KL08	MIC1	Microneme protein MIC1	HYTEEEGIR	0.57	7.91E-04	0.48	1.84E-05
V4Z6U4	ROP5	Rhoptry protein ROP5	EEELIGYCR	1.88	6.89E-04	1.59	4.40E-02
B9PSE4	HSP28	Heat shock protein 28	DHGEEDFVR	48.02	1.01E-07	2.96	2.34E-02
B9QDT9	GRA12	Dense granule protein GRA12	AATVAAGNELFK ASETGSGLAASFLNTVEVR	11.02	1.53E-05	1.59	2.74E-03

CAMs are distributed throughout regions of the plasma membrane and come in contact with other cells and extracellular matrix (Figliuolo da Paz et al., 2019). Herein the pathways of CAMs were significantly enriched and included neuronal growth regulator 1, ICAM1, syndecan-2, CD274, neural cell adhesion molecule L1, PTPRC, junctional adhesion molecule C, and ITGB2. ICAM1 is expressed on various cell types and participates in diverse cellular processes, including host–pathogen interactions (Bhalla et al., 2015). ICAM1 has been reported to interact with PfEMP1 in *Plasmodium falciparum* infection (Lennartz et al., 2019). Further, ICAM1 expression level has been noted to be upregulated upon host cell invasion by *T. gondii in vitro* and also during human toxoplasma uveitis *in vivo*, with ICAM1 interacting with TgMIC2 (Klok et al., 1999; Nagineni et al., 2000; Barragan et al., 2005). In this study, compared with uninfected HFFs, ICAM1 expression in infected cells was upregulated (1.96 fold). Blader et al. (2001) demonstrated that the ICAM1 gene was upregulated by at least 2-fold in HFFs after *T. gondii* invasion, and Nagineni et al. (2000) reported that ICAM1 secretion in human retinal pigment epithelial cells infected with *T. gondii* was augmented 5-fold over the uninfected control, and no significant changes in ICAM1 levels were found in human retinal pigment epithelial cells incubated with heat-killed *T. gondii*. These findings suggest that ICAM1 expression is derived from the interaction between host cells and the parasite.

Based on their roles in host–pathogen interaction, Spear et al. (2006) clustered host genes into three distinct classes: "pro-host", host genes required for host defense; "pro-parasite", host genes required for parasite growth; and "bystander", host genes incidentally regulated as a consequence of modulating the first two classes. In present study, we performed TMT-based proteomic analysis of *T. gondii* as well as host cells at 24 h of infection. The infection induced significant changes in the proteome of *T. gondii* and also led to the significant enrichment of immune-related signaling pathways in HFFs. Various DEPs, including cytoskeleton and immune-related proteins, were induced in response to host cell invasion, indicating that infection with *T. gondii* leads to considerable remodeling of the host cytoskeleton and also the crucial role played by the host immune system upon encountering *T. gondii*. Future studies are warranted to validate these proteins and to assess their clinical value.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

HS and JC designed this study and made the final revision. HS, JL and LW performed the experiments. KY, CX, GL and TX helped to create the tables and figures. BH and QW contributed to the reagents and materials. MG supervised the process. HS wrote the draft manuscript and JC revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Road Less Traveled? Unconventional Protein Secretion at Parasite–Host Interfaces

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Protein secretion in eukaryotic cells is a well-studied process, which has been known for decades and is dealt with by any standard cell biology textbook. However, over the past 20 years, several studies led to the realization that protein secretion as a process might not be as uniform among different cargos as once thought. While in classic canonical secretion proteins carry a signal sequence, the secretory or surface proteome of several organisms demonstrated a lack of such signals in several secreted proteins. Other proteins were found to indeed carry a leader sequence, but simply circumvent the Golgi apparatus, which in canonical secretion is generally responsible for the modification and sorting of secretory proteins after their passage through the endoplasmic reticulum (ER). These alternative mechanisms of protein translocation to, or across, the plasma membrane were collectively termed "unconventional protein secretion" (UPS). To date, many research groups have studied UPS in their respective model organism of choice, with surprising reports on the proportion of unconventionally secreted proteins and their crucial roles for the cell and survival of the organism. Involved in processes such as immune responses and cell proliferation, and including far more different cargo proteins in different organisms than anyone had expected, unconventional secretion does not seem so unconventional after all. Alongside mammalian cells, much work on this topic has been done on protist parasites, including genera *Leishmania*, *Trypanosoma*, *Plasmodium*, *Trichomonas*, *Giardia*, and *Entamoeba*. Studies on protein secretion have mainly focused on parasite-derived virulence factors as a main source of pathogenicity for hosts. Given their need to secrete a variety of substrates, which may not be compatible with canonical secretion pathways, the study of mechanisms for alternative secretion pathways is particularly interesting in protist parasites. In this review, we provide an overview on the current status of knowledge on UPS in parasitic protists preceded by a brief overview of UPS in the mammalian cell model with a focus on IL-1β and FGF-2 as paradigmatic UPS substrates.

Keywords: unconventional secretion, protist parasites, host–pathogen interface, moonlighting, glycolysis

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INTRODUCTION

A protein destined to be secreted carries a signal peptide or signal leader sequence and is often transported from the ER to the Golgi apparatus, to the trans-Golgi network for sorting, and finally to the plasma membrane via vesicular carriers (Palade, 1975; Rothman, 1994). This is a classic dogma of the cell biology of secretory protein trafficking and remains true for many proteins

leaving the intracellular space. However, more and more leaderless proteins are identified in the secretomes and surface proteomes of various species. These proteins reach the plasma membrane while bypassing classical secretory routes. Furthermore, some proteins with leader sequences were discovered to pass through the ER but to bypass the Golgi apparatus on their way to the plasma membrane. These non-canonical secretion pathways are collectively summarized under the term "unconventional protein secretion" (UPS) to distinguish them from classic canonical secretion following the ER–Golgi pathway (Rabouille et al., 2012).

Currently, there are mainly four described eukaryotic UPS pathways (**Figure 1** and **Table 1**). UPS type I is probably the best characterized and was mainly elucidated using fibroblast growth factor 2 (FGF2) as its model substrate (Wegehingel et al., 2008; La Venuta et al., 2015; Steringer et al., 2015; Legrand et al., 2020). In this secretion pathway, the leaderless substrate is translocated to the extracellular space with self-sustained formation of pores in the plasma membrane. The second described UPS pathway, type II, is mediated by ABC transporters, which transport leaderless acylated proteins across the plasma membrane. Although there are limited reports investigating the mechanisms of this pathway in-depth, several eukaryotes appear to employ this mode of secretion [mammals (Flieger et al., 2003), *Drosophila* (Ricardo and Lehmann, 2009), yeast (McGrath and Varshavsky, 1989), *Leishmania* (Denny et al., 2000; Stegmayer et al., 2005; MacLean et al., 2012), and *Plasmodium* (Möskes et al., 2004)]. This suggests that ABC transporter-mediated non-conventional secretion is conserved among eukaryotes (Ricardo and Lehmann, 2009). UPS type III in mammalian cells involves intracellular vesicular structures induced upon starvation (Bruns et al., 2011; Malhotra, 2013; Cruz-Garcia et al., 2020). As shown for IL-1β (Rubartelli et al., 1990; Andrei et al., 2004; Qu et al., 2007), AcbA in *Dictyostelium discoideum* (Kinseth et al., 2007; Cabral et al., 2010) and its yeast ortholog Acb1 (Duran et al., 2010; Manjithaya et al., 2010; Curwin et al., 2016) compartments (CUPS compartments for UPS, endosomes or exosome-like compartments) transport leaderless cargo proteins across the plasma membrane (Nickel and Rabouille, 2009; Curwin et al., 2016). UPS route type IV is the only known UPS pathway, which includes substrates presenting a leader sequence. These substrates are trafficked through the ER, but bypass the Golgi on their way to the plasma membrane (Gee et al., 2018; Kim et al., 2018). Even though not much is known about the machinery mediating the Golgi bypass, the best studied substrate of this pathway is the cystic fibrosis transmembrane conductance regulator (CFTR; Yoo et al., 2002; Gee et al., 2011; Rabouille et al., 2012). CFTR travels via the so-called pericentrosomal intermediate compartment, where it can reach recycling endosomes and is then transported to the plasma membrane (Marie et al., 2009; Prydz et al., 2012; Rabouille et al., 2012). Although the current UPS landscape appears complex, it is probably that more UPS routes remain to be discovered. Furthermore, it is also probable that the same substrate can be transported over several different routes, depending on cellular conditions.

With more and more published secretomes and surface proteomes and therein found leaderless proteins, the interest in studies on UPS is increasing. Involved in the most central mechanisms of disease such as inflammation and cancer cell proliferation and, specifically for this review, secretion of virulence factors in parasites, it is of crucial importance

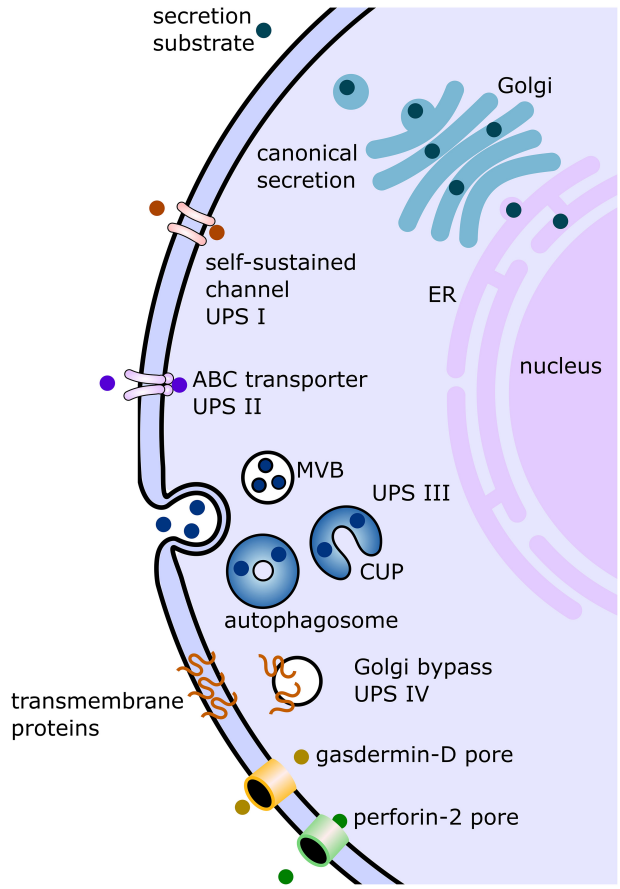

FIGURE 1 | Displayed are the canonical conventional as well as unconventional secretion pathways described in studies on mammalian cells and/or studies on protist parasites. In the conventional canonical secretion pathway, the secreted substrate passes through the endoplasmic reticulum (ER) to the Golgi apparatus (Golgi) and then to the plasma membrane via vesicular carriers. Several non-canonical pathways have been discovered and summarized under the term "unconventional protein secretion" (UPS). There are four pathway types formally described. UPS pathway I (UPS I) consists of substrates without a canonical signal sequence, transported via self-sustained channels. The best characterized substrate of this pathway is fibroblast growth factor. UPS route II is mediated by ABC transporters, as in the secretion of the leaderless Leishmania HASPB. UPS pathway III (UPS III) was mainly studied in mammalian cells under starvation conditions. In this pathway, leaderless substrates such as IL-1b are transported across the plasma membrane involving vesicular compartments for UPS (CUPS) which turn into autophagosomes. These autophagosomes then either fuse with multivesicular bodies (MVB) or fuse directly with the plasma membrane. The fourth described pathway (UPS IV) involves transmembrane proteins sequence for secretion, but the substrate (for example, cystic fibrosis transmembrane conductance regulator) bypasses the Golgi apparatus on the way from the ER to the plasma membrane. The two additional unconventional secretion pathways depicted in this figure involve the formation of pores as in UPS I, but these pores are not self-sustained and involve gasdermin-D (in case of IL-1b secretion) or perforin-2 facilitation (in case of IL-33).

TABLE 1 | Overview of UPS pathways including information on known machineries or components and relevant cargo examples.

UPS route type	Signal sequence for secretion	Known machineries/components	Examples of known cargo	Selected references
Type I	No	Self-sustained channels (binding to α1-subunit of Na,K-ATPase and $PI(4,5)P_2$)	FGF2, HIV-TAT	Temmerman et al., 2008; Zeitler et al., 2015; Legrand et al., 2020
Type II	No	ABC- transporters, N-terminal domain	HASPB, PfAK2	Denny et al., 2000; Stegmayer et al., 2005; MacLean et al., 2012; Thavayogarajah et al., 2015; Kim et al., 2018
Type III	No	Vesicles, inflammasome, heat shock protein, GRASP, ESCRTs	**IL-1β**, Acb1, HMGB1	Nickel and Rabouille, 2009; Dupont et al., 2011; Curwin et al., 2016; Cruz-Garcia et al., 2020; Kim et al., 2020
Type IV	Yes	Golgi bypass via pericentrosomal intermediate compartment, transmembrane domain	CFTR	Rabouille et al., 2012; Gee et al., 2018; Kim et al., 2018
Pore formation	No	Gasdermin D, perforin-2	**IL-1β**, IL-33	Martín-Sánchez et al., 2016; Heilig et al., 2017; Lieberman et al., 2019; Hung et al., 2020

IL-1β is highlighted to emphasize its known use of two different UPS pathways. FGF2, fibroblast growth factor 2; HASPB, hydrophilic acylated surface protein B; GRASP, Golgi reassembly stacking protein; $PI(4,5)P_2$, phosphatidylinositol-4,5-bisphosphate; CFTR cystic fibrosis transmembrane conductance regulator; PfAK2, Plasmodium falciparum adenylate kinase 2; ESCRT, endosomal sorting complex required for transport.

to understand the underlying mechanisms of unconventional secretion. Unraveling machineries involved in UPS will severely impact translational research and the development of therapeutic strategies in associated diseases, which are often caused by aberrant UPS-related processes (Kim et al., 2018).

In this short review, we will highlight the similarities and differences in UPS pathways and substrates in protist parasites and mammalian cells. Our objective is to provide the reader with a solid overview of developments in this fast growing, but still mostly underexplored, field of research.

UNCONVENTIONAL PROTEIN SECRETION IN MAMMALIAN CELLS

Secretion of Interleukin IL-1β

The roots of UPS research lie over 30 years back in time, in studies carried out primarily on mammalian cells. When the human cytokine interleukin 1 was sequenced, it was found to lack a leader sequence allowing this secreted molecule to enter the ER–Golgi pathway for secretion (Auron et al., 1984). The non-canonical secretion of cytokines has been studied since, often using interleukin 1 beta (IL-1β) as a model substrate (Rubartelli et al., 1990; Andrei et al., 2004; Daniels and Brough, 2017). IL-1β is a crucial cytokine in immune reactions and has been associated with many different states of disease, infection, and injury (Dinarello, 1996; Lopez-Castejon and Brough, 2011). The precursor form pre-IL-1β is converted to the active cytokine *via* the inflammasome apparatus (Dupont et al., 2011; Lamkanfi, 2011; Lamkanfi and Dixit, 2012). Although much effort has been invested in elucidating secretion mechanisms for IL-1β, they are not yet fully understood (Lopez-Castejon and Brough, 2011).

Several different pathways were suggested by which active IL-1β is translocated across the cell membrane. The release of IL-1β is induced by a variety of secretion stimuli: pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs; Takeuchi and Akira, 2010; Lopez-Castejon and Brough, 2011), depending on cell type and stimulus (Lopez-Castejon and Brough, 2011; Kim et al., 2018). One mechanism involves vesicular carriers as part of a UPS type III route for IL-1β release (Rabouille et al., 2012). As described in Dupont et al. (2011), IL-1β is captured by the autophagy-related process of sequestration of cytosol into a vesicular organelle called the autophagosome, which transports IL-1β to the plasma membrane for secretion. Some studies suggest that instead of directly fusing with the plasma membrane, the autophagosome fuses with a multivesicular body (MVB) to form an "amphisome" before fusing with the plasma membrane (Zhang et al., 2015). Even though not completely elucidated, the availability of Golgi reassembly stacking protein (GRASP) seems to be of great importance for successful translocation of IL-1β, since the knockdown of GRASP leads to strongly decreased secretion (Nickel and Rabouille, 2009; Dupont et al., 2011). Induction of autophagy appears to enhance inflammasome activity and, therefore, the production of active IL-1β. This suggests a strong link between autophagy and cytokine secretion, which was shown to influence severity of inflammation (Dupont et al., 2011). Zhang et al. (2015) investigated the translocation process of IL-1β into the autophagosomal compartment for UPS (CUPS) and found a strong dependency on a heat shock protein (HSP90) and GRASP proteins (Zhang et al., 2015). Additionally, Zhang et al. (2015) suggested in the same study that mature IL-1β targets a vesicular structure, which precedes sequestration into the autophagosome. This preceding step was now studied in greater detail (Zhang et al., 2020). This study suggests that the described vesicular structure corresponds to the ER–Golgi intermediate compartment (ERGIC), and IL-1β is transported into this compartment via a TMED10-mediated protein channel (Zhang et al., 2020).

Similar to IL-1β, AcbA (acylcoenzyme A-binding protein) and its yeast ortholog Acb1 have been shown to traffic along the UPS type III route (Kinseth et al., 2007; Cabral et al., 2010; Duran et al., 2010; Manjithaya et al., 2010; Curwin

et al., 2016). In yeast, Acb1 was found to be secreted during starvation of the cell, despite the lack of a signal sequence for secretion (Duran et al., 2010; Curwin et al., 2016). The machinery involving transport of the cargo via CUPS requires ESCRT proteins I, II, and III for successful secretion of Acb1 (Duran et al., 2010; Curwin et al., 2016). As ESCRT proteins are necessary to build up an MVB, it was hypothesized that UPS III involves secretion via MVBs (Duran et al., 2010). The results of an in-depth study by Curwin et al. (2016) found that even though certain components for the formation of a classic MVB are present, a part of the necessary machinery is lacking (ESCRT-0 or Vps4 proteins; Curwin et al., 2016). The authors suggest that instead of canonical MVBs, a specific doubled membrane-bounded multivesicular compartment is involved in Acb1 secretion (Curwin et al., 2016). They speculate that this compartment might be the functional ortholog of the amphisomes in IL-1β secretion (Zhang et al., 2015; Curwin et al., 2016). Studies on AcbA in *Dictyostelium* found that secretion involves cortical vesicles, which fuse with the surface membrane, or exocytosis of the vesicles from MVBs in a GRASP protein-dependent fashion (Kinseth et al., 2007; Cabral et al., 2010).

As Kim et al. (2020), showed in a recent study, the secretion of high-mobility group box 1 protein (HMGB1) represents another cargo of UPS pathway III. This protein, which is usually found in the nucleus where it acts as a DNA chaperone, has the function of a danger-associated molecular pattern when transported to the extracellular space and induces inflammation and cell migration (Andersson and Tracey, 2011). For its transport across the plasma membrane, a similar vesicular trafficking machinery is involved as has been found in IL-1b, including GRASP (GORASP2/GRASP55) and heat shock protein (HSP90AA1; Dupont et al., 2011; Zhang et al., 2015; Kim et al., 2020).

Another mode of IL-1β secretion does not involve vesicular carriers but the formation of pores in the plasma membrane (Martín-Sánchez et al., 2016; Heilig et al., 2017). IL-1β is also secreted by pyroptotic cells about to undergo inflammasome-controlled programmed cell death (Fink and Cookson, 2005; Lamkanfi and Dixit, 2012). The secretion of IL-1β by macrophage cells, seems to depend strongly on permeabilization of the plasma membrane (Martín-Sánchez et al., 2016). Gasdermin D has long been known as a regulator of pyroptosis, forming pores in the plasma membrane of a cell to induce programmed cell death (Ding et al., 2016). It was found that IL-1β secretion strongly depends on gasdermin Ds' pore-formation ability (He et al., 2015; Martín-Sánchez et al., 2016). However, whether IL-1β is secreted passively due to cell death, or actively by living cells, is a question that was only recently answered (Evavold et al., 2018; Kuriakose and Kanneganti, 2018; Lieberman et al., 2019). In a study by Evavold et al. (2018), gasdermin D pores were shown to be necessary for IL-1β transport across an intact plasma membrane of living macrophages. This suggests that gasdermin D is not only responsible for passive interleukin secretion during pyroptosis but also for its active transport in living cells (Heilig et al., 2017; Evavold et al., 2018; Lieberman et al., 2019). Even though this mode of secretion via pores has similarities with the UPS type I route, which was mainly studied in FGF2 (elaborated on in the section "Translocation and Binding of Fibroblast Growth Factor 2"), neither pro-IL-1β nor mature IL-1β was found to bind to membranes (Martín-Sánchez et al., 2016); hence, the transport is not mediated by the substrate itself. In this respect, this route does not fully match the description of UPS route type I and rather represents a separate UPS route. It is very likely that also other cargos employ more than one route of unconventional secretion or show secretion mechanisms, which do not fit in the previously described four routes of unconventional secretion as for IL-1β. Elucidating the mechanisms of non-canonical secretion of IL-1β boosts translational research on this molecule, which has shown to have not only positive effects as a mediator in immune response but, if found in elevated levels, can also have severe negative effects in conditions such as diabetes type II (Maedler et al., 2009).

Importantly, interleukin secretion in mammalian cells also plays a crucial role in the innate immune reaction against parasitic infections (Ryan et al., 2020). Interleukin 33 (IL-33) does not have a signal sequence, while it is still found to be secreted early on when a parasite is invading, involved in priming and/or modulating adaptive immune response of the host (Cayrol and Girard, 2018; Ryan et al., 2020). Similar to one of the pathways for IL-1β, IL-33 secretion has been brought into connection with cell death, while for both interleukins, cell death seems not to be a strict requirement for their release (Kouzaki et al., 2011; Chen et al., 2015; Molofsky et al., 2015; Daniels and Brough, 2017; Ryan et al., 2020). As Hung et al. (2020) could show in a recent study, in mouse dendritic cells, IL-33 export was facilitated by perforin-2, which forms pores in the plasma membrane, similar to gasdermin D.

Interleukin 33 has been shown to have a significant impact on different T cells, influencing their differentiation, behavior, and physiology (Alvarez et al., 2019; Ryan et al., 2020). This makes this interleukin an attractive potential target for translational research as an immunotherapy treatment in toxoplasmosis, malaria, and helminth infections (Jones et al., 2010; Yasuoka et al., 2011; Fairlie-Clarke et al., 2018; Ryan et al., 2020).

Translocation and Binding of Fibroblast Growth Factor 2

Fibroblast growth factor 2 (FGF2) is the substrate that quickly became the most studied UPS cargo in mammalian cells. The mechanisms by which it is secreted are by far the most elucidated and have been allocated to UPS route type I. FGF2 is an angiogenic mitogen with a wide variety of functions, from cell growth and tissue repair to differentiation of the nervous system. While its extracellular form shows pro-angiogenic and pro-inflammatory activity, FGF2 acts as a mitogenic stimulus when localized in the nucleus (Bikfalvi et al., 1997; Jeffery, 1999; Collingridge et al., 2010; Gómez-Arreaza et al., 2014; Yoon et al., 2018), thus, making FGF-2 an example of "moonlighting" protein, a protein with multiple functions (Jeffery, 2018). FGF2 was shown to be secreted directly and independently of ATP (Schäfer et al., 2004). The current state of knowledge concerning FGF2 secretion involves recruitment from the cytosol to the inner leaflet of the plasma membrane where it binds to

the α1-subunit of Na,K-ATPase and phosphatidylinositol-4,5-bisphosphate [PI(4,5)P_2] (Temmerman et al., 2008; Legrand et al., 2020). As Legrand et al. (2020) hypothesized in a recent study (Legrand et al., 2020), the α1-subunit of the Na,K-ATPase would accumulate FGF2 at the inner plasma membrane leaflet and, thus, facilitate PI(4,5)P_2-dependent secretion to the cell surface. When reaching the extracellular space, FGF2 seems to bind immediately to cell surface heparan sulfate proteoglycans (HSPGs), which are anchored in the plasma membrane and trap secreted FGF2 (Zehe et al., 2006; Wegehingel et al., 2008). The binding of FGF2 to HSPG serves a storage function as well as the protection of FGF2 against degradation and denaturation (Flaumenhaft et al., 1990; Coltrini et al., 1993). As Wegehingel et al. (2008) could show, FGF-2 is no longer functional if it is modified with a leader sequence to enter the canonical secretion pathway. The addition of a leader sequence leads to posttranslational modifications, which prevent FGF-2 from binding to heparan sulfate proteoglycans and, therefore, inhibits storage and signal transduction (Wegehingel et al., 2008). Secretion of HIV-TAT, an HIV-1 protein, which acts extracellularly as a viral toxin, has been suggested to use the same UPS core mechanism as FGF-2 (Zeitler et al., 2015). Here, too, oligomerization involving PI(4,5)P_2, led to the formation of plasma membrane pores through which HIV-TAT reached the extracellular space (Zeitler et al., 2015).

Significance of Unconventional Protein Secretion in Mammalian Cells

With IL-1β and FGF2 being the most studied, there are many other substrates in mammalian cells that were identified to be secreted unconventionally (Nickel, 2005; Kim et al., 2018). Galectins and annexins (Hughes, 1999; Popa et al., 2018), other interleukins such as IL-1α and IL-33 (Daniels and Brough, 2017), transmembrane proteins CFTR and Mpl (Gee et al., 2011; Cleyrat et al., 2014; Kim et al., 2016, 2018; Noh et al., 2018), and the multifunctional glycolytic enzyme GAPDH, which was found to be associated with exosomes (Théry et al., 2009; Mathivanan et al., 2010; Chauhan et al., 2015), represent a few examples. Studies performed on neuronal cell lines show that hundreds of neuronal surface proteins reach the plasma membrane in a UPS pathway bypassing the Golgi apparatus (UPS type IV; Hanus et al., 2016). All these cases in various cell types emphasize the significance of UPS in mammalian cell biology. The mechanisms by which these substrates are secreted are only partly understood. In mammalian cell model systems, mechanisms for all described UPS routes are suggested, depending on the cargo and secreting cell type (Evavold et al., 2018; Kim et al., 2018; Noh et al., 2018; Popa et al., 2018; Legrand et al., 2020). As recently reviewed by Kim et al. (2018), because of the tight association between UPS substrates and a wide variety of prevalent and severe diseases, UPS harbors great potential for drug targeting and therapeutic approaches, with tremendous potential impact on pharmaceutical intervention. Cancer, AIDS, cystic fibrosis, Alzheimer's and Parkinson's disease, and diabetes have all been found to be influenced by molecules secreted in a non-canonical fashion (Woodbury and Ikezu, 2014; Kim et al., 2016, 2018).

To study UPS in mammalian cells, different experimental setups have been explored. These include *in vivo* studies in mice (Dupont et al., 2011; Giuliani et al., 2011), *in vitro* studies on human cell cultures (Kim et al., 2016) on HeLa cells or on innovative artificial constructs such as inside-out vesicles as described in Schäfer et al. (2004), *in silico* analysis, and docking experiments to decipher secretion motifs and protein structure. In UPS *in vitro* studies, the chosen system and induction conditions are essential. The limitations lie in the fact that each treatment will disproportionately affect signaling pathways, leading to indirect effects (Giuliani et al., 2011).

Notably, even though much effort has been invested to elucidate UPS in mammalian cells, most molecular mechanisms remain unclear.

Unconventional Protein Secretion in Protist Parasites

Proteins that find their way to the cell surface without entering the ER–Golgi pathway are also present in parasitic protozoa. When investigating secretomes of several protist parasites, researchers have found substrates that do not carry any known leader sequences but are either exposed on the cell surface or found in the extracellular medium. UPS has specifically been studied in some of the most prevalent parasitic genera (**Table 2**): *Leishmania* (Cuervo et al., 2009; MacLean et al., 2012), *Trypanosoma* (Geiger et al., 2010; Neves et al., 2014), *Plasmodium* (Möskes et al., 2004; Thavayogarajah et al., 2015), *Trichomonas* (Gómez-Arreaza et al., 2014; Miranda-Ozuna et al., 2016), and *Giardia* (Eckmann et al., 2000; Ringqvist et al., 2008; Touz et al., 2008; Skarin et al., 2011). UPS cargo often presents moonlighting functions when transported to the extracellular space (Jeffery, 1999). Furthermore, it has been suggested that moonlighting and UPS are directly linked to each other, as posttranslational modifications seem to influence function as well as transport of a protein (Tristan et al., 2011). Especially in glycolytic enzymes, proteins that do not carry a leader sequence for secretion, moonlighting of extracellular proteins is strikingly common (Jeffery, 1999; Collingridge et al., 2010; Gómez-Arreaza et al., 2014). The diverse functions of these unconventionally translocated proteins, essential for the invasion and persistence of parasites, suggest that glycolytic enzymes may be among the most important UPS cargos in protist parasites.

Leishmania and *Trypanosoma*

As Silverman et al. (2008) have shown, secretion in *Leishmania* seems to involve UPS on a regular basis. The secretome analysis of *Leishmania donovani* revealed that the majority of secreted proteins do not carry leader sequences and must, therefore, be secreted unconventionally (Silverman et al., 2008). The same seems to be true for a close relative of *Leishmania*, as *Trypanosoma* has been suggested to employ UPS substantially (Geiger et al., 2010; Neves et al., 2014). Geiger et al. (2010) could demonstrate that only a small portion of secreted proteins carry secretion motives. It was proposed that proteins might be secreted via microvesicles, budding off at an organelle called the flagellar pocket (Geiger et al., 2010). In *Leishmania* as well as in

TABLE 2 | Overview of selected parasitic UPS cargo, including parasitic species and evidence for moonlighting functions.

Selected parasitic UPS cargo	Function	Selected studied organism/s	Evidence for functional moonlighting	Selected references
Enolase	Glycolytic enzyme, virulence factor	*Giardia*, *Entamoeba*, *Plasmodium*, *Trichomonas*	Yes	Tovy et al., 2010; Ghosh et al., 2011; Miura et al., 2012; Ahn et al., 2018
Triosephosphate isomerase (TvTIM)	Glycolytic enzyme, virulence factor	*Trichomonas*	Yes	Miranda-Ozuna et al., 2016
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Glycolytic enzyme, virulence factor	*Entamoeba*, *Trypanosoma*, *Trichomonas*, *Plasmodium*	Yes	Grébaut et al., 2009; Lama et al., 2009; Biller et al., 2014; Cha et al., 2016; Miranda-Ozuna et al., 2016
Translation elongation factor 1 alpha (TEF-1α)	Elongation factor, virulence factor	*Plasmodium*, *Leishmania*, *Trypanosoma*, *Giardia*	Yes	Nandan et al., 2002; Silverman et al., 2008; Grébaut et al., 2009; Skarin et al., 2011; Demarta-Gatsi et al., 2019
Hydrophilic acylated surface protein B (HASPB)	Parasite transmission, virulence factor	*Leishmania*	No	MacLean et al., 2012; Kim et al., 2018

Trypanosoma, there is clear evidence that UPS plays a crucial role in these species' virulence (Silverman et al., 2008; Geiger et al., 2010). Nevertheless, further insight into secretion machineries is still needed.

A relatively well-studied UPS substrate is translation elongation factor 1 alpha (TEF-1α). TEF-1α was found in the secretomes of *Trypanosomes* (Grébaut et al., 2009) as well as in *Leishmania* (Silverman et al., 2008). *Leishmania*-derived TEF-1α leads to deactivation of host macrophages (Nandan et al., 2002) and was used in an anti-leishmanial vaccine LeishDNAvax, as one of several antigens (Das et al., 2014; Naouar et al., 2016). Even though in *Leishmania* the secretion mechanism of TEF-1α has not yet been elucidated, this protein presents no classical secretory motifs in its amino acid sequence (Nandan et al., 2002). Another well studied translocation is the unconventional secretion of HASPB (hydrophilic acylated surface protein B) in *Leishmania* species. The function of HASPB is not fully elucidated, but it is suggested that it is involved in parasite transmission from the sand fly to the mammalian host or in establishing the parasite in the host macrophages (MacLean et al., 2012; Kim et al., 2018). The molecular machinery behind the secretion of HASPB is only partly understood. Rather than being transported by intracellular vesicles (Nickel, 2005), HASPB export involves ABC transporter-mediated processes, in a UPS type II route (Denny et al., 2000; Stegmayer et al., 2005; MacLean et al., 2012; Kim et al., 2018). While not carrying a classic secretory signal sequence, the N-terminal myristoylation of this protein is suggested to direct HASPB to the plasma membrane (Denny et al., 2000).

This N-terminal domain is necessary and sufficient since HASPB expressed heterologously in mammalian cells is correctly secreted, suggesting a highly conserved export machinery, which allows the export signal to be recognized by higher eukaryotes (Denny et al., 2000).

The glycolytic enzyme and widely studied UPS cargo GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was found in *Trypanosoma* exosomes (Geiger et al., 2010). In the secretome identification study of Grébaut et al. (2009), it was pointed out that, being involved in energy metabolism of the parasite, GAPDH is a virulence factor in Trypanosomes and a potential drug target (Grébaut et al., 2009).

Plasmodium

In the malaria parasite *Plasmodium*, protein secretion has been intensively studied because of its complexity due to several membranes a parasite protein has to travel through (Möskes et al., 2004). There are, for example proteins, that are targeted to the hosts red blood cells and have to be transported past the own cell membrane, then the parasitophorous vacuole membrane before even reaching the host cell. The unconventional secretion of enolase, a glycolytic enzyme, has been studied in a wide range of organisms. As experiments in yeast suggest, unconventional secretion might often be mediated by SNARE proteins (Miura et al., 2012). In *Plasmodium*, enolase seems to be of high importance for the parasite in the mosquito stage, while the secretion mechanisms are unclear (Ghosh et al., 2011). Ghosh et al. (2011) have studied *Plasmodium* ookinetes and found that enolase lines the ookinete surface and plays a crucial role in midgut invasion (Ghosh et al., 2011). While acting as an interaction ligand to the midgut epithelium of the mosquito, it also captures plasminogen, which in turn promotes the invasion machinery, both functions ultimately promoting the cell invasion process (Ghosh et al., 2011).

As mentioned for trypanosomes, GAPDH, is an extracellular glycolytic enzyme similarly widespread in parasites as enolase (Gómez-Arreaza et al., 2014). In *Plasmodium*, it plays a role in vesicular transport and host cell invasion (Daubenberger et al., 2003; Cha et al., 2016). It was found that GAPDH is located on the surface of *Plasmodium* sporozoites and mediates parasite traversal through Kupffer cells and infection of liver cells, therefore, performing a crucial role as virulence factor of this parasite (Cha et al., 2016). *Plasmodium* GAPDH is suggested as a promising vaccine candidate for a prehepatic vaccine antigen, as the sequence of *Plasmodium* GAPDH seems sufficiently different from mammalian GAPDH (Cha et al., 2016).

A protein not belonging to the group of glycolytic enzymes, which gained just as much interest in *Plasmodium* research as it has in Trypanosomes, is TEF-1α (Demarta-Gatsi et al., 2019). TEF-1α is another good example of an unconventionally secreted

moonlighting protein. As a study on *Plasmodium berghei* shows, in this parasite, TEF-1α is secreted via extracellular vesicles (Demarta-Gatsi et al., 2019). Plasmodia deficient in TEF-1α was no longer able to suppress T-cell response (Demarta-Gatsi et al., 2019). Furthermore, immunization of the host with this protein led to long-lasting protection from the parasite (Demarta-Gatsi et al., 2019).

The *Plasmodium falciparum* adenylate kinase 2 (PfAK2) secretion pathway shows a similar pattern to what was found in HASPB secretion in *Leishmania*, highlighting the importance of cotranslational modifications in targeting proteins for unconventional secretion pathways (Denny et al., 2000; Nickel, 2005; Thavayogarajah et al., 2015). Similar to HASPB, three N-terminal motifs found in PfAK2 were sufficient to target and translocate this protein across the parasite plasma membrane, an N-terminal myristoylation, a putative palmitoylation, and several basic amino acid residues in the 37 terminal amino acids (Thavayogarajah et al., 2015).

We attempted to reconstruct and compare the three-dimensional structure of the N-terminal 37 amino acids of PfAK2 as well as the N-terminal 55 amino acids of *Leishmania* HASPB using the Robetta web server.[1] Even though both modeled N-termini showed large helical structures, the obtained models were either low confidence ones (e-values of 0.41 for PfAK2, 0.28 for HASPB) in comparative modeling (Song et al., 2013) or presented very high angstrom error estimates using the TrRefineRosetta modeling method (Hiranuma et al., 2021; Yang et al., 2020). α-Helical structures are typical for classic secretory signal peptides (Emr and Silhavy, 1983); if present in UPS substrates, they may mimic membrane-association domains, which may favor membrane traversal.

Trichomonas

The sexually transmitted parasite *Trichomonas vaginalis* secretes virulence factors to the cell surface to adhere to the vaginal and cervical epithelia to be able to establish in this difficult microenvironment (Miranda-Ozuna et al., 2016). Several of these virulence factors have been studied for their secretion. TvTIM (triosephosphate isomerase) has been found on the surface of the parasite in addition to TvENO and seems to be an important part of host–pathogen interactions for this parasite (Miranda-Ozuna et al., 2016). TvTIM was studied specifically for its secretion pathways, and it was found that it might employ more than one route of UPS. In localization assays performed by Miranda-Ozuna et al. (2016), this protein was shown to be unconventionally secreted either via vesicles, or free of cytoplasmic vesicles, while the detailed underlying mechanism involved in its secretion could not be unraveled. TvGAPDH too is located on the cell surface and acts as a fibronectin-binding protein, being a crucial factor in host colonization and persistence (Lama et al., 2009; Miranda-Ozuna et al., 2016).

Giardia and *Entamoeba*

Giardia lamblia is an especially interesting organism when studying unconventional protein secretion. This organism lacks organellar structures reminiscent of a Golgi apparatus and is, therefore, an ideal system to study protein secretion independently of a classical ER–Golgi route (Palade, 1975; Rothman, 1994). The pathogenesis of this parasite is yet to be fully unraveled. During infection, *Giardia* trophozoites attach tightly to the intestinal walls of their host, but remain extracellular throughout their life cycle. The host experiences various symptoms such as nausea, vomiting, epigastric pain, and diarrhea (Ankarklev et al., 2010). Nutrient depletion of the host, caused by the feeding of *Giardia*, as well as secreted cargo by the parasite might be reasons for these symptoms. When comparing secretion in axenic cultures of *G. lamblia* trophozoites to cocultures with intestinal epithelial cells, giardial enolase was a protein that was considerably enriched in the medium of cocultured cells, suggesting that epithelial cells must have triggered enhanced *Giardia* enolase secretion in this model system (Eckmann et al., 2000; Ringqvist et al., 2008). Parasite-derived enolase, ornithine carbamoyltransferase (OCT), TEF-1 α, and arginine deiminase (ADI) were all found extracellularly in the absence of a signal sequence (Eckmann et al., 2000; Ringqvist et al., 2008; Touz et al., 2008; Skarin et al., 2011). The capability of enolase to show moonlighting functions is demonstrated in the intestinal parasite *Entamoeba histolytica*, where enolase shows a cytosolic as well as a nuclear localization (Tovy et al., 2010). In the nucleus, it interacts with, and regulates, Dnmt2 (cytosine-5 methyltransferase 2) activity, which catalyzes DNA methylation, whereas when secreted, enolase potentially functions as a virulence factor (Riahi et al., 2004; Tovy et al., 2010; Ahn et al., 2018). In *E. histolytica*, GAPDH seems likely to be another unconventionally secreted virulence factor playing a role in colonization of the host, influencing adhesion (Biller et al., 2014).

The role of ADI and OCT in *G. lamblia* host–pathogen interaction was specifically investigated in connection to local arginine depletion in the gut, as arginine depletion has a negative impact on host cell-dependent nitric oxide production and promotes parasite adhesion to the intestinal epithelium (Stadelmann et al., 2012; Banik et al., 2013). Alongside virulence factors, *G. lamblia* cells secrete large amounts of membrane-anchored variant surface antigens involved in antigenic variation for immune evasion (Gargantini et al., 2016). These variant surface proteins (VSPs) carry a leader sequence; however, since *Giardia* does not contain a Golgi apparatus, VSP secretion is effectively a Golgi bypass, UPS pathway type IV trafficking route (Gargantini et al., 2016). Secretome and surface proteome analysis of *Giardia* have revealed a large number of proteins, which are translocated across the plasma membrane without carrying a signal sequence, making these cargos highly interesting for studies on UPS machinery (Ma'ayeh et al., 2017; Dubourg et al., 2018; Davids et al., 2019).

OPEN QUESTIONS AND FUTURE PERSPECTIVES

Even though the first secreted proteins without a classical signal sequence for secretion have been known for over

[1] https://robetta.bakerlab.org/

three decades (Auron et al., 1984), the potential impact and benefits of deeper knowledge on UPS only just recently become more evident. Many open questions remain concerning the molecular mechanisms of translocation of most UPS cargos. In protist parasites, higher accessibility to whole secretomes and surface proteomes led to the discovery of a large number of putative UPS cargos (Silverman et al., 2008; Grébaut et al., 2009; Lama et al., 2009; Geiger et al., 2010; Biller et al., 2014; Cha et al., 2016; Miranda-Ozuna et al., 2016). Even though categorization of UPS substrates according to their secretion pathway has been attempted (Rabouille, 2017), a lack of knowledge on the variety and details of molecular translocation machineries make it difficult to find meaningful categories, which can be generally applied, as the example of IL-1β shows.

There are still many open questions, which will have to be solved for every cargo individually. How do proteins without a classic signal sequence enter a translocation pathway; what singles them out? Are there "unconventional" signal sequences for secretion? To solve these questions, what is sorely needed are *ad hoc in silico* prediction tools. Using an *in silico* program for sequence-based UPS prediction, such as SecretomeP, may aid the investigation of cargos of interest when studying unconventional secretion. Nevertheless, it is important to note that the algorithms in SecretomeP are trained in mammalian and bacterial proteins, thus, limiting the reliability of predictions in other lineages (Bendtsen et al., 2004; Lonsdale et al., 2016). Another set of questions should be asked concerning the evolution of unconventional secretion. UPS appears to be a widely distributed phenomenon across the evolutionary tree of life. Considering how many substrates, lineages, and mechanisms are involved, UPS may have evolved many times independently, although some pathways may be conserved. With regard to UPS cargo, the secondary structure of the cargos might play an even more important role here than peptide sequence *per se*. Might there be structural motifs that are preserved, acting as signals for secretion? Working toward answers for all these questions might turn out to be highly beneficial for translational research with UPS as a target for drug and vaccine development (Kim et al., 2018).

