An investigation of the role of lysosomes in amebic trogocytosis and cell killing

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Abstract

Entamoeba histolytica is a protozoan parasite that is prevalent in low-income countries where it causes potentially fatal diarrhea, dysentery and liver abscesses. The parasite ingests fragments of live host cells in a nibbling-like process termed amebic trogocytosis. Amebic trogocytosis is required for cell killing and contributes to tissue invasion, which is a hallmark of invasive amebic colitis. Trogocytosis has been observed in other organisms, however little is known about the mechanism in any system. In the current work, we show that acidified lysosomes are required for amebic trogocytosis and cell killing. Interfering with lysosome acidification using ammonium chloride, a weak base, or concanamycin A, a vacuolar H^+ ATPase inhibitor, decreased amebic trogocytosis and amebic cytotoxicity. Our data suggest that the inhibitors do not impair the ingestion of an initial fragment, but rather block continued trogocytosis and the ingestion of multiple fragments. The acidification inhibitors also decreased phagocytosis, but not fluid-phase endocytosis. These data suggest that amebic lysosomes play a crucial role in amebic trogocytosis, phagocytosis and cell killing.

I found that the disruption of lysosomal digestion by the inhibition of amebic cysteine proteases significantly reduced both amebic trogocytosis and cell killing. These observations provide further evidence that amebic lysosomes play an important role amebic trogocytosis and cell killing. They also suggest that amebic lysosomes are crucial for trogocytosis because they are required for

efficient degradation of ingested material. Additionally, I observed that there was a significant decrease in the amount of Gal/GalNAc lectin on the surface of inhibitor-treated parasite compared to control parasites following trogocytosis, implicating amebic lysosomes in the rapid recycling of internalized amebic surface receptors. Interestingly, I found that impairing lysosomal digestion through the inhibition of amebic cysteine proteases did not impact phagocytosis, suggesting that there may be distinct lysosomal functions required for amebic trogocytosis. This work will provide a foundation for future studies to understand the mechanism of amebic trogocytosis.

Table of Contents

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Chapter 1: Introduction to the biology and pathogenesis of *Entamoeba histolytica*

Entamoeba histolytica

Entamoeba histolytica is a single-celled eukaryotic parasite that causes amebiasis in humans. The parasite was first described in 1875 by Russian physician Fedor Lösch, who observed amebae in the stool and colonic ulcerations of a patient with fatal dysentery [1]. *E. histolytica* has a simple life cycle consisting of two stages: the cyst stage and the trophozoite stage [2, 3]. *E. histolytica* cysts are round, quadrinucleate cells. Amebic cysts are protected from the environment by a thick cell wall that likely contains chitin, chitin-binding Jacob and Jessie lectins and chitinases [1, 4]. The parasite cysts are ingested by the human host in contaminated food or water and pass through the host gastrointestinal (GI) tract to the small intestine where the parasite excysts to the trophozoite form [5]. In contrast to cysts, trophozoites are pleomorphic, highly motile and have a variable number of nuclei [1, 6]. The trophozoites pass from the small intestine into the large intestine where they can colonize and cause symptomatic disease. In the final stage of the life-cycle, an unknown trigger induces trophozoites to encyst and the cysts pass out of the host into the environment [5].

E. histolytica has variable ploidy, ranging from 1n to 8n [7]. The parasite has been observed to have different number of nuclei and variable DNA content per nuclei in different life cycle stages and different culture conditions [6]. Indeed, studies have shown that significant heterogeneity of DNA content exists within a single culture population [7]. The polyploid nature of *E. histolytica* has confounded efforts to knock out genes by homologous recombination. However, several RNAi-based methods for gene knockdown have been developed (reviewed in [3]).

Amebiasis

Entamoeba histolytica primarily infects humans and thus far no significant animal or environmental reservoirs have been found [3]. The parasite is common in low-income countries where it causes diarrhea, and potentially fatal dysentery and liver abscesses [8]. The prevalence of *E. histolytica* has been challenging to estimate due to limited surveillance and diagnostic capacity in most areas where the parasite is endemic [9]. However, in an urban slum of Dhaka, Bangladesh, it was shown that 80% of children are infected with *E. histolytica* at least once over a four-year period and 53% experience repeated infections [10].

E. histolytica is primarily transmitted fecal-orally via contaminated food or water, however in some high-income countries sexual transmission is more common, particularly in men who have sex with men (MSM) [11]. Infection with *E. histolytica* can have variable outcomes [12]. Asymptomatic colonization is the most common outcome of infection, but the most serious outcomes are invasive

colitis, which is seen in 10-25% of patients, and extra-intestinal abscesses, which occur in about 1% of patients and can be fatal [13-15]. These outcomes are not mutually exclusive and patients can progress from one manifestation to another [11, 14]. Indeed, studies have shown that 4-10% of patients with asymptomatic *E. histolytica* infections progress to symptomatic disease within a year [1]. Asymptomatic colonization occurs in children and adults of both genders, while amebic colitis is more common in males. Amebic liver abscess predominantly affects men between the ages of 18 and 50 [1, 16].

Variation in infection outcome is likely due to a combination of parasite genetics, human genetics, and environmental factors. Different parasite genotypes have been observed in paired colon and liver isolates from patients with amebic liver abscesses, suggesting that parasite genetics impact invasive potential [17]. Several sequence variations in the *E. histolytica* genome have been associated with different clinical outcomes of infection, including single nucleotide polymorphisms (SNPs) in the CYCLCIN2 and lectin genes and polymorphisms in a short-tandem repeat locus R-R [9, 18, 19]. Retrotransposons are more highly expressed in *E. histolytica* than in less virulent *Entamoeba* species, suggesting that rapid evolution of the parasite may promote more severe clinical outcomes [9].