CONCLUSION

The topic of UPS and its related mechanisms is gaining more and more interest in the field of protein research in protist parasites. One fundamental difference in unconventional secretion between mammalian cells and parasitic protists is that protist parasite cells secrete certain substrates unconventionally in normal growth conditions. In contrast, UPS in mammalian cells seems most often to be triggered by cellular stress, inflammation, starvation, ER stress, or mechanical stressors (Giuliani et al., 2011; Merani et al., 2015; Kim et al., 2016; Rabouille, 2017). The reason why mammalian cells are employing UPS when the cell is under a severe stressor is not fully understood. The strong connection in mammalian UPS to immune response and inflammation processes makes it likely that substrates of UPS have key roles for survival of the organism. When impaired, severe illness or death could be a likely outcome. Therefore, from an evolutionary point of view, the reduction in complexity of the pathway might be under strong selective pressure, while under normal conditions, there is no pressure to streamline the secretion process. Interesting findings in neuronal research suggest that Golgi bypassing, UPS type IV, is not only widespread in several neuron cell types but also affects a large amount of key membrane proteins on a regular basis, while the cells are not under stress conditions (Hanus et al., 2016; Kennedy and Hanus, 2019). This bypass, or the core glycosylation connected to the studied bypass, leads to the affected proteins being unusually fragile (Hanus et al., 2016). This instability reduces the distance the proteins are able to travel before degrading, limiting them to exploring synapses very locally (Hanus et al., 2016). It is possible that UPS in this case is a way of fine tuning the release of key membrane proteins, making them only available to few synapses for a short period of time (Hanus et al., 2016; Kennedy and Hanus, 2019). This example shows that certain mammalian cell types are employing UPS without the influence of stressors and suggests that UPS pathways are much more regularly used than initially thought.

Parasites are well known in evolutionary biology for being a good example of reductive evolution. The parasitic lifestyle allows these organisms to reduce certain metabolic pathways or even organelles, wherever the function can be replaced or hitchhiked from its host organism, making parasites very efficient life forms with the main focus of their cell functions to persist, reproduce, and spread to new hosts. The environmental changes a parasite faces urges the organism to adapt as quickly as possible to the new habitat as, if it fails to do so, it will have fatal consequences, and the parasite will not be able to establish in a host or its vector (Ginger, 2014). This is in line with the selection pressure to reduce complexity of protein secretion in parasitic protists.

Another advantage of unconventional secretion pathways is that, with separate pathways, the cell prevents unwanted interactions, for example, between glycoproteins and their ligands at the wrong time or location (Popa et al., 2018). While the ER–Golgi-dependent secretion pathway seems to be advantageous for the majority of proteins in mammalian cells under normal circumstances, UPS as a streamlined alternative seems to bring a selective advantage in a parasitic lifestyle and in mammalian cells under stress. Furthermore, many UPS studies concern cargos displaying moonlighting functions, suggesting that UPS and moonlighting are closely connected (Jeffery, 2018). Both unconventional secretion pathways as well as moonlighting functions of a protein are parsimonious changes to their classic alternatives and, therefore, represent a streamlining of this essential cellular process.

A better understanding of underlying secretion mechanisms could reveal possible ways to regulate or prevent export of UPS substrates involved in an astonishing variety of health and disease-related pathways. The fact that UPS plays an essential role in some of the most prevalent, most life-threatening diseases, points out the significance of research on UPS to

understand these unconventional pathways, which seem not so unconventional after all.

AUTHOR CONTRIBUTIONS

EB produced the illustration. Both authors wrote and revised the manuscript.

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REVIEW
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Recent Advances in the Roles of Autophagy and Autophagy Proteins in Host Cells During *Toxoplasma gondii* Infection and Potential Therapeutic Implications

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Toxoplasma gondii is an obligate intracellular protozoan that can cause encephalitis and retinitis in humans. The success of *T. gondii* as a pathogen depends in part on its ability to form an intracellular niche (parasitophorous vacuole) that allows protection from lysosomal degradation and parasite replication. The parasitophorous vacuole can be targeted by autophagy or by autophagosome-independent processes triggered by autophagy proteins. However, *T. gondii* has developed many strategies to preserve the integrity of the parasitophorous vacuole. Here, we review the interaction between *T. gondii*, autophagy, and autophagy proteins and expand on recent advances in the field, including the importance of autophagy in the regulation of invasion of the brain and retina by the parasite. We discuss studies that have begun to explore the potential therapeutic applications of the knowledge gained thus far.

Keywords: cell signaling, CD40, IFN-γ, macrophage, endothelial, epithelial

INTRODUCTION

Intracellular pathogens have developed a broad range of strategies to survive within host cells. *Toxoplasma gondii* is an important example of these pathogens. *T. gondii* secretes proteins during its interaction with host cells that: (i) Enable the formation of a parasitophorous vacuole (PV) that avoids the classical pathway of phagolysosomal fusion; (ii) Activate host cell signaling cascades that negatively regulate autophagic targeting of the PV. As a result, the parasite avoids lysosomal degradation mediated by constitutive autophagy and prevents its complete eradication when autophagy is stimulated by CD40; (iii) Impair the ability of interferon-gamma (IFN-γ) to activate autophagosome-independent effector mechanisms directed against the PV. We provide a brief overview of early work on the role of macroautophagy (referred herein as autophagy) and autophagy proteins in *T. gondii* infection and discuss in more detail recent discoveries that include the role of autophagy in the regulation of invasion of the brain and retina by *T. gondii*, identification of a signaling pathway that causes sustained blockade of autophagic targeting of the parasite,

identification of a molecular link between IFN-γ and autophagy proteins (ATG) in human cells as well as the role of ATG8 orthologs in the cell-autonomous control of *T. gondii* induced by IFN-γ. We also discuss the potential implications for new therapeutic approaches against toxoplasmosis.

Toxoplasma gondii INFECTION

Toxoplasma gondii is an apicomplexan protozoan of worldwide distribution. *T. gondii* is believed to remain in the infected host for life. One-third of the world population is chronically infected with *T. gondii* (Montoya and Liesenfeld, 2004). Although the infection is usually asymptomatic, *T. gondii* can cause ocular toxoplasmosis in immunocompetent and immunosuppressed patients and encephalitis in immunocompromised individuals (Montoya and Liesenfeld, 2004). In addition, pregnant women acutely infected with *T. gondii* can transmit the infection to the unborn baby (Montoya and Liesenfeld, 2004). Congenital toxoplasmosis can result in severe ocular and neurologic sequelae as well as abortion (Montoya and Liesenfeld, 2004). Most of the strains of *T. gondii* isolated in North America and Europe fall into three clonal lineages: I, II, and III. Atypical strains that can have a higher propensity to cause disease have been reported in South America (Gilbert et al., 2008).

There are various forms of the parasite during its life cycle that include: (i) the tachyzoite that infects almost any nucleated cell of the host; (ii) the tissue cyst (containing bradyzoites) that persists in tissues of infected hosts during the chronic phase of the infection; and (iii) the oocyst (containing sporozoites) that is produced in the intestine of felines (the definitive host) during the sexual cycle of the parasite. Humans and other intermediary hosts become infected by ingesting tissue cysts or oocysts. This is followed by the release of bradyzoites or sporozoites into the intestinal lumen, invasion of intestinal epithelial cells, transformation into tachyzoites, and dissemination *via* the blood and lymphatic.

Tachyzoites of *T. gondii* invade mammalian cells and form a PV within these cells. This process is dependent on the secretion of contents from parasite organelles: (i) Micronemes that secrete micronemal proteins (MICs), adhesins that bind host cell membrane receptors (Carruthers and Tomley, 2008). (ii) Rhoptries that secrete rhoptry neck proteins that form the moving junction (Bradley and Sibley, 2007). This structure anchors the parasite to the host cell cytoskeleton and excludes host cell type I transmembrane proteins from the membrane surrounding the parasite (Mordue et al., 1999). The exclusion of these proteins could explain the first strategy identified for parasite survival: avoiding the classical pathway of phagolysosomal fusion (Mordue et al., 1999). Rhoptries also inject rhoptry kinases (ROPs) into the host cell cytoplasm that manipulate host cell signaling (Hakimi et al., 2017). (iii) Dense granules that release GRA proteins that maintain the intravacuolar network, anchor to the PV membrane (PVM), and interact with the host cytosol (Cesbron-Delauw et al., 2008; Hakimi et al., 2017). As explained later, proteins from these organelles are also instrumental in setting in motion signaling cascades that subvert cell autonomous mechanisms of parasite eradication.

CD40 AND AUTOPHAGY-DEPENDENT KILLING OF *T. gondii*: RELEVANCE TO THE REGULATION OF INVASION OF THE BRAIN AND RETINA BY THE PARASITE

Studies in humans and mice revealed that CD40 and its main ligand CD154 are central for protection against cerebral and ocular toxoplasmosis (Subauste et al., 1999; Reichmann et al., 2000; Portillo et al., 2010). Although studies in patients with congenital deficiency of CD154 (X-linked hyper immunoglobulin M syndrome) linked defective T cell priming and type 1 cytokine production to susceptibility to toxoplasmosis (Subauste et al., 1999), work done in $CD40^{-/-}$ and $CD154^{-/-}$ mice showed that these animals had increased susceptibility to cerebral and ocular toxoplasmosis despite seemingly unimpaired expression of IFN-γ (Reichmann et al., 2000; Portillo et al., 2010). The likely mechanisms of protection against cerebral and ocular toxoplasmosis identified in mice are as follows: (i) CD40-induced toxoplasmacidal activity in macrophages/microglia (Reichmann et al., 2000; Portillo et al., 2010); (ii) recently identified induction of toxoplasmacidal activity in neural endothelial cells accompanied by reduced invasion of the brain and retina by the parasite (Portillo et al., 2019); (iii) reduced anti-*T. gondii* antibody production (Portillo et al., 2021); (iv) protection against $CD8^+$ T cell exhaustion (Bhadra et al., 2011). $CD8^+$ T cells, important mediators of protection against *T. gondii*, undergo exhaustion (loss of functional capacities) during the chronic phase of *T. gondii* infection, a process that is mediated by program death-1 (Bhadra et al., 2011). Blockade of program death-1 rescues $CD8^+$ T cells, an effect that is dependent on the CD40-CD154 pathway (Bhadra et al., 2011). However, it is important to note that cerebral and ocular toxoplasmosis in $CD40^{-/-}$ and $CD154^{-/-}$ mice precede the development of $CD8^+$ T cell exhaustion.

CD40 kills a type I strain of *T. gondii* in human and mouse macrophages, a process independent of IFN-γ and effector responses downstream of this cytokine, but is instead caused by autophagy-mediated degradation of the parasite (Andrade et al., 2006). The molecular events involved in autophagy, including the role of this process in *T. gondii* infection, have been reviewed elsewhere (Mizushima et al., 2010; Besteiro, 2019; Subauste, 2019; Melia et al., 2020; Nakatogawa, 2020). A schematic summary of autophagy is shown in **Figure 1**.

CD40 stimulates autophagy through events that include: (i) Activation of Unc-51-like kinase 1 (ULK1), the upstream kinase that stimulates autophagy, through a process dependent on calcium/calmodulin-dependent kinase kinase-β (a sensor of intracellular Ca^{2+}) and AMP-activated protein kinase (an energy and nutrient sensor) (**Figure 2**); (ii) dissociation of the autophagy protein Beclin 1 (ATG6) from its negative regulator Bcl-2; (iii) upregulation of Beclin 1; and (iv) activation of protein kinase double-stranded RNA-dependent (PKR) and the α subunit of

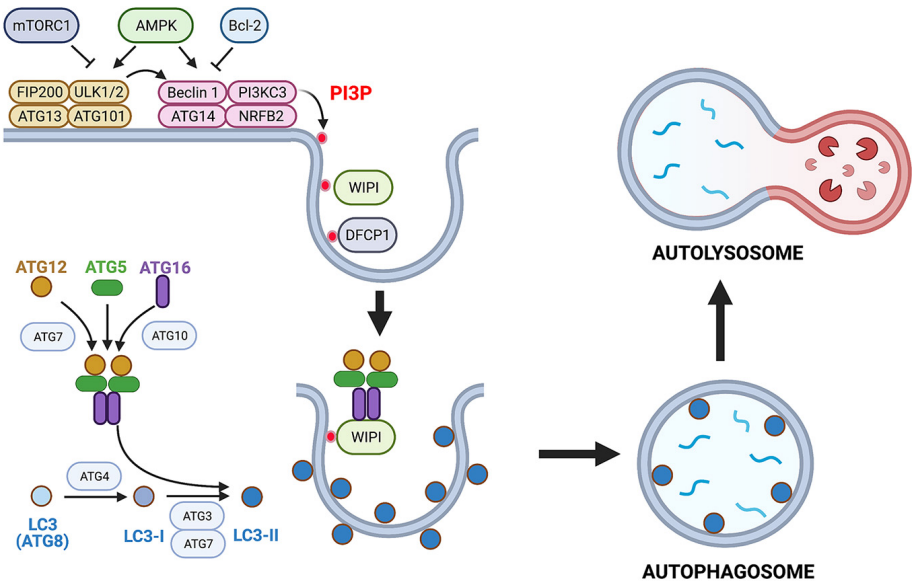

FIGURE 1 | Overview of the autophagy pathway. Autophagy is a constitutive and well-conserved process of lysosomal degradation. Autophagy is triggered by activation of ULK1 that forms a complex with ATG13, ATG101, and FIP200. ULK1 is under control of AMPK and mechanistic target of rapamycin complex 1 (mTORC1). A decrease in energy status activates ULK1 *via* AMPK leading to autophagy stimulation, whereas nutrient-rich conditions inhibit ULK1 *via* mTORC1. Membrane-associated ULK1 complex recruits and activates the PI3KC3 complex that also contains Beclin 1, ATG14, nuclear receptor binding factor 2 (NRBF2), as well as p150 and activating molecule in BECN1-regulated autophagy protein 1 (AMBRA1) (not shown). Bcl-2 inhibits pro-autophagic effect of Beclin 1. Activation of PI3KC3 complex causes local production of PI3P that recruits PI3P-binding proteins double FYVE domain-containing protein 1 (DFCP1) and WD-repeat protein interacting with phosphoinositides (WIPI). These scaffold proteins mediate membrane remodeling. Active PI3KC3 complex and WIPI promote expansion of isolation membrane through recruitment of ATG proteins. These proteins function as two ubiquitin-like conjugation systems: (i) ATG7 and ATG10 that conjugate ATG5 to ATG12 and ATG16. (ii) ATG3 and ATG7 together with ATG12–ATG5–ATG16 complex induce lipidation (phosphatidylethanolamine conjugation) of LC3 (ATG8). Lipidated LC3 (LC3-II) binds to several autophagy receptors promoting substrate uptake. Sequestration of the cargo by autophagosomes is followed by fusion with lysosomes resulting in formation of an autolysosome and lysosomal degradation of the cargo. This figure is a simplified overview of some of central components of autophagy pathway in mammalian cells. Figure was generated using BioRender.com.

eukaryotic initiation factor 2 α, proteins that stimulate autophagy (Ogolla et al., 2013; Liu et al., 2016; **Figure 2**). As a result, the autophagy protein LC3 (ATG8) is recruited around the PV, a process that is followed by vacuole–lysosomal fusion (VLF) as assessed by an accumulation of the late endosomal and lysosomal markers Rab7, mannose-6-phosphate receptor, CD63, lysosomal-associated membrane protein 1 (LAMP-1), and cathepsin D (Andrade et al., 2006). Parasite killing is dependent on ULK1, Beclin 1, and its interacting partner phosphatidylinositol 3-kinase catalytic subunit type 3 (PI3KC3), the autophagy proteins ATG5 and ATG7, and requires the activity of lysosomal enzymes, indicating that CD40 kills *T. gondii* in macrophages *via* autophagy (Andrade et al., 2006; Portillo et al., 2010; Liu et al., 2016). These findings have *in vivo* relevance because Beclin 1-deficient mice (*BECN1*$^{\pm}$), mice with deficiency of ATG7 in myeloid cells (Atg7$^{flox/flox}$-Lyz-M Cre mice) and mice deficient in PKR (PKR$^{-/-}$), exhibited increased susceptibility to cerebral and ocular toxoplasmosis (Portillo et al., 2010; Ogolla et al., 2013).

CD40 induces killing of type I and II strains of *T. gondii* in various human and mouse non-hematopoietic cells, including endothelial cells, through a mechanism dependent on the same components of the autophagy pathway identified in macrophages (Van Grol et al., 2013). As described later, consistent with the evidence that endothelial cells are the portal of entry into the central nervous system (CNS), CD40-driven autophagic killing in endothelial cells has been recently linked to regulation of invasion of the brain and eye, the two main organs affected by toxoplasmosis (Portillo et al., 2019).

Regardless of the route of infection, *T. gondii* circulates in the blood within infected leukocytes (including CD11b^{+} monocytes and dendritic cells) and as extracellular tachyzoites, a process that enables the parasite to invade the CNS (Courret et al., 2006; Lambert et al., 2006; Konradt et al., 2016). Neural endothelial cells become infected, and parasite replication within these cells followed by parasite egress into the neural parenchyma is likely the main mechanism of invasion of the CNS (Konradt et al., 2016). CD40$^{-/-}$ mice exhibited lower parasite load in the brain and retina since the initial stages of infection with *T. gondii*, suggesting that CD40 restricts invasion of these organs (Portillo et al., 2019). CD40$^{-/-}$ transgenic mice where CD40 expression is rescued in endothelial cells were used to examine further the role of endothelial CD40 during the development of cerebral and ocular toxoplasmosis. The presence of CD40 in these cells resulted in diminished parasite load and histopathology in the brain and retina after *T. gondii* infection (Portillo et al., 2019). Moreover, a lower parasite load was also detected after intravenous injection of *T. gondii*-infected CD11b^{+} monocytes

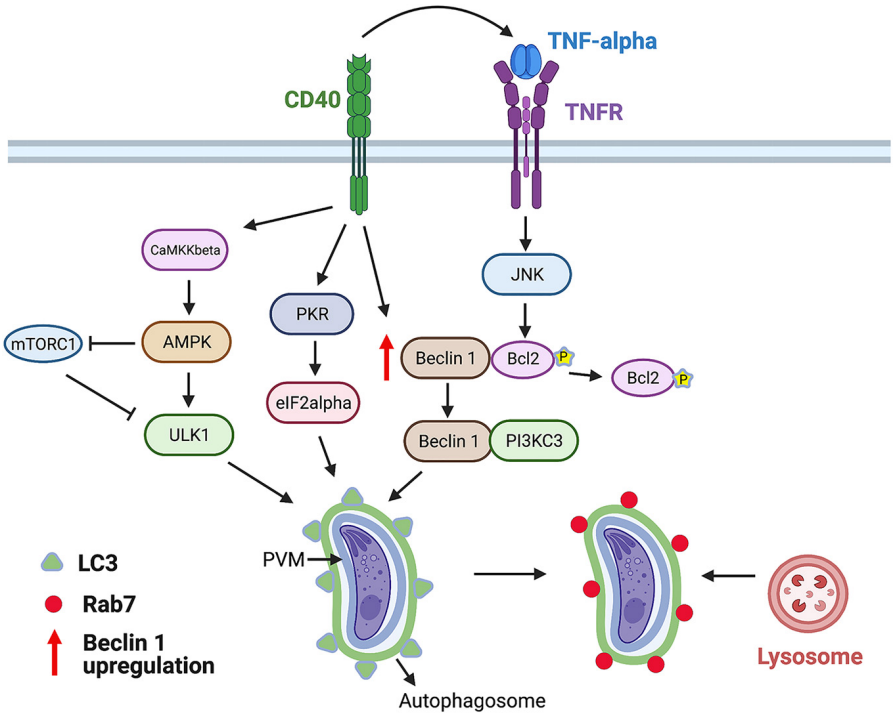

FIGURE 2 | CD40 induces killing of *T. gondii* via autophagy. CD40 ligation stimulates autophagy by activating ULK1, upstream kinase in the autophagy pathway. This process is dependent on calcium sensor CaMKKβ that activates energy/nutrient sensor AMPK. Besides activating ULK1, AMPK inhibits mTORC1, an inhibitor of autophagy. In addition, CD40 stimulates autophagy by triggering activation of pro-autophagy proteins PKR and eIF2α, upregulating autophagy protein Beclin 1, and releasing Beclin 1 from its negative regulator Bcl-2 after Bcl-2 phosphorylation induced by TNF receptor (TNFR)-JNK signaling. CD40 induces recruitment of LC3 around parasitophorous vacuole membrane (PVM) followed by Rab7-dependent fusion with lysosomes and killing of parasite. This process requires ULK1, Beclin 1, PI3KC3, ATG5, ATG7, and lysosomal enzymes. Figure was generated using BioRender.com.

or dendritic cells, indicating that endothelial cell CD40 reduced parasite invasion of the brain and retina (Portillo et al., 2019). The protective effect of endothelial CD40 was dependent on the presence of circulating infected leukocytes because no reduction in parasite load in the brain and retina was noted after challenge with extracellular tachyzoites (Portillo et al., 2019). Rather than reducing transmigration of infecting leukocytes across endothelial cells, CD40 likely conferred protection against cerebral and ocular toxoplasmosis because the presence of CD40 reduced the number of parasite foci in neural endothelial cells, diminishing invasion of the CNS (Portillo et al., 2019). *In vitro* studies revealed that endothelial cells acquired toxoplasmacidal activity upon interaction with *T. gondii*-infected dendritic cells or macrophages (Portillo et al., 2019). This effect was dependent on the expression of CD40, ULK1, and Beclin 1 in endothelial cells and led to the recruitment of LC3 and LAMP-1 around the PV of infected endothelial cells. During the interaction between infected leukocytes and endothelial cells, CD154 did not seem to be the major trigger for the autophagic killing of *T. gondii* in $CD40^{+}$ endothelial cells (Portillo et al., 2019). Rather, the expression on infected leukocytes of inducible heat shock protein 70, a protein reported to function as a ligand for CD40 (Becker et al., 2002; Shevtsov et al., 2014), seemed to induce toxoplasmacidal activity in endothelial cells (Portillo et al., 2019). Finally, the reduction in parasite invasion of the brain and retina was dependent not only on the expression of CD40 by endothelial cells but also on the expression of Beclin 1 and the expression of inducible heat shock protein 70 in dendritic cells (Portillo et al., 2019). These results indicate that CD40 and autophagy enhance protection against cerebral and ocular toxoplasmosis also by restricting parasite invasion of neural tissue.

Immune mechanisms of protection against pathogens, including *T. gondii*, do not fully overlap in humans and mice. For example, protection against *T. gondii* in mice requires IFN-γ and its downstream molecule signal transducer and activator of transcription 1 (STAT1) (Gavrielescu et al., 2004; Lieberman et al., 2004). A markedly different phenotype occurs in patients with an autosomal dominant defect in IFN-γR1 that results in deletion of the STAT1 binding site (Janssen et al., 2002). These patients are not susceptible to toxoplasmosis, although their macrophages are unable to display anti-*T. gondii* activity in response to IFN-γ (Janssen et al., 2002). Incubation with high concentrations of tumor necrosis factor-alpha (TNF-α) partially restored antimicrobial activity of macrophages from these patients, suggesting a protective role for TNF-α in these patients (Janssen et al., 2002). CD40 may contribute to IFN-γ-independent mechanisms of protection in humans (Subauste, 2009), as CD40 induces killing of *T. gondii* independently of IFN-γ, STAT1, and effector molecules downstream of IFN-γ [immunity-related GTPases (IRGs); nitric oxide synthase 2] and

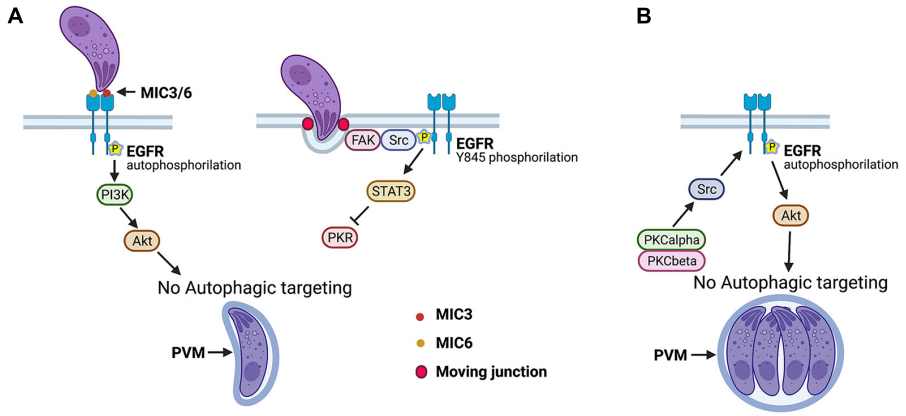

FIGURE 3 | *Toxoplasma gondii* manipulates host cell signaling cascades that prevent autophagic targeting of parasite. **(A)** Parasite adhesins MIC3 and MIC6 induce EGFR autophosphorylation that triggers PI3K-dependent activation of Akt, a negative regulator of autophagy. FAK, a cytoplasmic non-receptor tyrosine kinase, is activated during invasion of host cell at level of moving junction. This causes Src-dependent transactivation of EGFR (Y845 phosphorylation) and STAT3 signaling that prevents activation of pro-autophagy proteins PKR and eIF2α. **(B)** *T. gondii* maintains a blockade of autophagic targeting during its intracellular state by activating serine/threonine kinases PKCα/PKCβ that in turn maintain Src signaling, EGFR autophosphorylation, and Akt activation. Blockade of any of signaling molecules activated by *T. gondii* results in autophagic targeting of PV and killing of parasite. This process is dependent on ULK-1, Beclin 1, ATG7, and lysosomal enzymes. Figure was generated using BioRender.com.

CD40 induces TNF-α production (Andrade et al., 2005; Subauste and Wessendarp, 2006).

Toxoplasma gondii MANIPULATES HOST CELL SIGNALING TO AVOID AUTOPHAGIC TARGETING: MECHANISM OF PERSISTENT BLOCKADE OF AUTOPHAGIC DEGRADATION AND RELEVANCE TO INVASION OF THE BRAIN AND RETINA

Under basal conditions, *T. gondii*-infected cells accumulate $LC3^+$ structures around the PV without leading to autophagic targeting of the parasite (Wang et al., 2009), indicating that the parasite avoids targeting by autophagy. Indeed, *T. gondii* activates epidermal growth factor receptor (EGFR) and Src signaling during the invasion of an infected host cell to prevent its degradation by autophagy. MIC3 and MIC6, parasite proteins that contain EGF-like domains, cause rapid autophosphorylation of EGFR in human and mouse host cells followed by activation of phosphoinositide 3-kinase (PI3K), production of phosphatidylinositol 3,4,5 triphosphate around the PV, and activation of Akt, a negative regulator of autophagy (Muniz-Feliciano et al., 2013; **Figure 3A**). Blockade of any of the components of this cascade caused accumulation of LC3 and LAMP-1 around the PV, entrapment of the PV by a double membrane structure with characteristics compatible with those of an autophagosome, and killing of type I and II strains of *T. gondii* in human and rodent hematopoietic and non-hematopoietic cells that were dependent on the autophagy proteins ULK1, Beclin 1, ATG7, and lysosomal enzymes (Muniz-Feliciano et al., 2013).

The formation of the moving junction triggers a second signaling cascade that prevents autophagic targeting of *T. gondii*: activation of the cytoplasmic non-receptor tyrosine kinase focal adhesion kinase and its interacting partner Src that in turn causes ligand-independent transactivation of EGFR (Y845 phosphorylation of EGFR) (Portillo et al., 2017; **Figure 3A**). This pathway is responsible for the early phase of activation of STAT3, a negative regulator of autophagy (Portillo et al., 2017; **Figure 3A**). STAT3 acts by blocking the activation of the pro-autophagy protein PKR and its downstream signaling molecule eIF2α (Portillo et al., 2017). Inhibition of the focal adhesion kinase–Src–pY845 EGFR–STAT3 pathway results in the autophagic killing of type I, type II, and atypical strains of *T. gondii* in hematopoietic and non-hematopoietic human and rodent cells (Portillo et al., 2017).

Studies in transgenic mice that express a dominant-negative (DN) mutant of EGFR that impairs EGFR autophosphorylation and EGFR transactivation revealed the importance of manipulating host cell signaling in the development of cerebral and ocular toxoplasmosis. Expression of DN EGFR restricted to endothelial cells was accompanied by a reduction in parasite load and histopathology in the brain and retina after *T. gondii* infection (Lopez Corcino et al., 2019b). A lower parasite load in the CNS was also noted after intravenous challenge with infected dendritic cells or extracellular tachyzoites. The presence of DN EGFR reduced the number of foci of infection in neural endothelial cells (Lopez Corcino et al., 2019b). Moreover, DN EGFR in these cells led to spontaneous recruitment of LC3 around *T. gondii*, VLF, and parasite killing dependent on ULK1 and lysosomal enzymes (Lopez Corcino et al., 2019b). Autophagy likely explains the protective role of DN EGFR, as *in vivo* administration of the autophagy inhibitor 3-methyl adenine prevented DN EGFR mice from exhibiting reduced CNS invasion (Lopez Corcino et al., 2019b). Thus, EGFR enhances

T. gondii invasion of the CNS likely by promoting parasite survival in endothelial cells through avoidance of autophagic targeting. Moreover, these studies together with those on mice that express CD40 restricted to endothelial cells support the central role of autophagy as a process that regulates *T. gondii* invasion of the CNS.

The survival strategies described earlier are operative during the invasion of host cells by *T. gondii*. However, autophagy is a constitutive process, indicating that the parasite likely uses additional mechanisms to maintain a blockade of autophagic targeting. Indeed, *T. gondii* causes prolonged EGFR autophosphorylation in mammalian cells that is functionally relevant, as the addition of EGFR tyrosine kinase inhibitors (TKIs) 6 h after challenge with *T. gondii* resulted in the killing of the parasite (Lopez Corcino et al., 2019a). This process involved entrapment of the PV by a double-membrane structure compatible with an autophagosome, accumulation of LC3 and LAMP-1 around the PV, and pathogen killing dependent on ULK1, Beclin 1, and lysosomal enzymes (Lopez Corcino et al., 2019a). *T. gondii* induces prolonged EGFR autophosphorylation and activation of its downstream molecule Akt through a cascade that consists of the cytosolic serine/threonine kinases protein kinase C α (PKCα) and PKCβ that cooperate to sustain Src activation that drives prolonged EGFR autophosphorylation (Lopez Corcino et al., 2019a; **Figure 3B**). All these signaling molecules promote parasite survival, as not only inhibition of EGFR but also of PKCα, PKCβ, Src, and Akt in cells previously infected with *T. gondii* led to parasite killing (Lopez Corcino et al., 2019a). Thus, whereas *T. gondii* MIC3 and MIC6 act as an early switch for EGFR autophosphorylation, PKCα/β–Src signaling maintains EGFR autophosphorylation, ensuring the non-fusogenic nature of the PV and parasite survival.

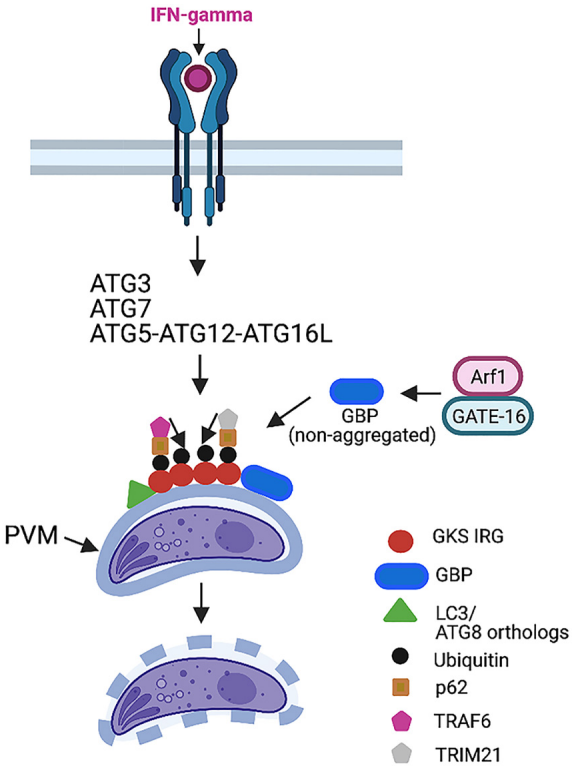

FIGURE 4 | IFN-γ-induces killing of type II and III strains of *T. gondii* in mouse cells. IFN-γ triggers recruitment of GKS IRGs to parasitophorous vacuole membrane (PVM), a process that requires ATG3, ATG7, and ATG12–ATG5–ATG16L. LC3 and ATG8 orthologs are recruited to PVM. IRGs promote deposition of ubiquitin, p62, and the E3 ubiquitin ligase TRAF6. p62 and TRAF6 further promote sustained decoration of PVM with ubiquitin. This process leads to delivery of GBPs to PVM. The end result is disruption of PVM and death of parasite. TRIM21 is another E3 ubiquitin ligase recruited by p62 that promotes ubiquitination. However, it does not seem to drive vesiculation of PVM. GATE-16 activates Arf1, enabling GBP to remain in a non-aggregated form, thus promoting GBP loading onto PVM. This process does not occur in cells infected with type I strains of *T. gondii* because ROP5/ROP18/GRA7 inactivate IRGs preventing their loading onto PVM. Figure was generated using BioRender.com.

INTERFERON-GAMMA RESTRICTS *T. gondii* CELLS THROUGH AUTOPHAGY-INDEPENDENT EFFECTS OF AUTOPHAGY PROTEINS: ROLE OF ATG8 ORTHOLOGS, UBIQUITIN, AND THE MOLECULAR LINK BETWEEN IFN-γ AND THE ATG PATHWAY

Mouse Cells

IFN-γ activates mechanisms that target the PV triggering cell-autonomous control of the parasite. In the case of mouse macrophages and fibroblasts (but not in human cells), certain autophagy proteins (ATG3, ATG5, ATG7, ATG12, and ATG16L) are required for recruitment to the PVM of effector GKS subfamily of IRGs (Zhao et al., 2008; Khaminets et al., 2010; Choi et al., 2014; Ohshima et al., 2014; **Figure 4**). This process induces deposition of ubiquitin and p62 on the PVM, followed by p62-dependent recruitment of guanylate-binding proteins (GBPs) (Haldar et al., 2015; **Figure 4**). The end result is vesiculation and rupture of the PVM and death of susceptible strains (types II and III) of *T. gondii* (Martens et al., 2005; Ling et al., 2006; Khaminets et al., 2010; Haldar et al., 2015). Type I strains avoid this process because ROP18, ROP5, ROP17, and GRA7 from these virulent strains prevent IRG recruitment to the PVM (Fentress et al., 2010; Fleckenstein et al., 2012; Etheridge et al., 2014; Reese et al., 2014). Disruption of the PVM in cells infected with type II and III strains of *T. gondii* does not represent *bona fide* autophagy, as it is not dependent on ULK1, ATG9, and ATG14L (Choi et al., 2014) and is not mediated by lysosomal degradation (Andrade et al., 2006; Van Grol et al., 2013; Choi et al., 2014).

Ubiquitin is a key component of the machinery that mediates the killing of *T. gondii* in IFN-γ-activated cells. Deposition of ubiquitin in the PVM was shown to be mediated not only by IRGs but also by p62 and the E3 ubiquitin ligase TNF receptor-associated factor 6 (TRAF6) (Haldar et al., 2015). Ubiquitin binds p62 that, in turn, recruits TRAF6 (Haldar et al., 2015; **Figure 4**). Both p62 and TRAF6 establish a positive feedback loop that

contributes to the rapid and sustained decoration of the PV with ubiquitin, resulting in the delivery of GBPs to the PV and parasite killing (Haldar et al., 2015). The tripartite motif protein 21 (TRIM21) is another E3 ubiquitin ligase that was recruited to the PV and promoted ubiquitin deposition in IFN-γ-treated cells (Haldar et al., 2015; Foltz et al., 2017; **Figure 4**). Whereas TRIM21 localized to GBP1-positive PV, TRIM21 did not promote vesiculation of the PVM (Foltz et al., 2017). However, TRIM21 was still able to restrict parasite replication (Foltz et al., 2017). In addition, studies in TRIM21$^{-/-}$ mice revealed that TRIM21 played an *in vivo* role in protection against *T. gondii* (Foltz et al., 2017). It is possible that the *in vivo* protective effect of this molecule is also mediated by the modulation of cytokine production (Foltz et al., 2017).

Mammalian cells express various ATG8 orthologs, including LC3A, LC3B, LC3C, gamma-aminobutyric (GABA)-A-receptor-associated protein (GABARAP), GABARAP-like1 (GABARAPL1), and GABARAPL2 (GATE-16). However, the PV is heavily decorated with LC3 in IFN-γ-activated mammalian cells; studies in mouse fibroblasts that lacked LC3A and LC3B revealed that LC3 proteins were dispensable for the control of *T. gondii* induced by IFN-γ (Sasai et al., 2017). In contrast, mouse fibroblasts that lacked GATE-16, GABARAP, and GABARAPL1 showed a marked defect in the recruitment of Irga6, ubiquitin, p62, and GBP1-5 to the PV as well as impaired parasite clearance after stimulation with IFN-γ (Sasai et al., 2017). The recruitment of GBP was dependent on the small GTPase ADP ribosylation factor 1 (Arf1) (Sasai et al., 2017). GATE-16 associated with Arf1 promoted its activation and, in turn, the recruitment of GBP (Sasai et al., 2017; **Figure 4**). Similar to mice with deficiency in ATG5, ATG7, or ATG16L targeted to phagocytes (Zhao et al., 2008; Choi et al., 2014), GATE-16-deficient mice died during the acute phase of *T. gondii* infection (Sasai et al., 2017). Thus, GATE-16 is central for resistance against *T. gondii* infection.

Human Cells

Although IFN-γ activates cell-autonomous control of *T. gondii* in mouse and human cells, there are significant differences in the effects of this cytokine between these two species. In this regard, IRGs and GBPs do not mediate anti-*T. gondii* activity in IFN-γ-stimulated human cells (Bekpen et al., 2005; Niedelman et al., 2013; Ohshima et al., 2014). Ubiquitination and some autophagy proteins are involved in the control of *T. gondii* induced by IFN-γ in human cells. However, these effector mechanisms are cell-type specific. In the case of human epithelial cells infected with type II or III (but not type I) strains of *T. gondii*, IFN-γ induces deposition of ubiquitin, p62, nuclear domain 10 protein 52 (NDP52), and LC3 around the PV (Selleck et al., 2015; **Figure 5A**). Although this process is dependent on ATG16L and ATG7, it does not represent autophagy, as it occurs independently of Beclin 1 and does not lead to VLF (Selleck et al., 2015). Although the integrity of the PV is maintained, a multilayer structure is formed around the PV that seems to explain the reduced growth rate of *T. gondii* (Selleck et al., 2015; **Figure 5A**).

IFN-γ also induces ubiquitination of the PV and recruitment of p62 and NDP52 in human endothelial cells (Clough et al., 2016; **Figure 5B**). However, in contrast to epithelial cells, LC3, GABARAP, and ATG16L are not recruited around the PV, and the induction of anti-*T. gondii* activity in endothelial cells is not dependent on ATG16L (Clough et al., 2016). Importantly, recruitment of ubiquitin and the adaptors p62 and NDP52 is followed by acidification of the PV and parasite killing (Clough et al., 2016; **Figure 5B**). This mechanism is operative against type II but not type I strains of *T. gondii* (Clough et al., 2016).

A recent study revealed that the presence of the ATG8 ortholog GABARAPL2 was key for the restriction of *T. gondii* replication in IFN-γ-activated HeLa cells (Zhang et al., 2020). GABARAPL2 accumulated around the PV in these cells in response to IFN-γ (Zhang et al., 2020). Similar to previous studies, IFN-γ induced deposition around the PV of ubiquitin and the adaptor proteins p62, NDP52, and optineurin (Zhang et al., 2020). These events are functionally relevant, as ubiquitination inhibitors or knockdown of the adaptor proteins impaired the recruitment of GABARAPL2 (Zhang et al., 2020). Moreover, knockdown of p62, NDP52, and optineurin impaired the ability of IFN-γ to reduce *T. gondii* replication. Finally, the recruitment of GABARAPL2 was dependent on the presence of ATG5, suggesting that lipidation of GABARAPL2 is required for its localization around the PV (Zhang et al., 2020). Thus, these studies, similar to those conducted in mice (Sasai et al., 2017), revealed the importance of GABARAPL2 in the control of *T. gondii* induced by IFN-γ.

Although IFN-γ sets in motion cell-autonomous immune responses to control *T. gondii* that is dependent on autophagy proteins, IFN-γ does not seem to upregulate ATG proteins. Recent studies were conducted to uncover the link between IFN-γ and the ATG pathway (Bhushan et al., 2020). IFN-γ upregulates IFN-stimulated genes (ISGs) (MacMicking, 2012). Work done in human epithelial cells revealed that ISG15 represents a molecular link between IFN-γ and the ATG pathway (Bhushan et al., 2020). ISG15 was markedly upregulated in IFN-γ-treated HeLa and A549 epithelial cells (Bhushan et al., 2020). Deletion of ISG15 partially inhibited the recruitment of p62, NDP52, and LC3 around the PV in IFN-γ-treated A549 cells without impairing ubiquitination of the vacuole (Bhushan et al., 2020; **Figure 5A**). The effect of ISG15 was selective, as the lack of this protein did not affect autophagy flux induced by rapamycin plus bafilomycin A1 (Bhushan et al., 2020). Moreover, ISG15 played an important role in the induction of IFN-γ-dependent restriction in *T. gondii* replication (Bhushan et al., 2020). Interestingly, ISG15$^{-/-}$ mice did not have increased susceptibility to *T. gondii* (Napolitano et al., 2018). This may be explained by the lack of a known role for ISG15 in the recruitment of IRGs and GBPs, the major mechanism that mediates cell-autonomous control of *T. gondii* in mice.

POTENTIAL THERAPEUTIC IMPLICATIONS

Current antibiotic regimens against toxoplasmosis have significant adverse effects and are not proven to be effective in the case of ocular toxoplasmosis. Compounds that stimulate autophagy could become part of treatment regimens against

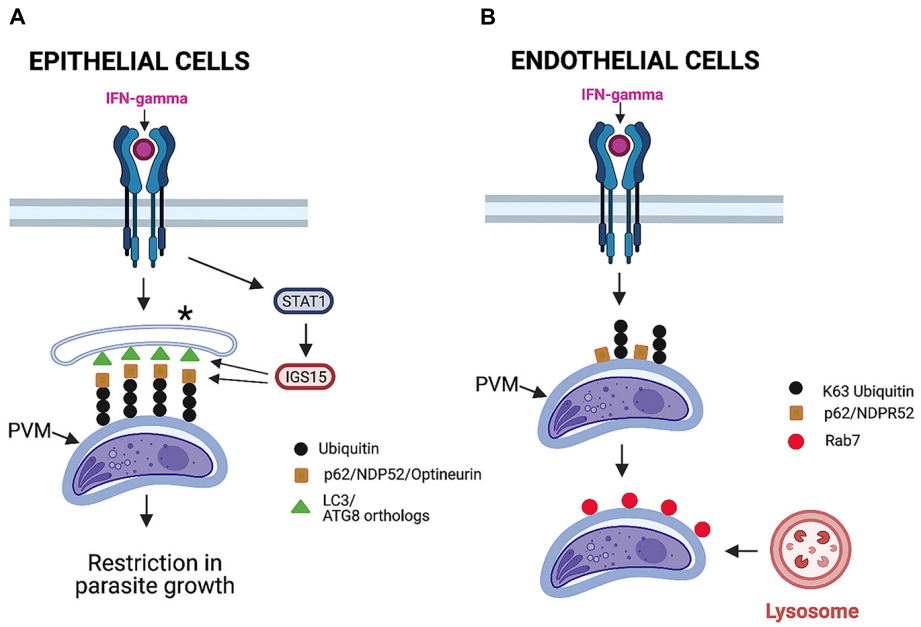

FIGURE 5 | Mechanisms induced by IFN-γ to restrict *T. gondii* in human cells. **(A)** In epithelial cells, IFN-γ promotes deposition of ubiquitin, adaptor proteins (p62, NDPR52, and optineurin), and LC3 around parasitophorous vacuole membrane (PVM). STAT1-dependent upregulation of ISG15 stimulates deposition of adaptor proteins around PVM. This process is independent of Beclin 1 and does not cause vacuole lysosomal fusion. Instead, it results in entrapment of parasitophorous vacuole by multilayer membrane structure (*) and inhibition of parasite growth. **(B)** In endothelial cells infected with a type II strain of *T. gondii*, IFN-γ induces deposition of ubiquitin, p62, and NDPR52. This is followed by recruitment of Rab7, fusion with lysosomes, and parasite killing. Importantly, LC3 is not recruited around PVM, and this process is not dependent on key autophagy molecule ATG16L. Figure was generated using BioRender.com.

T. gondii. Treatment of *T. gondii*-infected mammalian cells with rapamycin, a mTORC1 inhibitor and inducer of autophagy, triggered the killing of the parasite in a manner dependent on the presence of Beclin 1 (Andrade et al., 2006). Other studies evaluated the effects of docosahexaenoic acid (DHA), an omega-3 polyunsaturated fatty acid (Choi et al., 2019). DHA likely stimulated autophagy flux in mouse bone marrow-derived macrophages (BMDM) (Choi et al., 2019). In addition, DHA induced anti-*T. gondii* activity in BMDM that seemed to be mediated by autophagy, as DHA promoted the recruitment of LC3 around the PV, and 3-methyl adenine inhibited the anti-*T. gondii* activity induced by DHA (Choi et al., 2019). Similar findings were observed in BMDM from Fat-1 transgenic mice that synthesize omega-3 polyunsaturated fatty acid from ω6-PUFA (Choi et al., 2019). Moreover, Fat-1 mice infected with a type II strain of *T. gondii* exhibited lower tissue cyst counts in the brain (Choi et al., 2019).

More recent studies evaluated 4-hydroxybenzaldehyde (4-HBA) (Lee et al., 2020). 4-HBA-induced anti-*T. gondii* activity in BMDM seemed to be mediated by autophagy (Lee et al., 2020). 4-HBA seems to act *via* sirtuin-1 (SIRT1), a nicotine adenine dinucleotide-dependent protein deacetylase that can stimulate autophagy (Lee et al., 2020). 4-HBA upregulated SIRT1 protein levels, and SIRT1 inhibitors impaired the anti-*T. gondii* activity induced by 4-HBA. Although drugs that stimulate autophagy may facilitate the elimination of *T. gondii*, there are caveats with this approach. Autophagy regulates a broad range of homeostatic responses, including those in T cells, raising the concern that global stimulation of autophagy may have unintended negative consequences.

As part of an interesting approach, high-throughput screening of diverse small molecule libraries was performed in search of compounds that would enhance the anti-*T. gondii* activity of IFN-γ (Radke et al., 2018). A number of small molecules were identified that inhibited the growth of *T. gondii* at micromolar concentrations and exhibited increased potency in the presence of low concentrations of IFN-γ (Radke et al., 2018). Interestingly, some of these compounds increased the recruitment of LC3 around the PV, suggesting that they may act by potentiating the autophagy protein-mediated anti-*T. gondii* effects of IFN-γ (Radke et al., 2018). Compounds that synergize with immune mechanisms of control of *T. gondii* may improve the efficacy and tolerability of treatment regimens against toxoplasmosis.

The discovery that *T. gondii* manipulates host cell signaling to persistently block autophagic killing indicates that host-derived therapy may represent an attractive approach to improve the treatment of toxoplasmosis. Addition of Gefitinib, an EGFR TKI currently being used against cancer, to a broad range of human and rodent cells previously infected with *T. gondii* induced autophagic killing of type I and II strains of *T. gondii* (Lopez Corcino et al., 2019a). Importantly, Gefitinib was effective at concentrations up to ∼2 logs lower than those required for cytostatic activity against cancer cells (Lopez Corcino et al., 2019a). Gefitinib acted "on target" (required the presence of EGFR) and was active in the absence of immune activation

of host cells. Moreover, *in vivo* administration of a relatively low dose of Gefitinib (∼1/4th to 1/12th of those used in cancer models) to mice with preestablished ocular and cerebral toxoplasmosis resulted in control of the disease (Lopez Corcino et al., 2019a). The protective effect of Gefitinib was not mediated by enhanced cellular or humoral immunity against *T. gondii* but was dependent on the normal expression of Beclin 1 (Lopez Corcino et al., 2019a). These findings, together with the demonstration that inhibition of EGFR or the upstream inducers of EGFR signaling results in autophagic killing of type I, II, or atypical strains of *T. gondii* in various human and mouse cells (Muniz-Feliciano et al., 2013; Portillo et al., 2017; Lopez Corcino et al., 2019a), identified EGFR as a therapeutic target against toxoplasmosis. A combination of low-dose EGFR TKI with antibiotics may lead to an improved regimen against toxoplasmosis.

CONCLUSION

Maintaining the integrity of the PV is essential for the survival of *T. gondii*. CD40 stimulates autophagy that targets the PV resulting in the killing of *T. gondii*. Blocking the counterregulatory signaling induced by the parasite is sufficient to enable constitutive autophagy to target the PV and kill *T. gondii* (without the need for immune stimulation of autophagy). Studies to date indicate that in both scenarios, autophagic killing is not restricted to cell type, host species, or parasite strain. In contrast, IFN-γ triggers various autophagosome-independent mechanisms that attack the PV. The mechanisms activated by IFN-γ that have been identified thus far are not operative across species or parasite strains. Moreover, those uncovered in humans are not operative across cell types. Although much has been learned, further studies are needed to uncover the full spectrum of effector mechanisms induced by IFN-γ, especially in human cells, and identify the pathways of evasion utilized by *T. gondii*. This knowledge may contribute to the development of new approaches to improve the treatment of toxoplasmosis. The constitutive and conserved nature of autophagy already indicates that drugs that target components of the counterregulatory signaling pathway activated by *T. gondii* may become part of the novel and improved therapy against toxoplasmosis.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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To the Surface and Back: Exo- and Endocytic Pathways in *Trypanosoma brucei*

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Trypanosoma brucei is one of only a few unicellular pathogens that thrives extracellularly in the vertebrate host. Consequently, the cell surface plays a critical role in both immune recognition and immune evasion. The variant surface glycoprotein (VSG) coats the entire surface of the parasite and acts as a flexible shield to protect invariant proteins against immune recognition. Antigenic variation of the VSG coat is the major virulence mechanism of trypanosomes. In addition, incessant motility of the parasite contributes to its immune evasion, as the resulting fluid flow on the cell surface drags immunocomplexes toward the flagellar pocket, where they are internalized. The flagellar pocket is the sole site of endo- and exocytosis in this organism. After internalization, VSG is rapidly recycled back to the surface, whereas host antibodies are thought to be transported to the lysosome for degradation. For this essential step to work, effective machineries for both sorting and recycling of VSGs must have evolved in trypanosomes. Our understanding of the mechanisms behind VSG recycling and VSG secretion, is by far not complete. This review provides an overview of the trypanosome secretory and endosomal pathways. Longstanding questions are pinpointed that, with the advent of novel technologies, might be answered in the near future.

Keywords: cell surface, African trypanosomes, endocytosis, exocytosis, membrane recycling, Rab, clathrin

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INTRODUCTION

African trypanosomes rely on efficient strategies of immune evasion to successfully establish and maintain an infection in both their insect and mammalian hosts (Pays et al., 2014). In the bloodstream forms of *Trypanosoma brucei*, a dense coat of variant surface glycoprotein (VSG) constitutes the main virulence factor (Vickerman, 1969; Cross, 1975). The large repertoire of VSG genes coupled with periodic switches of the expressed VSG enables the parasites to change their surface epitopes, which allows an escape from the humoral immune response (Cross et al., 2014; Horn, 2014). VSG mRNA abundance varies slightly depending on the nature of the expressed VSG, but can amount to as much as ∼15% of total mRNA in *T. brucei* Lister 427 (Maudlin et al., 2021) and each trypanosome displays roughly 10^7 VSG monomers on its cell surface (Jackson et al., 1985; Bartossek et al., 2017). Thus, assuming a cell cycle of approximately 6 h, this would require the production of roughly 28,000 VSG molecules per minute. In steady-state cells, around 90% of the entire VSG is displayed on the cell surface with the remaining 10% found in intracellular compartments (Grünfelder et al., 2002). Most of the intracellular VSG cargo is found

in endosomes, which harbor 3-times as many VSG molecules than the biosynthetic organelles (Grünfelder et al., 2002).

A second trypanosome immune evasion strategy is based on the high mobility of the VSG coat, which affords resistance to low levels of VSG binding antibodies. VSG molecules are covalently bound to a glycosylphosphatidylinositol (GPI) anchor which mediates their attachment to the outer leaflet of the lipid bilayer and facilitates lateral diffusion (Bülow et al., 1988; Hartel et al., 2016). Since the single flagellum is attached over the entire length of the trypanosome cell body and constantly beats, the directional motion generates a hydrodynamic flow on the cell surface, which is directed toward the posterior of the cell. The fluid flow specifically drags antibody bound VSGs to the flagellar pocket (FP) (**Figure 1**; Engstler et al., 2007). The process of antibody clearance is very fast (30–60 s), with the predicted clearance time varying as a function of the size of the antibody (Engstler et al., 2007). The parasite cell is shaped by a dense subpellicular microtubule cytoskeleton. This cortex is broken only at the FP rendering this invagination of the plasma membrane the exclusive place for endo- and exocytosis (Overath et al., 1997; Gull, 2003).

Both, maintenance of the VSG coat and the high rate of endocytic bulk membrane flow demand efficient intracellular transport machineries (Engstler et al., 2004, 2007; Manna et al., 2014). The components of the trypanosome secretory and recycling machineries localize to the posterior part of the cell, filling the volume between nucleus and FP (Grünfelder et al., 2003; Engstler et al., 2004; Field and Carrington, 2009; Manna et al., 2014). The exception to this is the endoplasmic reticulum (ER), which displays tube-like connections to the flagellar attachment zone (FAZ) and, therefore, longitudinally spans the entire length of the cell body (Vickerman, 1969; Lacomble et al., 2012). The fact that trypanosomes possess strictly localized exo- and endocytosis, combined with a high degree of cell polarization and full genetic tractability, makes them an attractive cellular model system. Furthermore, trypanosomes are placed in the group Discoba and hence, have diverged earlier than Opisthokonta, to which mammals and yeast belong (Burki et al., 2020). This opens avenues for modern comparative cell biology. Here, we will review the mechanisms of VSG sorting and recycling. For this purpose, we summarize the main aspects of the corresponding pathways in yeast and mammals first and subsequently highlight differences and gaps in our knowledge of the exo- and endocytosis machineries in *T. brucei*. In addition, we pinpoint how the advent of new and improved technologies might contribute to answering the remaining open questions.

BIOSYNTHESIS AND SORTING OF SURFACE MOLECULES

The biosynthesis of molecules inside a cell involves many complex and highly regulated steps, which are collectively known as the biosynthetic pathway. Despite molecule specific differences, newly synthesized proteins follow a general route to their target compartments. During passage through the biosynthetic pathway, nascent proteins are modified and folded into their three-dimensional conformations.

Entry Into the Endoplasmic Reticulum

Protein secretion starts with the targeted import of nascent polypeptides into the ER. In mammals and yeast, two main ways of protein import into the ER have been described: the co-translational and post-translational pathways (reviewed in Aviram and Schuldiner, 2017). In the co-translational pathway, the interaction between a signal peptide of the nascent protein with the signal recognition particle (SRP) targets the ribosome-nascent chain complex to the translocon (Akopian et al., 2013). In contrast, the post-translational translocation of proteins from the cytoplasm to the ER is SRP-independent. In this pathway, cytosolic chaperons bind to the nascent, signal peptide containing proteins and direct them to the translocon (Rapoport, 2007). The translocon is a conserved heterotrimeric membrane protein complex which transports the nascent proteins into the ER (Osborne et al., 2005). In *T. brucei*, both co- and post-translational pathways have been observed for the translocation of signal peptide containing proteins into the ER (Boothroyd et al., 1981; Lustig et al., 2007; Goldshmidt et al., 2008), suggesting conservation of the ER-import mechanisms. However, the post-translational pathway may be favored for GPI-anchored proteins (Goldshmidt et al., 2008) while co-translational translocation may be more important for polytopic membrane proteins (Boothroyd et al., 1981; Lustig et al., 2007).

Processing and Quality Control in the ER

Once inside the ER lumen, the immature protein is exposed to ER-resident enzymes and undergoes several modifications assisted by chaperones and folding factors, which are usually referred to as the ER quality control system (Ellgaard and Helenius, 2003). A modification that occurs in nearly all glycoproteins, and represents a major function of the ER, is N-glycosylation (Helenius, 1994). Polypeptides bearing the glycosylation consensus sequence (N-X-S/T, where X can be any amino acid except for proline) serve as acceptors for a preassembled oligosaccharide (Welply et al., 1983; Mohanty et al., 2020). The reaction is catalyzed by oligosaccharyltransferase (OST) which transfers $Glc_3Man_9GlcNAc_2$ *en bloc* to the asparagine side chain of the acceptor polypeptide (Mohanty et al., 2020). Processing of the glycan begins immediately after this transfer reaction: glucosidase I hydrolyzes the outermost glucose, which is followed by the sequential removal of the remaining two glucose residues through glucosidase II (Grinna and Robbins, 1979). Monoglucosylated oligosaccharides are recognized by the ER lectin-like chaperones calnexin and/or calreticulin (Williams, 2006; Rutkevich and Williams, 2011), which prevent aggregation, oligomerization and formation of non-native disulfide bonds (Moremen and Molinari, 2006). Trimming of the innermost glucose residue by glucosidase II releases the protein from calnexin/calreticulin. UDP-glucose/glycoprotein glucosyl transferase (UGGT) senses the folding state of released glycoproteins and, if the correct conformation has not been achieved, UGGT reglucosylates the N-glycan to enable another cycle of calnexin/calreticulin assisted protein folding

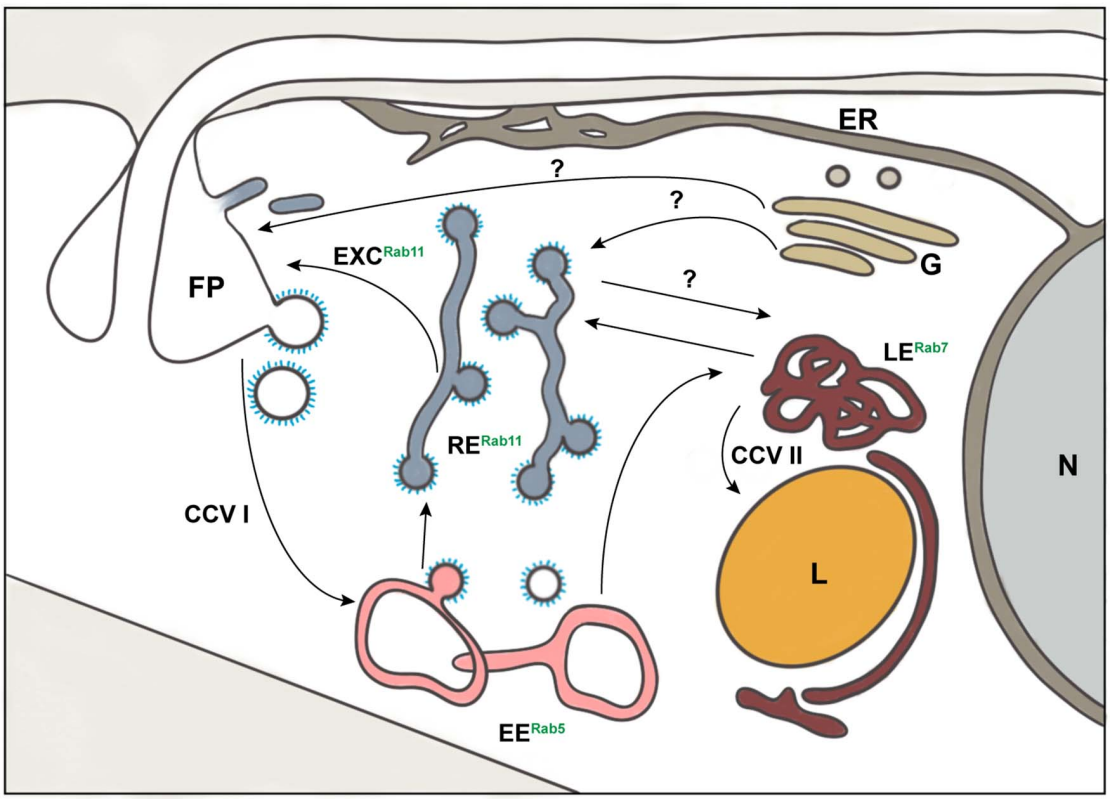

FIGURE 1 | Schematic representation of exo- and endocytic pathways in *Trypanosoma brucei*. The known organelles involved in the processes of exo- and endocytosis in *T. brucei* are depicted: class I clathrin-coated vesicles (CCV I), class II clathrin-coated vesicles (CCV II), early endosomes (EE), endoplasmic reticulum (ER), exocytic carrier (EXC), flagellar pocket (FP), Golgi apparatus (G), lysosome (L), late endosomes (LE), nucleus (N), and recycling endosomes (RE). The endocytic compartment is marked by the presence of small GTPases of the Rab family: Rab5 (EE), Rab7 (LE), and Rab11 (RE). The arrows represent the direction of exo- and endocytic cargo of *T. brucei*. The question marks indicate pathways that may exist but are still unknown.

(Solda et al., 2007; D'Alessio et al., 2010). Finally, correctly folded proteins are released from the cycle (Lederkremer, 2009). The remarkable ability of UGGT to bind to misfolded or incompletely folded glycoproteins was already reported in the early 1990s (Sousa et al., 1992), yet the complete recognition mechanism still remains unknown.

Proteins that are unable to acquire their native structure must be degraded to prevent accumulation of misfolded polypeptides in the ER. This degradation process is known as ER-associated degradation (ERAD), which usually involves recognition and retro-translocation from the ER to the cytosol, followed by ubiquitinoylation and proteasomal degradation (Brodsky, 2012). A special form of ERAD was reported for some misfolded GPI-anchored proteins in rat kidney cells (Satpute-Krishnan et al., 2014; Sikorska et al., 2016). Under acute ER stress, misfolded GPI-anchored proteins were exported from the ER, along the secretory pathway, to the plasma membrane from where they were eventually targeted to lysosomes for degradation (Satpute-Krishnan et al., 2014). This pathway was termed rapid ER stress-induced export (RESET) but may also operate constitutively in unstressed cells (Satpute-Krishnan et al., 2014).

Trypanosomatid protozoa are unable to synthesize dolichyl phosphate glucose (de la Canal and Parodi, 1987). Thus, while eukaryotic cells usually transfer the oligosaccharide $Glc_3Man_9GlcNAc_2$ to their proteins, trypanosomatids attach unglucosylated glycans to the polypeptide chains (Parodi, 1993). In most eukaryotic organisms, the required OST consists of a multi-subunit protein complex with STT3A and STT3B as the catalytic domains. In contrast, *T. brucei* possesses three STT3 paralogs, with different acceptor specificities, but no other subunits of the OST complex (Izquierdo et al., 2009b). Thus, trypanosomes extended their glycosylation ability by duplication of the STT3 gene and diversification of STT3 specificity (Izquierdo et al., 2009b; Schwarz and Aebi, 2011). In addition, the recent finding of O-glycosylation of VSGs might indicate further unidentified biochemical diversity in protein processing factors in *T. brucei* (Pinger et al., 2018).

The orthologs of ER quality control proteins that were found and characterized in *T. brucei* include the reglucosylating UGGT, calreticulin, and glucosidase II (Conte et al., 2003; Jones et al., 2005; Izquierdo et al., 2009a). Although it is likely that the ER resident quality control machinery contributes to VSG folding, there is an ongoing discussion as to whether VSG folding is inherently energetically favorable and may therefore, not require chaperones (Rehaber et al., 1990). While some evidence suggests that VSGs might be synthesized in 2–3-fold excess, with their

relative abundance regulated by an active ERAD (Field et al., 2010; Manna et al., 2014), other studies found no evidence for rapid degradation and promote a model whereby VSG synthesis is precisely regulated (Tiengwe et al., 2016). Interestingly, recent findings are adding a new piece to this puzzle. Aroko et al. (2021, preprint) demonstrated that targeting of several abundantly expressed proteins to the ER leads to a downregulation of mRNA levels of the endogenous VSG. Thus, they suggested that feedback generated at the ER has a central role in regulating VSG mRNA amounts.

Export From the ER

Proteins exit the ER at specific locations named ER exit sites (ERES) via vesicles coated with coat protein (COP) II. The highly conserved and essential COP II coatomer is generally composed of two protein complexes Sec23/Sec24 and Sec13/Sec31, and the small GTPase Sar1 (Lopez et al., 2019). Vesicle formation is initiated by Sec12, a guanine exchange factor localized in the ER, which activates Sar1 (Nakano and Muramatsu, 1989; Barlowe and Schekman, 1993; Futai et al., 2004). Activated Sar1 embeds into the ER membrane and recruits the first heterodimer Sec23/24 via a specific interaction with Sec23 to the ERES (Bi et al., 2002; Fath et al., 2007). This "prebudding complex" selectively binds secretory cargo mainly via interaction with Sec24 and to a lesser extent with Sec23 (Miller et al., 2003, 2005; Cai et al., 2007; Farhan et al., 2007). Following this, the heterotetramer Sec13/Sec31 is recruited, which stimulates membrane deformation and vesicle fission from the ERES (Matsuoka et al., 1998). While transmembrane secretory cargo is selectively recruited via direct interactions between their cytosolic motifs and Sec24 (Aridor et al., 1998; Kuehn et al., 1998; Miller et al., 2003), soluble and GPI-anchored proteins cannot interact directly with the COP II machinery (Barlowe, 2003). These proteins require transmembrane cargo receptors, such as p24, which link them to the COP II coat (Schimmoller et al., 1995). The subsequent transport of COP II vesicles from the ER to the Golgi is likely to be mediated by the small GTPases Rab1 and Rab2 (Tisdale et al., 1992).

In *T. brucei*, two isoforms each of Sec23 and Sec24 have been found, with TbSec23.2 and TbSec24.1 responsible for VSG export (Sevova and Bangs, 2009). While the abundance of synthesized and transported VSG may explain the requirement for an additional specific heterodimer, the mechanism by which VSGs are selectively incorporated into TbSec23.2/TbSec24.1 COP II vesicles remains unknown. Recently, it was hypothesized that p24 orthologs in *T. brucei* may facilitate VSG incorporation into COP II vesicles (Kruzel et al., 2017). Studies with TbRab1 and TbRab2 have validated the role of these GTPases in ER to Golgi and *intra*-Golgi transport (Dhir et al., 2004).

Golgi Traffic

The Golgi apparatus is a central membrane organelle that has a function in glycan maturation, trafficking and sorting. Each Golgi stack is formed by several tightly aligned flattened cisternae, which are referred to as *cis*-, medial- or *trans*-compartments. While the *cis*-Golgi network receives cargo from the ER, the medial-Golgi cisternae contain glycosylation enzymes and process cargo proteins and lipids, and the *trans*-Golgi network (TGN) sorts cargo molecules for delivery to different destinations (e.g., to the cell surface or to vacuolar or lysosomal compartments) (reviewed in Di Martino et al., 2019; Huang and Wang, 2017). In addition, the TGN can also be separated from the Golgi stack and may act as an early endosome in yeast and plants, suggesting that this compartment might be an independent organelle that is distinct from the Golgi apparatus (Uemura et al., 2004; Dettmer et al., 2006; Staehelin and Kang, 2008; Day et al., 2018).

Surprisingly, the mechanism of transport through the Golgi, is still controversial and different models that are not mutually exclusive have been suggested. The most prominent models suggest stable cisternae with COP I vesicles transporting cargo between them or cisternal maturation with progressive movement of cisternae toward the *trans* face (reviewed in Glick and Luini, 2011). COP I vesicles are also responsible for Golgi retrograde transport which returns ER resident proteins to the ER lumen (Maier et al., 2001). However, the exact mechanism of delivery and the extent to which this occurs both remain unclear.

In *T. brucei*, the stable cisternae model offers the most likely explanation for *intra*-Golgi transport (Warren, 2013). TbRab18 (Jeffries et al., 2002), and TbRabX2 (also called TbRab31) (Field et al., 2000) have been associated with the Golgi apparatus and may be related to Golgi dependent transport pathways. In TrypTag.org, a project that aims to determine the localization of every trypanosome protein within the cell (Dean et al., 2017), TbRab6 (Tb927.2.2130) was also found to be located in the region of the Golgi.

Post-Golgi Transport to the Plasma Membrane

Cargo proteins destined for the plasma membrane are loaded into post-Golgi carriers and follow the secretory pathway (Luini et al., 2005). In *Saccharomyces cerevisiae*, the molecules involved in this transport were identified by the isolation of *sec* mutants that were unable to secrete the extracellular enzyme invertase (Novick et al., 1980, 1981). Subsequent studies in yeast have strongly indicated an additional role of the Rab11 GTPase family in Golgi exit and transport to the sites of exocytosis (Jedd et al., 1997; Morozova et al., 2006; Lipatova et al., 2008). Secretory carriers are directed by tropomyosin-actin cables and delivered to the plasma membrane to which they are tethered by the exocyst complex prior to SNARE complex dependent fusion in a Sec4 GTPase manner (TerBush et al., 1996; Guo et al., 1999).

In *T. brucei*, the mechanism of transport from the Golgi to the FP remains elusive and it may be possible that different carriers, perhaps containing different cargos, are involved (**Figure 1**). The role and involvement of TbRab11 in the secretory pathway is still contentious. VSG has been demonstrated to be transported inside TbRab11-enriched exocytic carriers during VSG recycling using immunoelectron microscopy (Grünfelder et al., 2003). However, RNAi silencing of TbRab11 showed no effect on exocytosis of newly synthesized VSG in pulse-chase radiolabel experiments (Hall et al., 2005). In accordance with these results, another study, also using pulse-chase radiolabeling of VSGs, validated

that depletion of TbRab11 had no impact on the transport of newly synthesized VSG (Umaer et al., 2018). However, no reports of TbRab11 negative secretory carriers can be found in the literature. This could be explained by the existence of a yet unidentified route from the Golgi to the FP that could easily have been overlooked due to the comparatively small proportion of biosynthetic VSG in the total intracellular VSG pool. Furthermore, the exocyst has been shown to be an important mediator of the late steps of exocytosis. This complex presents one subunit (Sec15) which interacts with TbRab11 (Boehm et al., 2017), highlighting once more the importance of this GTPase in the exocytosis process of *T. brucei*. Altogether, these findings suggest the need to characterize the role of TbRab11 in a more detailed manner. Is TbRab11 involved in post-Golgi transport to the FP? Is this transport mediated via endosomes? Does TbRab11 distinguish between VSG and other cargo transport? Another intriguing aspect regarding Golgi transport to the FP is related to the role of the cytoskeleton. The export of newly biosynthesized VSG from the Golgi has been reported to be independent of actin (Nolan and Garcia-Salcedo, 2008). No further reports about the involvement of cytoskeleton components in cargo transport exist, but it is unlikely that these pathways can be entirely independent of interactions with the cytoskeleton. Thus, the role of the cytoskeleton remains largely undefined.

ENDOCYTOSIS AND MEMBRANE RECYCLING

Endocytosis allows eukaryotic cells to internalize plasma membrane proteins and extracellular molecules for various physiological processes, including nutrient uptake, degradation, cell signaling and recycling. The endocytosis processes are generally divided into fluid-phase and receptor-mediated endocytosis. The first refers to the non-specific uptake of extracellular material, and the second is characterized by the triggered uptake of a ligand after binding to its receptor. Different endocytic pathways exist in eukaryotes, such as caveolae-mediated (Kiss and Botos, 2009) and raft-dependent endocytosis (Lajoie and Nabi, 2007). However, the major endocytic pathway is mediated by clathrin (Bitsikas et al., 2014), which explains why the term endocytosis is often used synonymously with clathrin-mediated endocytosis. In *T. brucei*, all endocytic processes, for both fluid phase and receptor-mediated uptake, are clathrin-mediated (Allen et al., 2003; Grünfelder et al., 2003; Engstler et al., 2004).

Clathrin-Mediated Endocytosis and Endocytic Trafficking in Mammals and Yeast

Clathrin molecules are trimers composed of three heavy chains, each associated with one light chain, connected through a central core (Crowther and Pearse, 1981; Ungewickell and Branton, 1981). Due to the architecture of the molecule, clathrin trimers are called triskelions (Ungewickell and Branton, 1981). Clathrin triskelions can connect with one another to form flat lattices or a cage-like structure (Crowther and Pearse, 1981; Kirchhausen and Harrison, 1981; Sochacki and Taraska, 2019). The curvature of clathrin lattices linked to the membrane contributes to the forces required for local deformation, that culminates in pit formation and vesicle budding (Kirchhausen and Harrison, 1981; Kaksonen and Roux, 2018; Sochacki and Taraska, 2019). Flat hexagonal clathrin lattices need to reorganize to form the curved mix of pentagonal and hexagonal lattices (Sochacki and Taraska, 2019). The organization of clathrin triskelions into flat lattices has also been shown to occur spontaneously *in vitro* (Dannhauser et al., 2015). However, the conformational change from flat to curved clathrin lattices *in vivo* most likely demands energy and requires the participation of accessory molecules (reviewed in Kaksonen and Roux, 2018; Sochacki and Taraska, 2019). The recruitment of clathrin to the plasma membrane requires several proteins that form the pioneer module, a complex with several characterized components, such as F-BAR domain only protein 1 and 2 complex (FCHO1/2), adaptor protein 2 (AP-2), and epidermal growth factor receptor substrate 15 (EPS15) (Shih et al., 1995; Ma et al., 2016; Kaksonen and Roux, 2018). In addition, several molecules such as actin-related protein 2/3 complex (Arp2/3), protein-rich protein Las17 (Las17), type I myosins (Myo3/5), Sla2 and epsin 1 (Ent1) (Galletta et al., 2008; Cheng et al., 2012; Feliciano and Di Pietro, 2012; Kaksonen and Roux, 2018; Lizarrondo et al., 2021), contribute to polymerization of the actin cytoskeleton. Finally, the progressive invagination of the membrane is associated with a constriction process, which culminates in membrane scission (Kaksonen and Roux, 2018). Constriction involves actin, dynamin, and $PI(4,5)P_2$ (reviewed in Mettlen et al., 2009). This phosphoinositide can bind to the epsin N-terminal homology (ENTH) and AP180 N-terminal homology (ANTH) domains of proteins and is considered critical for the recruitment of adaptors and formation of clathrin coated pits (CCP) (Wendland et al., 1999; Zoncu et al., 2007; Kaksonen and Roux, 2018), which bud as clathrin coated vesicles (CCVs). These CCVs are then uncoated in a process involving dephosphorylation of $PI(4,5)P_2$ by inositol 5-phosphatases and disruption of the clathrin–clathrin interactions by HSC70 (Prasad et al., 1993; Ungewickell et al., 1995; Kaksonen and Roux, 2018). The uncoated vesicles are able to fuse with endosomes, a polymorphic, dynamic endomembrane system, commonly divided into subpopulations named early (or sorting) endosomes (EEs), late endosomes (LEs), and recycling endosomes (REs) (Goldenring, 2015; Naslavsky and Caplan, 2018). These different subclasses can be distinguished according to marker proteins enriched in their membranes, especially those belonging to the Rab family, as well as by differences in luminal pH (Feng et al., 1995; Rink et al., 2005; Wandinger-Ness and Zerial, 2014; Raiborg et al., 2015; Naslavsky and Caplan, 2018) which is controlled by a V-ATPase pump (Hurtado-Lorenzo et al., 2006; Scott et al., 2014).

Endocytosed cargo enters EEs first, from where the molecules are sorted for different fates, either degradation or recycling. When destined for degradation, the cargo passes through LEs before arriving in the lysosome. In the recycling pathway, there are two possible routes: anterograde transport, which returns

the molecules to the cell surface, and retrograde transport, which transports cargo to the TGN (Cullen and Steinberg, 2018; Naslavsky and Caplan, 2018). The dynamics of cargo transport is still a topic of debate and distinct models have been proposed. The vesicular-transport model suggests that vesicles budding from steady-state organelles move their cargo to the different endocytic compartments. However, studies on living mammalian cells have suggested the existence of a maturation process in which these organelles/compartments are formed from progressive differentiation of endocytic vesicles (Rink et al., 2005; Scott et al., 2014; Langemeyer et al., 2018; Trivedi et al., 2020).

In mammals, the EEs have been characterized to be a vacuolar organelle associated with tubular membranous extensions (Cullen and Steinberg, 2018). They are further characterized by the presence of Rab4, Rab5, Rab10, Rab14, Rab21, and Rab22, which are found in distinct microdomains of the EE membrane (Scott et al., 2014; Naslavsky and Caplan, 2018). Inside the EEs, molecules destined for degradation are sorted with the participation of the endosomal sorting complex required for transport (ESCRT) into luminal invaginations of the EE membrane. These invaginations pinch off into the EE luminal space, thereby forming intralumenal vesicles (ILVs). The ILVs accumulate in vacuolar regions of EEs, which will detach and become free multivesicular bodies (MVBs) or endosomal carrier vesicles (ECVs). The MVBs/ECVs are subsequently transported on microtubules to a different location in the cell (Scott et al., 2014; Raiborg et al., 2015). Interestingly, live-cell microscopy studies suggested the maturation of these Rab5-positive MVBs/EVCs into Rab7-positive LEs, with Rab5 gradually replaced by Rab7 (Rink et al., 2005; Scott et al., 2014; Langemeyer et al., 2018). Thus, the LEs are mature MVBs with a different set of marker proteins and a more acidic pH. The morphology of LEs, with tubular-cisternae and multivesicular regions, is similar to that of EEs, which are also formed through ESCRT sorting into ILVs (Scott et al., 2014; Cullen and Steinberg, 2018). From the LEs, the cargo can follow either the degradation or the recycling pathway. For degradation, LEs fuse with lysosomes, forming a hybrid organelle referred to as the endo-lysosome. This transient organelle matures into the classical lysosome, which can be distinguished from LEs through its spherical and electron-dense structure (Rink et al., 2005; Scott et al., 2014). Cargo from the LEs that is not destined for the lysosome, can be routed to the cell surface through tubular-vesicular carriers which fuse with the plasma membrane (van Weering et al., 2012; Cullen and Steinberg, 2018). Another path back to the cell surface is via REs, which also have contact sites with EEs. This compartment is composed of highly dynamic tubular structures that are characterized by the presence of Rab11 and Rab8 (reviewed in Hsu and Prekeris, 2010; Scott et al., 2014; Goldenring, 2015). Interestingly, in polarized cells, Rab11 seems to participate in direct recycling of cargo to the plasma membrane while Rab8 functions in transport via the TGN (Hsu and Prekeris, 2010; Cullen and Steinberg, 2018). Rab11-positive vesicles that bud from REs are directed to the plasma membrane through interaction with actin-motor proteins (Ji et al., 2019). Thus, actin is not only involved in CCV budding but also in the transport of endocytic cargo. The retrograde transport of molecules to the TGN is mediated by the retromer complex. This complex is formed by at least three cargo selection molecules (Vps35, Vps26 and Vps29) that interact with members of the sorting nexin (SNX) family (reviewed in Scott et al., 2014).

Endocytosis in *T. brucei*

T. brucei restricts both endo- and exocytosis to the FP (**Figure 2**; Vickerman, 1969; Overath et al., 1997; Gull, 2003; Engstler et al., 2004; Field and Carrington, 2009). Uptake from the FP involves the formation of CCPs (Morgan et al., 2001; Allen et al., 2003; Grünfelder et al., 2003; Engstler et al., 2004), which rapidly pinch off as CCVs with a diameter of 135 nm, known as class I CCV (CCV I) (Grünfelder et al., 2003; Overath and Engstler, 2004).

Clathrin can be recruited to the entire FP membrane with the exception of the region of the microtubule quartet (4MT) (Gadelha et al., 2009). This recruitment involves many proteins, which will interact with each other as well as with clathrin, cargo, other adaptors, and phosphoinositides. TbEpsinR and TbCALM are two characterized proteins that possess a phosphoinositide binding domain (ENTH and ANTH, respectively) and are critical for the recruitment of adaptors as well as for formation of CCPs (Gabernet-Castello et al., 2009; Manna et al., 2015). While TbEpsinR has been shown to colocalize with clathrin (Gabernet-Castello et al., 2009), the colocalization of clathrin and TbCALM has been inferred from their individual localization to the cytoplasmic side of the FP (Manna et al., 2015). Interestingly, TbEpsinR was distributed throughout the cytoplasm in clathrin-depleted cells leading to the proposition of targeting-dependency between TbEpsinR and clathrin (Gabernet-Castello et al., 2009). The depletion of either TbEpsinR or TbCALM led to a small reduction in receptor-mediated endocytosis and morphological aberrations in both CCP and CCVI (Manna et al., 2015). The simultaneous knockdown of both proteins led to FP enlargement and inhibition of endocytosis, suggesting redundancy in the roles of the two proteins (Manna et al., 2015).

The ATPase TbHsc70 co-precipitates with clathrin and colocalizes with clathrin-enriched structures (Adung'a et al., 2013). Knockdown of TbHsc70 reduces FITC-concanavalin A (ConA) trafficking into late endosomal compartments (Adung'a et al., 2013), suggesting a probable involvement in uncoating as observed *in vitro* and *in vivo* for mammals and yeast (DeLuca-Flaherty et al., 1990; Rapoport et al., 2008; Yim et al., 2010). Currently, no other proteins involved in clathrin uncoating have been detected in *T. brucei*.