As early as 1914 a small human challenge study clearly demonstrated that participants infected with parasites from the same stool sample could show completely different clinical outcomes, suggesting that not all humans are equally susceptible to *E. histolytica* infection [12]. More recently, a prospective

observational study of Bangladeshi children found that while *E. histolytica* infection was common, a subset of children did not become infected [10]. Subsequent work has found that a genetic polymorphism in the human leptin receptor (Q223R) correlates with increased susceptibility to *E. histolytica* diarrhea in children, and amebic liver abscess in adults [20]. Leptin is a nutritional hormone that plays an important role in regulating food intake and has been implicated in regulation of the immune system. Studies in mice have demonstrated that leptin plays protective role in *E. histolytica* infection, as mice lacking leptin or the leptin receptor developed invasive amebic disease [21]. Malnutrition is also associated with increased susceptibility to *E. histolytica* infection. Interestingly, circulating leptin levels are low in malnourished children [20].

In the lumen of the gut, the interaction between *E. histolytica* trophozoites and the host microbiome likely influences infection outcome. Studies in Indian adults and Bangladeshi children have found that dysbiosis is associated with symptomatic *E. histolytica* infection [22, 23]. In Bangladeshi children, the anaerobic gram-negative bacterium *Prevotella copri* was associated with amebic colitis, suggesting that specific components of the microbiome may influence disease progression [24]. Causality is difficult to establish in human population-based studies thus model organisms have been used to investigate the interaction between *E. histolytic* and the host microbiome. Antibiotic-treated mice develop more severe amebic colitis and exhibit delayed clearance of the parasite while colonization with a single bacterial species, *Clostridia*-related segmented

filamentous bacterium, is sufficient to induce resistance to amebiasis [22, 25]. In both of these studies, changes to the microbiome resulted in changes to the mouse immune system. Taken together, these findings suggest that the interplay between the host immune system and the microbiome plays an important role in host susceptibility to *E. histolytica* infection.

Massive tissue destruction is the hallmark of both amebic colitis and amebic extraintestinal abscesses. As described by Stanley [1], the pathology of amebic colitis can include mucosal thickening, multiple ulcers separated by regions of normal-appearing colonic mucosa, diffusely inflamed and edematous mucosa, as well as necrosis and perforation of the intestinal wall. Indeed, the gross findings of amebic colitis are often reminiscent of those seen in inflammatory bowel disease. Patients with amebic colitis classically present with bloody diarrhea, abdominal pain and abdominal tenderness [1].

In approximately 1% of patients, parasites may escape the colon to form extra-intestinal abscesses, most commonly in the liver, although abscesses at other sites including the brain, lung and abdominal wall have been reported [26-28]. Amebic liver abscesses are typically well-circumscribed regions of dead cells and cellular debris surrounded by a thick ring of connective tissue. In contrast to amebic colitis, which is characterized by a strong inflammatory response, the parenchyma surrounding an amebic abscess usually appears normal, with few inflammatory cells or amebic trophozoites. Patients with amebic liver abscess commonly present with right upper quadrant pain and tenderness. Some patients may also have amebic colitis, but most do not. The onset of symptoms is typically

acute but may also be chronic, in which case weight loss and loss of appetite are common [1].

To date there is no vaccine for amebiasis. Nitroimidazole antibiotics are used to treat invasive amebiasis, but can have toxic side effects and 40-60% of patients require additional drugs to cure the infection [13]. The continued morbidity and mortality due to amebiasis indicate that current treatment and prevention strategies are inadequate [14].

Virulence

Cytotoxicity

E. histolytica is highly cytotoxic to a wide range of human cells, and parasite cytotoxic activity is likely to drive tissue destruction. The parasite is able to rapidly (within minutes) kill host cells in a contact-dependent manner [29, 30]. It has been shown that parasite attachment to host cells via the parasite Gal/GalNAc lectin is required for host cell killing [29, 31]. Intact, viable parasites are also required for cell killing, as neither parasite supernatants nor extracts are cytotoxic [29]. The parasite cytoskeleton is necessary for cytotoxicity, suggesting that host cell killing is an active process [31]. Functioning lysosomes are also necessary for host cell killing, as perturbing amebic lysosomal pH decreases cytotoxicity [32]. Finally, amebic cysteine proteases and amoebapores (pore-forming proteins) have also been implicated in cell killing, though direct evidence is lacking [33-35]. These findings led to a prevailing model of host cell killing, in which the parasite attaches to the host cell via the Gal/GalNAc lectin, and then

induces host cell death by an unknown mechanism that requires parasite cytoskeletal remodeling and lysosome function [14].