Intriguingly, AP-2, one of the major clathrin recruiters in opisthokonts, has not been identified in the genome of African trypanosomes although it is present in the genome of other trypanosomes, such as *Trypanosoma cruzi*, *Trypanosoma theileri*, *Trypanosoma grayi*, and *Trypanosoma carassii* (Morgan et al., 2002; Manna et al., 2013). The authors suggested that the high density of the VSG coat would not require cargo selection/accumulation to be coordinated by AP-2. They also proposed that the absence of this protein could increase the speed of endocytosis by reducing the need for dephosphorylation reactions in the clathrin uncoating processes (Manna et al., 2013). However, clathrin uncoating is essential for cargo progression to the endosomal system. Therefore, dephosphorylation reactions

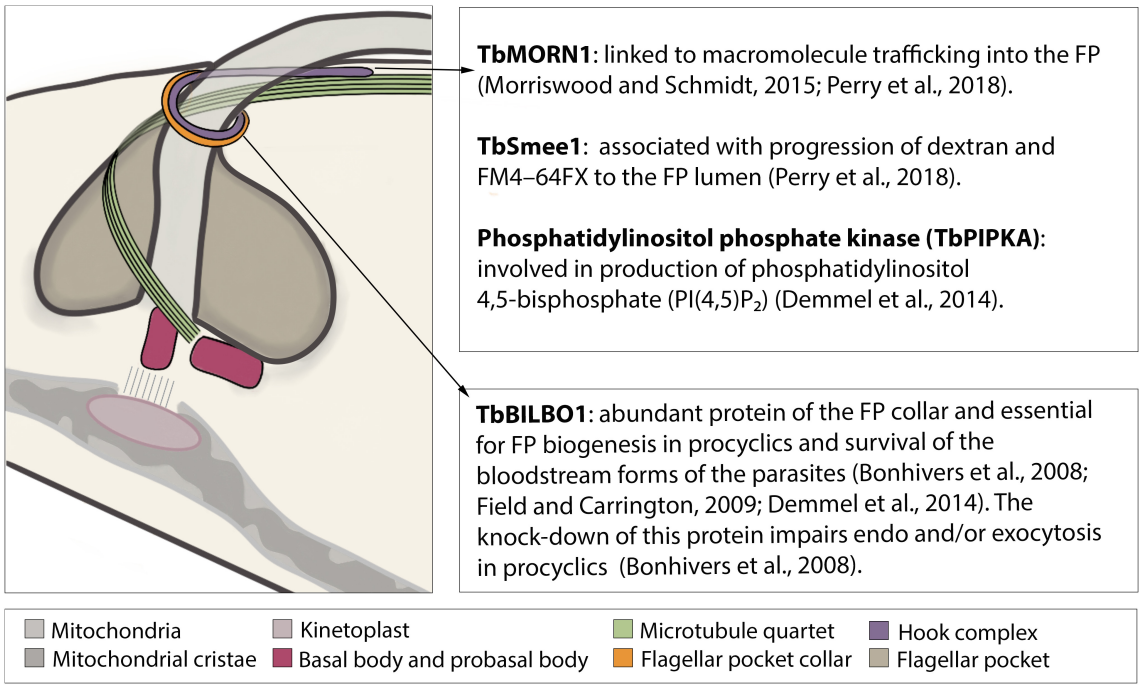

FIGURE 2 | Schematic structure of the flagellar pocket with emphasis on its cytoskeleton components involved in endocytosis. The flagellar pocket (FP) of *Trypanosoma brucei* is a subdomain of the plasma membrane, corresponding to approximately 2–5% of its total area (Grünfelder et al., 2002; Engstler et al., 2004; Field and Carrington, 2009). On its most external face lies the flagellar pocket collar (FP collar) of which TbBILBO1 is the main component (Sherwin and Gull, 1989; Bonhivers et al., 2008; Lacomble et al., 2009). On top of the FP collar lies the hook complex (HC), a multiprotein structure, of which three components (TbMORN1, TbSmee1, and TbPIPKA) have been shown to be involved in endocytosis (Morriswood et al., 2009; Zhou et al., 2010; Esson et al., 2012; Demmel et al., 2014; Morriswood and Schmidt, 2015; Perry et al., 2018; Sajko et al., 2020).

are necessary in the presence of any clathrin-coat accessory molecules that bind to phosphoinositide, such as PI(4,5)P_2 that is present in the FP membrane of *T. brucei* (Demmel et al., 2014). Thus, the abolishment of AP-2 dephosphorylation alone might not explain a faster endocytosis rate.

A proteomic approach revealed the existence of *T. brucei* clathrin-associating proteins (TbCAP) that had not been identified through *in silico* screening (Adung'a et al., 2013). Of these, eight were found to be exclusive to trypanosomatids: TbCAP116, TbCAP118, TbCAP125, TbCAP161, TbCAP186, TbCAP292, TbCAP334, and TbTOR-like 1 (Adung'a et al., 2013). Interestingly, knockdown of TbCAP100, TbCAP116, TbCAP161 and TbCAP334 decreased endocytosis of FITC-ConA at early time-points and led to FP enlargement, suggesting their involvement in clathrin assembly and VSG trafficking (Adung'a et al., 2013).

The cytoskeleton is another important factor in endocytosis. Recently, genetic screening confirmed the presence of actin and actin-related proteins in *T. brucei* (reviewed in Gupta et al., 2020). In bloodstream forms, actin is localized in the posterior region of the cell body, between the nucleus and kinetoplast, and its depletion causes impairment of endocytosis and enlargement of the FP (Garcia-Salcedo et al., 2004). The actin cytoskeleton also seems to play a role in the polarization of the mechanoenzyme TbMyo1 in the posterior region of bloodstream form cells (Spitznagel et al., 2010). Immunofluorescence assays on fixed cells revealed differences in both the number and the fluorescence intensity of TbMyo1 spots inside the population suggesting a dynamic nature of the protein (Spitznagel et al., 2010). Knockdown of TbMyo1 led to the impairment of endocytosis and changes in the subcellular distribution of clathrin, which clearly demonstrates its role in endocytosis (Spitznagel et al., 2010). Previous studies had suggested that dynamin, involved in CCV budding in mammals and yeast, was not involved in endocytosis in *T. brucei* (Morgan et al., 2004). However, the single dynamin-like protein of *T. brucei* has two paralogs (TbDLP1 and TbDLP2), which were found to have distinct expression patterns and functions depending on the life cycle stage of the parasite (Benz et al., 2017).

Compared to mammals and yeast, both the description and characterization of clathrin endocytosis in *T. brucei* lack details and require further analysis to be better understood. Among the questions to be elucidated are, for example, how clathrin recruitment and association with the CCP is coordinated and how cytoskeleton elements participate in vesicle budding from the FP membrane and in their subsequent transport. The use of proteomic methods to investigate the cohort of molecules involved in clathrin assembly in trypanosomes (Adung'a et al., 2013) highlighted the low conservation of the components compared to other eukaryote supergroups. This can either be explained by a reduced clathrin system in *T. brucei*, as suggested

previously, or it demonstrates the need for new experimental strategies to complement *in silico* homology searches.

Endosomal Trafficking in *T. brucei*

In *T. brucei*, all endosomal compartments are in the posterior part of the cell (**Figure 1**). All endocytosed cargo, VSG and fluid phase, pass through EEs (Grünfelder et al., 2003; Engstler et al., 2004). Following uptake, VSG starts to colocalize with EEs after 2.2 s and finally fills up to 85% of the compartment volume (Engstler et al., 2004). From this compartment, 53% of the VSG pool moves to the REs and returns to the surface, a route known as the fast route, which is completed within approximately 10 s (Engstler et al., 2004). Alternatively, 47% of VSGs are recycled through the slow route, which takes approximately 50 s to complete (Engstler et al., 2004). Here, VSG passes first from the EE to the LE, a path also followed by the fluid phase cargo, and then returns to the surface via the RE. Interestingly, while the fluid phase cargo moves from the LE to the lysosome for degradation no VSG was detected inside this organelle (Engstler et al., 2004). The retrograde transport of cargo to the TGN has not yet been characterized in *T. brucei*. Studies on the endosomal compartment of *T. brucei* have only been conducted on fixed samples. This, allied to the transient nature of the maturation model, limits our comprehension of the endosomal compartment in this organism. Nevertheless, the endocytic compartment of *T. brucei* also has the characteristic tubular-vesicular shape found in other organisms (Engstler et al., 2004). One molecule, TbMBAP1, was found in all endosomal membranes (Engstler et al., 2005). Four EE markers in this parasite have been identified through homology searches: one homolog of Rab4 (TbRab4), two homologs of Rab5, TbRab5A, and TbRab5B (Field and Boothroyd, 1995; Field et al., 1998), and one homolog of Rab21, TbRab21 (Ali et al., 2014). Studies using epifluorescence microscopy have suggested TbRab4 and both subpopulations of TbRab5 to be present in vesicles in the cytoplasm of bloodstream forms (Field et al., 1998). These vesicles were localized between nucleus and kinetoplast, which is where the endosomal compartment of *T. brucei* is found, and some overlap was observed (Field et al., 1998). However, it is crucial to remember that the resolution of widefield microscopy combined with the close proximity of different endocytic compartments does not allow an accurate localization. In addition, it is tempting to ask if these markers might all be part of a single structure with distinct subdomains. If that were the case, how is the cargo sorted inside this structure? To answer these questions (**Figure 3**), it is crucial to use super-resolution microscopy.

In bloodstream forms TbRab4 acts in fluid phase transport to the lysosome and in the accumulation of the constitutive transmembrane glycoprotein p67 (Hall B. S. et al., 2004; Peck et al., 2008). TbRab5A was related to IgG, transferrin, VSG, and invariant surface glycoprotein (ISG) 65 transport, while TbRab5B has so far only been linked to ISG100 (Field et al., 1998; Chung et al., 2004; Hall B. et al., 2004).

The expression level of TbRab21 is low in the bloodstream forms of the parasite, but the overexpression of tagged TbRab21 revealed its localization in the posterior part of the cell (Ali et al., 2014). Immunofluorescence assays showed a partial overlap of TbRab21 with clathrin, TbRab5A and TbRab11, and juxtaposition with p67. However, some variation in the relative position of TbRab21 to TbRab11 and p67-positive structures was observed within the population (Ali et al., 2014). These variations in position could again be related to the limited resolution of the microscopy techniques used in the study along with cell-to-cell expression level variations. Furthermore, despite partial colocalization with TbRab5A, knockdown of TbRab21 did not have an impact upon the early steps of endocytosis, though it did have an effect on the degradation pathway. These observations together with the colocalization of TbRab21 with TbRab28 and TbVps23 might suggest a role in late steps of endocytosis (Ali et al., 2014).

Orthologs of the ESCRT machinery, such as TbVps4, TbVps23, TbVps24, and TbVps28 were found in *T. brucei* (Leung et al., 2008; Silverman et al., 2013; Gilden et al., 2017; Umaer and Bangs, 2020). These ESCRT components as well as TbFab1 kinase and its product $PI(3,5)P_2$, one of the ligands of TbVps24, localize in LE membranes of *T. brucei* (Leung et al., 2008; Silverman et al., 2013; Gilden et al., 2017; Umaer and Bangs, 2020). Despite the role of ESCRT in ILV formation and MVB development in mammalian cells, *T. brucei* lacks a well-defined MVB (Silverman et al., 2013). Another typical membrane-bound protein of LEs is TbRab7 (Engstler et al., 2004). TbRab7 depletion does not affect the endocytosis rate, but leads to complete cessation of delivery of the endocytosed cargo to the lysosome, a similar effect to that observed upon silencing of TbVps23 and TbVps4 (Silverman et al., 2011, 2013). Interestingly, while TbRab7 has no influence on biosynthetic trafficking of the lysosomal markers p67 and TbCathepsin L (TbCatL), the ESCRT components TbVps4, TbVps23 and TbVps24 do (Silverman et al., 2011, 2013; Umaer and Bangs, 2020). Another Rab, TbRab28, seems to be involved in transport to the lysosome. Depletion of the protein by RNAi resulted in reduced ConA transport to the lysosome in 80% of the cells (Lumb et al., 2011). LEs and lysosome localize proximal to the nucleus (Engstler et al., 2004). The transport from EEs to LEs and/or lysosome is performed by class II clathrin-coated vesicles (CCV II) found budding from endosomes (Grünfelder et al., 2003). CCVs II are 50–60 nm in diameter and are depleted in VSG and enriched in the fluid-phase markers ferritin and horseradish peroxidase (Grünfelder et al., 2003; Engstler et al., 2004). Interestingly, a detailed study of the kinetics of endocytosis in *T. brucei* showed that the fluid-phase marker dextran and biotinylated VSG (VSG_{biotin}) were endocytosed at the same time and then were gradually segregated, reaching a maximum of spatial separation after approximately 1 min (Engstler et al., 2004). At steady state 37% of the intracellular VSG_{biotin} did not lie on the endocytic route of internalized dextran (Engstler et al., 2004). The authors suggested that the separation of VSG from the fluid-phase marker occurred concurrently with the biphasic filling of the RE (Engstler et al., 2004).

The RE is a compartment where cargo transported from EEs and LEs can be redirected to the plasma membrane (Grünfelder et al., 2003; Engstler et al., 2004; Overath and Engstler, 2004). This endosomal compartment is predominantly marked by TbRab11 and has, to a certain extent, an interface with EEs (Grünfelder et al., 2003; Chung et al., 2004; Engstler et al., 2004;

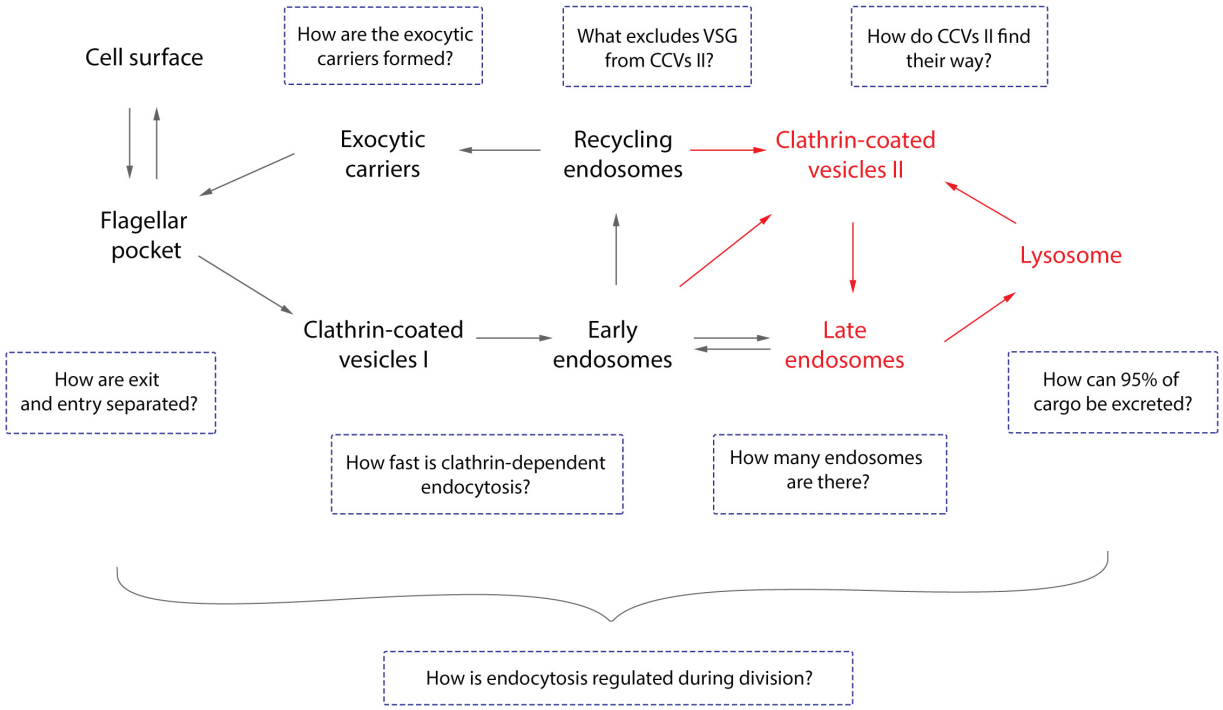

FIGURE 3 | Open questions about endocytosis of trypanosomes. The general route of endocytosis and recycling in *Trypanosoma brucei* is depicted. The gray arrows represent the direction of all cargo while the red arrows refer solely to fluid-phase cargo. Endo- and exocytosis is restricted to a single site in trypanosomes: the flagellar pocket. Thus, the first intriguing question is how does the cell discriminate between cargo entering and leaving the pocket? All molecules are endocytosed via clathrin mediated endocytosis (CME), the recycling rates are incredibly fast, and *T. brucei* lacks AP-2 (Grünfelder et al., 2002; Morgan et al., 2002; Engstler et al., 2004). Therefore, the naturally following questions are: how fast is CME and does the lack of AP-2 influence this process? VSGs have never been observed in the lysosome and the route to this organelle is likely to be mediated by class II clathrin-coated vesicles (CCV II). So, the remaining questions here are how VSG is excluded from CCV II sorting and how these vesicles are routed to the lysosome. Furthermore, the number of endosomes in *T. brucei* cells and the origin of exocytic carriers remain elusive as does the mechanism of excretion of 95% of the cargo. Overall, the essentiality of the VSG coat for trypanosomes allied to the natural increase of volume of the cell during the cell cycle advocates for a precise regulatory mechanism to maintain the dense surface coat. However, such a mechanism has not yet been elucidated.

Field et al., 2009). From the REs, the recycling cargo is destined for the FP via TbRab11-positive exocytic carriers. These disk-shaped carriers, with a diameter of ca. 154 nm and thickness of approximately 34 nm, were found close to both the FP and the cisternae-shaped endosomal compartment near the lysosome (Grünfelder et al., 2003; Engstler et al., 2004). Interestingly, electron microscopy revealed both VSG and horseradish peroxidase within the EXCs (Grünfelder et al., 2003; Engstler et al., 2004). Thus, TbRab11 has been proposed to be involved in both fluid-phase and receptor-mediated cargo recycling to the cell surface. Consistent with these results, TbRab11 RNAi depleted cells showed an approximately 80% reduction in recycling of transferrin (Hall et al., 2005). However, conflicting observations were reported in a further study that used the same knockdown strategy. In this study, TbRab11 depleted and control cells were treated with cycloheximide to flush nascent VSG from the exocytic pathway and free surface amino groups were blocked by acetylation at 4°C (Umaer et al., 2018). Subsequently, shifting to 37°C allowed the recycling of internal unblocked VSG, which is susceptible to surface biotinylation. Flow cytometry revealed an increase of about 10% in surface biotinylation 5 min after the temperature shift (Umaer et al., 2018). The authors considered this to be similar to the kinetics of exocytosis in control cells. This led to the proposition of the existence of a redundant mechanism to recycle VSG to the cell surface (Umaer et al., 2018). However, the endocytosis kinetics published by Engstler et al. (2004) reports that around 10% of total VSG (equivalent to the intracellular VSG pool) is recycled per minute. Consequently, the measurement after 5 min can mask an up to five times slower recycling kinetics and the possibility of a functional, but less efficient, recycling pathway without TbRab11. Another possibility could be related to the activity of residual TbRab11 following RNAi, which might still be contributing to VSG transport. It would be desirable to look at earlier timepoints (e.g., 1 min) to establish differences in kinetics of TbRab11 depleted and control cells. Therefore, the existence of a redundant mechanism in VSG recycling remains controversial.

A third class of clathrin-coated vesicles was observed close to the outermost *trans*-Golgi cisterna, which was proposed to be the TGN equivalent in *T. brucei* (Grünfelder et al., 2003). The TGN-homolog marker, TbGRIP70, was localized in the outer Golgi cisterna of the trypanosomatid *Leishmania mexicana* that had been genetically modified to express TbGRIP70, through immunoelectron microscopy (McConville et al., 2002).

The same localization was observed in *T. brucei* through immunofluorescence assays using widefield microscopy (Sealey-Cardona et al., 2014). However, whether this third class of clathrin vesicles in trypanosomes is part of the biosynthetic route, as observed in HeLa cells infected with varicella-zoster virus (Alconada et al., 1996), or of the retrograde recycling route is not clear. Furthermore, homologs of the retromer complex subunits, associated with the retrograde transport in other organisms, were found in *T. brucei* (TbVps26, TbVps29, and TbVps35) as was one homolog of the SNX family interactor (TbVps5) (Koumandou et al., 2011). Immunofluorescence assays suggested a close association of the *T. brucei* retromer complex and the endocytic apparatus components (Koumandou et al., 2011). However, a structural characterization is not available to date. The depletion of TbRab28 led to a decrease in the expression of TbVps26 and the ESCRT components TbVps23 and TbVps28 suggesting that these components may have a functional connection (Lumb et al., 2011). Notably, the retrograde recycling mechanism is not associated with VSG (Engstler et al., 2004).

CONCLUSION

In trypanosomes, most studies to identify adaptor/accessory proteins of the endocytic/exocytic machinery have employed homology searches based on genes found in mammals and yeast. Based on this, the trypanosome machinery has been deemed to be simpler than the machineries of the opisthokont models (Manna et al., 2014). However, genomic BLAST is limited to similarity searches, indicating not the complexity of the characteristics of different organisms but the conservation between them. Therefore, it is interesting and not entirely unexpected that studies using direct screening for trypanosome-specific proteins have led to the discovery of new components involved in distinct steps within the endocytic/exocytic machinery (Adung'a et al., 2013; Boehm et al., 2017).

Investigations into the maturation model and evidence of endosomal mobility are still missing from *T. brucei* cell biology. For example, our knowledge of the contribution of the cytoskeleton to vesicle budding and transport is limited. Consequently, it would be interesting to analyze transport mechanisms for directed vesicle motion in *T. brucei*. In addition, specific VSG sorting at different trafficking steps is likely to exist but remains to be studied.

The methods used for subcellular localization and morphological descriptions of the endocytic/exocytic apparatus are another important aspect to be considered. The compartment markers have usually been located via widefield fluorescence microscopy and characterized via RNAi knockdown. The use of electron microscopy has been restricted to the morphological analysis of the FP. However, considering the high dynamicity observed in endosomal tubules of living cells, analyses of fixed samples could give the impression of a fragmented morphology (Baetz and Goldenring, 2013; Goldenring, 2015). Furthermore, microscope resolution is an important limiting factor that could also lead to inaccuracies in morphological descriptions, as overlapping signals in epifluorescence microscopy can be resolved into subdomains inside an organelle when investigated by super-resolution microscopy (Baetz and Goldenring, 2014).

Therefore, our understanding of the divergent endomembrane system of *T. brucei* still lags behind that of the classical opisthokont systems. A greater focus on trypanosomes could contribute to a broader comprehension of cell biology. In fact, a more wholesome understanding of the biodiversity requires an in depth look at a range of diverged organisms. Detailing the dynamics of endocytic processes with nanoscale resolution using novel and emerging technologies, such as live correlative light and electron microscopy (live CLEM) (Fu et al., 2019) or expansion microscopy (Chen et al., 2015), will be the next step for all cell biology model systems, and this time there is no reason for the trypanosome model to lag behind.

AUTHOR CONTRIBUTIONS

AB and FL wrote the manuscript. AB designed the figures. ME and NJ provided conceptual input and contributed to writing. All authors read and approved the final manuscript.

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REVIEW
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Advances in Understanding *Leishmania* Pathobiology: What Does RNA-Seq Tell Us?

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Leishmaniasis is a vector-borne disease caused by a protozoa parasite from over 20 *Leishmania* species. The clinical manifestations and the outcome of the disease vary greatly. Global RNA sequencing (RNA-Seq) analyses emerged as a powerful technique to profile the changes in the transcriptome that occur in the *Leishmania* parasites and their infected host cells as the parasites progresses through their life cycle. Following the bite of a sandfly vector, *Leishmania* are transmitted to a mammalian host where neutrophils and macrophages are key cells mediating the interactions with the parasites and result in either the elimination the infection or contributing to its proliferation. This review focuses on RNA-Seq based transcriptomics analyses and summarizes the main findings derived from this technology. In doing so, we will highlight caveats in our understanding of the parasite's pathobiology and suggest novel directions for research, including integrating more recent data highlighting the role of the bacterial members of the sandfly gut microbiota and the mammalian host skin microbiota in their potential role in influencing the quantitative and qualitative aspects of leishmaniasis pathology.

Keywords: *Leishmania*, RNA-Seq, gene expression, promastigotes, amastigotes, macrophages, skin lesions, metatranscriptomics

INTRODUCTION

Leishmaniasis is caused by a parasitic protozoan carried by over 90 sandfly species which are known to transmit more than 20 species of *Leishmania* parasites to humans, through either zoonotic or anthroponotic infection cycles (Ready, 2013; Rostamian and Niknam, 2019; World Health Organization [WHO], 2019). Three main forms of the disease exist and range in severity from mutilating cutaneous leishmaniases (CL) causing skin lesions and ulcers, mucocutaneous leishmaniasis (MCL) leading to a partial or total destruction of mucous membranes of the nose, mouth and throat and visceral leishmaniasis (VL), also known as kala-azar, affecting the spleen and liver and causing mortality in over 95% of untreated cases (World Health Organization [WHO], 2019).

Leishmania parasites, when alternating between the sandfly vector and the mammalian host, have two major corresponding life stages: promastigotes living in the sandfly's gut and

amastigotes residing inside mammalian macrophages (**Figure 1**). Following a blood meal, the sandfly ingests a macrophage containing *Leishmania* amastigotes. Once liberated from the macrophage inside the sandfly midgut, amastigotes differentiate into procyclic promastigotes. Then, the procyclic promastigotes become nectomonad promastigotes, which are able to cross the protective peritrophic matrix of the sandfly gut and attach to the microvilli of the epithelial cells of the midgut (Bates, 1994; Gossage et al., 2003; Bates, 2007; Sunter and Gull, 2017). From there, they can migrate to the thoracic midgut and stomodeal valve where they differentiate into leptomonad promastigotes. The leptomonad promastigotes can eventually differentiate into either haptomonad promastigotes, which attach to the stomodeal valve, or metacyclic promastigotes, which are the mammalian infective form. Metacyclic promastigotes are transmitted to the host by the sandfly during the next blood meal (Bates, 1994; Sacks et al., 2000; Rogers et al., 2002; Bates, 2007; Dillon et al., 2015a).

The diverse pathologies associated with *Leishmania* infections develop following excessive inflammatory responses by the infected tissues due to dis-regulated immune responses, which are increasingly appreciated to be triggered by complex network of interactions between the parasites, host immunocytes, the bacteria from the human skin microbiota and sandfly vector gut microbiota in addition to *Leishmania* RNA viruses (LRV) known to infect some *Leishmania* species (Ives et al., 2011; Brodskyn and de Oliveira, 2012; Gimblet et al., 2017; Cruz and Freitas-Castro, 2019) (**Figure 2**). Understanding the complex interplay between these factors involved in the host-parasite-microbiota interactions during *Leishmania* infection is crucial to refine the design of *in vivo* and *in vitro Leishmania* infection models and assays to study the biology of these parasites in more details and to eventually develop more effective prophylactic and therapeutic strategies with minimal or no side effects (**Figure 2**).

Although numerous studies have employed microarrays and RT-PCR to profile gene expression changes in *Leishmania* and host macrophages following infection (McNicoll et al., 2006; Srividya et al., 2007; Guerfali et al., 2008; Lin et al., 2008; Kumari et al., 2008; y Fortéa et al., 2009; Alcolea et al., 2010, 2011; Adaui et al., 2011; Probst et al., 2012; Rabhi et al., 2012, 2013; Beattie et al., 2013), global RNA sequencing (RNA-Seq) analyses emerged as a powerful methodology to generate a more global and systematic analyses of the transcriptome of the parasite and relevant cells from the concomitant insect vector and mammalian host. This review focuses on RNA-Seq based analyses of the transcriptome of *Leishmania*-hosts interactions and summarizes the main findings extracted using this technology. This includes the more detailed understanding of the interactions between human macrophages and *Leishmania* parasites and the differentiation of the parasite along its various cellular forms characteristic of its complex life cycle. By doing so we will highlight current caveats in our understanding of the parasite's pathobiology and speculate on how RNA-Seq investigations will be able to further contribute at reducing our knowledge gap on the molecular and cellular basis of host-parasite interactions among these fascinating and complex parasites along with their microbial endosymbionts (e.g., LRV) and microbial neighbors (insect gut and mammalian skin microbiotas). This will integrate the more recent insights into the potential role played by other microbes associated with *Leishmania* environment, including bacteria members of the insect gut microbiota and the mammalian host skin microbiota.

CONTROL OF GENE EXPRESSION IN *Leishmania*

Leishmania and related trypanosomatids have unusual mechanisms to control gene expression (Clayton, 2016). Little transcriptional regulation appears to exist as non-functionally related genes arranged in large clusters are constantly transcribed into long polycistronic precursor RNAs (Martınez-Calvillo et al., 2003). Large polycistronic transcription units (PTUs) may be comprised of more than hundred genes with no obvious/apparent functional relationship (Beverley, 2003; Ivens et al., 2005). Kinetoplastid parasites rely almost exclusively on post-transcriptional gene regulation because of their constitutive transcription of RNA polymerase II (pol II)-driven polycistronic gene arrays (de Pablos et al., 2016). Accordingly, RNA binding proteins (RBPs) act as primary gene regulators and are overrepresented in the proteome (de Pablos et al., 2019). RNA binding proteins dynamically bind to mRNA forming ribonucleoprotein complexes (mRNPs) and regulate the trafficking and processing of mRNA molecules from synthesis to decay (Gehring et al., 2017). Moreover, it has been shown that in *Leishmania mexicana* parasites, mRNA levels are not a strong predictor of whole cell RNA expression or RNA binding potential of encoded proteins (de Pablos et al., 2019). Thus, the mechanisms mRNA stability, decay or translation are controlled in near absence of transcriptional regulation are still not fully elucidated (de Pablos et al., 2016).

Following transcription, precursor transcripts are processed into mature mRNAs coding for individual proteins through an unusual process known as *trans*-splicing (Matthews et al., 1994; Liang et al., 2003; Kramer and Carrington, 2011). During *trans*-splicing at specific positions, a polycistronic precursor is removed and a 39 bp long mini-exon is added to the 5'end of all mRNAs. The added mini-exon is also known as the spliced leader (SL) (LeBowitz et al., 1993). Then, a poly-A tail is added to the 3′-end of the mRNA. As such, the polyadenylation of the upstream gene is directed by and coupled to the *trans*-splicing of the downstream gene (LeBowitz et al., 1993). The mRNA is then exported to the cytoplasm where it can be recognized by the translation machinery by a highly modified 5′-cap structure on the mini-exon part (Freire et al., 2017). Notably, despite of the polycistronic transcription approach being used, adjacent genes often do not have the same levels of gene expression (Rastrojo et al., 2013). Indeed, some collinear mRNAs can have highly distinct steady-state expression levels (Rastrojo et al., 2013).

Despite these challenging and interesting/unusual features of the *Leishmania* transcriptome, variation in

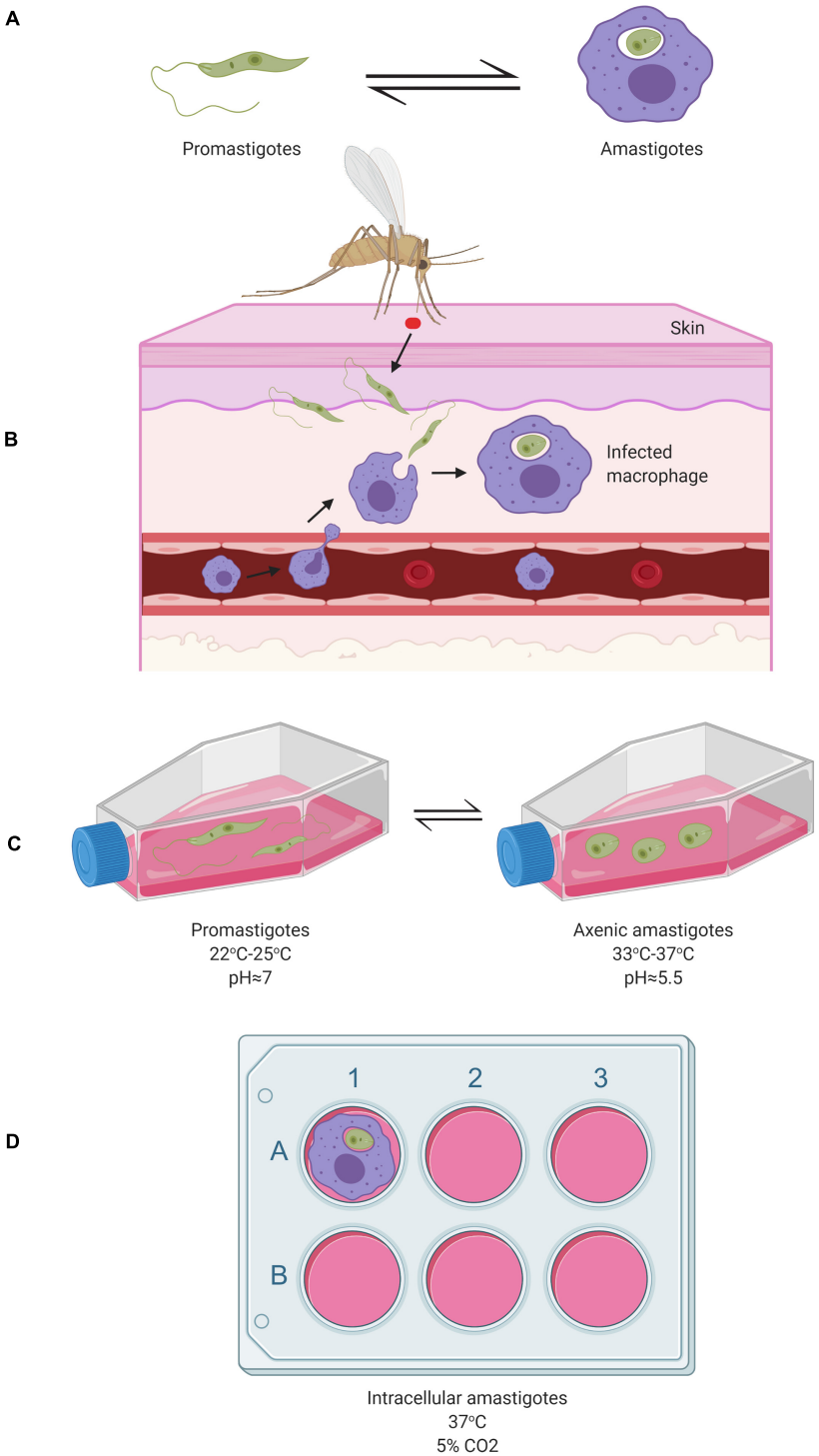

FIGURE 1 | Schematic representation of the two major *Leishmania* cellular forms during their digenic life cycle *in vivo* and their study in corresponding *in vitro* model systems. **(A)** The flagellated motile promastigotes form (left) in the gut of a sandfly and the round intracellular amastigotes form residing inside infected mammalian macrophages (right), represent the two major cellular forms of *Leishmania* species. **(B)** Under *in vivo* conditions, sandfly vectors inject *Leishmania* promastigotes into the host's skin following a blood meal which are then taken up by macrophages where they differentiate into amastigotes; **(C)** Under *in vitro* conditions, axenic promastigotes can be maintained in cell culture flasks in a rich, 10%- Fetal Bovine Serum (FBS)-supplemented medium (e.g., RPMI 1640, Schneider, Grace, or M199) at 22–25°C (left). These can be differentiated *in vitro* into axenic amastigotes in a medium at an acidic pH~5.5 (Achieved through the addition of HCl) at 33–37°C (right) (Teixeira et al., 2002); **(D)** Macrophages (e.g., from human or mouse), can be seeded in 6-well cell plates in RPMI media supplemented with 10% FBS at 37°C to which the parasites are added resulting in macrophages infection within 4 to 24 h. More detailed aspects of the *Leishmania* life cycle are reviewed by Sunter and Gull (2017). Image created with BioRender.com.

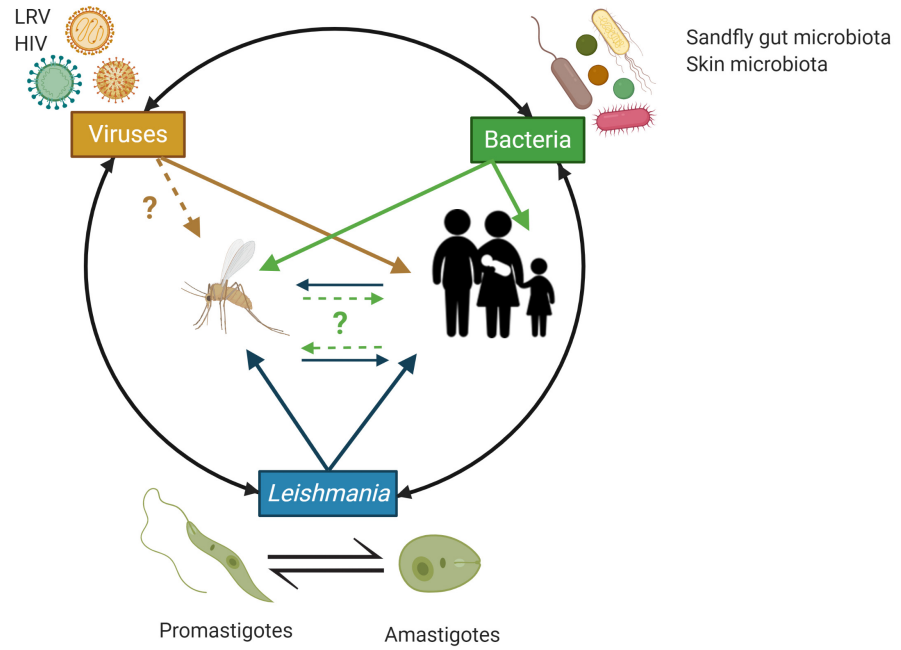

FIGURE 2 | Schematic overview of the potential trans-kingdom cross talk between *Leishmania*, viruses (including *Leishmania* RNA virus – LRV) and bacteria from the implicated animal hosts microbiota and their impact on the human host. Animal hosts (here, sand flies and humans) are increasingly recognized to be supra-organisms made of animal cells/tissues/organs and their associated microbiota (e.g., associated with the human skin and mucosal surfaces) that through complex, continuous and highly dynamic interactions contribute to the host phenotype in both health and disease. In the context of infections by *Leishmania*, initiated by the bite of a sand fly, bacteria from the insect gut microbiota, the human skin microbiota and *Leishmania* RNA virus (LRV), which can infect *Leishmania*, have all be implicated in regulating the inflammatory tone associated with the infection by *Leishmania* and by doing so to the various pathobiologies associated with *Leishmania* infections. HIV is also indicated as AIDS can facilitate/worsen the pathobiology or *Leishmania* infections through weakening the host immune response and represent an important case of co-infections with negative impact on the human host. Image created with BioRender.com.

mRNA abundance has been observed between different life stages of the digenic infection cycles where mRNA levels were not considered as a strong predictor of the whole cell expression and protein levels (Lahav et al., 2011; de Pablos et al., 2016, 2019).

ADVANTAGES AND LIMITATIONS OF RNA-SEQ TECHNOLOGY

Global RNA sequencing and transcriptomics analysis illustrates significant variations in gene expression occurring during host infection with *Leishmania*, its survival inside the sandfly vector gut and during its differentiation between its two life forms as amastigotes and promastigotes. The implementation of RNA-Seq for studying gene expression in *Leishmania* culminated in a rapid expansion of the available knowledge with regard to the host parasite interactions, the existing variations, the relationship between the parasite and its vector and the differentiation of the parasite (Shadab et al., 2019). Haydock et al. (2015) developed the RNA-Seq-based protocol to exploit the 39 bp SL found at the 5′-end of all *Leishmania* mRNA transcripts (Haydock et al., 2015).

Numerous studies on gene expression profiling were performed in *Leishmania* under various conditions and on its different life stages (McNicoll et al., 2006; Srividya et al., 2007; Guerfali et al., 2008; Lin et al., 2008; Kumari et al., 2008; y Fortéa et al., 2009; Alcolea et al., 2010, 2011; Adaui et al., 2011; Probst et al., 2012; Rabhi et al., 2012, 2013; Beattie et al., 2013). RNA-Seq provided, however, several advantages over other less advanced gene expression profiling approaches such as RT-PCR and microarrays (Nowrousian, 2010). As opposed to hybridization-based methodologies, RNA-Seq can be used without the availability of previous genomic data, which is particularly useful in studying non-model organisms (Vera et al., 2008). It is also less dependent on complete gene annotations as it can identify new genes missed in initial annotations of the genomes of interest (Dillon et al., 2015a). Additionally, RNA-Seq has very low background signal when compared to DNA microarrays having a large dynamic range of expression levels without an upper limit for quantification (Wang et al., 2009). It also provides more accurate quantification of expression levels which could be confirmed through qPCR (Nagalakshmi et al., 2008). Finally, less starting material, total RNA, is required and the results obtained can be highly reproducible for technical and biological replicates (Nagalakshmi et al., 2008; Wang et al., 2009). The most frequently used parameter to measure mRNA abundance based on RNA-Seq data is fragments–or reads-per kilobase of transcript per million mapped reads (FPKM). FPMK represents transcript abundance by considering the

RNA length and the total read number in the measurement (Mortazavi et al., 2008). However, other units have been shown to be more effective at contrasting mRNA abundance between samples, including transcripts per millions reads (TPM) (Wagner et al., 2012)

With the vast amount of data generated, there are many opportunities to "translate" this data into forms that can be exploited for drug and vaccine development. The comparison of results between various RNA-seq studies has been hindered by differences in the parasite host, developmental stages of the parasite and experiment lab conditions (for instance, hours of infection) (Dillon et al., 2015a) as such various variables must be addressed and standardized during experimental design to allow for accurate comparative studies of differential gene expression.

Notably, RNA-Seq experiments can also be used to perform metatranscriptomics analyses, reviewed below, at the infection sites and covering the variations in host skin microbiota and insect gut microbiota can provide additional insights into the complex relationships between the bacterial members of the sandfly gut microbiota and the mammalian host skin microbiota in shaping the disease outcome as well as the potential role of LRV present in at least some species of *Leishmania*.

SANDFLY VECTOR TRANSCRIPTOMICS

Isolating promastigotes from the natural microenvironment (i.e., the vector host) is one potentially useful approach but technically challenging (Alcolea et al., 2016). Previously, *in vitro* infectivity and differential gene expression have been studied in *Leishmania infantum* promastigotes isolated from the stomodeal valve of the sandfly *Phlebotomus perniciosus* (Alcolea et al., 2016). Differential gene expression was determined by RNA shotgun genome microarray hybridization analysis and showed that most differentially expressed genes were involved in regulation of gene expression, intracellular signaling, amino acid metabolism and biosynthesis of surface molecules (Alcolea et al., 2016). Microarray hybridization analysis, however, cannot account for the presence of bacterial or viral genes (Schulze and Downward, 2001). Thus, metatranscriptomics analysis using RNA-Seq on *Leishmania* promastigotes isolated from the inside of the stomodeal valve of the sandfly of the vector could potentially provide more information on the complex relationship existing between parasites, viruses and bacteria co-existing in the vector.

The diversity of the natural gut microbiota of sandflies is acquired from several sources, including feeding on their respective animal and plant sources of blood and sugar, or re-colonization of the gut by the microbes ingested by the terrestrial dwelling larval stages (Dillon et al., 1996; Hillesland et al., 2008; Mukhopadhyay et al., 2012; Peterkova-Koci et al., 2012; Sant'Anna et al., 2012).