Amebic Trogocytosis

Recently, it was discovered that *E. histolytica* is able to kill by ingesting fragments of live human cells, in a process termed amebic trogocytosis, from the Greek *trogo* “to nibble” [30]. Trogocytosis is distinguished from other methods of intercellular transfer, such as phagocytosis, by the transfer of pieces of cell material (including intact proteins but not whole cells), the requirement for close cell-cell contact, and the rapid rate of uptake (within minutes) [36]. Amebic trogocytosis begins with the attachment of the parasite to the host cell, mediated largely by the parasite Gal/GalNAc lectin (Figure 1.1) [29-31]. Following attachment, the parasites ingest fragments of the host cell. These fragments appeared to be surrounded by two membranes, an outer membrane derived from the amebic plasma membrane and an inner membrane derived from the host plasma membrane. The contents of the ingested fragments included host cell cytoplasm, membrane and mitochondria. The parasites continued ingesting pieces of the host cell until the host eventually died. Notably, while amebic trogocytosis was initiated rapidly, host cells initially maintained plasma membrane integrity and host cell death did not occur for several minutes, after the amebae had ingested multiple fragments. A rapid rise in intracellular Ca^{2+} was observed in the host cell following the initiation of trogocytosis, but prior to host cell death [30], likely indicating activation of host membrane repair pathways (membrane repair reviewed in [37]). The number of ingested bites is likely critical

for eliciting host cell death, since pharmacological and genetic inhibitors that quantitatively reduced the number of ingested fragments almost completely inhibited host cell death. These data suggest that cell death results after a threshold of physical damage has been crossed. Amebic trogocytosis was observed when parasites were incubated with Jurkat T cells, Caco-2 colonic epithelial cells, and human erythrocytes, as well as *ex vivo* mouse intestinal tissue, suggesting that amebic trogocytosis likely contributes to tissue damage *in vivo* [30].

Amebic trogocytosis (ingestion of cell fragments) shares many similarities with another endocytic process, phagocytosis (ingestion of whole cells). Both amebic trogocytosis and phagocytosis are receptor-dependent processes, requiring attachment to a target cell via the amebic Gal/GalNAc lectin [30, 31]. These processes also require amebic actin rearrangement and PI3K signaling [30, 38, 39]. However, the precise mechanism of amebic trogocytosis is unknown.

Trogocytosis in human lymphocytes

Trogocytosis has been observed in a number of eukaryotes, ranging in complexity from protozoa to mammals, but has been most extensively studied in human lymphocytes. Human lymphocytes, including T, B, natural killer (NK) and dendritic cells (DCs) undergo a process of intercellular transfer that has been described as trogocytosis. Lymphocyte trogocytosis usually involves the transfer of plasma membrane fragments (containing membrane and surface proteins) from an antigen-presenting cell to a lymphocyte [36, 40].

Lymphocyte trogocytosis has been implicated in cell-cell communication and modulating the immune response to different insults [36, 41]. It has been shown that human DCs can acquire MHC-peptide complexes from melanoma cells and present the complexes to T-cells, implicating trogocytosis in the initiation of immune responses [41, 42]. T-cells have also been shown to acquire peptide-MHC complexes from antigen presenting cells [43]. T-cells can present these complexes to other T-cells, leading to different responses depending on the activation of the T-cells. T-cells that acquired peptide-MHC complexes and co-stimulatory CD80 from DCs via trogocytosis, were shown to act as antigen presenting cells for resting T-cell, resulting in expansion of the T-cell population [44]. These data suggest a role for trogocytosis in propagating an immune response. In contrast, T-cells that acquired peptide-MHC complexes and presented them to activated T-cells were shown to induce both apoptosis and anergy [45]. In NK cells, trogocytosis of MHC class I chain-related molecule (MIC), an NKG2D ligand, results in decreased NK cell cytotoxicity [46]. Taken together, these observations suggest that trogocytosis can play a role in dampening the immune response to an insult. Lymphocyte trogocytosis has also been implicated in limiting or terminating immune responses. Cytotoxic T-lymphocytes (CTLs) that acquire peptide-MHC complexes from APCs become susceptible to cytolysis by neighboring CTLs [41]. Le Maoult et al. report that CD4+ T cells that trogocytosed HLA-G, a non-classical MHC I protein, became unresponsive to further stimuli and appeared to function as regulatory T cells.

HLA-G is expressed in the human placenta, thus the authors speculate that trogocytosis of HLA-G may contribute to maternal tolerance to fetal tissue [47].

In humans, trogocytosis plays an important role in the susceptibility of immune cells to infection and has been implicated in exacerbating disease severity. Trogocytosis of CCR5, a cytokine receptor and HIV co-receptor, by CCR5 negative cells renders the cells susceptible to HIV infection *in vitro* [41]. Human macrophages can also undergo trogocytosis involving the transfer of cytoplasm from multiple types of cells to macrophages. Intracellular bacteria, including *Francisella tularensis* and *Salmonella enterica*, have been show to exploit this process to facilitate direct cell to cell spread from infected cells to uninfected cells [48]. A trogocytosis-like process observed in *Plasmodium falciparum*-infected red blood cells has been implicated in the pathogenesis of cerebral malaria [49].

The precise mechanism of trogocytosis in lymphocytes is unclear. In T- and NK-cells, trogocytosis is a metabolically active process that requires signaling in the acceptor cell, and modulation of both the actin cytoskeleton and intracellular Ca^{2+} (minireview [50]). The small GTPases TC21 and RhoG, and phosphatidylinositide 3-kinase (PI3K), were identified as key players in T-cell trogocytosis [51]. In contrast, B-cell trogocytosis does not appear to require active actin polymerization or kinase signaling [52].

Trogocytosis in other organisms

A trogocytosis-like process has recently been observed in *Caenorhabditis elegans* endodermal cells. During embryonic development *C. elegans* primordial

germ cells (PGCs) extend lobes, which are actively removed and digested by endodermal cells [53]. The authors described this process as “cannibalism,” but it fits the definition of trogocytosis. In this case, trogocytosis involved the ingestion of PGC plasma membrane, cytoplasm and organelles including P granules and a significant percentage of the PGC mitochondria. Endodermal cell trogocytosis is an active process requiring CED-10 (Rac1) and LST-4 (sorting nexin, SNX9) dependent accumulation of actin and DYN-1 (dynamin) [53]. However, as with human lymphocyte trogocytosis, the mechanism has not been elucidated.