A recent 16S rRNA sequence profiling of the gut microbiota from *Lutzomyia longipalpis*, the primary vector of VL in Brazil, revealed 13 distinct bacterial genera (*Bacillus*, *Enterococcus*, *Erwinia*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Lysinibacillus*, *Pseudocitrobacter*, *Providencia*, *Pseudomonas*, *Serratia*, *Staphylococcus,* and *Solibacillus*) (Campolina et al., 2020). Following a co-cultivation of the identified bacteria with various *Leishmania* species in *in vitro* conditions, a growth reduction in all tested parasites was observed suggesting a potential role of the gut microbiota in hindering parasite transmission by the sandfly vector (Campolina et al., 2020). In contrast, the bacterial communities naturally present in the *Phlebotomus duboscqi* sandfly midgut were shown to be essential for the colonization of the midgut by infective stage, metacyclic *Leishmania major* promastigotes (Louradour et al., 2017).

Although insects are hosts to a vast variety of viruses (Telleria et al., 2018), relatively little is known about viruses infecting sandflies (Depaquit et al., 2010). These include phleboviruses such as Toscana viruses (TOSV) (Depaquit et al., 2010). As such, a recent metatranscriptomics analysis of individual mosquitoes unveiled their blood meal sources and uncovered a rich microbial cargo consisting of eukaryotes, prokaryotes, and a high frequency of viral co-infection with 70 known and novel viral species (Batson et al., 2020).

The salivary transcriptome of the *Nyssomyia neivai* sandfly, one of the main vectors of tegumentary leishmaniasis in Brazil, has also been recently performed and highlighted the abundances of several *N. neivai* salivary proteins which can be used as biomarkers of *N. neivai* (Vernal et al., 2020). Thus, looking into the microbial diversity of sandfly vectors gut is of particular interest. Such experiments could also compare and contrast various species of sandfly vectors.

FINDINGS IN *Leishmania* GLOBAL TRANSCRIPTOMICS

The first RNA-Seq global gene expression profiling was performed on *L. major* in 2013 (Rastrojo et al., 2013). A time-based evolution of RNA-Seq experiments conducted on *Leishmania* parasites is presented in **Figure 3**. Rastrojo et al. (2013) investigated the transcriptome of *L. major* axenic promastigotes and identified a total of 10,285 transcripts including 1,884 novel transcripts that did not match genes previously annotated. They observed and reported the presence of extensive heterogeneity in the SL and polyadenylation addition sites. A sequence comparison of ribosomal protein L23 showed that both genes have identical coding regions, but marked differences both in length and sequence in the 3′-UTRs., which could be linked to the efficiency of the mRNA translation (Rastrojo et al., 2013). Most abundant transcripts included cytosolic heat shock protein 70 (HSP70), various ribosomal proteins, nucleoside transporters, histone H4, peptidases, cyclophilin, *Leishmania*-activated C-kinase antigen (LACK), amastin-like surface protein and alpha tubulin (Rastrojo et al., 2013).

Later Fiebig et al. (2015) used RNA-Seq to characterize and compare the transcriptomes of *L. mexicana* promastigotes, axenic and intracellular amastigotes. A comparative analysis of gene expression between promastigotes and amastigotes revealed 3,832 significantly differentially expressed among 9,169 protein-coding genes. Genes associated with the motility of the flagellum were mainly downregulated while those linked

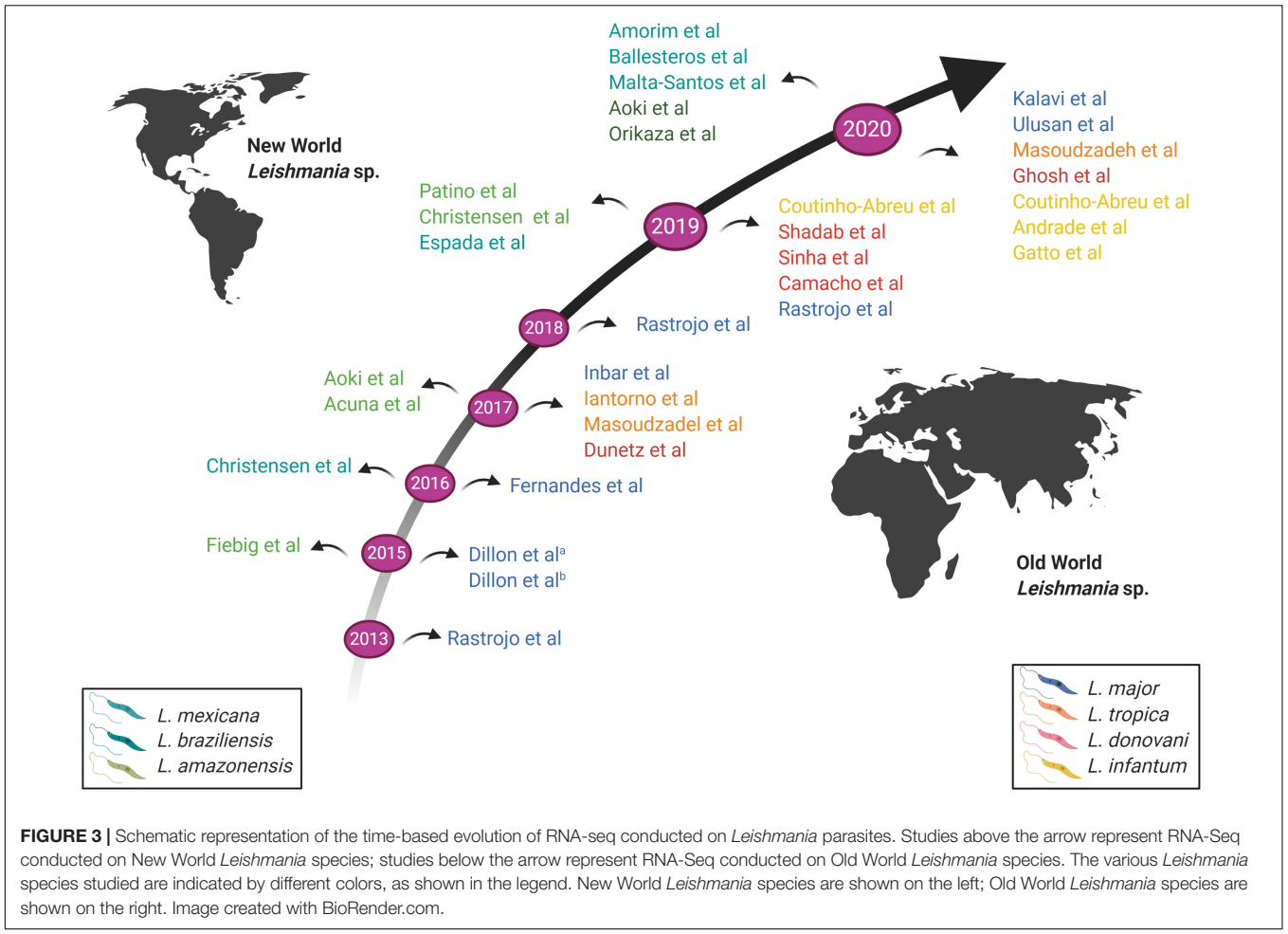

FIGURE 3 | Schematic representation of the time-based evolution of RNA-seq conducted on *Leishmania* parasites. Studies above the arrow represent RNA-Seq conducted on New World *Leishmania* species; studies below the arrow represent RNA-Seq conducted on Old World *Leishmania* species. The various *Leishmania* species studied are indicated by different colors, as shown in the legend. New World *Leishmania* species are shown on the left; Old World *Leishmania* species are shown on the right. Image created with BioRender.com.

to cell surface proteins, transporters, peptidases, and a number of uncharacterized proteins were upregulated in amastigotes. Around 936 novel transcripts were identified providing the first evidence of a link between the whole chromosome duplication event and adaptation to the vertebrate host in this group. Gene ontology-term (GO-term) enrichment analyses exploit the GO system of classification to link genes based on functional characteristics (Bauer et al., 2008). This approach revealed that unfolded protein binding, protein folding, aminoacyl-tRNA ligase activity and tRNA aminoacylation (for protein translation and microtubule motor activity) were preferentially expressed in promastigotes compared to intracellular amastigotes in *L. mexicana*. Genes related to the cytoplasm, calmodulin binding, microtubule-based flagellum, protein folding, and aminoacyl-tRNA ligase activity were preferentially expressed in promastigotes when compared to axenic amastigotes. Chromosome 30 carried genes the expression of which was upregulated in amastigotes suggesting a link between chromosome duplication, gene expression, and parasite host adaptation. Finally, genes having a GO-term annotation related to nucleosome, nucleosome assembly, DNA binding, and DNA replication were preferentially expressed in axenic amastigotes versus intracellular amastigotes (Fiebig et al., 2015).

The differences in gene expression occurring as *Leishmania* acquires its infectivity *in vitro* were also assessed. Dillon et al. (2015a) conducted a global transcriptomic analysis on *L. major* as it gained its infectivity comparing non-infective procyclic promastigotes and infective metacyclic promastigotes. In doing so, they detected 3,138 differentially expressed genes that included iron/zinc transporters, histone H4, adenine amino hydrolysases and 1,044 novel open reading frames (ORFs). Using GO analysis, they revealed that ATP synthesis coupled proton transport and cytoplasm related genes were among the downregulated genes in metacyclic promastigotes. On the other hand, genes encoding for protein kinases and ATP binding were upregulated during differentiation from non-infective procyclic promastigotes and infective metacyclic promastigotes. A widespread alternative *trans*-splicing and polyadenylation was also detected in 8,981 genes (94.2% of a total of 9,530 genes). 5′ and 3′ UTR boundaries for a majority of genes were also identified. No association was found between stage-specific preferential *trans*-splicing or polyadenylation sites and differentially expressed genes. As a result, differences in gene expression levels could not be attributed to stage-regulated alternative mRNA processing (Dillon et al., 2015a).

Furthermore, Iantorno et al. (2017) combined genome-wide high-throughput DNA sequencing with RNA-seq and could identify correlation between chromosome aneuploidy (somy), CNVs, and SNPs across 14 *Leishmania tropica* isolates with total gene expression at the promastigotes stage grown *in vitro*. They found that gene dosage, at the level of individual genes or chromosomal "somy" resulted in more than 85% of total gene expression variation in genes with a two-fold or greater change in expression. Genes encoding membrane-bound transporters were among the most highly upregulated genes. Biopterin transporter BT1 and folate transporter FT1, aquaglyceroporin genes responsible for transport of trivalent antimonials into the cell and the LiMT/LiRos3 transport system, were all previously linked to drug resistance (Marquis et al., 2005; Mandal et al., 2015; Imamura et al., 2016). Notably, in *L. tropica* LRC-L810 recovered from a distinct sandfly vector (*Phlebotomus arabicus* rather than the more commonly known vector *Phlebotomus sergenti*) (Soares et al., 2004) showed a very distinct expression profile (Iantorno et al., 2017). The most highly upregulated gene in isolate LRC-L810 encoded for a receptor-type adenylate cyclase (Iantorno et al., 2017) that has been previously linked to differentiation in African trypanosomes (Fraidenraich et al., 1993), inhibition of the host immune response in *Trypanosoma brucei* (Salmon et al., 2012), and motility in the insect stages (Lopez et al., 2015). *L. tropica* species remains understudied using RNA-seq. As opposed to other *Leishmania* species such as *L. major* (Rastrojo et al., 2013; Dillon et al., 2015a), *L. mexicana* (Fiebig et al., 2015; Patino et al., 2019) and others (**Figure 3**), very few studies addressed differences in gene expression in *L. tropica* (Iantorno et al., 2017).

Dumetz et al. (2017) also correlated chromosome aneuploidy to changes in gene expression in *Leishmania donovani* during their adaptation to *in vivo* conditions while mimicking natural vertebrate and invertebrate host environments. To mimic the various life stages of the parasite, they used Syrian golden hamsters and sandflies to investigate *in vivo* amastigote and promastigote stages, respectively, and compared the results to *in vitro*-maintained parasites. Aneuploidy was highly variable and correlated with the corresponding transcript levels although aneuploidy-independent regulation of gene expression was also observed. A total of 589 genes were upregulated in promastigotes compared to amastigotes. These mainly belonged to carbon, lipid, and fatty acid metabolism, translation, protein modification, membrane transport, DNA replication and nucleosome assembly, and the function of the flagellum. On the other hand, only 261 genes encoding for amastins and amastin-like proteins, among others were upregulated in amastigotes (Dumetz et al., 2017).

Camacho et al. (2019) also generated the transcriptome of *L. donovani* HU3 promastigotes and identified 2,410 novel transcripts 1,513 of which were homologs to transcripts annotated in *L. major* (Rastrojo et al., 2013). Histone transcripts, alpha tubulin genes, ribosomal proteins, HSP70, and kinetoplastid membrane protein 11 (KMP11) were found to be among the most abundant transcripts (Camacho et al., 2019).

Inbar et al. (2017) described the transcriptome dynamics of *L. major* as it develops inside its natural vector *P. duboscqi* by examining global gene expression in procyclic (PP), nectomonad (NP), and metacyclic promastigotes (MP) compared to BALB/c mice lesion-derived amastigotes (AM) and culture-derived metacyclic promastigotes (CMP). Notably, the greatest number of differentially expressed genes was observed during early transformation from AM to PP in the blood-fed midgut where amastins were down-regulated while multiple cell surface proteins, sugar and amino acid transporters, and genes related to glucose metabolism and cell cycle progression were upregulated. The NP stage revealed changes in genes acting in cell cycle arrest and the upregulation of genes associated with starvation and stress, including autophagic pathways of protein recycling. Maturation to the infective MP showed amastigote-like profiles of surface proteins and metabolism-related genes. The results obtained from comparing gene expression profiles of sandfly derived and culture-derived MP revealed similar results except for an upregulation of transcripts associated with nutrient stress *in vivo* (Inbar et al., 2017).

Coutinho-Abreu et al. (2020b) performed RNA-seq on unaltered midguts of infected *L. longipalpis* sandflies, the vector of *L. infantum*. The sequences obtained from procyclic, nectomonad, leptomonad or metacyclic promastigote stages were grouped into distinct populations based on principal component analysis, with the procyclic stage being the most distinct. A total of 836 genes were differentially expressed between procyclic and nectomonad promastigotes, 113 between nectomonad and leptomonad promastigotes and 302 between leptomonad and metacyclic promastigotes. Most of the differentially expressed genes were uniquely expressed in each stage and not in other stages. Stage-specific markers included genes encoding a zinc transporter in procyclics, a beta-fructofuranidase in nectomonads, a surface antigen-like protein in leptomonads, and an amastin-like surface protein in metacyclics (Coutinho-Abreu et al., 2020b). Conversely, the presence of *L. infantum* in *L. longipalpis* sandflies produced a limited change in the sandfly's transcript expression profile (Coutinho-Abreu et al., 2020a). As such, the parasite appeared to modulate gene expression early on in the developmental cycle (Days 1 to 4) in order to overcome the barriers imposed by the midgut of the insect, then most of the differentially expressed transcripts were up-regulated with small fold changes at later time points (Day 6 and onward) with only slight changes observed in midgut gene expression (Coutinho-Abreu et al., 2020a). The most abundant transcripts in promastigotes and amastigotes of various *Leishmania* species are summarized in **Tables 1**, **2**, respectively.

HOST CELL'S RESPONSE TO INFECTION

Another advantage of using RNA-Seq is to be able to look into gene expression changes in the host cell following parasitic infections in so called dual RNA-Seq experiments (Dillon et al., 2015b; Fernandes et al., 2016). Dillon et al. (2015b) aimed at identifying global changes in gene expression using murine macrophages from C57BL/6 mice and *L. major* at 4, 24, 48, and 72 h post-infection (hpi). The most significant response to infection by the macrophage was observed at 4 hpi, with 6,897 mouse genes significantly differentially expressed between

TABLE 1 | Top twenty most abundant transcripts in promastigotes of various *Leishmania* species.

Reference	**Rastrojo et al. (2013)[a]**		**Dillon et al. (2015b)[b]**		**Fiebig et al. (2015)[c]**		**Dumetz et al. (2017)[d]**	
Species	***L. major***		***L. major***		***L. mexicana***		***L. donovani***	
#	**Gene ID**	**Product description**	**Gene ID**	**Product description**	**Gene ID**	**Product description**	**Gene ID**	**Product description**
1	LmjF.28.T2770	HSP70–II	LmjF.12.0940	PSA2**	LmxM.01.0410	–	LdBPK_230 013000	PAP2 superfamily, putative
2	LmjF.35.T0240	ribosomal protein L30	LmjF.12.1000	PSA2	LmxM.03.0250	ribosomal protein L38, putative	LdBPK_040 015700	COPI associated protein, putative
3	LmjF.28.T2780	HSP70–II	LmjF.12.0980	PSA2	LmxM.03.0430	60S acidic ribosomal protein P2, putative	LdBPK_350 038000	cystathione gammalyase, putative
4	LmjF.36.T1940	inosine–guanosine transporter (NT2)	LmjF.34.3645	hypothetical protein (pseudogene)	LmxM.04.0750	60S ribosomal protein L10, putative	LdBPK_310 032500	3′–nucleotidase/nuclease precursor, putative
5	LmjF.31.T0900	hypothetical protein	LmjF.12.0910	PSA	LmxM.06.0010	histone H4	LdBPK_020 011100	hypothetical protein, conserved
6	LmjF.28.T2205	ribosomal protein S29	LmjF.20.0150	hypothetical protein	LmxM.06.0570	60S ribosomal protein L23a, putative	LdBPK_360 045100	EF–hand domain pair, putative
7	LmjF.35.T2220	KMP11*	LmjF.12.0920	PSA	LmxM.07.0680	40S ribosomal protein S9, putative	LdBPK_340 015900	amastin–like protein
8	LmjF.19.T0983	–	LmjF.12.1040	surface antigen protein putative	LmxM.08_29.2461	60S ribosomal protein L13, putative	LdBPK_120 012700	–
9	LmjF.35.T0600	ribosomal protein L18a	LmjF.12.1020	surface antigen protein putative	LmxM.09.1340	histone H2B	LdBPK_120 014800	PSA2
10	LmjF.06.T0010	histone H4	LmjF.14.1360	inositol-3-phosphate synthase (INO1)	LmxM.10.0070	ribosomal protein l35a, putative	LdBPK_260 017500	HSP70
11	LmjF.35.T3800	ribosomal protein L23	LmjF.12.0860	surface antigen protein putative	LmxM.11.0970	40S ribosomal protein S5	LdBPK_180 021700	hypothetical protein
12	LmjF.36.T3620	hypothetical protein, conserved	LmjF.12.1060	surface antigen protein putative	LmxM.13_158814	novel transcript	LdBPK_120 013500	PSA2
13	LmjF.35.T2210	KMP11*	LmjF.07.0745	hypothetical protein	LmxM.13.0280	alpha tubulin	LdBPK_200 018100	small myristoylated protein-1
14	LmjF.28.T2460	ribosomal protein S29	LmjF.12.0780	surface antigen protein 2 precursor	LmxM.13.0560	60S ribosomal protein L18, putative	LdBPK_190 014100	ATG8/AUT7/APG8/ PAZ2,putative
15	LmjF.20.T1285	–	LmjF.28.1570	hydrolase alpha/beta fold family putative	LmxM.13.1670	60S ribosomal protein L44, putative	LdBPK_190 020100	hypothetical protein
16	LmjF.31.T0964	–	LmjF.32.2260	HSP20	LmxM.14.1270	ubiquitin/ribosomal protein S27a, putative	LdBPK_030 011600	Uroporphyrinogen-III synthase HemD, putative
17	LmjF.31.T0895	–	LmjF.26.0640	10 kDa heat shock protein putative	LmxM.15_455510	novel transcript	LdBPK_360 057700	paraflagellar rod component, putative
18	LmjF.35.T3290	ribosomal protein L31	LmjF.07.0745	hypothetical protein	LmxM.15.0010	histone H4	LdBPK_360 076600	tartrate-sensitive acid phosphatase acp-3.2, putative

(Continued)

TABLE 1 | Continued

Reference		Rastrojo et al. (2013)[a]		Dillon et al. (2015b)[b]		Fiebig et al. (2015)[c]		Dumetz et al. (2017)[d]	
Species		*L. major*		*L. major*		*L. mexicana*		*L. donovani*	
#	Gene ID	Product description	Gene ID	Product description	Gene ID	Product description	Gene ID	Product description
19	LmjF.13.T0570	ribosomal protein S12	LmjF.36.4050	hypothetical protein	LmxM.15.1160	tryparedoxin peroxidase	LdBPK_250026700	2,4-dihydroxyhept-2-ene-1,7-dioicacidaldolase, putative
20	LmjF.35.T3790	ribosomal protein L23	LmjF.31.1440	hypothetical protein	LmxM.15.1240	nucleoside transporter 1, putative	LdBPK_310040800	iron/zinc transporter protein-like protein

**Kinetoplastid membrane protein-11; **Promastigote surface antigen protein 2.*

[a]In Rastrojo et al. (2013), promastigotes were grown to mid log phase by seeding cultures at 1 × 10^6 cells/mL, and collected for RNA isolation when the culture density reached 6.1 × 10^6 cells/mL (mid-logarithmic phase of growth).

[b]In Dillon et al. (2015b), murine macrophages from C57BL/6 mice were infected with L. major metacyclic promastigotes at a ratio of 5 parasites per macrophage for 4 h. L. major promastigotes were not split for more than 5 passages. List of upregulated genes was obtained by comparing metacyclic promastigotes to amastigotes at 4 hpi.

[c]In Fiebig et al. (2015), promastigotes were harvested in late exponential growth phase (around 1 × 10^7 cells/ml) and collected for RNA isolation.

[d]In Dumetz et al. (2017), promastigotes were harvested in logarithmic phase (day 4). List of upregulated genes was obtained by comparing differential expression in metacyclic promastigotes to amastigotes.

uninfected and infected cells. Genes related to both pro- and anti-inflammatory immune responses and glycolysis were substantially upregulated, and genes related to lipid metabolism, biogenesis, and Fc gamma receptor-mediated phagocytosis were downregulated in the murine macrophages. Genes linked to the mitigation of oxidative stress by the host immune system were upregulated while genes related to translation, cell signaling, fatty acid biosynthesis, and flagellum structure were downregulated in *L. major* amastigotes. KEGG enrichment analysis revealed that the differentially expressed genes in macrophages were related to immune response (cytokine-cytokine receptor interactions and arginine and proline metabolism) and glycolysis. The immunoregulatory activity detected in macrophages infected with *L. major* has been previously also observed in macrophages stimulated with lipopolysaccharide (LPS) (Fleming et al., 2015). Dillon et al. (2015b) also observed a total of 2,962 genes that were differentially expressed in metacyclic promastigotes compared to amastigotes. These were mostly thought, based on their annotation, to be involved in reducing the effects of an oxidative stress response exerted on the parasite by the innate immune response taking place in the phagosome (Zhang et al., 2010). Heat shock proteins, especially HSP83, multiple tryparedoxin peroxidase family members, and multiple cyclophilins were all upregulated upon entry of metacyclic promastigotes into host cells (Dillon et al., 2015b). Human monocytes infected with *L. major* also revealed an upregulation of pro-inflammatory cytokine and cytokines receptors including IL1A, IL1RN, IL6, and IL6R (Kalavi et al., 2020).

It's noteworthy that Fernandes et al. (2016) used a dual transcriptome approach to profile gene expression in human $CD14^+$ monocytes infected by *L. major* and *Leishmania amazonensis* at different time points (4, 24, 48, and 72 hpi). A temporal expression pattern was observed in both the macrophage and the parasite as macrophage response and parasite transformation stabilized shortly (4 hpi) after entry of the *Leishmania* into the host. No significant difference was observed in the parasite species transcriptomes or the macrophage response between the two different *Leishmania* species. Following infection with *L. major*, 5,713 human genes were differentially expressed between uninfected and infected macrophages. Similar results were obtained with *L. amazonensis*, mainly detecting genes encoding for inflammatory cytokines (IL-1β, TNF, TNF superfamily members, and IL-6) and a number of immunomodulators. Immunomodulators included: prostaglandin-endoperoxide synthase 2 (PTGS2), colony-stimulating factors 1 and 2 (CSF1 and CSF2), and superoxide dismutase 2 (SOD2). SOD2 was upregulated only following infection with *L. major*. Of interest were the host metallothionein 1 family members with their role in the disease outcome requiring further attention. On the parasite side, genes encoding for amastin, *gp63*, kinesin, flagellar attachment zone protein, AAT family members, dynein and cysteine peptidase B were all upregulated post-infection in both *Leishmania* species (Fernandes et al., 2016).

Moreover, Shadab et al. (2019) investigated host-specific and parasite-specific factors modulating the host-parasite interaction in *L. donovani* AG83 strain. They infected murine peritoneal

TABLE 2 | Top twenty most abundant transcripts in amastigotes of three major *Leishmania* species.

Reference	Dillon et al. (2015b)[a]		Fiebig et al. (2015)[b]		Dumetz et al. (2017)[c]	
Species	***L. major***		***L. mexicana***		***L. donovani***	
#	**Gene ID**	**Product description**	**Gene ID**	**Product description**	**Gene ID**	**Product description**
1	LmjF.12.1060	surface antigen protein putative	LmxM.01.0410	unspecified product	LdBPK_260011800	Thioredoxin, putative
2	LmjF.12.1020	surface antigen protein putative	LmxM.03.0250	ribosomal protein L38, putative	LdBPK_090017600	DNA-directed RNApolymerase III subunit, putative
3	LmjF.12.0780	surface antigen protein 2 precursor	LmxM.03.0430	60S acidic ribosomal protein P2, putative	LdBPK_350057300	hypothetical protein
4	LmjF.12.0920	PSA	LmxM.04.0750	60S ribosomal protein L10, putative	LdBPK_190015500	FYVE zinc finger containing protein, putative
5	LmjF.12.1040	surface antigen protein putative	LmxM.06.0010	histone H4	LdBPK_300018500	hypothetical protein
6	LmjF.12.0910	PSA	LmxM.06.0570	60S ribosomal protein L23a, putative	LdBPK_160011500	RNA recognition motif
7	LmjF.12.0810	PSA2	LmxM.06.0580	60S ribosomal protein L23a, putative	LdBPK_340024100	Amastin surface glycoprotein, putative
8	LmjF.12.0860	surface antigen protein putative	LmxM.07.0680	40S ribosomal protein S9, putative	LdBPK_290023800	MutS-like protein
9	LmjF.12.0940	PSA2	LmxM.08_29.1090	ribosomal protein L1a, putative	LdBPK_200019600	hypothetical protein
10	LmjF.12.0890	surface antigen protein 2 putative	LmxM.08_29.1740	histone H2A, putative	LdBPK_350041100	Pre-rRNA-processing protein PNO1, putative
11	LmjF.12.1000	PSA2	LmxM.08_29.1800	40S ribosomal protein S15A, putative	LdBPK_320045600	hypothetical protein
12	LmjF.12.0980	PSA2	LmxM.08_29.2461	60S ribosomal protein L13, putative	LdBPK_160010000	hypothetical protein
13	LmjF.12.0960	surface antigen protein 2 putative	LmxM.08.1030	unspecified product	LdBPK_110016600	hypothetical protein
14	LmjF.04.0040	hypothetical protein	LmxM.08.1030a	unspecified product	LdBPK_300028700	Zinc finger
15	LmjF.25.1120	aldehyde dehydrogenase mitochondrial precursor (ALDH2)	LmxM.08.1070	cathepsin L-like protease, putative	LdBPK_300010200	hypothetical protein
16	LmjF.26.1340	DNA ligase k alpha putative	LmxM.09.1340	histone H2B	LdBPK_040017600	casein kinase I, putative
17	LmjF.12.0830	surface antigen protein 2 putative	LmxM.10.0070	ribosomal protein l35a, putative	LdBPK_310015600	KIAA1430 homolog, putative
18	LmjF.34.2940	hypothetical protein conserved	LmxM.13.0280	alpha tubulin	LdBPK_110016700	hypothetical protein
19	LmjF.24.1280	amastin-like surface protein-like protein	LmxM.13.0450	hypothetical protein, conserved	LdBPK_230018900	5-formyltetrahydrofolatecyclo-ligase family, putative
20	LmjF.27.1080	hypothetical protein conserved	LmxM.13.1670	60S ribosomal protein L44, putative	LdBPK_210015500	hypothetical protein

[a] *In Dillon et al. (2015b), murine macrophages from C57BL/6 mice were infected with L. major metacyclic promastigotes at a ratio of 5 parasites per macrophage for 4 h. L. major promastigotes were not split for more than 5 passages. List of upregulated genes was obtained by comparing amastigotes at 4 hpi vs. 24 hpi.*

[b] *In Fiebig et al. (2015), to generate intracellular amastigotes, promastigotes were left to grow into stationary phase (from 1 × 10^5 to 2 × 10^7 cells/ml) and then incubated with mouse macrophages at a parasite to macrophage ratio of 20 to 1. Leishmania-infected macrophages were harvested 24 h post-infection and collected for RNA isolation.*

[c] *In Dumetz et al. (2017), amastigotes were recovered from infected hamsters and resuspended at 2 × 10^8 amastigotes per ml in phosphate-buffered saline (PBS) for RNA extraction. List of upregulated genes was obtained by comparing differential expression in amastigotes to metacyclic promastigotes.*

macrophages with either the virulent or a non-virulent variant of the parasite derived from the AG83 strain. Infection with virulent *L. donovani* strains revealed suppression of many important cellular processes, including protein synthesis. Genes encoding virulence factors and those important for parasite survival were significantly upregulated in the intracellular virulent amastigotes. In contrast, genes involved in the immune stimulations and negative regulation of the cell cycle and transcriptional regulation were also all upregulated in the non-virulent strains. The non-virulent *L. donovani* AG83 strain was generated by

Sinha et al. (2018) through continuous *in vitro* passages and revealed global changes in the genome and transcriptome of the serially passaged *L. donovani* AG83 promastigotes. No massive transcript expression changes between the virulent and non-virulent AG83 promastigotes were observed and among the few differentially expressed genes were the one coding for acid phosphatase playing a role in virulence and endosome sorting (Katakura and Kobayashi, 1988), and cyclin-dependent kinase pho85-like protein. Homologs of the latter gene was previously implicated environmental signaling in yeast in response to stressful environmental conditions (Carroll and O'Shea, 2002).

Dual RNA sequencing of both enucleated fibroblasts (cytoplasts) and intracellular *L. amazonesis* has been recently performed to gain further insights in the parasite's control over the host cell (Orikaza et al., 2020). This experimental system demonstrated that the parasite multiplication and biogenesis of large parasitophorous vacuoles were independent of the host cell nucleus (Orikaza et al., 2020). Infected cytoplast transcripts were enriched in phagolysosome-related pathway, pro-survival, and SerpinB-mediated immunomodulation compared to control non-infected cytoplasts. Notably, these results suggested that a parasite-mediated control of host cell transcripts half-life was beneficial to the parasite's intracellular multiplication and evasion of the host immune response (Orikaza et al., 2020).

L. infantum did not activate the inflammasomes in the THP-1 human macrophage infected cells (Gatto et al., 2020). In fact, *L. infantum* triggered a gene expression pattern more similar to non-infected THP-1 cells yet very different from LPS-stimulated cells. Some of the most up-regulated genes in *L. infantum*-infected cells were related to: cell cycle (CDC20, CDKN2C, CNNL2), glycolysis/gluconeogenesis (ENO1), metabolism of carbohydrates (AGRN), signal transduction (RIT1, RPS6KA1, ILR6, SFPQ), MAPK signaling (RPS6KA1), gene expression (NOTCH2), ubiquitin mediated proteolysis (CDC20) and interleukin signaling (TNFRSF14, TNFRSF1B, ILR6, CSF1). Genes associated with the inflammasome signaling pathway were not differentially expressed and caspase-1 activation and IL-1β production were absent following infection (Gatto et al., 2020). Genes significantly upregulated or downregulated in macrophages following infection with various *Leishmania* species are summarized in **Tables 3**, **4**, respectively.

ASSESSMENT OF METABOLIC PATHWAYS

RNA-Seq was also used to assess the importance of L-arginine pathway in *L. amazonensis* promastigotes and axenic amastigotes (Aoki et al., 2017). The L-arginine pathway in *Leishmania* is important during the parasite life cycle and infection of the mammalian macrophages (Cunningham, 2002). Nitric oxide (NO) production is a defense mechanism used by macrophages being produced by nitric oxide synthase 2 (NOS2) in the presence of the amino acid L-arginine as substrate (Cunningham, 2002). Arginase on the other hand, reduces NO production by limiting the availability of L-arginine and favoring the survival of *Leishmania* in the macrophage (Camargo et al., 1978; Aoki et al., 2020). Aoki et al. (2017) studied gene expression differences between *L. amazonensis* wild-type (*La*-WT) and *L. amazonensis* arginase knockout (*La*-arg$^-$) promastigotes and axenic amastigotes. In doing so, they identified a total of 8,253 transcripts in both strains of which 60% encoded hypothetical proteins, 443 were novel transcripts that did not match any previously annotated genes, and 85% were constitutively expressed. WT amastigotes had lower levels of arginase and higher levels of glutamate-5-kinase compared to WT promastigotes. *La*-arg$^-$ promastigotes had increased levels of pyrroline 5-carboxylate reductase, but decreased levels of arginosuccinate synthase, pyrroline 5-carboxylate dehydrogenase, acetylornithine deacetylase and spermidine synthase compared to the WT. Thus, arginase activity is important in *Leishmania* gene expression modulation during its differentiation and adaptation to environmental changes. Acuña et al. (2017) also described an arginase-dependent NOS-like activity in *L. amazonensis* which could be important to trigger parasite differentiation during infection of macrophages. They suggested that NO production could be arginase-dependent and higher levels of NO were produced in axenic amastigotes compared to promastigotes. Aoki et al. (2019) further expanded the study of the role of arginase in the modulation of virulence factors involved in parasite recognition, growth and differentiation in *L. amazonensis*. Arginase was found to upregulate membrane markers that affect parasite recognition, autophagy-related genes implicated in parasite differentiation and amastins that impact amastigote replication and survival, while modulating oxidative-stress related genes (Aoki et al., 2019). The parasite's arginase activity also appeared to modulate gene expression in the host macrophage including in the immune response and amino acid transport and metabolism (Aoki et al., 2020).

Recently, Malta-Santos et al. (2020) investigated the importance of the polyamine biosynthetic pathway in CL lesions. Diffuse CL was associated with higher concentrations of amino acids, polyamines and its substrate transporters compared to MCL or localized CL. The most upregulated transcripts in diffuse CL lesions were CAT2A (isoform encoded by SLC7A2), ARG1 and SMS compared to MCL lesions acting in the regulation of arginine availability. A differential gene expression of polyamine metabolism-related genes of patients' lesions was associated with parasite loads and the leishmaniasis disease outcome (Malta-Santos et al., 2020).

DIRECT APPLICATION TO SKIN BIOPSIES

RNA-Seq can also be directly applied on human tissues such as skin biopsies. Christensen et al. (2016, 2019) studied gene expression changes in the host and the parasite using skin biopsies from *Leishmania braziliensis*-infected patients with, respectively, localized cutaneous leishmaniasis (LCL) and diffuse cutaneous leishmaniasis (DCL). In Christensen et al. (2016), detectable parasite transcripts in only one

TABLE 3 | Top twenty upregulated genes in macrophages following infection with various *Leishmania* species.

Reference	**Dillon et al. (2015b)[a]**		**Fernandes et al. (2016)[b]**		**Shadab et al. (2019)[c]**	
Cell Types	**C57BL/6 mice peritoneal macrophages**		**Human macrophages**		**C57BL/6 mice peritoneal macrophages**	
Species	***L. major***		***L. major***		***L. donovani***	
#	**ID**	**Product description**	**ID**	**Product description**	**MGI ID**	**Product description**
1	ENSMUSG00000031762	metallothionein 2	ENSG00000168334	xin actin-binding repeat containing 1	MGI:1340899	chitinase-like 1
2	ENSMUSG00000031765	metallothionein 1	ENSG00000122641	inhibin beta A	MGI:2685490	transmembrane protein 132E
3	ENSMUSG00000010051	hyaluronoglucosaminidase 1	ENSG00000164400	colony stimulating factor 2 (granulocyte-macrophage)	MGI:2135946; MGI:2443796	CD163 antigen; CD163 molecule -like 1
4	ENSMUSG00000045362	tumor necrosis factor receptor superfamily member 26	ENSG00000010310	gastric inhibitory polypeptide receptor	MGI:2676631	interleukin 17F
5	ENSMUSG00000050914	ankyrin repeat domain 37	ENSG00000125144	metallothionein 1G	MGI:107364; MGI:2676631	interleukin 17A; interleukin 17F
6	ENSMUSG00000037709	family with sequence similarity 13 member A	ENSG00000205364	metallothionein 1M	MGI:96062; MGI:2685715	histidine decarboxylase; hdc homolog, cell cycle regulator
7	ENSMUSG00000000794	potassium intermediate/small conductance calcium-activated channel subfamily N member 3	ENSG00000169908	transmembrane 4 L six family member 1	MGI:2146080	shisa family member 8
8	ENSMUSG00000031444	coagulation factor X	ENSG00000169715	metallothionein 1E	MGI:1352744; MGI:2672905	deltex 1, E3 ubiquitin ligase; deltex 4, E3 ubiquitin ligase
9	ENSMUSG00000089929	B cell leukemia/lymphoma 2 related protein A1b	ENSG00000115009	chemokine (C-C motif) ligand 20	MGI:1916978	caspase recruitment domain family, member 11
10	ENSMUSG00000078566	BCL2/adenovirus E1B interacting protein 3	ENSG00000073861	T-box 21	MGI:1858224	carbohydrate (chondroitin 6/keratan) sulfotransferase 3
11	ENSMUSG00000029321	solute carrier family 10 (sodium/bile acid cotransporter family) member 6	ENSG00000181773	G protein-coupled receptor 3	MGI:1306780	early growth response 3
12	ENSMUSG00000025161	solute carrier family 16 (monocarboxylic acid transporters) member 3	ENSG00000149635	osteoclast stimulatory transmembrane protein	MGI:1917066	marginal zone B and B1 cell-specific protein 1
13	ENSMUSG00000062345	serine (or cysteine) peptidase inhibitor clade B member 2	ENSG00000125084	wingless-type MMTV integration site family member 1	MGI:105086	POU domain, class 2, associating factor 1
14	ENSMUSG00000031709	TBC1 domain family member 9	ENSG00000166670	matrix metallopeptidase 10 (stromelysin 2)	MGI:101774	CD79A antigen (immunoglobulin-associated alpha)
15	ENSMUSG00000035105	egl-9 family hypoxia-inducible factor 3	ENSG00000128342	leukemia inhibitory factor	MGI:96543; MGI:96547	interleukin 1 beta; interleukin 1 receptor antagonist
16	ENSMUSG00000024730	membrane-spanning 4-domains subfamily A member 8A	ENSG00000117090	signaling lymphocytic activation molecule family member 1	MGI:1919299	tumor necrosis factor receptor superfamily, member 13c
17	ENSMUSG00000039753	F-box and leucine-rich repeat protein 5	ENSG00000205358	metallothionein 1H	MGI:88319	CD19 antigen
18	ENSMUSG00000056054	S100 calcium binding protein A8 (calgranulin A)	ENSG00000166923	gremlin 1 DAN family BMP antagonist	MGI:2443630	sialic acid binding Ig-like lectin G

(Continued)

TABLE 3 | Continued

Reference	Dillon et al. (2015b)[a]		Fernandes et al. (2016)[b]		Shadab et al. (2019)[c]	
Cell Types	C57BL/6 mice peritoneal macrophages		Human macrophages		C57BL/6 mice peritoneal macrophages	
Species	*L. major*		*L. major*		*L. donovani*	
#	ID	Product description	ID	Product description	MGI ID	Product description
19	ENSMUSG00000024679	membrane-spanning 4-domains subfamily A member 6D	ENSG00000129521	egl-9 family hypoxia-inducible factor 3	MGI:2138647	Fc receptor-like A
20	ENSMUSG00000019916	procollagen-proline 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase) alpha 1 polypeptide	ENSG00000164181	ELOVL fatty acid elongase 7	MGI:96941	chymase 1, mast cell

[a] *In Dillon et al. (2015b), peritoneal macrophages were isolated from C57BL/6 mice and infected with L. major metacyclic promastigotes at a ratio of 5 parasites per macrophage. The list of up-regulated genes shows top genes consistently upregulated across all timepoints upon infection by L. major at 4, 24, 48, and 72 hpi.*

[b] *In Fernandes et al. (2016), human macrophages were derived from purified monocytes and infected using a ratio of 5 L. major parasites per macrophage for 4 h. List of upregulated genes was obtained by comparing differential gene expression in L. major-infected human macrophages relative to uninfected controls at 4 hpi.*

[c] *In Shadab et al. (2019), peritoneal C57BL/6 mice macrophages were infected at a parasite to macrophage ratio of 10:1 for 12 h. List of top upregulated genes was obtained by comparing differential gene expression in L. major-infected human macrophages relative to uninfected controls.*

group of patients were found in which an increase in B lymphocyte-specific and immunoglobulin transcripts in the lesions, and an upregulation of immune inhibitory molecules was observed. Patients negative for parasite transcripts had a decrease in B cell activation, but increased expression of antimicrobial genes and genes encoding skin barrier functions, and which were different from transcripts expressed in human macrophages *in vitro*. Therefore, the differences in gene expression observed *in vitro* in macrophages does not accurately reflect the one observed from skin lesions, as expected as in the skin biopsies one collects multiple cells. Hence it is not possible/very difficult to compare the transcriptome of macrophages in cell culture versus mix of cells, including macrophages, from a biopsy. Highly expressed genes in the parasite included those encoding for cysteine peptidase and synthase, glycerophosphoryl phosphodiesterase family protein and transport protein SEC13 among others. Upregulated genes in lesions with detectable parasite transcripts encoded immunoglobulin fragments, CXCL8 (encoding chemokine IL-8, granulocyte and neutrophil chemotaxis), IL-21 (B cell proliferation), and granulysin (cellular cytotoxicity) encoding genes (Christensen et al., 2016).

Skin lesions from BALB/c mice infected with *L. major* showed an upregulation of FCGR4, CCL4, CXCL9, Arg1, and IL-1β and an enrichment of the Triggering Receptor Expressed on Myeloid Cells 1 (TREM1) signaling pathway (Ulusan et al., 2020).