Trogocytosis has been observed in the human pathogen and protozoan parasite *Trypansoma cruzi* during invasion of host cells and during parasite-parasite interactions. Similar to human lymphocyte trogocytosis, *T. cruzi* trogocytosis involves the transfer of membrane and certain glycoproteins from the parasite to the host cell [54]. The mechanism of trogocytosis in *T. cruzi* is unclear.

A process referred to as trogocytosis also been observed in the free-living ameba *Naegleria fowleri,* a human parasite that may use this process to destroy host cells [55-57]. In *N. fowleri* trogocytosis is an active process requiring actin polymerization but again the mechanism is unknown [58].

Amebic Effectors

Gal/GalNAc Lectin

The parasite surface protein, Gal/GalNAc lectin, plays a crucial role in adherence and host cell killing. The lectin is composed of three subunits: a 170-

kDa heavy subunit (HgL) and a 35kDa light subunit (LgL), which together form a disulfide-linked heterodimer, and a noncovalently associated 150kDa intermediate subunit (IgL) [59-61]. Gal/GalNAc lectin binds to galactose (Gal) and *N*-acetyl-D-galactosamine (GalNAc) residues on mucus and host cells via the HgL carbohydrate recognition domain (CRD) [62, 63]. It has been shown that the addition of Gal, GalNAc or anti-Gal/GalNAc lectin antibodies reduces, but does not completely abolish, parasite adherence, demonstrating that the Gal/GalNAc lectin is responsible for the majority, but not all, host cell binding [31, 64].

Treatment with Gal, GalNAc or anti-Gal/GalNAc lectin antibodies reduces amebic trogocytosis and cell killing, and mutant host cells that lack surface glycosylation are resistant to parasite killing, indicating that adherence via the lectin is crucial for cell killing [29-31, 64, 65]. Notably, one anti-Gal/GalNAc lectin antibody (3F4) was shown to enhance parasite adherence to host cells, but block cell killing, suggesting the lectin may play a more direct role in cell killing, independent of its role in adherence [64].

Amoebapores

Amoebapores are a family of three pore-forming proteins that are believed to play a central role in host cell killing by *E. histolytica*. These proteins have been thought to be secreted and to kill host cells by directly modifying their membranes. The proteins have sequence similarity to pore-forming proteins produced by mammalian lymphocytes [66]. Amoebapore A (ApA) is the most highly expressed and well studied of the three amoebapores. The purified

amoebapores are toxic to both gram-positive bacteria and eukaryotic cells, however high concentrations are required to kill eukaryotic cells and the dying cells do not exhibit the same features as cells directly killed by the amebae [67, 68]. It was found that epigenetic gene silencing of ApA resulted in decreased cytotoxicity [69]. However, the silencing approach that was used affected chromatin structure and altered the expression of many genes [70]. Therefore, it is not clear if the phenotype is due to silencing of ApA. Furthermore, while secretion is a prerequisite for the proposed role of the amoebapores in host cell killing, secretion has not been experimentally demonstrated, nor have any of the amoebapores been demonstrated to traffic to host cell membranes. Amoebapores absolutely require low pH (~pH 5.2) for pore-forming activity [71]. Additional studies have shown that amoebapores localize to lysosomes and co-localize with ingested bacteria [72, 73]. It was also noted that ApA-silenced parasites degraded ingested bacteria less efficiently [73]. Together, the existing data are not sufficient to support the proposed role of the amoebapores as secreted cell killing effectors. Instead, the evidence suggests that amoebapores may function within the amebic lysosome.

Cysteine proteases

Cysteine proteases (CPs) are believed to play a crucial role in tissue invasion and destruction by degrading colonic mucus and the extracellular matrix (ECM). The *E. histolytica* genome encodes more than 50 cysteine proteases [74, 75]. Under culture conditions, the majority (>95%) of CP activity is attributed to

four CPs: EhCP1, EhCP2, EhCP5, and EhCP7 [76]. These four CPs are structurally similar to human Cathepsin-L and appear to have Cathepsin-B like activity [77]. Interestingly, two of these CPs are either not encoded (EhCP1) or not expressed (EhCP5) in the less pathogenic strain *E. dispar,* suggesting that they may function as virulence factors [78, 79].

EhCP5, the best studied of the amebic CPs, was shown to alter the host collagen matrix and activate host metalloproteases involved in ECM degradation [80, 81]. Together these data support a role for amebic CPs in ECM degradation. Amebic CPs have also been shown to cleave MUC2, the major structural component of colonic mucin [82, 83]. Similarly, epigenetic silencing of EhCP5 resulted in parasites that were unable to degrade a mucus barrier and failed to disrupt the underlying cell monolayer [84]. Silencing EhCP5 also resulted in decreased invasion in human colonic explants, providing strong evidence that CPs play a key role in tissue invasion [85]. However, the silencing approach used in these studies altered the expression of several other genes, notably ApA, complicating the interpretation of the results [70, 84, 85]. A number of studies have suggested that CPs are involved in cell killing [86-88]. However, these studies used *in vitro* monolayer destruction as a proxy for cell killing and thus do not distinguish between monolayer release due to ECM degradation and cell death [14]. Finally, amebic CPs have been shown to cleave host antimicrobial peptides and degrade human antibodies and complement, suggesting that they may also play a role in immune evasion [35, 89].