To date, very few metatranscriptomics studies have been performed on *L. tropica*. These included RNA-Seq performed on skin biopsies in *L. tropica* infected patients (Masoudzadeh et al., 2017). Unfortunately, the usefulness and application of this study for downstream comparative analysis remains limited due to the unavailability of reads from *L. tropica* which were not analyzed nor deposited in a public database. Notably, transcription profiling of *L. tropica*-infected skin identified over 5,000 differentially regulated human genes compared to uninfected controls (Masoudzadeh et al., 2017). Gene ontology enrichment analysis indicated that upregulated genes were mostly involved in immune response activation, extracellular matrix degradation, inflammatory cell recruitment, cytotoxicity and antimicrobial peptides (Masoudzadeh et al., 2017). Similarly to the previous report by Christensen et al. (2016), they observed an increase in B lymphocyte-specific (immunoglobulin lambda-like polypeptide 5, IGLL5) and immunoglobulin receptor (Fc Fragment of IgG Receptor Ia, FCGR1A) transcripts. These findings highlight the role of B cells at the infection site. Downregulated genes on the other hand, encoded mainly for structural proteins associated with muscle contraction (e.g., myosin 2, alpha 1 actin, myosin 1, and troponin C type 2) (Masoudzadeh et al., 2017). Furthermore, shared and unique functional transcriptional pathways in *L. tropica* infected skin lesions of ulcerative CL (UCL) and non-ulcerative CL (NUCL) patients were investigated using RNA-Seq (Masoudzadeh et al., 2020a). Inflammatory cytokines and chemokines were differentially expressed in the UCL and NUCL lesions. Transcriptional pathways for Fcγ receptor dependent phagocytosis were enriched in both conditions (Masoudzadeh et al., 2020a).

TABLE 4 | Top twenty down-regulated genes in macrophages following infection with various *Leishmania* species.

Reference	**Dillon et al. (2015a)[a]**		**Fernandes et al. (2016)[b]**		**Shadab et al. (2019)[c]**	
Cell Types	**57BL/6 mice peritoneal macrophages**		**Human macrophages**		**57BL/6 mice peritoneal macrophages**	
Species	***L. major***		***L. major***		***L. donovani***	
#	**ID**	**Product description**	**ID**	**Product description**	**MGI ID**	**Product description**
1	ENSMUSG00000013236	protein tyrosine phosphatase receptor type S	ENSG00000121933	adenosine A3 receptor	MGI:102709	caveolin 1, caveolae protein
2	ENSMUSG00000036067	solute carrier family 2 (facilitated glucose transporter) member 6	ENSG00000220008	leucine rich repeat and Ig domain containing 3	MGI:2136773	CWC22 spliceosome-associated protein
3	ENSMUSG00000029581	fascin homolog 1 actin bundling protein	ENSG00000127533	coagulation factor II (thrombin) receptor-like 3	MGI:2145242	arrestin domain containing 3
4	ENSMUSG00000021451	sema domain immunoglobulin domain (Ig) transmembrane domain (TM) and short cytoplasmic domain (semaphorin) 4D	ENSG00000150681	regulator of G-protein signaling 18	MGI:107422	heat shock protein 4 like
5	ENSMUSG00000053063	C-type lectin domain family 12 member a	ENSG00000179144	GTPase IMAP family member 7	MGI:88590	cytochrome P450, family 1, subfamily b, polypeptide 1
6	ENSMUSG00000076431	SRY (sex determining region Y)-box 4	ENSG00000137834	SMAD family member 6	MGI:2449975	IQ motif containing GTPase activating protein 2
7	ENSMUSG00000091747	–	ENSG00000245848	CCAAT/enhancer binding protein (C/EBP) alpha	MGI:2443069	peptidylprolyl isomerase domain and WD repeat containing 1
8	ENSMUSG00000025574	thymidine kinase 1	ENSG00000257108	NHL repeat containing 4	MGI:96969; MGI:1913910; MGI:1915147	met proto-oncogene; SAFB-like, transcription modulator; RNA (guanine-7-) methyltransferase
9	ENSMUSG00000002602	AXL receptor tyrosine kinase	ENSG00000107719	phosphatase domain containing paladin 1	MGI:2443342	post-GPI attachment to proteins 1
10			ENSG00000180340	frizzled class receptor 2	MGI:1095419	lysine (K)-specific demethylase 6A
11			ENSG00000152804	hematopoietically expressed homeobox	MGI:2183747	FYVE, RhoGEF, and PH domain containing 4
12			ENSG00000136457	chondroadherin	MGI:107483; MGI:108427; MGI:1924705	ral guanine nucleotide dissociation stimulator-like 2; insulin-like 3; rearranged L-myc fusion sequence
13			ENSG00000234432	–	MGI:1196332	Rho GTPase activating protein 6
14			ENSG00000134222	proline/serine-rich coiled-coil 1	MGI:1914829	cytoplasmic polyadenylation element binding protein 4
15			ENSG00000163606	CD200 receptor 1	MGI:1931053	membrane-associated ring finger (C3HC4) 7
16			ENSG00000004799	pyruvate dehydrogenase kinase isozyme 4	MGI:107448	lysosomal trafficking regulator
17			ENSG00000177599	zinc finger protein 491	MGI:1923036	cytoskeleton associated protein 5
18			ENSG00000204131	NHS-like 2	MGI:1913975	leucine-rich repeat kinase 2

(Continued)

TABLE 4 | Continued

Reference	Dillon et al. (2015a)[a]		Fernandes et al. (2016)[b]		Shadab et al. (2019)[c]	
Cell Types	57BL/6 mice peritoneal macrophages		Human macrophages		57BL/6 mice peritoneal macrophages	
Species	*L. major*		*L. major*		*L. donovani*	
#	ID	Product description	ID	Product description	MGI ID	Product description
19			ENSG00000196934	NA	MGI:96562	interleukin 7 receptor
20			ENSG00000213203	GTPase IMAP family member 1	MGI:1914769	DENN/MADD domain containing 4C

[a]*In Dillon et al. (2015b), peritoneal macrophages were isolated from C57BL/6 mice and infected with L. major metacyclic promastigotes at a ratio of 5 parasites per macrophage. The list consists of nine down-regulated genes consistently downregulated across all timepoints upon infection by L. major at 4, 24, 48, and 72 hpi.*

[b]*In Fernandes et al. (2016), human macrophages were derived from purified monocytes and infected using a ratio of 5 L. major parasites per macrophage for 4 h. List of down-regulated genes was obtained by comparing differential gene expression in L. major-infected human macrophages relative to uninfected controls at 4 hpi.*

[c]*In Shadab et al. (2019), peritoneal C57BL/6 mice macrophages were infected at a parasite to macrophage ratio of 10:1 for 12 h. List of top down-regulated genes was obtained by comparing differential gene expression in L. major-infected human macrophages relative to uninfected controls.*

Notably, Christensen et al. (2019) using meta-transcriptomic analysis of biopsies from patients with DCL, demonstrated an infiltration of atypical B cells producing a dominance of the IgG4 isotype in these *L. amazonensis* infected patients. They additionally, revealed the absence of cytotoxic and T_H2 cell responses. A regulatory phenotype was observed in macrophages with some genes being actively expressed such as an ATP-binding cassette, subfamily B, member 5 (ABCB5), dendritic cell specific transmembrane protein (DC-STAMP), and a secreted phosphoprotein 1 (SPP1) among others. Gene expression in DCL lesions resembled patterns obtained from *in vitro* parasite growth in resting macrophages. Furthermore, 336 genes in *L. amazonensis* were upregulated in LCL compared to DCL. Among these upregulated genes, seven potential virulence factors and four stress response genes were detected, including GP63, proteins containing leucine-rich repeats, a cyclophilin protein, a protein containing a RmlC-like jelly roll fold domain, and a Betv1-like superfamily protein.

The blood of *L. braziliensis* infected patients was also recently analyzed using RNA-seq to determine systemic responses that might be influencing the disease (Amorim et al., 2020). A strong interferon stimulated gene (ISG) signature has been identified which was correlated with an increase in circulating monocytes and macrophages (Amorim et al., 2020). A cytotoxicity signature was correlated with an increase in cytolytic cells (Amorim et al., 2020).

Similarly, the blood of VL-HIV patients was studied through transcriptional profiling (Adriaensen et al., 2020). A downregulation of genes associated with host cellular activity and immunity, and upregulation of antimicrobial peptide activity in phagolysosomes was observed in patients that were successfully treated with AmBisome or AmBisome/miltefosine, in contrast, no pathway enrichment among differentially expressed genes was observed in treatment failure patients (Adriaensen et al., 2020).

ASSESSING DRUG RESISTANCE

The appearance of drug resistance phenotypes is a major concern hindering available anti-leishmanial treatment (Sundar and Singh, 2016). Rastrojo et al. (2018) identified genomic and transcriptomic alterations associated with experimental resistance in *L. donovani*, *L. infantum* and *L. major* promastigotes and amastigotes in response to common drugs used against VL including: trivalent antimony (S line), amphotericin B (A line), miltefosine (M line), and paromomycin (P line). In total, 1,006 differentially expressed transcripts were identified in the S line, 379 in the A line, 146 in the M line, and 129 in the P line. Changes in chromosomal aneuploidy and amplification/deletion of particular regions were correlated with resistance. A series of genes were identified as possible drivers of the resistance phenotype. The S line included peptidyl dipeptidase, amastin, and amastin-like surface proteins among others. The M line included protein kinase, carboxypeptidase and amino acid transporter AAT1.4

among others. The P line included flavoprotein subunit-like protein, amastin-like protein, protein associated with differentiation and iron/zinc transporter protein-like protein (LIT1) among others. Finally, the A line included a sterol 24-c- methyltransferase and phosphatidylinositol 4- kinase alpha among others.

RNA-Seq experiments became more frequently used in 2019 where shifts in transcriptional responses were measured in various *Leishmania* species and under more variable conditions. Patino et al. (2019) used RNA-Seq to analyze transcriptome profiles between antimony-resistant and antimony-sensitive *L. amazonensis* promastigotes. They identified a total of 723 differentially expressed genes between lines that were resistant or sensitive to trivalent sodium stibogluconate (Sb^{III}). These genes encoded proteins associated with various biological processes, like adhesion, metabolism, cell cycle, autophagy, structural organization and stress response. Some mRNA encoding amastin proteins were overexpressed in resistant lines, whereas the downregulated protein-encoding genes encoded putative superoxide dismutase, other subset of the amastin family and transporter proteins, which included a folate/biopterin transporter, pteridine transporter and an ABC transporter (Patino et al., 2019).

Differential gene expression analysis between wild-type and Sb^{III}-resistant *L. infantum* lines revealed 933 differentially expressed transcript including 837 upregulated and 96 downregulated transcripts (Andrade et al., 2020). Upregulated transcripts in the Sb^{III}-resistant line were associated with protein phosphorylation, microtubule-based movement, ubiquitination, host–parasite interaction, cellular process, and other categories while downregulated transcripts were associated with ribonucleoprotein complex, ribosome biogenesis, rRNA processing, nucleosome assembly and translation (Andrade et al., 2020).

Global transcriptomics studies were similarly performed in *L. braziliensis* promastigotes and intracellular amastigotes. Espada et al. (2019) investigated the pathways related to intrinsic miltefosine tolerance in *L. braziliensis* clinical isolates and found upregulated Ros3 mRNA levels in sensitive strains compared to resistant ones and suggested that miltefosine transporter (MT)-Ros3 was responsible for the observed drug sensitivity in *L. braziliensis*. Drug efflux and compartmentalization were similar in resistant and sensitive strains and drug susceptibility did not correlate with SNPs in the MT-Ros3 (Espada et al., 2019).

Mechanisms underlying artemisinin resistance in *L. donovani* have been recently investigated using comparative genomic and transcriptomic analyses (Ghosh et al., 2020). A dependency on lipid and amino acid metabolism, a reduced DNA and protein synthesis leading to parasites in the quiescence state, and an active drug efflux have been observed in resistant isolates. Upregulated genes included those encoding cathepsin-L like protease, amastin-like surface protein, and amino acid transporter, while downregulated genes include ABCG2, pteridine receptor, adenylatecyclase-type receptor, phosphoaceylglucosamine mutase, and a collection of hypothetical proteins (Ghosh et al., 2020).

ASSESSING THE EFFECTS OF ENVIRONMENTAL VARIATIONS

Leishmania parasites alternate between poikilothermic and homoeothermic hosts. Sudden temperature variations are natural events in their life cycles as the transmission from insect vector to the mammalian host includes a drastic increase over ambient temperature by more than 10 °C, which is in addition to the variation in temperature the insects experience in their habitats (Requena et al., 2012). The transcriptome of *L. major* promastigotes following a moderate heat shock from 26°C to 37°C was determined (Rastrojo et al., 2019). Following a moderate heat shock, Rastrojo et al. (2019) observed that the upregulated transcripts were heat shock proteins, amastigote-specific proteins and several hypothetical proteins. Downregulated transcripts, however, were associated with transporters, proteins involved in RNA metabolism or translational factors. Putative long non-coding RNAs were also identified among the differentially expressed transcripts and temperature-dependent changes in the selection of the SL addition sites were observed. Accordingly, alternative *trans*-splicing was suggested as an additional mechanism altering gene expression in *Leishmania* (Rastrojo et al., 2019).

Moreover, minor temperature shifts from 26°C to 24°C, 28°C, and 30°C) did not affect chromosomal ploidy but caused transcriptomic changes in *L. braziliensis* promastigotes grown *in vitro* (Ballesteros et al., 2020). Amastin surface-like proteins were downregulated under the three temperatures compared with the control (Ballesteros et al., 2020).

FUTURE DIRECTIONS

Global RNA-Seq and transcriptome analysis has gained significant attention as a tool to unveil *Leishmania* species molecular biology and their pathogenicity, differentiation into various life stages and interplay with the host immune response (Dillon et al., 2015b; Shadab et al., 2019). The simultaneous analysis of the gene expression profiles of parasites and hosts is a critical step in our understanding of the disease (Dillon et al., 2015b; Masoudzadeh et al., 2020b). However, with the colossal amount of data generated, there is a need to "translate" these complex datasets into forms that can be exploited for drug and vaccine development.

Various species such as *L. tropica* remain understudied using RNA-seq, which mostly include data derived from *in vitro* cultures. Also, in order to perform an accurate comparative analysis using global RNA-Seq data, experimental design should be reproducible and cover various *Leishmania* species grown *in vitro* under similar well-defined conditions and infected in human macrophages similarly maintained under constant

growth conditions and followed up in time course experiments to gain insights into the dynamic process of the infection. The outcome of such analysis should be further enriched and validated *in vivo* with transcriptomics data derived from promastigotes isolated directly from the sandfly gut and from amastigotes collected from induced lesions in mice models.

Reports suggesting only a moderate correlation between transcript abundance and cellular protein levels in *Leishmania* species requires further attention (de Pablos et al., 2019). Proteomics allow the analysis of expression levels, post-translational modifications, interactions, structure and subcellular distribution of proteins (Cox and Mann, 2007). Proteomics have been widely used to generate a proteome map providing an overall picture of gene expression at a given point in time (Sundar and Singh, 2016). In *Leishmania*, the comparison of proteomes has been employed to provide a better understanding of the parasite's life cycle, host–pathogen interactions, protein–protein interactions and drug resistance mechanisms for different *Leishmania* species (Capelli-Peixoto et al., 2019). Several key candidates for vaccine development have also been identified through proteomics investigations (Sundar and Singh, 2018; Sundar et al., 2019).

Despite the low observed correlation between RNA and protein abundance in *Leishmania* (Leifso et al., 2007; de Pablos et al., 2019), proteomic technologies could complement genomics and transcriptome profiling studies to characterize specific gene products (Sundar and Singh, 2016). Furthermore, complementing genome sequence with transcriptomics and proteomics data enable more accurate assembly and annotation of newly sequenced genomes (Cox and Mann, 2007; Prasad et al., 2017).

Moreover, the control of mRNA stability, decay and translation in the near absence of transcriptional regulation is not well understood (de Pablos et al., 2016). Also, the dynamics and regulation of messenger ribonucleoprotein complexes (mRNPs) in *Leishmania* species or differences in translational regulation from ribosomal profiling of the mRNAs from each life cycle stage are not yet elucidated (de Pablos et al., 2016).

Potentially sinefungin could be used to examine gene transcription. Sinefungin is a naturally occurring nucleoside isolated from *Streptomyces griseolus* and *S. incarnates* bacteria with structural similarities with S-adenosyl-1-methionine (Bachrach et al., 1980; Dube et al., 1983; McNally and Agabian, 1992). Sinefungin has antitrypanosomal activity and inhibits SL *trans*-splicing in these organisms (Bachrach et al., 1980; Dube et al., 1983; McNally and Agabian, 1992; Pandarakalam et al., 2019).

The role of adenylate cyclase in *Leishmania* parasites residing inside insect hosts requires further investigations (Iantorno et al., 2017). Also, more studies should focus on the role of amastins in infection, survival and host-parasite interactions.

Another interesting target is to investigate the roles of non-coding RNAs (ncRNAs) in regulating *Leishmania* mRNA transcription (Castro et al., 2017; Ruy et al., 2019). ncRNAs emerge as key players in a variety of regulatory processes including mRNA processing, mRNA stability in addition to DNA replication, chromosome maintenance and transcriptional regulation in various organisms (Mattick, 2005; Morris and Mattick, 2014). In *L. major* and *L. donovani*, 26 and 30 putative ncRNAs were identified, respectively, with the majority arising from UTRs (Castro et al., 2017). The biological function of these ncRNA and their regulatory roles in transcription deserve further investigation.

Moreover, a detailed metatranscriptomics analysis *in situ* in the skin and in the insect vector covering the insect gut microbiota and the human skin microbiota, respectively, could aid in enriching our understanding of the factors that govern the complex host–parasite-microbiota interplay and their potential impact and relationship with the different forms of leishmaniasis, CL, VL, and MCL. Skin microbiota dysbiosis associated with CL requires further attention modulating the pathobiology of *Leishmania* infections and appears, importantly, as a potential novel therapeutic target (Gimblet et al., 2017). Notably, the mammalian skin and insect gut microbiota are also potentially relevant to trigger and modulate the shift from CL to VL (Gimblet et al., 2017). Moreover, the impact of LRV and other viruses, such as the Lymphocytic choriomeningitis virus (LCMV), on the pathobiology of various *Leishmania* species would also be of great interest to investigate with more integrative approaches and model systems (Ives et al., 2011; Crosby et al., 2015). Lymphocytic choriomeningitis virus, for instance, is a rodent-borne, Old World arenavirus that causes asymptomatic or mild, self-limited illness in otherwise healthy humans (Zhou et al., 2012). It is a known cause of aseptic meningitis and rarely fatal (Fischer et al., 2006). It has been reported that an increased recruitment of neutrophils and more severe lesions were observed in mice co-infected with *L. major* and LCMV (Crosby et al., 2015). More recently, a ssRNA *Leishmania*-infecting leishbunyavirus (*Lmar*LBV1) has been isolated from *Leishmania martiniquensis* (Grybchuk et al., 2020). Thus, the diversity and implications of viruses on leishmaniasis outcome and disease progression are other lines of research of great interest to investigate.

Gut microbes from the sandfly are egested into the host skin alongside *Leishmania* parasites at the bite sites (Dey et al., 2018). These have been shown to augment the severity of VL caused by *L. donovani* via inflammasome-derived IL-1β production (Dey et al., 2018).

Although much remains to be elucidated on the effects of skin microbiota and sandfly gut microbes on the progression of leishmaniasis, understanding the complex interactions between *Leishmania*, skin microbiota and the sandfly gut microbes in disease outcome appears as a potential therapeutic target and an important parameter to untangle the complex immunological cross-talks occurring between the parasite and the host skin and/or sandfly gut microbiota.

AUTHOR CONTRIBUTIONS

TS: conceptualization, investigation, and writing—original draft and review and editing. TS, ST, and RH: data curation, investigation, and writing—original draft and review and editing. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The Glycosylphosphatidylinositol Anchor: A Linchpin for Cell Surface Versatility of Trypanosomatids

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The use of glycosylphosphatidylinositol (GPI) to anchor proteins to the cell surface is widespread among eukaryotes. The GPI-anchor is covalently attached to the C-terminus of a protein and mediates the protein's attachment to the outer leaflet of the lipid bilayer. GPI-anchored proteins have a wide range of functions, including acting as receptors, transporters, and adhesion molecules. In unicellular eukaryotic parasites, abundantly expressed GPI-anchored proteins are major virulence factors, which support infection and survival within distinct host environments. While, for example, the variant surface glycoprotein (VSG) is the major component of the cell surface of the bloodstream form of African trypanosomes, procyclin is the most abundant protein of the procyclic form which is found in the invertebrate host, the tsetse fly vector. *Trypanosoma cruzi*, on the other hand, expresses a variety of GPI-anchored molecules on their cell surface, such as mucins, that interact with their hosts. The latter is also true for *Leishmania*, which use GPI anchors to display, amongst others, lipophosphoglycans on their surface. Clearly, GPI-anchoring is a common feature in trypanosomatids and the fact that it has been maintained throughout eukaryote evolution indicates its adaptive value. Here, we explore and discuss GPI anchors as universal evolutionary building blocks that support the great variety of surface molecules of trypanosomatids.

Keywords: cell surface proteome, evolution, GPI-anchor, Kinetoplastea, *Trypanosoma, Leishmania*

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INTRODUCTION

Cell membranes are the interface between cells and their environment. Therefore, the architecture of the cell membrane that is exposed to the environment defines how the cell interacts with external influences. Membranes are covered with a plethora of different proteins that can be attached to the lipid bilayer in different ways. While integral proteins are inserted into the membrane via intrinsic hydrophobic regions and usually, but not always, span the entire membrane, peripheral proteins are entirely exposed at the cytoplasmic or extracellular face of the plasma membrane, where they are attached via weak interactions or a covalently bound lipid anchor, which integrates into one leaflet of the lipid bilayer (Singer and Nicolson, 1972; Stillwell, 2016). Whereas integration into the cytoplasmic side of the plasma membrane is mediated for instance by myristoyl, palmitoyl, and prenyl groups, there is only one structure that attaches proteins to the outer leaflet of the

plasma membrane, which is the glycosylphosphatidylinositol (GPI) anchor (Stillwell, 2016). This anchor is preassembled and attached post-translationally to several hundred known proteins and it is considered to be ubiquitous among eukaryotes (Kinoshita, 2020; Liu and Fujita, 2020). GPI-anchored proteins have diverse functions, including regulation of the complement system and acting as receptors, antigens and enzymes (Paulick and Bertozzi, 2008; Kinoshita, 2020). Hence, defects in the biosynthesis of GPI-anchored proteins cause severe diseases known as inherited GPI deficiencies (IGDs) (reviewed in Bellai-Dussault et al., 2019).

Despite the essential roles of GPI-anchored proteins in both mammals and yeast, these proteins constitute only a minor proportion of the proteome of these organisms. In mammalian cells, the total number of GPI-anchored proteins rarely exceeds 10^5 molecules (Ferguson et al., 1994). In contrast, the pathogens *Trypanosoma brucei*, *Trypanosoma cruzi* and species of the *Leishmania* genus are covered by a dense glycocalyx of GPI-anchored molecules, which function as key virulence factors (Ferguson et al., 2015). In the bloodstream of the vertebrate host, the surface coat of *T. brucei* consists nearly entirely of homodimeric variant surface glycoprotein (VSG) made up of an impressive number of 10^7 monomers, which constitutes around 10% of the proteome and 90–95% of proteins found on the cells surface, therefore highlighting the importance of this GPI-anchored protein to the parasite (Cross, 1975; Jackson et al., 1985; Bartossek et al., 2017). Furthermore, the great variety of GPI-anchored molecules in *T. cruzi* and *Leishmania* spp. (El-Sayed et al., 2005) and evidence for prevalence of GPI-anchored surface molecules in other trypanosomatids suggests that GPIs might be linked to the success of parasitism in this large family of unicellular eukaryotes.

Trypanosomes and *Leishmania* spp. are causative agents of neglected tropical diseases (WHO, 2020) and are also found in wildlife, where they infect a vast range of vertebrates and invertebrates. For the genus *Trypanosoma* alone at least 500 species have been described that infect all classes of vertebrates (Spodareva et al., 2018). The close interaction between human populations and parasite reservoirs (Hamill et al., 2013; Jansen et al., 2018; Medkour et al., 2019, 2020), along with the disruption of the ecological equilibrium, can cause the spread of the previously confined wildlife parasites to humans (Thompson, 2013; Cable et al., 2017). This, for example, is considered to be the starting point for human trypanosomiasis in the Americas (reviewed in Jansen et al., 2020). Thus, infections by trypanosomatids are a prime example of the One Health concept (Gruetzmacher et al., 2021), which recognizes the impact of the interaction of all living organisms and the surrounding environment on human health.

This review aims to highlight the importance of GPI anchored molecules, which form the interface between the trypanosomatids and their environment, as an essential factor for their evolutionary success. For this purpose, we first describe the biochemistry of GPI synthesis in *T. brucei* in comparison to that in mammalian and yeast cells. Then we consider the relationship/interaction of the GPI-anchored molecules found in the human pathogens *T. brucei*, *T. cruzi*, and *Leishmania* spp. with their distinct host microenvironments. Finally, we summarize the current knowledge of GPI-anchored molecules in other species of *Trypanosoma*.

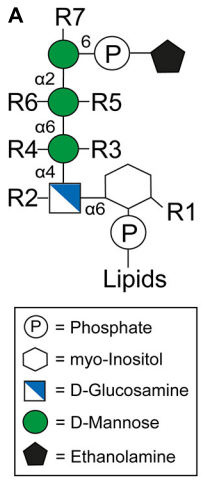
GPI ANCHORS

Glycosylphosphatidylinositol-anchored proteins were discovered in mammalian cells in the late 1970s, as a result of their hydrolytic release mediated by phospholipase (PLC) (Ikezawa et al., 1976; Low and Finean, 1977). The release of proteins without cell lysis led to the suggestion that these molecules were covalently attached to the outer leaflet of the lipid bilayer via a phosphatidylinositol molecule. In 1985, the chemical compositions of two GPI anchors were published; one, that of the VSG found in *T. brucei*, the other, that of Thy-1 found in rat brains (Ferguson et al., 1985; Tse et al., 1985). The first structural details of these GPI-anchors followed in 1988, when the complete structures were determined using a combination of different methods, including NMR spectroscopy and mass spectrometry (Ferguson et al., 1988; Homans et al., 1988).

The Composition and Structure of GPI Anchors

Glycosylphosphatidylinositol anchors consist of a glycan and a lipid part. With the single exception of *Entamoeba* proteophosphoglycan (Moody-Haupt et al., 2000), all known protein-linked GPI anchors possess the same conserved core glycan structure composed of mannose(α1-2)mannose(α1-6)mannose(α1-4)glucosamine(α1-6)*myo*-inositol (**Figure 1A**). The core glycan can be modified by side chains, with the nature of these modifications varying between species and even within different life cycle stages of the same organism (Ferguson et al., 2015). Further, the extent of some of these modifications can also be protein specific (Ferguson et al., 2015). Typical modifications are mannose, galactose, and phosphoethanolamine residues as well as sialic acids. **Figure 1B** summarizes the GPI anchor modifications of prominent surface molecules of mammals and human infective trypanosomatids. For example, *T. brucei* adds different amounts of galactose side chains to nearly all GPI anchors of VSGs (Zamze et al., 1991), whereas branched poly-*N*-acetyllactosamine (poly-NAL) repeats capped by sialic acid residues are added to the anchor of procyclins, which form the major surface coat in a different life cycle stage (Engstler et al., 1993; Ferguson et al., 1993; Treumann et al., 1997; Mehlert et al., 1998). In *T. cruzi*, the attachment of aminoethylphosphonic acid to the core glucosamine represents a modification that is exclusive to this species (Macrae et al., 2005; Paulick and Bertozzi, 2008). Furthermore, a fourth mannose is found in all *T. cruzi* GPI anchors (Heise et al., 1996; Ferguson et al., 2015), a feature also present in the anchors of yeast and some mammalian proteins (Paulick and Bertozzi, 2008; Ferguson et al., 2015). The purpose of these modifications remains to be elucidated. In contrast, *Leishmania* species entirely lack side chain modifications of the GPI anchor (Ferguson et al., 2015).

The GPI anchor is generally linked to the protein through a peptide bond between the amino group of phosphoethanolamine



B

	Lipids	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Mammalian protein	alkylacylglycerol /diacylglycerol	± acyl	OH	± β4-GalNAc ± (Gal-Neu5Ac)	EtN-P	OH	± EtN-P	± α2-Man
T. brucei VSG	diacylglycerol	OH	OH	± α3-Gal-(α2-Gal)-α6-Gal-(α2-Gal)	OH	± β3-Gal	OH	± α2-Gal
T. brucei procyclin	*lyso*-acylglycerol	acyl	OH	OH	OH	average: $Neu5Ac_5Gal_9GlcNAc_9$	OH	OH
T. cruzi mucins	ceramide/ alkylacylglycerol	OH	AEP	*	*	OH	OH	α2-Man
Leishmania GP63	alkylacylglycerol	OH	OH	OH	OH	OH	OH	OH

GlcNAc = N-Acetylglucosamine Neu5Ac = N-Acetylneuraminic acid Man = D-Mannose * = unspecified hexose
EtN-P = Ethanolamine phosphate AEP = Aminoethylphosphonic acid Gal = D-Galactose

FIGURE 1 | General structure of the GPI anchor and their side chain modifications. **(A)** Structure of the conserved glycan core with the different side chain modifications. The respective positions of the modifications are indicated by R_x. **(B)** Comparison of different side chain modifications (R_x) and lipid moieties for selected GPI anchored proteins in mammals, *Trypanosoma brucei*, *Trypanosoma cruzi,* and *Leishmania*. The positions of R1-7 and the lipids in the GPI anchor are indicated in panel **(A)**. The nature and position of linkage of an additional hexose (*) at the first mannose of the GPI anchor of *T. cruzi* mucins is not yet known (Serrano et al., 1995). This figure was modified from Figure 1 of a review by Fujita and Kinoshita (2010).

(EtN-P) and the C-terminal carboxyl group of the polypeptide (Ferguson et al., 1988). However, an alternative attachment via aminoethyl phosphonate (AEP) instead of EtN-P has been identified for proteins in *T. cruzi* (Heise et al., 1996).

At the opposite end of the GPI anchor, a phospholipid mediates attachment to the cell membrane by insertion of its tail into the membrane's outer leaflet. The composition of this tail depends on the species of origin and varies between diacylglycerols, *lyso*-acylglycerols, alkylacylglycerols or ceramides (McConville and Ferguson, 1993). In addition, the lipids vary in length, ranging from 14 to 26 carbons, and can be either saturated or unsaturated (Ferguson et al., 2015). In some cases an additional fatty acid may be attached to the 2-hydroxyl of the inositol residue, which is known as inositol acylation (Ferguson et al., 2015). This modification can be found in several mammalian GPI anchors and it is also present in some GPI anchors of trypanosomatids (**Figure 1**) (Ferguson et al., 2015). Interestingly, GPI anchors containing an inositol acylation are resistant to PLC cleavage (Roberts et al., 1988).

Glycosylphosphatidylinositol-anchoring is not restricted to proteins, underlining the versatility of this mode of attachment. Non-protein linked GPI molecules include glycoinositolphospholipids (GIPLs) and lipophosphoglycans (LPGs), with anchors that are either identical to those of protein-linked GPIs or contain compositional and structural modifications. For example, type-1 GIPLs contain the Manα1-2Manα1-6Manα1-4GlcNα1-6PI sequence common to the protein-linked GPIs whereas type-2 GIPLs contain a Manα1-3Manα1-4GlcNα1-6PI motif and others possess hybrid structures presenting the branched motif (Manα1-6)Manα1-3Manα1-4GlcNα1-6PI (**Figure 2**) (McConville and Ferguson, 1993). While GIPLs in mammalian cells likely follow the same structural remodeling pathway as protein-linked GPIs (Wang et al., 2019), GIPLs in *Leishmania* and *T. cruzi* may share a common precursor but most likely represent the product of different biosynthetic pathways (Heise et al., 1996; Ralton and McConville, 1998; Ilgoutz and McConville, 2001).

GPI Biosynthesis

Current understanding of GPI biosynthesis has been gained mostly from studies on mammalian and yeast cells. This bias can be explained by the traditional focus on opisthokont models. Moreover, the mutations of GPI biosynthetic enzymes, used to elucidate these biosynthetic pathways, are lethal in other organisms, such as bloodstream form *T. brucei* (Nagamune et al., 2000). Therefore, we first summarize the current knowledge of GPI biosynthesis in mammals and then compare this mammalian pathway to that found in trypanosomatids. As GPI assembly in *T. brucei* is much better understood than in any other trypanosomatid species, we will mainly focus on *T. brucei* and, whenever possible, provide information available on *T. cruzi* and *Leishmania*.

Glycosylphosphatidylinositol biosynthesis is a sequential addition of sugars and ethanolamine to phosphatidylinositol (PI). While the initial steps take place on the outer membrane of the endoplasmic reticulum (ER), the final assembly is performed on the luminal side of the ER (Kinoshita and Fujita, 2016; Liu and Fujita, 2020). Once the GPI precursor has been synthesized inside the ER, it is attached *en bloc* to a protein in exchange for its GPI-signal sequence (Kinoshita and Fujita, 2016; Liu and Fujita, 2020). Following this, maturation of the GPI anchor includes lipid remodeling reactions and side chain glycosylation, which can occur at different time points and either in the ER or the Golgi apparatus, depending on the organisms (Ferguson, 1999; Fujita and Kinoshita, 2010; Kinoshita and Fujita, 2016).

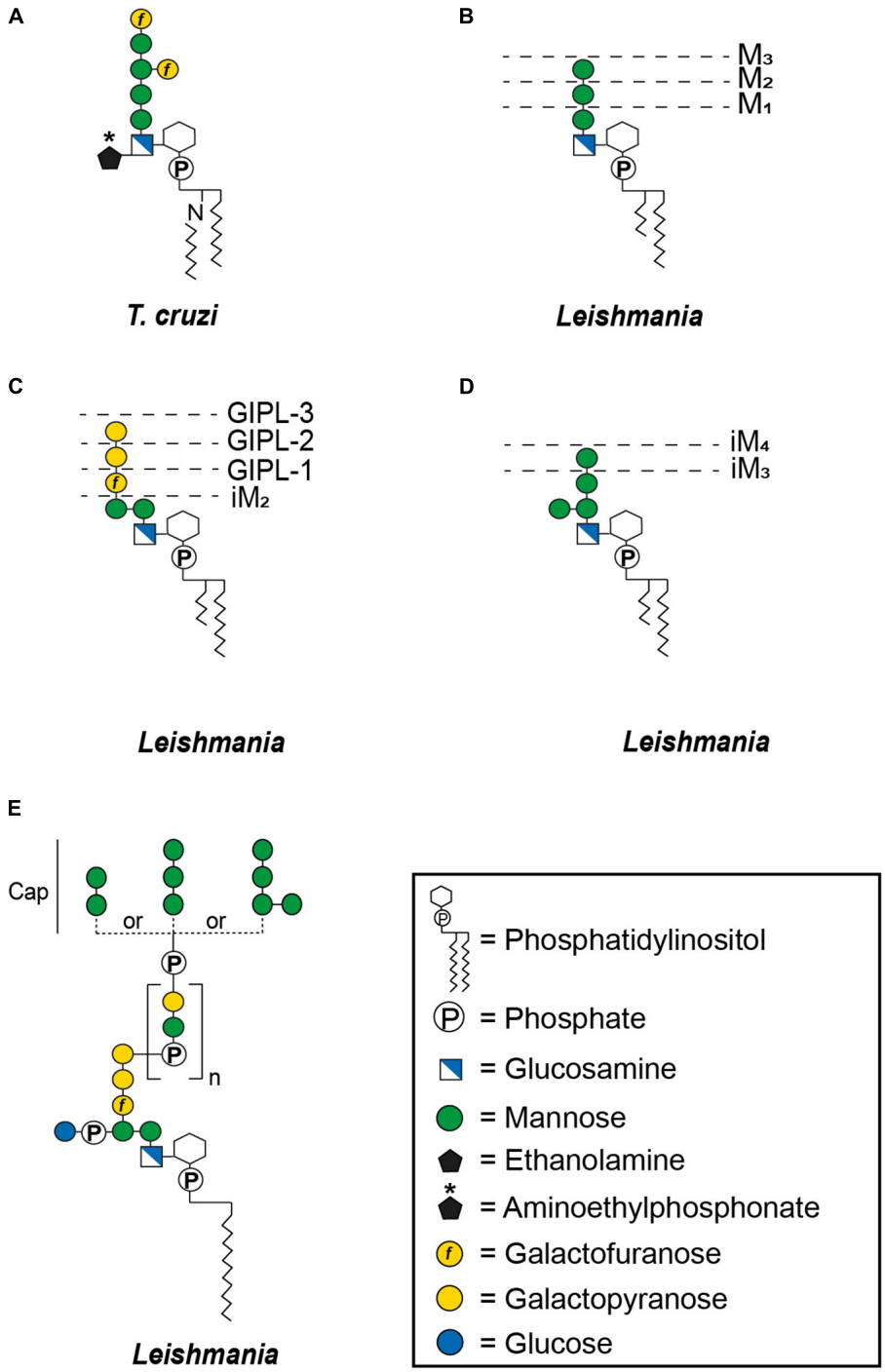


FIGURE 2 | General structures of free GPIs (GIPLs) and LPGs. The dashed lines indicate smaller GIPL species in *Leishmania* (McConville and Ferguson, 1993). M_X indicates the number of mannoses and iM the unusual α1-3 binding of these mannoses. The number of phosphosaccharide repeats (n) of *Leishmania* LPGs is stage and species specific (Forestier et al., 2014). **(A)** *Trypanosoma cruzi* Type-1 GIPL, **(B)** *Leishmania* Type-1 GIPLs, **(C)** *Leishmania* Type-2 GIPLs, **(D)** *Leishmania* hybrid GIPLs, and **(E)** *Leishmania* LPGs.

In mammals, GPI biosynthesis starts on the cytoplasmic side of the ER membrane with the transfer of *N*-acetylglucosamine (GlcNAc) from uridine-diphosphate-*N*-acetylglucosamine to PI, generating GlcNAc-PI (**Figure 3A**, step 1). This step is catalyzed by the multi-subunit enzyme GPI-GlcNAc transferase (PIG-A, PIG-C, PIG-H, PIG-P, PIG-Q, PIG-Y, and DPM2) (Fujita and Kinoshita, 2010). Once GlcNAc-PI has been formed, it is deacylated to glucosaminyl-PI (GlcN-PI) by

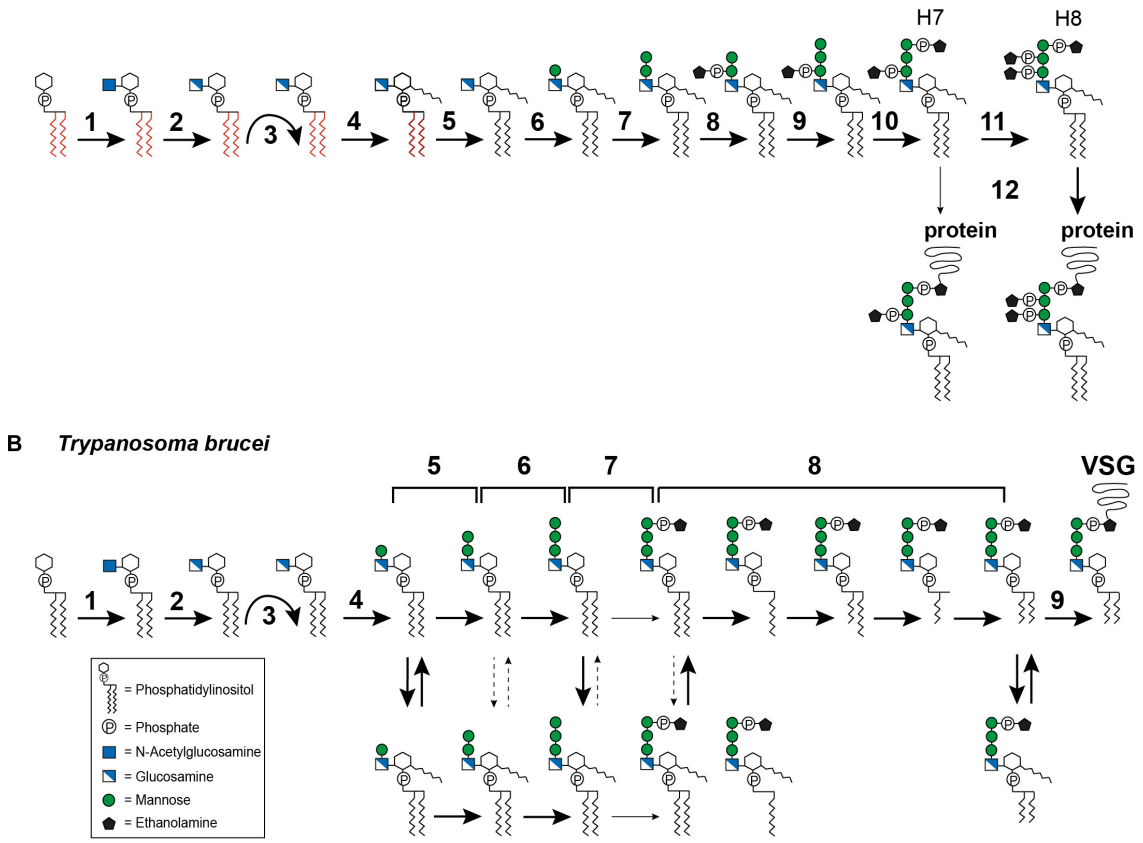


FIGURE 3 | Glycosylphosphatidylinositol biosynthesis pathway up to the point of protein attachment. **(A)** Mammalian GPI biosynthesis steps at and within the endoplasmic reticulum (ER). The reaction steps are numbered and are described in detail in the text: (1) transfer of *N*-acetylglucosamine (GlcNAc) to phosphatidylinositol (PI), (2) deacylation of GlcNAc-PI, (3) flipping of GlcN-PI into the ER lumen, (4) inositol acylation, (5) lipid remodeling, visualized by the color change from red to black, (6–7) addition of mannose, (8) addition of ethanolamine phosphate (EtN-P), (9) addition of mannose, (10) addition of EtN-P, (11) addition of EtN-P, (12) attachment of the GPI anchor to the protein. The occasionally observed addition of a fourth mannose is not depicted. **(B)** GPI biosynthesis steps at and within the ER in *Trypanosoma brucei*. The reaction steps are numbered and are described in detail in the text: (1) transfer of *N*-acetylglucosamine (GlcNAc) to phosphatidylinositol (PI), (2) deacylation of GlcNAc-PI, (3) flipping of GlcN-PI into the ER lumen, (4–6) addition of mannose, (7) addition of EtN-P, (8) lipid remodeling, (9) attachment of the GPI anchor to the protein. The broad solid arrows indicate reactions for which direct evidence exists. The dashed arrows indicate conversions that may exist. The light solid arrows indicate reactions that are not frequently observed. The curved arrows indicate the flipping reaction into the ER lumen.

an *N*-deacetylase, PIG-L (**Figure 3A**, step 2) (Nakamura et al., 1997; Watanabe et al., 1999). GlcN-PI is then flipped to the luminal side of the ER, an energetically costly process (Pomorski and Menon, 2006). Although flipping has been demonstrated to be bidirectional and independent of ATP (Vishwakarma and Menon, 2005) no GPI flippase has yet been identified. The most promising candidate, ARV1, has been described for *Saccharomyces cerevisiae* with evidence suggesting that ARV1-linked human diseases result from defective GPI anchor synthesis (Okai et al., 2020).

After flipping into the ER lumen (**Figure 3A**, step 3), GlcN-PI is acylated by an acyltransferase, PIG-W, at the 2-hydroxyl residue of the inositol ring (**Figure 3A**, step 4) (Doerrler et al., 1996; Murakami et al., 2003). Subsequent lipid remodeling reactions occur on GlcN-(acyl)-PI, leading to the replacement of the diacyl PI moiety (displayed in red in **Figure 3A**) with 1-alkyl-2-acyl-PI (visualized in black in **Figure 3A**), with the mechanism and enzymes involved remaining elusive (**Figure 3A**, step 5) (Kanzawa et al., 2009). The following steps are the sequential transfer of two mannose molecules from dolichol-phosphate-mannose to GPI intermediates via different glycosidic linkages (DeGasperi et al., 1990). The first mannose is transferred by GPI mannosyltransferase I, PIG-M and PIG-X, to the 4-hydroxyl residue of GlcN (**Figure 3A**, step 6) (Maeda et al., 2001; Ashida et al., 2005), and the second mannose is transferred by GPI mannosyltransferase II, PIG-V, to the 6-hydroxyl residue of the first mannose (**Figure 3A**, step 7) (Kang et al., 2005). Following the transfer of the second mannose, the first mannose receives an EtN-P side chain modification, at the 2-hydroxyl residue (**Figure 3A**, step 8). This step is catalyzed by GPI-EtN-P transferase I, PIG-N (Hong et al., 1999). Following this, a

third mannose is transferred by mannosyltransferase III, PIG-B, to the 2-hydroxyl residue of the second mannose (**Figure 3A**, step 9) (Takahashi et al., 1996). The third mannose is then modified by GPI-EtN-P transferase III (PIG-O and PIG-F) which transfers the "bridging" EtN-P that later connects the GPI anchor to the protein (**Figure 3A**, step 10) (Inoue et al., 1993; Hong et al., 2000). The resulting EtN-P-Man-Man-(EtN-P)Man-GlcN-(acyl)PI, also known as H7, is now ready for protein attachment (Hirose et al., 1992), though usually another EtN-P is attached to the second mannose to generate EtN-P-Man-(EtN-P)Man-(EtN-P)Man-GlcN-(acyl)PI, known as H8 (Hirose et al., 1992). This reaction is mediated by GPI-EtN-P transferase II, PIG-G and PIG-F (**Figure 3A**, step 11) (Shishioh et al., 2005). A fourth mannose may be attached to the 2-hydroxyl residue of the third mannose by mannosyltransferase IV, PIG-Z (not shown in **Figure 3A**) (Taron et al., 2004).

Attachment of the GPI anchor to a protein is mediated by an enzyme complex termed GPI transamidase, which consists of PIG-K, GPAA1, PIG-S, PIG-T and PIG-U (**Figure 3A**, step 12) (Hamburger et al., 1995; Yu et al., 1997; Ohishi et al., 2000, 2001; Hong et al., 2003). The complex can recognize and cleave the C-terminal GPI signal sequence of the protein and replace it with the preassembled GPI anchor. Once the GPI anchor has been attached to a protein, further lipid and glycan remodeling reactions take place. Most of these occur within the Golgi apparatus (reviewed in Kinoshita, 2020; Liu and Fujita, 2020), but inositol deacylation (Liu et al., 2018) and EtN-P removal (Fujita et al., 2009) are performed in the ER lumen.

This completes the summary of events that lead to GPI-anchoring of a protein in mammals. How does this process differ in trypanosomatids?

In *T. brucei*, the first two steps (**Figure 3B**, steps 1–2) of GPI biosynthesis are comparable with those in mammals. Several homologs of the GPI-GlcNAc transferase subunits have been reported, including TbGPI1, TbGPI2, TbGPI3, and TbGPI19 (Fujita and Kinoshita, 2010). The GlcNAc-PI de-*N*-acetylase homolog in *T. brucei* is TbGPI12 (Chang et al., 2002). After the precursor has been flipped to the luminal side of the ER (**Figure 3B**, step 3), the pathways diverge. Inositol acylation occurs only after the first mannosylation, indicating a different substrate specificity for the mannosyltransferase I, TbGPI14, in this parasite (**Figure 3B**, step 4) (Güther and Ferguson, 1995). In addition, it has been shown that the inositol acyltransferase requires a hydroxyl group at the fourth position on the first mannose and a free amine on the glucosamine residue (Urbaniak et al., 2008). The same requirement for mannosylation was also reported for *Leishmania* (Smith et al., 1997).

The transfer of the second mannose is mediated by the homolog of mannosyltransferase II, TbGPI18 (**Figure 3B**, step 5) (Fujita and Kinoshita, 2010), and TbGPI10, the homolog of mannosyltransferase III, transfers the third mannose (**Figure 3B**, step 6) (Nagamune et al., 2000). Among trypanosomatids, the addition of a fourth mannose seems to be unique to *T. cruzi*, but no homologs for a mannosyltransferase IV have been identified so far. In *T. brucei*, several GPI intermediates bearing one to three mannoses are in a dynamic equilibrium between inositol acylated and non-acylated states (Menon et al., 1990; Güther and Ferguson, 1995) (**Figure 3B**, equilibrium arrows). The equilibrium is maintained by a diisopropylfluorophosphate (DFP)-sensitive inositol deacylase together with a phenylmethylsulfonyl fluoride (PMSF)-sensitive inositol acyltransferase (Güther and Ferguson, 1995). Whether such an equilibrium exists in *Leishmania* and *T. cruzi* is still controversial (Heise et al., 1996; Ralton and McConville, 1998; Hilley et al., 2000; Bertello et al., 2004).

Following the assembly of the mannoses, an EtN-P bridge is added to the third mannose (**Figure 3B**, step 7). This reaction might be mediated by TbGPI13, which has been suggested to be an EtN-P transferase III homolog (Fujita and Kinoshita, 2010). Inositol acylation of the Man_3GlcN-PI intermediate has been reported to enhance the efficiency of EtN-P addition (Güther and Ferguson, 1995). Subsequently, fatty acid remodeling reactions occur (**Figure 3B**, step 8) that can be described as sequential deacylations and reacylations. In addition, inositol deacylation is thought to be a prerequisite for complete fatty acid remodeling (Güther and Ferguson, 1995). In the bloodstream form of *T. brucei* the remodeling of the GPI anchor was studied for its major surface protein, the VSG. It is initiated by removal of the sn-2 fatty acid followed by acylation of myristate using myristoyl-CoA as the donor (Masterson et al., 1990; Hong and Kinoshita, 2009). The following steps of sn-1 deacylation and a second myristate incorporation lead to the formation of the mature GPI precursor (Masterson et al., 1990; Hong and Kinoshita, 2009). So far, this exclusive use of myristate has only been found in *T. brucei* (Ferguson et al., 1988). TbGup1, was shown to mediate the myristate transfer steps during the remodeling reactions (Jaquenoud et al., 2008). The GPI anchor of procyclin, the abundant surface molecule of *T. brucei* procyclic forms, retains the inositol acylation throughout synthesis and in the mature form (Treumann et al., 1997; Hong et al., 2006). Lipid remodeling has also been reported for *T. cruzi* (Heise et al., 1996) and *Leishmania* (Ralton and McConville, 1998). In *T. cruzi*, two different remodeling reactions occur: conversion of glycerolipid to ceramide and fatty acid remodeling (Heise et al., 1996; Bertello et al., 2004; De Lederkremer et al., 2011). The metacyclic forms of *T. cruzi* contain inositolphosphoceramide in the lipid part of their GPI anchored glycoproteins, which represents a stage specific modification (Serrano et al., 1995). Another organism that utilizes inositolphosphoceramide rather than glycerolipids in their GPI anchors is *S. cerevisiae* (Conzelmann et al., 1992). In this organism, the ceramide conversion most likely takes place after the GPI anchor is transferred to the protein (Sipos et al., 1997). Both chronology and location of ceramide conversion in *T. cruzi* remain unclear.

As in mammals, the preassembled GPI anchor is attached to the protein by a GPI transamidase complex (**Figure 3B**, step 9). In *T. brucei*, this complex is formed by two trypanosome-specific components, TAA1 and TAA2, plus three subunits that have homologs in mammals and yeast, TbGAA1, TbGPI8, and TbGPI16 (Nagamune et al., 2003). A direct comparison of homologous key enzymes in selected organisms is provided in **Table 1**.

TABLE 1 | List of proteins involved in the GPI biosynthesis pathway.

Enzyme type	*Homo sapiens*		*Saccharomyces cerevisiae*		*Trypanosoma brucei*		*Leishmania mexicana*		*Trypanosoma cruzi*	
	Name	**UniProtKB**	**Name**	**UniProtKB**	**TriTrypDB**	***e*-value**	**TriTrypDB**	***e*-value**	**TriTrypDB**	***e*-value**
GPI-GlcNAc transferase	PIG-A	P37287	GPI3	P32363	Tb927.2.1780	1E-150	LmxM.32.1670	2E-146	TcCLB.510259.18	3E-151
	PIG-C	Q92535	GPI2	P46961	Tb927.10.6140	1E-30	LmxM.36.1710	9E-30	TcCLB.507639.100	5E-38
	PIG-H	Q14442	GPI15	P53961						
	PIG-P	P57054	GPI19	Q04082	Tb927.10.10110	4E-12	LmxM.36.4750	6E-11	TcCLB.508307.100	4E-12
	PIG-Q	Q9BRB3	GPI1	P53306	Tb927.3.4570	8E-13	LmxM.08_29.2030	3E-14	present in non-reference strains	
	PIG-Y	Q3MUY2	ERI1	P62651						
	DPM2	O94777			Tb927.9.6440	1E-3	LmxM.15.0815	3E-6	TcCLB.506579.119	6E-4
									TcCLB.510043.29	6E-4
GlcNAc-PI de-*N*-acetylase	PIG-L	Q9Y2B2	GPI12	P23797	Tb927.11.12080	3E-36	LmxM.09.0040	7E-37	TcCLB.504005.20	8E-33
Inositol acyltransferase	PIG-W	Q7Z7B1	GWT1	P47026						
Mannosyl-transferase I	PIG-M	Q9H3S5	GPI14	P47088	Tb927.6.3300	3E-64	LmxM.29.2030	9E-62	TcCLB.503909.100	1E-64
	PIG-X	Q8TBF5	PBN1	P25580						
Mannosyl-transferase II	PIG-V	Q9NUD9	GPI18	P38211	Tb927.10.13160	1E-9	LmxM.18.0960	3E-8	TcCLB.506359.30	2E-11
EtN-P transferase I	PIG-N	O95427	MCD4	P36051	Tb927.11.5070	1E-4	LmxM.24.0340	2.5E-2	TcCLB.507667.11	9.9E-2
Mannosyl-transferase III	PIG-B	Q92521	GPI10	P30777	Tb927.10.5560	5E-47	LmxM.36.1200	9E-25	TcCLB.503527.40	3E-43
EtN-P transferase III	PIG-O	Q8TEQ8	GPI13	Q07830	Tb927.11.5070	1E-44	LmxM.24.0340	4E-41	TcCLB.507667.11	6E-37
	PIG-F	Q07326	GPI11	Q06636						
EtN-P transferase II	PIG-G	Q5H8A4	GPI7	P40367	Tb927.11.5070	4E-28	LmxM.24.0340	4E-33	TcCLB.507667.11	3E-28
	PIG-F	Q07326	GPI11	Q06636						
Mannosyl-transferase IV	PIG-Z	Q86VD9	SMP3	Q04174						
Lyso-GPI acyltransferase	PGAP2	Q9UHJ9	CWH43	P25618			LmxM.27.1770	1.7E-2	TcCLB.504153.120	2E-11
			GUP1	P53154	Tb927.10.15910	2E-42	LmxM.19.1347	5E-50	TcCLB.511355.40	1E-51
GPI transamidase	PIG-K	Q92643	GPI8	P49018	Tb927.10.13860	8E-63	LmxM.18.0360	3E-57	present in non-reference strains	
	GAA1	O43292	GAA1	P39012	Tb927.10.210	7.4E-2				
	PIG-S	Q96S52	GPI17	Q04080						
	PIG-T	Q969N2	GPI16	P38875	*					
	PIG-U	Q9H490	GAB1	P41733						
					Tb927.11.15760		LmxM.31.2560		TcCLB.511545.190	
					Tb927.10.5080		LmxM.36.0650		TcCLB.510293.79	

All known proteins involved in the biosynthesis of mammalian (e.g., Homo sapiens*) and yeast (*Saccharomyces cerevisiae*) GPIs were BLASTed for homology in* Trypanosoma brucei, Trypanosoma cruzi, *and* Leishmania mexicana*. The BLAST was performed with the annotated protein sequences of* H. sapiens *acquired from UniProtKB against all trypanosomatid protein sequences currently annotated in TriTrypDB. In the case of GUP1, which is not present in the mammalian biosynthesis pathway, the protein sequence from* Saccharomyces cerevisiae *was used. All Gene-IDs and respective e-values given refer to the current reference strains. *Our BLAST did not find a homolog for the GPI transamidase subunit PIG-T. However, a previous study found an ortholog of mammalian PIG-T and yeast GPI16 in* T. brucei *(Nagamune et al., 2003).*

Clearly, several steps involved in GPI biosynthesis are common to mammals, yeast and trypanosomatids, though there are also marked differences. These include the chronological order of inositol acylation and lipid remodeling as well as the details of further modifications in the Golgi apparatus. In mammals and yeast, lipid remodeling occurs after inositol acylation in the ER as well as in later steps in the Golgi apparatus (reviewed in Kinoshita, 2020; Liu and Fujita, 2020). In contrast, lipid remodeling in trypanosomatids has been reported to take place on GPI precursors within the ER and, except for ceramide remodeling in *T. cruzi*, occurs directly before protein attachment (Masterson et al., 1990; Hong and Kinoshita, 2009; De Lederkremer et al., 2011). Interestingly, an alternative myristoylation pathway, called lipid exchange, has been identified exclusively in the bloodstream form of *T. brucei* (Buxbaum et al., 1994, 1996). This pathway was reported to be mechanistically similar to lipid remodeling but involves a distinct set of enzymes and appears to operate in a post-ER secretory compartment, possibly the Golgi (Paul et al., 2001). The authors suggested that lipid exchange might be a proofreading mechanism to ensure

that all lipids on the VSG anchor consist of myristate. This may highlight once again the importance of this fatty acid for the VSG GPI anchor. Although the GPI biosynthesis steps are well studied, we pinpointed some questions that remain unanswered, especially for trypanosomatids.

The Influence of the Biophysical Properties of GPI Anchors on Their Respective Proteins

The function of GPI anchors does not solely lie in connecting proteins to the membrane. In fact, this anchor has been implicated in increasing lateral mobility of proteins and in targeting of proteins to special microdomains, named lipid rafts, as well as being subject to cleavage mediated by activation of specific GPI cleaving enzymes (GPIases), which leads to the release of the surface protein.

Early studies using fluorescence recovery after photobleaching (FRAP) on GPI-anchored proteins, such as Thy-1, placental alkaline phosphatase and Ly6E, reported diffusion coefficients comparable to those of membrane lipids and 2- to 5-times faster than those of transmembrane proteins (Ishihara et al., 1987; Zhang et al., 1991). In *T. brucei*, the lateral mobility of the VSG was found to be comparable to the mobility of other membrane-bound glycoproteins, but slower than that of phospholipids (Bülow et al., 1988). However, FRAP measurements are limited by poor spatial resolution, inherent averaging of the dynamics of multiple individual molecules, and a possible convolution of diffusion and protein interactions (Saha et al., 2016). To resolve these inherent limitations of the FRAP technique, studies of the dynamics of single molecules or small groups thereof have been employed to gain a more accurate picture of the diffusion process. Pioneering single particle tracking (SPT) studies on GPI-anchored proteins, such as Thy-1 and NCAM-125, revealed different diffusion modes for these molecules, as evidenced by their individual trajectories (Sheets et al., 1995; Sheets et al., 1997). In *T. brucei*, the trajectories revealed that single VSG molecules diffused freely in artificial membranes, as well as on living cells (Hartel et al., 2016). In addition, the authors were able to detect a specific molecular crowding threshold that limits diffusion and affects protein function. To the best of our knowledge, no data on surface molecule mobility has been reported to date for *T. cruzi* or *Leishmania* spp.

Although several different experiments have suggested that GPI-anchored proteins diffuse freely as individual molecules over large length scales, a dynamic partitioning into lipid rafts has also been proposed (Simons and Ikonen, 1997; Kenworthy et al., 2004; Komura et al., 2016; Kinoshita et al., 2017). Lipid rafts are microdomains enriched with sphingolipids and cholesterol (Simons and Ikonen, 1997; reviewed in Levental et al., 2020). Saturated lipid chains are critical for the lipid-lipid interactions between sphingolipids and GPI anchors (Schroeder et al., 1994, 1998). As the GPI anchor does not extend through the lipid bilayer, lipid rafts might function as binding hubs for GPI-anchored proteins and receptors involved in intracellular signaling pathways (Stefanova et al., 1991). Other postulated functions include apical and basolateral sorting as well as export mechanisms of GPI-anchored proteins from the trans-Golgi network in polarized cells (Lisanti et al., 1988; Muniz and Riezman, 2016; Lebreton et al., 2019). It has been suggested that, during Golgi transit, where the sterol content increases, proteins with shorter anchors are retained and ultimately targeted for ER-associated degradation, while the ones with longer anchors progress toward the plasma membrane (Bagnat et al., 2000; Simons and Sampaio, 2011; Spira et al., 2012). Studies on lipid rafts in protozoan parasites indicate that they may be possible factors involved in parasite–host interactions, including host cell signaling, cell adhesion and invasion as well as vesicle trafficking, release and motility (Goldston et al., 2012). In *T. cruzi*, an increasingly popular hypothesis describes the surface coat as a rather highly organized "patchwork quilt"-like structure, instead of a continuum of glycoconjugates (Mucci et al., 2017). The proposed structure is composed of multiple nanoscale membrane domains (10–150 nm) bearing different compositions of proteins and probably also of lipids (Lantos et al., 2016; Mucci et al., 2017). However, the size, function, lifespan, and even existence of such domains, in general, remains controversial (reviewed in Levental et al., 2020).

Another important characteristic of GPI-anchored proteins is their controlled shedding from the cell surface through the action of specific GPIases (Kinoshita, 2020). The shedding of GPI-anchored proteins triggers diverse responses and is implicated in essential cellular functions, such as neuronal differentiation (Sabharwal et al., 2011; Park et al., 2013), promotion of endothelial cell migration (Watanabe et al., 2007), and fertilization competence of spermatozoa (Fujihara et al., 2013; Fujihara et al., 2014).

In *T. brucei*, soluble VSG (sVSG) is shed from the cell surface by GPI-PLC mediated hydrolysis of the GPI-anchor (Bülow et al., 1989; Garrison et al., 2021). In addition, membrane form VSG (mfVSG) containing the intact GPI-anchor is released via direct shedding (Garrison et al., 2021). Although GPI-PLC is involved in VSG turnover, its exact function and even localization is still unclear. While the locations reported in the literature include the cytoplasmic leaflet of the plasma membrane (Cardoso De Almeida et al., 1999), the flagellar membrane (Grab et al., 1987; Hanrahan et al., 2009; Sunter et al., 2013), and the cell surface of short-stumpy forms (Gruszynski et al., 2003), the proposed functions include the stimulation of endocytosis of the transferrin receptor (TfR) (Subramanya et al., 2009), cleavage of misfolded GPI-anchored proteins prior to ER-associated degradation (Tiengwe et al., 2018), and VSG release during differentiation (Gruszynski et al., 2003). In contrast, the shedding of mfVSG containing an intact GPI anchor might be a direct consequence of the unusual lipid composition of the VSG GPI anchor, which exclusively contains dimyristoyl glycerol (Ferguson et al., 1988). This lipid shows a high off rate from biological membranes at 37°C (Silvius and Leventis, 1993; Silvius and Zuckermann, 1993; Garrison et al., 2021), which might explain why VSG molecules have been found to integrate into the plasma membrane of erythrocytes (Rifkin and Landsberger, 1990).

While the evolutionary advantage of enzymatic shedding of trypanosomatid surface molecules is still not clear, the release of virulence factors in extracellular vesicles has been shown to influence parasite–host-interactions (reviewed in Szempruch et al., 2016). Therefore, it is tempting to speculate that VSG shedding from doomed cells is a final attempt to modulate host defenses, with the released antigens acting as a decoy to bind antibodies, thus rendering the latter refractory to interacting with VSGs in living cells. Such altruistic behavior has recently been demonstrated for *Escherichia coli* populations, in which mass cell suicide was detected as a defense strategy in bacterial warfare (Granato and Foster, 2020). It has already been speculated that an altruistic form of programmed cell death has a function in life cycle progression of African trypanosomes (Duszenko et al., 2006; Welburn et al., 2006). However, it remains to be determined whether a unicellular organism can undergo a process that is considered altruistic.

SURFACE MOLECULES OF THE HUMAN PATHOGENIC TRYPANOSOMATIDS

While the majority of trypanosomatids are monoxenic parasites of insects, *Trypanosoma* and *Leishmania* species have largely adopted a dixenic lifestyle by successfully infecting and proliferating in vertebrate hosts (Lukeš et al., 2014, 2018; Adl et al., 2019). The dixenic lifestyle can be seen as beneficial to the parasites, which started exploiting an additional host that provides different nutrient resources and, potentially, less competition (Ricklefs, 2010; Lukeš et al., 2014). However, living in such distinct microenvironments represents a challenge that requires constant adaptation from the parasites for their survival.

Within the vertebrate host, pathogens are exposed to a complex and orchestrated immune response. As a result, trypanosomatids have developed a range of strategies to overcome the attack by humoral and cellular components of both the innate and adaptive immune systems of their hosts and to maximize the probability of being transmitted to another host (Cardoso et al., 2015; Geiger et al., 2016). Inside the invertebrate host, trypanosomatids are confronted with harsh physiological conditions (acidic pH as well as proteolytic and hydrolytic activities), have to handle innate immune responses and must cross physical barriers to ensure infection of a specific tissue (e.g., gut, salivary glands) that enhances the chance of further transmission (Caljon et al., 2016). The microbiome of the vector and symbiotic associations are likely to play an additional role in infection resistance (Cirimotich et al., 2011; Weiss and Aksoy, 2011). Thus, parasites must overcome several bottlenecks to successfully complete their life cycle.

In all life cycle stages, with their vastly varying microenvironments, it is the cell surface of the parasite that represents the interface for interactions with the host or insect vector. Therefore, one hallmark of trypanosome developmental progression is the changing of the molecular composition of their glycocalyx (Acosta-Serrano et al., 2007; de Souza et al., 2010). In the following subsections we give an overview of the life cycles of different human infective trypanosomatids, after which we indicate how the parasites employ GPI-anchored surface molecules to adapt to their diverse microenvironments in order to facilitate endurance in such contrasting surroundings. Since the repertoire of expressed surface molecules varies greatly, we will focus on highly abundant GPI-anchored molecules.

Trypanosoma brucei

The Life Cycle

Inside the vertebrate host, *T. brucei* is found exclusively in extracellular fluids and in two morphologically distinct forms: the proliferative slender and the cell cycle arrested stumpy trypomastigotes. Infection of the tsetse starts when the fly takes a bloodmeal from an infected mammalian host and ingests these bloodstream forms. Classically, the stumpy form has been described as 'preadapted' to survive in the fly and, hence, was long considered to be the only fly infective form (Robertson and Bradford, 1912; Rico et al., 2013; Smith et al., 2017; Szöőr et al., 2020). However, recent findings have shown slender forms to be equally competent for tsetse passage (Schuster et al., 2021). Once inside the fly, trypanosomes pass through the crop to the tsetse midgut. Here, they elongate and differentiate into the proliferative procyclic forms (Vickerman, 1969; Turner et al., 1988). After having established themselves in the midgut as procyclics, the trypanosomes must then cross the peritrophic matrix, a protective, chitinous barrier that separates the bloodmeal from the midgut tissue (Lehane et al., 1996; Rose et al., 2014; Rogerson et al., 2018). In order to cross this barrier, procyclic trypanosomes must swim back in the direction they came from, to reach the site of peritrophic matrix synthesis, the proventriculus. Here, they can swim through the peritrophic matrix in its immature state (Rose et al., 2020). After entering the endotrophic space, procyclic trypanosomes can either continue to colonize the ectotrophic midgut or elongate in the anterior midgut to become cell-cycle arrested mesocyclic trypanosomes, which then invade the proventriculus (Vickerman, 1985; Van Den Abbeele et al., 1999). In the proventriculus, trypanosomes develop into the long, proliferative epimastigote forms (Vickerman, 1985; Van Den Abbeele et al., 1999; Sharma et al., 2008; Rose et al., 2020). While undergoing an asymmetric division to create a long and a short daughter cell, the epimastigote forms migrate to the salivary glands. Though very difficult to confirm experimentally, it is thought that upon entry into the salivary gland, the long daughter cell dies while the short daughter cell attaches to the gland epithelium via its flagellum (Vickerman, 1969). Once attached, the trypanosomes either divide symmetrically to generate more attached epimastigotes, or they undergo an asymmetric division. This asymmetric division results in the formation of the cell cycle arrested, free-swimming metacyclic form. With the next bite of the tsetse fly, the metacyclic trypomastigotes infect the mammalian host and subsequently differentiate to the proliferative slender bloodstream stage (Vickerman, 1985; Schuster et al., 2017; Szöőr et al., 2020). An overview of the life cycle showing the parasite stages in their mammalian hosts and respective insect vectors can be found in **Figure 4A**.

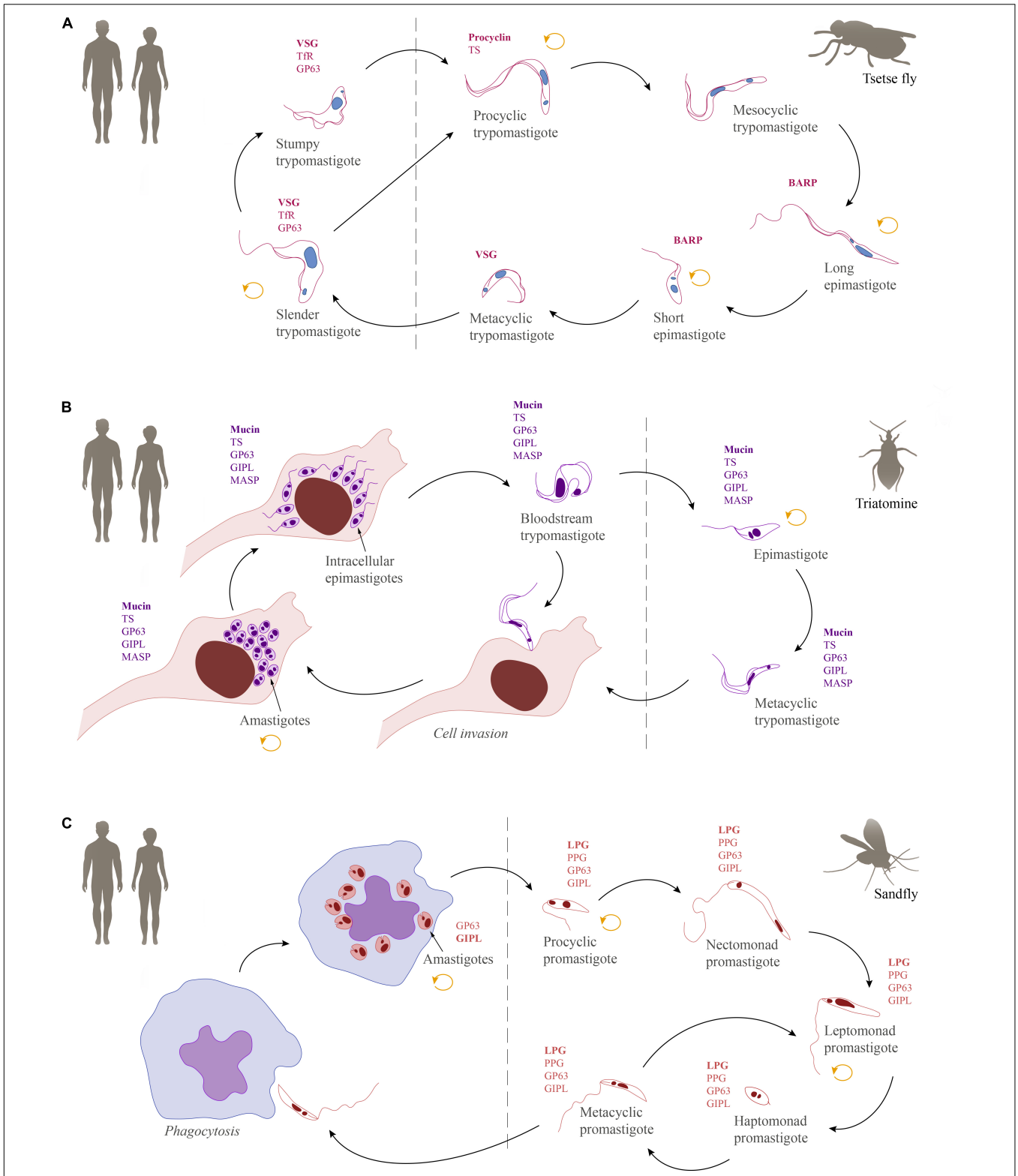


FIGURE 4 | Schematic overview of the life cycles of human-infective trypanosomatids together with their most abundant GPI-anchored molecules. **(A)** *Trypanosoma brucei*, **(B)** *Trypanosoma cruzi*, and **(C)** *Leishmania*. The replicative stages are indicated by a circular arrow (orange). GPI-anchored surface molecules: variant surface glycoprotein (VSG), transferrin receptor (TfR), GP63, procyclin, *trans*-sialidase (TS), *brucei* alanine rich protein (BARP), mucin, glycoinositolphospholipid (GIPL), mucin-associated surface protein (MASP), lipophosphoglycan (LPG), proteophosphoglycan (PPG).

GPI-Anchored Molecules in Mammalian Host Stages

The mammalian host reacts to infections by African trypanosomes with the full spectrum of immune responses, activating both cellular and humoral components (reviewed in Onyilagha and Uzonna, 2019). However, due to the extracellular lifestyle of these trypanosomes, humoral responses constitute the most prominent line of defense against the parasite. To outwit the host defenses, the parasite employs a sophisticated mechanism of antigenic variation and antibody clearance revolving around its GPI-anchored major surface glycoprotein, the VSG (**Table 2**), which forms a dense monolayer on the cell surface. VSG monomers in *T. brucei* have a molecular mass of approximately 55 kDa (Cross, 1975). Based on studies on the VSG N-terminal domain from the 1990s, VSGs were long thought to invariably form homodimers of very similar structure (Freymann et al., 1990; Blum et al., 1993) with a more recently solved VSG structure also showing very similar structural traits (Bartossek et al., 2017). Other recent findings, however, suggest that the members of the large VSG family are structurally more diverse than previously thought, with some believed to form trimers on the cell surface (Pinger et al., 2018; Umaer et al., 2021; Zeelen et al., 2021). VSG homodimers are attached to the plasma membrane via two GPI anchors, with each one covalently linked to the C-terminus of one monomer (Ferguson et al., 1988). Usually, each monomer carries at least one N-linked oligosaccharide (Zamze, 1991; Mehlert et al., 1998).

The cell surface of the bloodstream form of *T. brucei* is covered by a glycocalyx composed of about 10^7 VSG monomers, creating a dense monolayer (Cross, 1975). The amino-terminal domains of the VSGs, which constitute about 75% of the mature protein, show high sequence diversity. This diversity is ensured by a large repertoire (>1000) of VSG coding genes, of which approximately 80% are pseudogenes or incomplete genes that are used to expand the variability through recombination events (Cross et al., 2014). Out of this variety, the parasites express just one VSG at a time, and sporadic switches in VSG expression form the basis for antigenic variation (Mugnier et al., 2016). The process of VSG switching is thought to represent the only occasion when more than one VSG isoform is displayed on the cell surface (Horn, 2014). Furthermore, the VSG molecules are randomized on the cell surface by lateral diffusion (Hartel et al., 2016) and conformational changes in VSG molecules have been suggested to contribute to an adaptive packing (Bartossek et al., 2017). The latter mechanism ensures that the covering of the plasma membrane is not compromised by slight fluctuations of the amount of proteins in the VSG surface coat, as might occur during antigenic variation and during the course of the cell cycle (Bartossek et al., 2017). In this way, the flexible and dense VSG coat always shields the plasma membrane and invariant molecules efficiently from immune recognition.

The high VSG mobility conferred by the GPI-anchor is also essential for antibody clearance. The incessant and directional movement of trypomastigotes generates hydrodynamic flow forces on the cell surface that drag VSG-antibody complexes to the posterior region of the cell (Engstler et al., 2007), where they are internalized through the flagellar pocket by a very efficient endocytosis machinery (reviewed in Overath and Engstler, 2004; Link et al., 2021). Another important skill for any parasite is nutrient scavenging from the host. For example, the transferrin receptor (TfR) (**Table 2**) is responsible for iron uptake in *T. brucei*. TfR is a heterodimer consisting of ESAG6 and ESAG7 and is attached to the plasma membrane by a single GPI anchor on ESAG6 (Trevor et al., 2019). The localization of TfR is usually restricted to the flagellar pocket (FP), but under iron starvation TfR expression has been shown to be upregulated and the receptors escape from the FP and cover the entire cell surface (Mussmann et al., 2003, 2004). In this scenario, it is likely that *T. brucei* employs the same principle of GPI-anchor mobility for iron endocytosis. Interestingly, a study that analyzed TfR trafficking was able to highlight the importance of the GPI anchor as an intracellular sorting signal in trypanosomes (Tiengwe et al., 2017). The authors showed that ESAG7 homodimers, which contain no GPI anchor, are transported to the lysosome while ESAG6 homodimers, which display two GPI anchors, are carried to the cell surface. In addition, they created a modified TfR heterodimer with two GPI anchors, which was found to localize to the cell surface. These results indicate that the attachment of two GPI anchors might be a requirement for proteins to be translocated to the cell surface in *T. brucei*.

Although other stage-specific surface proteins exist, their detection is hampered by the high abundance of VSG molecules (Shimogawa et al., 2015). Due to the lethality of VSG deletion to the parasite, the biological role of most of these other molecules is still not understood.

GPI-Anchored Molecules in Invertebrate Host Stages

In the tsetse fly midgut, the ingested bloodstream forms of *T. brucei* are exposed to proteases that promote the differentiation to procyclic forms (Sbicego et al., 1999). During differentiation, it is assumed that the GPI anchored GP63 (or major surface protease - MSP) and GPI-PLC synergistically cleave VSG from the surface (**Table 2**) (Bülow et al., 1989; Gruszynski et al., 2003, 2006; LaCount et al., 2003; Grandgenett et al., 2007). Simultaneously, a new, stage-specific coat consisting of GPI-anchored procyclins (**Table 2**) is established (Bülow et al., 1989; Gruszynski et al., 2003, 2006; Grandgenett et al., 2007). Distinct classes and isoforms of procyclins are differentially expressed by the trypanosomes. The EP procyclins contain repeats of a dipeptide composed of glutamic acid (E) and proline (P), while the GPEET procyclins consist of pentapeptide repeats of glycine (G), proline (P), two glutamic acids (EE), and threonine (T) (Roditi et al., 1998). While the polypeptide backbone of the procyclins may be modified with phosphate groups (Bütikofer et al., 1999; Mehlert et al., 1999), the C-terminal GPI anchor is furnished with large and branched poly-NAL glycans (Ferguson et al., 1993) and its inositol acylation makes it resistant to cleavage by GPI-PLC (Field et al., 1991). The poly-NAL chains on the GPI anchor are further capped with sialic acid by a GPI anchored *trans*-sialidase (TS; **Table 2**), conferring additional negative charges to the procyclin coat (Engstler et al., 1993; Pontes de Carvalho et al., 1993). However, it has not yet been demonstrated whether sialic acid capping is important for parasite survival.

TABLE 2 | Glycosylphosphatidylinositol-anchored surface molecules of trypanosomatids.

Organism	Molecule	Stage	Function	References
Trypanosoma brucei	VSG	BSF	Immune evasion	Cross, 1975; Cross et al., 2014; Mugnier et al., 2016
	TfR	BSF	Transferrin uptake	Steverding et al., 1994; Mussmann et al., 2003, 2004
	GPI-PLC	BSF	Phospholipase, implicated in VSG shedding	Bülow et al., 1989; Gruszynski et al., 2003; Garrison et al., 2021
	GP63	BSF	Metalloprotease, implicated in VSG shedding	LaCount et al., 2003; Grandgenett et al., 2007
	Procyclin	PCF	Implicated in proteases resistance	Roditi et al., 1998; Acosta-Serrano et al., 2001b
	TS	PCF	Sialyation of procyclin	Engstler et al., 1993; Pontes de Carvalho et al., 1993
	BARP	E		Urwyler et al., 2007
Trypanosoma cruzi	Mucin	All	Immune evasion and cell attachment	Almeida et al., 2000; Pereira-Chioccola et al., 2000; Buscaglia et al., 2006
	TS	All	Cell attachment and complement resistance	Schenkman et al., 1991a; Frevert et al., 1992; Lantos et al., 2016
	GP63	All, highest in A	Implicated in cell adhesion	Grandgenett et al., 2000; Cuevas et al., 2003; Rebello et al., 2019
	MASP	All, highest in BSF	Implicated in immune evasion	De Pablos et al., 2011, 2016; De Pablos and Osuna, 2012
	GIPL	All, highest in E	Implicated in cell attachment and host cell recruitment	Golgher et al., 1993; Previato et al., 2004; Nogueira et al., 2007
Leishmania spp.	LPG	P	Cell attachment and complement resistance	Turco and Descoteaux, 1992; Forestier et al., 2014
	PPG	P	Protease's resistance	Ilg et al., 1999a; Secundino et al., 2010
	GP63	All, highest in P	Metalloprotease, implicated in complement resistance and cell attachment	Bouvier et al., 1985; Brittingham et al., 1995; Olivier et al., 2012
	GIPL	All, highest in A	Implicated in modulation of host cell signalling	McConville et al., 1990; Suzuki et al., 2002; Chawla and Vishwakarma, 2003
Trypanosoma congolense	VSG	BSF	Immune evasion	Jackson et al., 2013
	GARP	PCF, E, M		Eyford et al., 2011; Jackson et al., 2013
	CESP	E		Sakurai et al., 2008; Jackson et al., 2013
	GP63	?	Metalloprotease	Jackson et al., 2013; Jackson, 2015
	TS	?	Sialyation	Jackson et al., 2013; Jackson, 2015
	Procyclin	?		Jackson et al., 2013; Jackson, 2015
Trypanosoma vivax	VSG	BSF	Immune evasion	Jackson et al., 2013; Jackson, 2015; Silva Pereira et al., 2020
	BARP/ GARP-like	?		Jackson et al., 2013; Jackson, 2015
	MASP-like	?		Jackson et al., 2013; Jackson, 2015
	GP63	?	Metalloprotease	Jackson et al., 2013; Jackson, 2015
	TS	?	Sialyation	Jackson et al., 2013; Jackson, 2015
Trypanosoma rangeli	GP63	?	Metalloprotease	Wagner et al., 2013; Bradwell et al., 2018
	TS	?	Sialyation	Wagner et al., 2013; Bradwell et al., 2018
	Mucin	?	Implicated in immune evasion	Wagner et al., 2013; Bradwell et al., 2018
	GIPL	?	Implicated in modulation of host cell signalling	Gazos-Lopes et al., 2012
Trypanosoma conorhini	GP63	?	Metalloprotease	Wagner et al., 2013; Bradwell et al., 2018
	TS	?	Sialyation	Wagner et al., 2013; Bradwell et al., 2018
	Mucin	?	Implicated in immune evasion	Wagner et al., 2013; Bradwell et al., 2018
Trypanosoma theileri	GP63	?	Metalloprotease	Kelly et al., 2017
	TS	?	Sialyation	Kelly et al., 2017
	TTPSP	?		Kelly et al., 2017
Trypanosoma grayi	TS	?	Sialyation	Coding genes are annotated in the reference strain ANR4 in TriTrypDB
Trypanosoma carassii	Mucin-like	?		Lischke et al., 2000; Aguero et al., 2002
	TS	?	Sialyation	Aguero et al., 2002
Paratrypanosoma	GP63	?	Metalloprotease	Jackson et al., 2016
Crithidia	GP63	?	Metalloprotease	Jackson et al., 2016
Bodo saltans	GP63	?	Metalloprotease	Jackson et al., 2016

Summary of the GPI-anchored surface molecules discussed in this paper. For each molecule, the life cycle stages in which they are expressed, and the proposed functions are provided. Question marks are used when no information regarding the corresponding life cycle stage is available. GPI-anchored surface molecules: variant surface glycoprotein (VSG), transferrin receptor (TfR), glycosylphosphatidylinositol-phospholipase C (GPI-PLC), GP63, procyclin, trans-sialidase (TS), brucei alanine rich protein (BARP), mucin, mucin-associated surface protein (MASP), glycoinositolphospholipid (GIPL), lipophosphoglycan (LPG), proteophosphoglycan (PPG), glutamine alanine rich protein (GARP), congolense epimastigote specific protein (CESP), Trypanosoma theileri *putative surface protein (TTPSP), promastigote surface antigen (PSA). Life cycle stages: bloodstream form (BSF), procyclic form (PCF), metacyclic (M), epimastigote (M), amastigote (A), promastigote (P).*

All procyclin isoforms are resistant to cleavage by GP63 and tsetse midgut proteases (Acosta-Serrano et al., 2001b; Liniger et al., 2003) and their expression is temporally regulated. Directly after differentiation is induced, all three EP isoforms (EP1, EP2, EP3) and GPEET are expressed (Vassella et al., 2001). In the first days, GPEET synthesis is increased, making it the predominant component of the early procyclic surface coat (Acosta-Serrano et al., 2001b; Vassella et al., 2001). After a few days, GPEET is repressed, indicating the transition to late procyclic forms (Vassella et al., 2001). These findings led to the postulation that GPEET might be important for survival in the midgut, while EP represents a better coat for parasite survival on the way to the salivary glands. However, experiments with EP/GPEET null mutants indicated that procyclins are not essential for procyclic forms *in vitro* (Vassella et al., 2003) and cyclical transmission by the tsetse fly was also not negatively affected (Haenni et al., 2006; Vassella et al., 2009). Interestingly, analysis of the null mutant revealed that in the absence of procyclin polypeptide precursors, free GPI anchors formed a glycocalyx on the surface (Vassella et al., 2003). Thus, the concrete functions of procyclins remain elusive, but it might be possible that they are required for infections in the wild, where infection levels are very low. In addition, they might be important for migration to the salivary glands, as EP procyclins are also expressed by the mesocyclic forms in the anterior midgut, and by trypomastigotes in the proventriculus (Sharma et al., 2008).