Amebic CPs may be secreted via EhRab11B-mediated exocytosis, trafficked to the cell surface, trafficked to lysosomes via EhRab7A, B, D, E or H, trafficked to phagosomes via EhRab7A and B [76, 88]. A novel class of transmembrane cysteine protease receptors, CP-binding family (CPBFs), appear to regulate CP trafficking in *E. histolytica* [76].

Endocytosis and Vesicular Trafficking

Endocytosis is a basic biological process that cells use to internalize a range of extracellular material, from plasma membrane proteins and lipids to pathogens and cellular debris [90-92]. A number of internalization pathways have been identified that differ in the cargo to be transported and the machinery used [90]. *E. histolytica* is highly endocytic – the parasite has been shown to internalize a large variety of molecules, particles and cells [93]. Endocytosis is likely crucial to both the growth and pathogenesis of the parasite.

Fluid-phase endocytosis

Fluid-phase endocytosis (FPE) is receptor-independent ingestion of fluids and solutes. FPE is generally used interchangeably with the term pinocytosis, however the term FPE can also be used to include macropinocytosis [94]. Pinocytosis involves the receptor-independent ingestion of small amounts of fluids or solutes. This uptake may be clathrin-dependent or clathrin-independent, and usually occurs independent of actin polymerization [95]. In contrast, macropinocytosis refers to the ingestion of large amounts of fluid through the

action of cytoplasmic projections [95]. Similar to phagocytosis, macropinocytosis is dependent on actin-mediated remodeling of the plasma membrane [90]. Both pinocytosis and macropinocytosis have been described in *E. histolytica* and likely play an important role in nutrient acquisition by the parasite [94].

Phagocytosis

Phagocytosis involves the ingestion of large particles (>0.5μM) and thus the internalization of large amounts of membrane >1μm [90, 92]. Unlike fluid-phase endocytosis, phagocytosis requires recognition and binding of the target by cell surface receptors [92]. *E. histolytica* has been shown to phagocytose a wide range of targets, including beads, bacteria, yeast and dead human cells [30, 96-98]**.**

Earlier studies suggested that phagocytosis of host cells played a crucial role in *E. histolytica* cell killing and tissue destruction [29, 38, 98]. However, with the discovery that *E. histolytica* is able to kill human cells via amebic trogocytosis and the observation that the parasite will preferentially phagocytose (ingest whole) dead human cells and trogocytose (ingest fragments) live human cells, it is likely that amebic trogocytosis is primarily responsible for human cell death [30].

Vesicular Trafficking

Rab GTPases are small GTPases that regulate the formation, trafficking and fusion of vesicles and thus play a crucial role in all endocytic processes. Rabs act as molecular switches, cycling between an active, GTP-bound form and an inactive, GDP-bound form (Figure 1.2). The exchange of GDP for GTP is catalyzed by guanine nucleotide exchange factors (GEFs), which facilitate GDP release. GTP binds as soon as GDP has been released due to the high cytosolic concentration of GTP [99]. The GTP-bound Rab is then able to recruit and/or activate effectors, such as molecular tethers, motors, sorting adapters and kinases [100-103]. Hydrolysis of GTP to GDP is facilitated by GTPase-activating proteins (GAPs) [104]. GDP-bound Rabs are sequestered in the cytosol through interaction with Rab GDP-dissociation inhibitors (GDIs). GTP binding and hydrolysis involve major conformational changes in the Rab protein (reviewed in [105] and [99]).

Higher eukaryotes generally encode a larger number of Rabs, however, over 100 Rabs have been identified in *E. histolytica*, compared to 66 encoded in the human genome and just 11 identified in *S. cerevisiae* [106-108]. The expansion of Rab GTPases in *E. histolytica* suggests that the endocytic pathways in *E. histolytica* are complex and highly regulated. Approximately 20 genes with sequence similarity to mammalian GEFs, 50 genes with sequence similarity to mammalian GAPs and 2 putative GDIs have been identified in *E. histolytica*, but none have yet been experimentally confirmed [109].

Thus far only a small number of *E. histolytica* Rabs have been studied. EhRab7A and EhRab7B are amebic Rab GTPases with a high degree of sequence identity to human Rab7A. Mammals typically encode one or two Rab7 isoforms (Rab7A and Rab7B), which localize to late endosomes, lysosomes and autophagosomes [110-112]. Mammalian Rab7A and Rab7B have been implicated in early to late endosome transport, late endosome to Golgi transport, lysosome biogenesis and autophagosome maturation [110, 111, 113-116]. In contrast, *E. histolytica* encodes 9 Rab7 isoforms (EhRab7A-I) [72]. EhRab7A, B, C, D, and E have been associated with the amebic phagosome [117-119]. Only two of these EhRabs, EhRab7A and EhRab7B have been studied in detail and they appear to have distinct functions. EhRab7A has been localized to both phagosomes and the prephagososmal vacuole (PPV), a unique structure that forms upon the initiation of phagocytosis and is believed to serve as a hub for the processing, activation and storage of digestive proteins before transport to the phagosome [120, 121]. EhRab7A has also been shown to form a complex with amebic retromer components Vps26, Vps29 and Vps35 [120]. EhRab7A is believed to play an important role in trafficking proteins from the PPV and phagosomes to the Golgi apparatus.