Another stage-specific molecule anchored by GPI is known as the *T. brucei* alanine-rich protein (BARP) (**Table 2**). BARPs are expressed by epimastigotes attached to the epithelium of the salivary glands, but their biological role is still unknown (Urwyler et al., 2007).

Trypanosoma cruzi

The Life Cycle

Trypanosoma cruzi infects the mammalian host when the metacyclic trypomastigotes, which are present in the feces of the triatomine vector, enter the body through wounds or mucosa. In contrast to African trypanosomes, the metacyclic forms of *T. cruzi* attach to and invade a variety of host cells (Chagas, 1909; Schenkman et al., 1991b; Yoshida, 2006). The strategies used by *T. cruzi* for cell entry are diverse (reviewed in Walker et al., 2014). Once inside the cells, they are initially confined within a membrane-bound compartment, the parasitophorous vacuole, which later fuses with the lysosome, facilitating the escape of the parasites to the cell cytoplasm and triggering the differentiation into amastigotes (Andrews, 1993). In the cytoplasm, amastigotes proliferate and differentiate into the intracellular trypomastigotes (Chagas, 1909). During differentiation, an intracellular epimastigote-like stage is observed, which represents an intermediate stage preceding the maturation into trypomastigotes (Almeida-de-Faria et al., 1999). The intracellular amastigotes and trypomastigotes can escape to the extracellular environment where they can infect neighboring host cells (Dvorak and Hyde, 1973; Ferreira et al., 2012; Arias-Del-Angel et al., 2020). In addition, trypomastigotes can invade the bloodstream, where they are accessible for uptake by the hematophagous triatomine vectors (Salassa and Romano, 2019). After ingestion, most of the trypomastigotes are broken down in the stomach of the insect while the surviving parasites differentiate into epimastigotes (Ferreira et al., 2016). Epimastigotes move to the intestine where they proliferate, attach to the perimicrovillar membranes, and pass through metacyclogenesis, which is the transformation of non-infective epimastigotes into highly infective metacyclic trypomastigotes (Schaub, 1989; Goncalves et al., 2018). There is evidence suggesting that a microenvironmental shift in the concentration of oxidants and antioxidants may influence both the proliferation of epimastigotes and the differentiation into metacyclics (Nogueira et al., 2015). A schematic overview of the *T. cruzi* life cycle is provided in **Figure 4B**.

GPI-Anchored Molecules in Mammalian Host Stages

In contrast to *T. brucei*, *T. cruzi* also invades cells of their vertebrate host next to being found in the bloodstream. Cell invasion is a well-known strategy to avoid humoral immune responses. Nevertheless, hiding inside a cell triggers other components of host immunity: effectors of the cellular response, such as $CD8^+$ cells (reviewed in Cardoso et al., 2015).

The major surface glycoprotein of all life-cycle stages of *T. cruzi* is the mucin (also known as mucin-like glycoprotein). Mucins (**Table 2**) are GPI anchored, distributed over the entire plasma membrane and play a key role in parasite protection, infectivity and immune modulation during all *T. cruzi* life cycle stages (Mortara et al., 1992; Moreno et al., 1994; Ruiz et al., 1998; Acosta-Serrano et al., 2001a; Almeida and Gazzinelli, 2001; Buscaglia et al., 2006). These molecules contain a polypeptide backbone with Thr-rich domains that are extensively modified with short O-linked glycans (Schenkman et al., 1993; Almeida et al., 1994; Pereira-Chioccola et al., 2000). Two major gene families, called TcSMUG and TcMUC encode for mucins (Di Noia et al., 1995; Buscaglia et al., 2006). When in the mammalian host, genes of the TcMUC family are expressed (Campo et al., 2004; Buscaglia et al., 2006; Urban et al., 2011; Pech-Canul et al., 2017).

Mucin molecules of mammalian stages range from 60 to 200 kDa in molecular weight, share the sialic acid containing epitope Ssp-3, and present terminal Gal(α1,3)Gal epitopes (Schenkman et al., 1991a; Almeida et al., 1994; Tomlinson et al., 1994; Buscaglia et al., 2006). The Ssp-3 epitope is implicated in mammalian cell attachment and invasion and has been suggested to be involved in diverting the complement cascade (Schenkman et al., 1991a; Tomlinson et al., 1994; Buscaglia et al., 2006). The terminal Gal(α1,3)Gal epitopes are a main target of antibody responses. To evade the vertebrate immune response, these saccharides are masked by sialic acid molecules scavenged from the host (Previato et al., 1985; Pereira-Chioccola et al., 2000). In addition, the sialylation of mucins inside the parasitophorous vacuole transfers sialic acid from LAMP proteins to the parasite, which contributes to the rupture of the vacuole and invasion of the cytoplasm by the parasite (Hall et al., 1992; Albertti et al., 2010; Cardoso et al., 2015). The GPI anchor of most mammal-derived mucins contains

alkylacylglycerol with predominantly unsaturated fatty acids at the *sn*-2 position (Serrano et al., 1995). This feature most likely correlates with an induced production of proinflammatory cytokine interleukin-12 (IL-12) and tumor-necrosis factor α (TNFα) (Almeida et al., 2000; Almeida and Gazzinelli, 2001; Previato et al., 2004). In contrast, approximately 70% of the GPI anchor of metacyclic mucins contain inositolphosphoceramide in their phospholipid tail, which is thought to increase the mucin shedding rate (Schenkman et al., 1993; Serrano et al., 1995). Mucin shedding was hypothesized to play a role in the clearance of surface immunocomplexes (Buscaglia et al., 2006). In the metacyclic trypomastigote, mucins have also been proposed to have a function in cell attachment and invasion of mammalian host cells, including induction of intracellular Ca^{2+} signaling (Moreno et al., 1994; Ruiz et al., 1998).

Another important component of the *T. cruzi* surface is TS (**Table 2**). Like mucins, these GPI anchored glycoproteins are distributed over the entire plasma membrane of *T. cruzi* (Frevert et al., 1992; Lantos et al., 2016). Although all life cycle stages show TS at the cell surface, the functions vary immensely (Pech-Canul et al., 2017). The bloodstream trypomastigotes possess proteins with TS and/or neuraminidase activities (Schenkman et al., 1991a, 1992). The TS activity is responsible for the transfer of sialic acid from host glycoconjugates to mainly mucin O-linked glycans, enabling epitopes to be camouflaged (Schenkman et al., 1993; Pereira-Chioccola et al., 2000), as explained above in the context of mucins. The neuraminidase activity is used to remove sialic acids from the parasite surface and/or from the host cells, thereby facilitating the internalization of the parasite (Velge et al., 1988; Schenkman et al., 1992). Interestingly, the inhibition of TS activity in metacyclic trypomastigotes reduced the activation of Ca^{2+} signaling pathways (Ruiz et al., 1998). Considering that mucins have been linked to Ca^{2+} activity during cell invasion, it is likely that the orchestrated work of TS and mucins contributes to cell invasion through this pathway. This mechanism is activated by protein tyrosine phosphorylation (Favoreto et al., 1998). The TS of mammalian host stages are GPI anchored while the TS presented by insect stage epimastigotes are predicted to have a transmembrane domain (Briones et al., 1995b). Although the GPI anchoring of this molecule may be an adaptation of the mammalian host stages (Briones et al., 1995a; Rubin-de-Celis et al., 2006), the biological implications of this are still not clear. In amastigotes, SA85 is one of the few characterized TS molecules. This molecule is a ligand for the mannose receptor of macrophages, which has been suggested to increase the amastigote's potential for cell invasion (Kahn et al., 1995).

As is the case for mucins and TS, the GPI-anchored surface metalloprotease, also termed GP63 (or major surface protease; Table 2), is present in all life cycle stages of *T. cruzi* (Cuevas et al., 2003). However, it is more abundant in amastigotes than in epimastigotes or trypomastigotes (Grandgenett et al., 2000; Cuevas et al., 2003). Although this suggests different functional importance, the major role of GP63 is still elusive (Grandgenett et al., 2000; Cuevas et al., 2003; Kulkarni et al., 2009).

The fourth group of surface molecules belongs to the MASP multigenic family (**Table 2**) which is specific to *T. cruzi* and contains more than 1300 genes characterized by conserved N- and C-termini and a highly variable central region (El-Sayed et al., 2005). Although preferentially expressed in the bloodstream trypomastigotes, all life cycle stages express members of the MASP family (Atwood et al., 2005; De Pablos et al., 2011; De Pablos and Osuna, 2012). The well characterized MASP52 is upregulated in metacyclic and bloodstream trypomastigotes. Assays using antibodies raised against the ATP/GTP binding motif decreased cell invasion by *T. cruzi in vitro* (De Pablos et al., 2011). The predicted GPI anchoring of MASP was confirmed by its release following PLC treatment (Bartholomeu et al., 2009). Although little is known about MASPs, their release into the extracellular environment can trigger a humoral immune response (De Pablos et al., 2016), which suggests it could have a similar role in host evasion to that of TS.

The last group of surface molecules of *T. cruzi* are the GIPLs. Initially called lipopeptidophosphoglycans (LPPGs; **Table 2**) (De Lederkremer et al., 1976), these molecules were originally not considered to be GIPLs due to co-extraction with NETNES glycoprotein (Macrae et al., 2005) giving the impression that these molecules were not "naked." Interestingly, all GIPLs, characterized in *T. cruzi*, contain a type 1 conserved glycan core (**Figure 2**), like that found in GPI-anchored glycoproteins (Previato et al., 1990; de Lederkremer et al., 1991). Immunoassays with anti-GIPL serum demonstrated that the expression of this molecule is significantly decreased and heterogeneously distributed in the trypomastigote population when compared to the epimastigote stage (Golgher et al., 1993). This suggests a developmental regulation of its expression. The concrete function of mammalian-stage derived GIPLs is not completely understood but they may act in TNF-α induced neutrophil recruitment (Oliveira et al., 2004; Medeiros et al., 2007).

GPI-Anchored Molecules in Invertebrate Host Stages

Whereas the mammalian stages of *T. cruzi* express mucins of the TcMUC family, the insect stages express mucins from the TcSMUG family (Campo et al., 2004; Buscaglia et al., 2006; Urban et al., 2011; Gonzalez et al., 2013; Pech-Canul et al., 2017). These mucins are smaller, ranging between 35 and 50 kDa in molecular weight, and have a significant similarity in their amino-acid and carbohydrate composition (Yoshida et al., 1989; Mortara et al., 1992; Schenkman et al., 1993; Previato et al., 1994; Acosta-Serrano et al., 2001a). Epimastigote mucins do not act as sialic acid acceptors (Urban et al., 2011; De Pablos and Osuna, 2012). This correlates with different TS activities (Frasch, 2000) and might indicate that sialic acids are required for immune evasion within the mammalian host and play a less important role within the insect vector. Epimastigote mucins primarily have a protective role against proteases that are present in the intestinal tract of the insect vector (Mortara et al., 1992).

The role of GP63 in the invertebrate host has been studied less than in the mammalian host (d'Avila-Levy et al., 2014). A recent study investigated the effect of metal chelators as well as the effect of antibodies raised against GP63 on the interaction of *T. cruzi* with its principal triatomine vector *Rhodnius prolixus*. Both treatments reduced the interaction of the parasite with the explanted guts of the insect, indicating a possible function of

GP63 in adhesion (Rebello et al., 2019). However, the precise molecular mechanism of the vector interaction remains elusive.

Glycoinositolphospholipids molecules are abundant on the cell surface of *T. cruzi* epimastigotes (Golgher et al., 1993). Immunoelectron microscopy has shown that GIPLs form a homogeneous surface coat with an estimated number of 1.5×10^7 molecules/cell (de Lederkremer et al., 1991; Golgher et al., 1993). GIPLs are likely to be one of the components involved in the adhesion of *T. cruzi* to the luminal insect midgut surface and possibly one of the determinants of parasite infection in the insect vector (Nogueira et al., 2007).

Surprisingly, purified GIPLs from *T. cruzi* were reported specifically to suppress nitric oxide (NO) production within the salivary glands of the triatomine vector (Gazos-Lopes et al., 2012). Since salivary glands play no part in the life cycle of *T. cruzi*, the actual biological role remains unclear.

Leishmania spp.

The Life Cycle

The major transmission route for *Leishmania* is mediated by sand flies of the genera *Lutzomyia* and *Phlebotomus* (Marinkelle, 1980; Killick-Kendrick, 1999). Flies become infected by the ingestion of macrophages harboring amastigotes (Alexander and Russell, 1992; Bates, 2018). The environmental changes experienced by these parasite stages in the fly midgut, such as shifts in temperature and pH, stimulate their differentiation into promastigotes. Due to morphological differences found in the promastigote population, the insect forms are subdivided into procyclic, nectomonad, leptomonad, haptomonad, and metacyclic promastigotes. The first form found in the midgut is the procyclic form, a proliferative stage with a short flagellum and weak motility. After 48–72 h of proliferation, these forms differentiate into nectomonad promastigotes, a life cycle stage with a longer flagellum and higher motility (Rogers et al., 2002). The nectomonad forms migrate to the anterior portion of the midgut, where they differentiate into leptomonad promastigotes (Walters, 1993). These forms can either initiate further cycles of proliferation or differentiate into haptomonad promastigotes, which attach to the surface of the anterior midgut, or metacyclic promastigotes, the infective forms for vertebrates (Sacks, 1989; Dostalova and Volf, 2012; Bates, 2018). Interestingly, in all sand flies examined to date a gel-like plug, the parasite-derived promastigote secretory gel, blocks the anterior midgut, which forces infected insects to regurgitate parasites into the skin before they can take a blood meal (Rogers et al., 2004). Recently, it has been suggested for *Leishmania infantum* and *Leishmania major* that metacyclic promastigotes, which were not transmitted into the mammalian host de-differentiate into retroleptomonads, which starts a new cycle of proliferation and differentiation, which enhances the parasitic load and the potential for transmission (Bates, 2018; Serafim et al., 2018). This boost is likely to be important for infections in the wild, where flies will initially become infected with very small numbers of parasites by feeding on an infected vertebrate host (Doehl et al., 2017). Finally, inside the vertebrate hosts, the metacyclic promastigotes will be phagocytosed by macrophages where they differentiate into the proliferative and fly infective amastigotes (Barak et al., 2005; Rogers et al., 2009; Mollinedo et al., 2010). The life cycle is shown in **Figure 4C**.

GPI-Anchored Molecules in Mammalian Host Stages

In *Leishmania*, LPG (**Table 2**) is one of the major surface glycoconjugates of promastigotes (5×10^6 copies/cell) (Turco and Descoteaux, 1992; Forestier et al., 2014). Structurally, LPG is a highly complex macromolecule with four domains: a type-2 GIPL anchor, a glycan core, a linear phosphoglycan chain and a terminal oligosaccharide cap (**Figure 2E**) (Turco and Descoteaux, 1992; McConville et al., 1993; McConville and Ferguson, 1993; Forestier et al., 2014). The anchor possesses only one saturated C24-26 aliphatic chain (Forestier et al., 2014). The attached glycan core comprises two galactopyranosides, one galactofuranoside and one mannose. The phosphoglycan chain contains 15–40 phosphodisaccharide (Galβ1-4Manα1-PO_4) units (Forestier et al., 2014) with species-specific side chain modifications (Turco et al., 2001; de Assis et al., 2012). Lastly, a species-specific di-, tri-, or tetrasaccharide cap structure assembled as Manα1-2Manα1 or Galβ1-4(Manα1-2)Manα1 is attached (Forestier et al., 2014). Inside the vertebrate host, the long LPG of metacyclic promastigotes gives them an advantage in avoiding lysis by the complement system (Puentes et al., 1988, 1989). In promastigotes, LPG also delays phagosome maturation and acidification by impairing recruitment of lysosomal markers. This prevents the parasite from being killed inside macrophages, which allows their differentiation into the resistant amastigotes (Desjardins and Descoteaux, 1997; Holm et al., 2001; Vinet et al., 2009). In amastigotes, the expression of LPG is downregulated (Moody et al., 1993; Ilg, 2000).

Another important GPI-anchored surface molecule belongs to the proteophosphoglycans (PPGs; **Table 2**). PPGs contain a large polypeptide backbone, which is modified with a range of complex phosphoglycan chains (Ilg et al., 1999a,b). While some PPGs contain a GPI anchor and are present at the cell surface (mPPG), others lack a GPI attachment signal (Lovelace and Gottlieb, 1986; Stierhof et al., 1994; Ilg et al., 1995) and are secreted (sPPG), sometimes as large filamentous complexes (fPPG) that are assembled in the flagellar pocket (Stierhof et al., 1994). These different forms of PPGs have an important role in the establishment of *Leishmania* infections, including macrophage recruitment and modulation of host arginase activity to inhibit the production of harmful NO (Rogers et al., 2009). In addition, sPPG was found to increase interferon-γ (INF-γ) stimulated NO production (Piani et al., 1999). This suggests that PPG, on the one hand, may contribute to binding of *Leishmania* to host cells and, on the other hand, may play a role in downregulation of macrophage pro-inflammatory responses.

The zinc-dependent and GPI-anchored metalloprotease GP63 (also called leishmanolysin, MSP, or PSP; **Table 2**) represents another major surface antigen of *Leishmania* species (Bouvier et al., 1985; Bianchini et al., 2006). GP63 is a 60 kDa enzyme modified with *N*-glycosylated high mannose glycans (Ilgoutz and McConville, 2001). The structure predominantly contains β-sheets (Schlagenhauf et al., 1998). The N-terminal domain of GP63 displays the catalytic domain of a zinc

proteinase while the C-terminal domain is connected to the GPI anchor (Schlagenhauf et al., 1998). While GP63 is abundant in promastigotes (approximately 5×10^5 copies/cell), it is downregulated in amastigotes (Bouvier et al., 1985; Schneider et al., 1992; Bianchini et al., 2006). However, due to the simultaneous absence of LPG on the amastigote surface, the GP63 enzymes might have better access to their target molecules and therefore may be sufficient for modulation of host responses (Pimenta et al., 1991). Given its presence on both parasite forms combined with different expression levels, it is likely that GP63 fulfills a number of different functions, depending on the parasite stage. For example, the presence of GP63 on the metacyclic promastigote surface is connected to resistance to complement lysis by conversion of C3b into C3bi (Brittingham et al., 1995). C3bi is a ligand to CR3 complement receptors on the surface of macrophages, which is important for facilitating the parasite's entry into these cells as well as for inhibiting the interleukin-12 production leading to a deficiency in intracellular pathogen responses (Blackwell et al., 1985; Kimura and Griffin, 1992; Carter et al., 2009). In addition, GP63 can interact with the fibronectin receptor of mammalian cells, indicating that the receptors for complement and fibronectin may cooperate to mediate the efficient adhesion of parasites to macrophages (Brittingham et al., 1999). In amastigotes, GP63 plays a role in protection from phagolysosomal degradation (Chaudhuri et al., 1989; Seay et al., 1996; Chen et al., 2000) as well as alteration of macrophage signaling thereby favoring *Leishmania* survival and persistence within the host (Olivier et al., 2005, 2012). Consequently, this molecule not only actively protects the parasites in the extracellular environment, but also has a role in invasion and survival of *Leishmania* inside macrophages, which is essential for life cycle progression and a successful infection.

Glycoinositolphospholipids (**Table 2**) are also present on the *Leishmania* cell surface. These molecules have a similar abundance as LPGs (McConville and Bacic, 1990). These glycolipids may form a densely packed glycocalyx on the plasma membrane. The amount and type of GIPL displayed by *Leishmania* can vary according to the species of the parasite (McConville et al., 1990; McConville and Blackwell, 1991; McConville and Ferguson, 1993; Schneider et al., 1993, 1994; Winter et al., 1994). Their anchors can have the same structure as the GPI protein anchor (type 1), the LPG anchor (type 2) or contain motifs in common with both anchors (hybrid type) (**Figure 2**). In contrast to LPG or GPI-anchored glycoproteins, GIPL expression remains high in amastigotes indicating a possible function in intracellular survival (McConville and Blackwell, 1991; Bahr et al., 1993). GIPLs effectively deactivate the protein kinase C (PKC) cascade, which impairs the production of reactive oxygen species that could kill the parasites inside macrophages (Chawla and Vishwakarma, 2003). In addition, 85% of *Leishmania braziliensis* GIPLs are present in membrane microdomains and disruption of these domains leads to a significantly decreased macrophage infectivity (Yoneyama et al., 2006). Another report also indicated that glycosylation of GIPLs in *L. major* might be important for invasion of macrophages (Suzuki et al., 2002).

GPI-Anchored Molecules in Invertebrate Host Stages

Studies on surface molecules that might play a role in defending the parasite against the hostile conditions within the sand fly have mainly focused on glycoconjugates, including LPG and PPG. The LPG of *Leishmania* promastigotes can show stage specific adaptations. For example, in *L. major* and *L. donovani* the average length of the LPG phosphoglycan chain is more than doubled when proliferative procyclics differentiate to non-dividing metacyclics (McConville et al., 1992). The stage and species dependent changes in LPG structure are thought to be important for the attachment of haptomonad promastigotes to epithelial cells in the sand fly midgut, which is essential for avoiding elimination by peristaltic forces during colonization of non-permissive vectors (Pimenta et al., 1992; Butcher et al., 1996; Secundino et al., 2010; Dostalova and Volf, 2012; Volf et al., 2014). Subsequently, metacyclic LPGs were shown to be subject to conformational changes that impair efficient binding to the sand fly midgut, a key step in the release of mammalian infective forms (Sacks et al., 1995). It is tempting to speculate that the detachment of parasites from the midgut during development might also be explained by enzyme driven shedding of the LPG that is involved in binding.

In the insect stages of *Leishmania*, the PPGs have also been reported to be important factors for life cycle progression. The fPPGs were described as mucin-like glycoproteins which are one component of the gel-like matrix that blocks the passage to the midgut of the flies forcing them to regurgitate between blood meals, thus increasing the efficiency of transmission (Ilg, 2000; Rogers et al., 2004). In contrast, mPPG was reported to be a key molecule, protecting the fully developed procyclic promastigotes by conferring resistance to the activity of digestive enzymes present in the sand fly midgut (Secundino et al., 2010).

Despite the considerable amount of GP63 molecules present in promastigotes the deletion of this molecule in *L. major* did not alter the growth and development of the parasite within the insect vector (Joshi et al., 1998). Thus, GP63 does not appear to be needed to confer resistance to proteolytic enzymes in the gut.

EVOLUTION AND CELL SURFACE COMPOSITION OF *TRYPANOSOMA*

As discussed in the previous section, the medically relevant trypanosomatids exploit their surface molecules to interact with both vertebrate and invertebrate hosts. It is clear that these parasites rely on their diverse repertoires of GPI-anchored molecules to survive and thrive while residing in very different microenvironments during the course of their respective life cycles. Interestingly, their glycocalyces seem to be composed of a mixture of very specific molecules, such as VSGs, mucins and LPGs, as well as ubiquitous molecules, such as TS and GP63 (**Figure 4**). To increase our understanding of host-parasite interactions and gain insight into the essential features that were positively selected for over time, it is necessary to analyze the glycocalyx composition of species that are usually not in the spotlight. Thus, by broadening our perspective we can begin to comprehend the factors leading to the success of

parasitism of trypanosomatids. In the following, we aim to give an overview of the glycocalyx composition of other *Trypanosoma* species, focusing on their GPI-anchored molecules (**Table 2**) and correlating their surface composition with the evolutionary story of the group.

Trypanosoma is a monophyletic genus that can be divided into 17 subgenera of two lineages: aquatic and terrestrial (Kostygov et al., 2021). The human parasites *T. brucei* and *T. cruzi* are both of terrestrial lineage and have independent evolutionary stories (Hamilton and Stevens, 2017; Borges et al., 2021; Kostygov et al., 2021). This independence can be observed to a certain extent in their specific repertoires of surface molecules, which are related to their distinct lifestyles, survival strategies, and interactions with their hosts.

Closely related to *T. brucei*, other African trypanosomes are of socio-economic importance, such as *Trypanosoma congolense*, *Trypanosoma vivax*, *Trypanosoma evansi*, and *Trypanosoma equiperdum* (Hoare, 1972; Stevens and Gibson, 1999; Borges et al., 2021; Kostygov et al., 2021). Due to phylogenetic similarities, *T. evansi* and *T. equiperdum* were suggested to be subspecies of *T. brucei* (Lai et al., 2008; Carnes et al., 2015; Kamidi et al., 2017; Borges et al., 2021). However, such a taxonomical change is not allowed by the International Code of Zoological Nomenclature (ICZN, 1999; Molinari and Moreno, 2018). Thus, in this review, we will adhere to the conventionally used species names.

A common, and specific, characteristic of the African trypanosomes is the VSG coat that covers the cell surface of their bloodstream forms (Uzcanga et al., 2004; Jackson et al., 2013, 2015; Carnes et al., 2015; Carrasquel et al., 2017). Due to the close relationship between *T. brucei*, *T. evansi*, and *T. equiperdum*, a high degree of similarity in their surface molecules is not unexpected. For example, while VSGs of *T. congolense* and *T. vivax* lack a C-terminal domain (Rausch et al., 1994; Gardiner et al., 1996), *T. evansi* and *T. brucei* possess a conserved C-terminal domain through which the VSG is connected to the GPI anchor (Carrington et al., 1991; Chattopadhyay et al., 2005; Jones et al., 2008; Jia et al., 2011). In addition, the VSG repertoire as well as all VSG N-terminal subtypes are conserved between *T. evansi* and *T. brucei* (Carnes et al., 2015). However, the C-terminal structure of *T. evansi* VSGs can differ from *T. brucei* VSGs by the absence of cysteine residues (Jia et al., 2011). Larger differences in this coat can be found in *T. vivax*, an African trypanosome with earlier divergence and the highest evolutionary rates (Stevens and Rambaut, 2001).

In *T. vivax*, for instance, VSG transcript abundance, though high, has been reported to be lower than in *T. brucei* (Greif et al., 2013; Jackson et al., 2013, 2015). In addition, the VSG-coat of *T. vivax* is probably less dense than that of other African trypanosomes as suggested by the successful immunization of mice using an invariant surface protein of *T. vivax* (Autheman et al., 2021). Furthermore, the reduced recombination of *T. vivax* VSG genes (Silva Pereira et al., 2020) and the presence of *T. vivax*-specific putative membrane protein families in its bloodstream forms (Jackson et al., 2013, 2015) indicate other means of interaction between this parasite and the vertebrate host. The positive selection of VSGs and the expansion of VSG genes in *T. congolense* and *T. brucei*/*T. evansi* (Silva Pereira et al., 2020) advocates the importance of this GPI-anchored molecule for survival in the vertebrate host. Interestingly, *T. evansi* and *T. equiperdum* lost their capacity to infect and reproduce inside an invertebrate host, becoming the only known examples of monoxenic trypanosomes (Borst et al., 1987; Lai et al., 2008; Desquesnes et al., 2013). This specialization to the vertebrate host is linked to partial or total loss of maxicircles of kinetoplast DNA (kDNA), which carry information on the respiratory chain components that are required for mitochondrial metabolism and ATP production in the insect forms (Vickerman, 1965; Flynn and Bowman, 1973; Borst et al., 1987; Lai et al., 2008; Dewar et al., 2018).

Despite reports of mechanical transmission through distinct vectors, *T. congolense* and *T. vivax* are mainly transmitted by the tsetse fly, i.e., by the same invertebrate host as *T. brucei* (Peacock et al., 2012; Ooi et al., 2016). The insect stages of *T. congolense* produce the species-specific molecules *T. congolense* epimastigote-specific protein (CESP) and glutamic acid and alanine-rich protein (GARP), which is analogous to *T. brucei*'s BARP. CESP is exclusively expressed in epimastigotes and has been suggested to contribute to the adhesion of these stages to the proboscis, where the differentiation into metacyclics occurs (Sakurai et al., 2008; Peacock et al., 2012; Jackson et al., 2013). GARP is found in epimastigotes, procyclics, and metacyclics and has been proposed to protect the parasites against digestion in the midgut as well as to influence the migration of the parasites to different organs, but no concrete evidence for these biological function exists so far (Beecroft et al., 1993; Hehl et al., 1995; Sakurai et al., 2008; Eyford et al., 2011; Jackson et al., 2013). Procyclin homologs were found in *T. congolense* but not in *T. vivax* (Jackson et al., 2013, 2015), suggesting that procyclins appeared later in the evolution of African trypanosomes. Considering that *T. vivax* development in the tsetse fly is restricted to the mouth parts (Ooi et al., 2016) while *T. congolense* and *T. brucei* pass through the midgut and other organs (Peacock et al., 2012; Schuster et al., 2017, 2021; Rose et al., 2020), it is likely that procyclin is related to the development of a complex life cycle inside the fly. Because *T. vivax* possesses BARP/GARP-like genes (Jackson et al., 2013, 2015) these proteins may have an important biological role, at least, for the passage through the mouth parts of the fly. The presence of GP63 and TS has also been detected in the genome of both *T. vivax* and *T. congolense* (Jackson et al., 2013, 2015), and genes for both are annotated in the genome of *T. evansi* in TriTrypDB[1], pointing at a wide distribution in trypanosomatids beyond the medically relevant species. The presence of MASP-like proteins, similar to the abundantly expressed MASP of metacyclic stages of *T. cruzi*, was also detected in *T. vivax* (Jackson et al., 2013, 2015). However, their biological role is still unknown.

The other important human pathogen, *T. cruzi*, is closely related to other parasites of mammals, such as *Trypanosoma rangeli* and *Trypanosoma conorhini* (Hoare, 1972; Stevens and Gibson, 1999; Borges et al., 2021; Kostygov et al., 2021). Genomic analyses of *T. rangeli* and *T. conorhini* strains revealed a similar

[1]http://tritrypdb.org

number of GPI-anchored proteins and the presence of multigene families. As in *T. cruzi*, the surface molecules with the highest gene expansion in both species were TS and GP63 (Wagner et al., 2013; Bradwell et al., 2018). However, the presence of mucin genes was less frequent and no homology to other multigenic families present in *T. cruzi* was detected (Wagner et al., 2013; Bradwell et al., 2018). The proposed biological roles of mucins in *T. cruzi* vary from cell invasion in the vertebrate host to protection against lysis in the invertebrate host (see section "*Trypanosoma cruzi*"). Considering the extracellular lifestyle of *T. rangeli* and *T. conorhini* in the vertebrate host, it is tempting to suggest that the protective role of mucins represents an ancestral characteristic linked to survival inside the triatomine host. In this scenario, the role in cell invasion would be the result of a change of function promoted by the genetic expansion of mucins in *T. cruzi*. The presence of GIPLs on the cell surface of *T. rangeli* was also detected and it has been shown to downregulate NO synthesis (Gazos-Lopes et al., 2012), which is one of the key mechanisms of invertebrate immune response.

Trypanosoma theileri is a ubiquitous parasite of cattle and is closely related to crocodilian trypanosomes and ancestral to *T. cruzi*, *T. rangeli,* and *T. conorhini* (Hamilton et al., 2009; Kelly et al., 2017). The genome of *T. theileri* contains homologs of GP63-like surface protease and TS, but no mucin orthologs were detected (Kelly et al., 2017), suggesting the appearance of mucin in a later differentiation event and highlighting, once more, TS and GP63 as common GPI-anchored proteins among trypanosomes. In addition, four large groups of proteins, putatively containing conserved N-terminal signals and C-terminal GPI-addition sequences, were found. These proteins are considered to be exclusive to *T. theileri* and were provisionally named *T. theileri* putative surface protein (TTPSP) (Kelly et al., 2017). Together, these TTPSP and GP63-like proteins account for approximately 10% of the genome (Kelly et al., 2017).

Trypanosoma grayi is an extracellular parasite found in the bloodstream of crocodiles and is transmitted by the feces of tsetse flies (Hoare, 1931; Kelly et al., 2014). It occurs in Africa but is closely related to other crocodilian trypanosomes from South America, such as *Trypanosoma kaiowa* (Fermino et al., 2019). Phylogenomic and phylogenetic analyses show that this species is more closely related to *T. cruzi* than to African trypanosomes (Kelly et al., 2014). BLAST and OrthoMCL analyses of the genome sequence and predicted gene models did not reveal the presence of VSG surface antigens or mucin (Kelly et al., 2014). The hypothesis of independent evolution of African trypanosomes suggests separate events of colonization of the tsetse fly during the evolution of *Trypanosoma* (Hamilton and Stevens, 2017). The lack of VSG in a tsetse transmitted trypanosome corroborates this hypothesis. While the position of *T. grayi* in *Trypanosoma* is still under debate, the lack of mucins suggests that it may have diverged earlier than *T. cruzi*, *T. rangeli*, and *T. conorhini*. Genes coding for TS and GP63 are annotated in the *T. grayi* reference strain ANR4 in TriTrypDB (see footnote 1).

So far, we have focused on trypanosomes of the terrestrial lineage. Compared to these, even less is known of species of the aquatic lineage. An electron microscopy study of *Trypanosoma fallisi*, an anuran trypanosome, suggested that surface coat components were secreted inside vesicles detected around and within the flagellar pocket (Martin and Desser, 1990). However, these components were not characterized further. The presence of polysaccharides on the surface of epimastigotes of *Trypanosoma rotatorium*, another anuran trypanosome, was observed using Thiery's silver proteinate method (Desser, 1976), but no other information on the nature of these molecules is available. *Trypanosoma carassii* is a fish parasite with a glycocalyx composed of GPI-anchored mucin-like proteins similar to *T. cruzi* (Lischke et al., 2000; Overath et al., 2001; Aguero et al., 2002). The mucin-like molecules of *T. carassii* are sialylated (Lischke et al., 2000) with reported activity of TS, which transfers sialic acids from sialyllactose to a lactose acceptor in cell fraction extracts (Aguero et al., 2002). However, detailed analyses on the functional groups of *T. carassii* TS are lacking. Due to the extracellular lifestyle of *T. carassii* (Dóró et al., 2019), it is possible to suggest that the mucin-like coat of *T. carassii* acts only in parasite protection. Although still under debate, it is likely that the aquatic lineage has diverged later in the evolution of *Trypanosoma* as a split from a terrestrial species (Hamilton and Stevens, 2017; Borges et al., 2021). The presence of mucin-like proteins in *T. carassii* could be explained by this hypothesis, but this can only be confirmed by ancestral character reconstruction.

Ancestral reconstruction studies are still at an early stage for trypanosomatids. However, by reviewing the GPI-anchored molecules displayed by trypanosomes we could highlight parallels with the evolutionary story of the group. In addition, it is evident that a set of surface molecules has been maintained during evolution and is shared among different species, namely the GP63 proteases and TS (Bouvier et al., 1985; Schenkman et al., 1991a; Cuevas et al., 2003; Engstler et al., 1993; LaCount et al., 2003; Jackson et al., 2013; Wagner et al., 2013; Bradwell et al., 2018). From the above, it is clear that a broader focus on understanding the molecular composition of the cell surface of different trypanosome species could help to fill in the gaps in the evolutionary story of this group, which has a direct impact on developmental cell biology research and could also influence evolution-based drug discovery. However, studies connecting the biochemical composition of the cell surface with the evolutionary story of the group are ongoing. An expansion of such a perspective, including *Leishmania* and other genera, could contribute even more to the knowledge of this important group of parasites and we encourage the scientific community to adopt such an approach.

CONCLUSION

Since the discovery of the GPI anchor (Ferguson et al., 1985; Tse et al., 1985), a plethora of reviews have summarized information on biosynthesis, trafficking, structure, and functions of this anchor. However, the majority of these have emphasized the relationship between GPI deficiency and disease development in mammals.

Compared to mammalian cells, the cell surface of trypanosomatids contains exceptionally high numbers of

GPI-anchored molecules making it reasonable to suggest that this anchor has brought advantages to the parasites. One of these advantages is the rapid transport of these molecules to the cell surface promoted by the *en bloc* transfer of the GPI anchor to the C-terminal residue of the polypeptide, enabling high production rates of the wide range of surface molecules. In addition, the biophysical properties of the anchor can be extended to the anchored molecules and can be exploited by the parasites in different ways, such as antibody clearance through endocytosis (facilitated by mobility of the anchor) or overstimulation of the immune system (connected to the shedding of anchored molecules). Hence, by relying on GPIs, trypanosomatids have ensured a fast, stable, and efficient way to assemble a range of different molecules on their cell surfaces.

The GPI-anchored proteins of trypanosomatids are diverse and the genetic expansion of such molecules is usually linked to multigenic families providing variability inside the population. Despite the very little information available for wildlife trypanosomatids, it is evident that GPI-anchored molecules expressed by the human pathogens, such as GP63 and TS, are shared by many other species. The importance of these molecules becomes evident when we consider their positive selection and their presence in a range of different trypanosomatid species. Homologs of GP63 (**Table 2**) are found in the monoxenic trypanosomatids *Paratrypanosoma* and *Crithidia* (Inverso et al., 1993; Cuevas et al., 2003; El-Sayed et al., 2005; Venkatesh et al., 2018) as well as in the free-living kinetoplastid *Bodo saltans* (Jackson et al., 2016). The presence of GP63 is less enriched in *B. saltans* (**Table 2**) than in trypanosomes, suggesting a possible change of function of this ancestral protease in parasites, which could be related to their survival inside the invertebrate host (Jackson et al., 2016). The biological role of TS in the transfer of sialic acid to mucin molecules is well-exploited by *T. cruzi*, as discussed above (see section "*Trypanosoma cruzi*"). However, other species only have a few or no mucin-like genes. This apparent lack of mucins can indicate either a different function for TS in these organisms or low conservation of mucin-like genes in these species. Another biological role of TS is in cell-to-cell interaction, facilitating the invasion of macrophages by *T. cruzi* (see section "*Trypanosoma cruzi*"). In this sense, the attachment of *T. carassii* to blood vessels and other cells (Dóró et al., 2019) could be related to TS, but suggests that this molecule alone is not enough to guarantee cell invasion. Thus, the genetic expansion of both GP63 and TS in some species could mirror their functional diversity and is likely linked to the trypanosomatids' adaptation to different microenvironments.

The macroevolution of trypanosomatids is likely to be accompanied by host-switching and geographical dispersion (Hoberg and Brooks, 2008; Lukeš et al., 2014). Notably, host switches are considered to be one of the major processes in the emergence of zoonotic diseases (Webster et al., 2016). Thus, it is intriguing that these parasites are still being overlooked by the research community. Although infections caused by protozoans represent only around 10% of the emerging infectious disease cases, once the infection barrier is crossed, diseases caused by these organisms tend to become established in the population due to difficulties in developing vaccination strategies or lack of efficient drug treatment to completely eliminate the parasite (Robertson et al., 2014). Last, but not least, molecular evidence indicating cattle infections by *T. grayi* on the African continent could be indicative of an imminent host switch (Ngomtcho et al., 2017; Paguem et al., 2019).

Overall, the widespread distribution of trypanosomatids and their adaptation to diverse vertebrate host species are the result of an unprecedented evolutionary success story. These parasites have found opportunities to pass to new hosts by acquiring means to survive and proliferate inside these and ultimately adapting to allow the coexistence of host and parasite (Araujo et al., 2015). This review has summarized how trypanosomatids synthesize and utilize one biochemical feature, namely the GPI anchor, to mediate the attachment of a staggering variety of proteins that form the respective cell surface coats. This fascinating example of evolutionary click-chemistry might have contributed to the astonishing adaptive radiation of trypanosomatids. The vast repertoire of surface molecules combined with the biophysical properties of the GPI anchors might have maximized their chance of success inside different hosts. Unraveling their complete biological roles is necessary for a complete understanding of parasite–host interactions, which might impact the development of drugs by turning "their weapons against them."

AUTHOR CONTRIBUTIONS

AB and FL wrote the manuscript. FL and AB designed the figures and tables. ME and NJ provided conceptual input and contributed to writing. All the authors read and approved the final manuscript.

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