EhRab7B is implicated in lysosomal maturation and late endosome/lysosome fusion [72]. Previous work has shown that constitutive expression of a mutated GTP-bound form (H69L) of EhRab7B (EhRab7B-GTP) results in moderately decreased phagocytosis, phagosome acidification and

degradation of phagocytosed cells [72]. This may be due to decreased efficiency of recruitment of V-ATPases to phagosomes and lysosomes [72].

Figure 1.1

Model for Amebic Trogocytosis. Amebic trogocytosis begins with attachment of the ameba (green) to the host cell (blue) via the parasite surface receptor Gal/GalNAc lectin. Following attachment, the parasites ingest fragments of the host cell. This process requires both phosphatidylinositide 3-kinase (PI3K) signaling and actin rearrangement. The ingested fragments appear to be surrounded by two membranes, an outer membrane derived from the amebic plasma membrane and an inner membrane derived from the host plasma membrane. After the initiation of trogocytosis, a rapid rise in intracellular Ca^{2+} is observed in the host cell. The parasites continue ingesting pieces of the host cell until the host eventually died. At this point the parasites detach from the dead host cell. Notably, host cell death does not occur for several minutes, after the amebae have ingested multiple fragments. Image adapted from Ralston, *Curr Opin Microbiol,* 2015 [122].

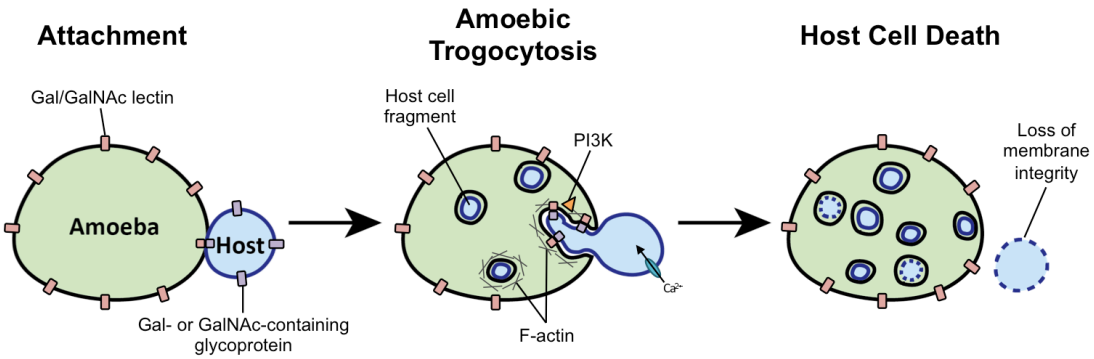
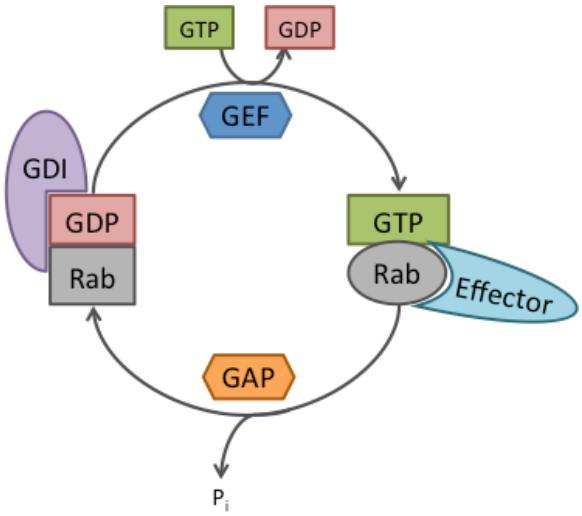

Attachment
Amoebic Trogocytosis
Host Cell Death
Gal/GalNAc lectin
Amoeba
Host
Gal- or GalNAc-containing glycoprotein
Host cell fragment
PI3K
F-actin
Loss of membrane integrity

Figure 1.2

Cycling of Rab GTPases. GDP-bound Rab (GDP, red; Rab, grey rectangle) is sequestered in the cytosol through its interaction with a Rab GDP-dissociation inhibitor (GDI, purple). Guanine nucleotide exchange factors (GEFs, blue) facilitate GDP release. GTP (green) immediately binds the free Rab due to the high cytosolic concentration of GTP. The GTP-bound Rab (Rab, grey oval) is then able to recruit and/or activate effectors (light blue). Hydrolysis of GTP to GDP is facilitated by GTPase-activating proteins (GAPs, orange). Image adapted from Stenmark, *Nat Rev Mol Cell Biol*, 2009 [99].


GTP
GDP
GEF
GDI
GDP
Rab
GTP
Rab
Effector
GAP
P_i

Chapter 2: Inhibition of Amebic Lysosomal Acidification Blocks Amebic Trogocytosis and Cell Killing

This chapter has been adapted from the publication authored by Allissia A. Gilmartin, Katherine S. Ralston, and William A. Petri, Jr. mBio (2017).

Introduction

Entamoeba histolytica is a protozoan parasite that is prevalent in low-income countries. In humans, the parasite causes potentially fatal invasive colitis, which is seen in 10-25% of patients, and extra-intestinal abscesses, which occur in about 1% of patients [13, 14]. Worldwide, diarrheal disease is the second leading cause of death for children under five years old [123]. In an urban slum of Dhaka, Bangladesh, we have found that 80% of children are infected with *E. histolytica* at least once over a four-year period and 53% experience repeated infections [10]. Repeated infections in children are particularly serious as they are associated with chronic malnourishment, stunting and cognitive defects [124].

Tissue destruction is the hallmark of invasive *E. histolytica* infection, manifesting as massive intestinal ulceration or abscesses in other sites. *E. histolytica* is highly cytotoxic to a wide range of human cells, and parasite cytotoxic activity is likely to drive tissue destruction. It was recently discovered that *E. histolytica* kills by ingesting fragments of live host cells, which has been termed amebic trogocytosis [30]. This process begins with attachment of the

parasite to the host cell, which is mediated in large part by the parasite Gal/GalNAc lectin [29-31]. Following attachment, the parasites ingest fragments of the host cell. These fragments were shown to contain host cell membrane, cytoplasm, and mitochondria. The parasites continue ingesting fragments of the host cell until the host cell eventually dies. Notably, it has been demonstrated that, while amebic trogocytosis initiated rapidly, host cell death did not occur until several minutes later, after the amebae had ingested multiple fragments. The number of ingested fragments is likely critical for eliciting host cell death, since pharmacological and genetic inhibitors that quantitatively reduced the number of ingested fragments almost completely inhibited host cell death [30]. These data suggest that cell death results after a threshold of physical damage has been crossed. However, we currently lack an understanding of the mechanism that underlies amebic trogocytosis and cell killing.

A process morphologically similar to amebic trogocytosis has been observed in other organisms. Human lymphocytes, including T, B, natural killer, dendritic cells and macrophages undergo a process that has also been called trogocytosis [36]. In lymphocytes, trogocytosis has been implicated in cell-cell communication [36]. The process is distinguished from other methods of intracellular transfer, such as phagocytosis, by the transfer of fragments of cell material (including intact proteins but not whole cells), the requirement for close cell-cell contact, and the rapid rate of uptake (within minutes), all of which are reminiscent of amebic trogocytosis [36]. In T- and NK-cells, trogocytosis is a metabolically active process that requires signaling in the acceptor cell, and

modulation of both the actin cytoskeleton and intracellular Ca^{2+} (minireview [50]). The small GTPases TC21 and RhoG, and phosphatidylinositide 3-kinase (PI3K), were identified as key players in T-cell trogocytosis [51]. A process referred to as trogocytosis also been observed in the free-living ameba *Naegleria fowleri,* a human parasite that may use this process to destroy host cells, but again the mechanism is unknown [55-57]. Finally, a trogocytosis-like process observed in *Plasmodium falciparum*-infected red blood cells has been implicated in the pathogenesis of cerebral malaria [49].

Previous work demonstrated that amebic trogocytosis leads to an irreversible elevation of host cell calcium, followed by death [30]. It was shown that increasing levels of amebic trogocytosis correspond with increasing levels of host cell death. Moreover, inhibition of amebic trogocytosis using a range of methods, including PI3K inhibition and blockade of the amebic surface receptor Gal/GalNAc lectin, resulted in a concomitant decrease in cell killing. These data indicate that amebic trogocytosis results in cell killing [30]. It has been observed that host cell death occurs after sustained ingestion of many fragments [30]. Importantly, the numerous ingested cell fragments acquired during amebic trogocytosis must be processed. In eukaryotes, lysosomes are critical for the degradation of ingested macromolecules. Lysosomal function is therefore likely to be essential to support continued trogocytosis and, consequently, host cell killing. It has been demonstrated that elevated amebic lysosomal pH decreases cytotoxicity, suggesting that functional lysosomes are required for cell killing [32]. In addition, work on EhRab7B, an amebic Rab GTPase that is implicated in

lysosomal maturation and late endosome/lysosome fusion, has shown that interfering with EhRab7B results in decreased phagocytosis, phagosome acidification and degradation of ingested cells [72]. These findings suggest that there is a crucial role for the degradation pathway in cell killing. Interestingly, previous work also found that phagosomes in *E. histolytica* were acidified much more quickly and reached a lower pH than phagosomes in the less virulent *E. dispar* suggesting that phagosome acidification, and therefore amebic lysosomes, may play a role in amebic virulence [125].

Based on these findings, we hypothesized that amebic lysosomes are essential for continued amebic trogocytosis and host cell killing. In this study we examined the impact of impaired lysosomal acidification on amebic trogocytosis and cell killing. Using imaging flow cytometry to quantitatively assess the rates of trogocytosis and host cell killing, we found that inhibition of lysosomal acidification significantly decreased amebic trogocytosis, phagocytosis and cell killing indicating a crucial role for amebic lysosomes in these processes. This work sheds new light on an observation, first made 30 years ago, that weak bases inhibit amebic killing of human cells, by demonstrating that acid vesicle neutralization acts through the inhibition of trogocytosis.

Results

Acidification inhibitors decrease amebic trogocytosis and cell killing.

E. histolytica can kill human cells through amebic trogocytosis [30]. Previous work has shown that elevated amebic lysosomal pH decreased

cytotoxicity, suggesting that functional lysosomes are required for cell killing [32]. To determine whether acidification of amebic lysosomes is required for trogocytosis and cell killing, we used two independent pharmacological inhibitors of lysosomal pH: concanamycin A and ammonium chloride. Concanamycin A is a specific inhibitor of vacuolar H^+ ATPases (V-ATPases). V-ATPases function as proton pumps and their inhibition has been shown to block acidification of vesicles in *E. histolytica* [126-128]. In ciliated protozoa, V-ATPases are also required for targeting and subsequent tethering of the acidified vesicle to its target membrane (review [129]). Treatment of *E. histolytica* with concanamycin A has been shown to inhibit acidification of phagosomes and degradation of ingested bacteria and yeast [125]. Ammonium chloride, a weak base, also raises the pH of amebic lysosomes, and was previously shown to inhibit host cell killing [32].

CMFDA-labeled parasites were treated with either concanamycin A or ammonium chloride for 1 h. Following this incubation, the concanamycin A-treated parasites were washed extensively to remove free inhibitor, then co-incubated with DiD-labeled human Jurkat T-cells for 5, 20 or 40 min in media alone. Thus only the amebae, not the Jurkat T-cells were exposed to concanamycin A. Previous studies have shown that *E. histolytica* is able to rapidly re-acidify lysosomes within minutes of ammonium chloride removal, therefore ammonium chloride was maintained in the media throughout the co-incubation with human cells [32]. After co-incubation of the parasites with the human cells, all cells were stained with the permeability dye Live/Dead Violet and

fixed. We then assessed trogocytosis and cell killing using imaging flow cytometry (Fig. 2.1). To quantify trogocytosis, we measured fragmentation of the human material within the amebae, as previously described by Ralston et al. (Fig 2.1A, Fig 2.2 shows the full gating strategy).

In untreated amebae the percentage of amebae that ingested a high number of human fragments increased over time from 10.0% at 5 min to 67.0% at 40 min. However, the inhibitor-treated parasites showed significantly less ingestion, with only 4.4% of parasites treated with 100mM ammonium chloride ingesting a high number of human fragments after 40 min (Fig 2.1A). Indeed, treatment with either ammonium chloride (Fig 2.1B) or concanamycin A (Fig 2.1C) significantly decreased amebic trogocytosis in a dose-dependent manner, as measured by the percentage of amebae that ingested a high number of human cell fragments. As expected, treatment with these inhibitors also decreased cell killing (Fig 2.1 D and 2.1E). Treatment with ammonium chloride alone did not directly cause human cell death (Fig 2.1D). Human cells were not exposed to concanamycin A in this assay, therefore we assessed the impact of incubation in media alone and found that it also did not cause human cell death (Fig 2.1E). Treatment with either of the inhibitors alone did not directly cause amebic death (Fig 2.3). Together, these data suggest a crucial role for amebic lysosomes in amebic trogocytosis.

Acidification inhibitors do not impair initiation of amebic trogocytosis.

To understand how amebic lysosomes participate in amebic trogocytosis, we first sought to determine when during the process of amebic trogocytosis inhibitor-treated parasites were impaired. Amebic trogocytosis entails a series of events that result in human cell death: attachment to the human cell, internalization of a human cell fragment and then continued ingestion of multiple fragments. It is possible that the inhibitors impaired the ability of the parasites to attach to human cells and initiate trogocytosis. To determine whether the acidification inhibitors impaired initiation of trogocytosis we assessed whether there was a difference in the ability of inhibitor-treated parasites to ingest any amount of human material, as compared to control parasites. We found that the percentage of control parasites that had internalized human material increased over time from 19.9% at 5 min to 77.9% at 40 min. Interestingly, similar percentages of parasites treated with 100mM ammonium chloride 79.1% had internalized human material by 40 min (Fig 2.4A), indicating that a majority of amebae was able to ingest some human material by 40 min, regardless of ammonium chloride treatment. Overall, there was no difference in the percentage of inhibitor-treated parasites that internalized any human material compared to control parasites (Fig 2.4B and 2.4C). These data indicate that the acidification inhibitors did not impair the initiation of trogocytosis. However, the inhibitors did decrease in the percentage of parasites that ingested a high number of fragments (Fig 2.1B and 2.1C), suggesting that the parasites were impaired in their ability to continue to trogocytose many fragments.

Acidification inhibitors also decrease phagocytosis, but not fluid-phase endocytosis

Amebic trogocytosis (ingestion of cell fragments) shares many similarities with another endocytic process, phagocytosis (ingestion of whole cells). Both amebic trogocytosis and phagocytosis are receptor-dependent processes, requiring attachment to a target cell via the amebic Gal/GalNAc lectin [30, 31]. These processes also require amebic actin rearrangement and PI3K signaling [30, 38, 39]. Thus far, no pathways unique to trogocytosis have been discovered in any organism, therefore, we assessed the impact of the acidification inhibitors on phagocytosis. It has been demonstrated that *E. histolytica* will preferentially phagocytose (ingest whole) dead human cells and trogocytose (ingest fragments) live human cells [30]. To measure the impact of acidification inhibitors on phagocytosis, we co-incubated ammonium chloride-treated, concanamycin A-treated and control parasites with CMFDA-labeled heat-killed human Jurkat T-cells at 37°C (Fig 2.5A). Amebae pre-treated with ammonium chloride were co-incubated with dead human cells in media containing ammonium chloride, while amebae pre-treated with concanamycin A were washed and co-incubated with dead human cells in media without inhibitor. Following co-incubation, all cells were fixed and phagocytosis was assessed using confocal microscopy as previously described [38]. Consistent with previous work [32], we found that treatment with either ammonium chloride or concanamycin A decreased phagocytosis (Fig 2.5B and 2.5C).

In contrast to both amebic trogocytosis and phagocytosis, fluid-phase endocytosis is not receptor dependent and does not require large actin rearrangements. To determine whether the inhibition of lysosomal acidification impaired fluid-phase endocytosis we incubated ammonium chloride-treated parasites with RITC-dextran for 40 min in media containing ammonium chloride and assessed ingestion of the dextran using a fluorescence plate reader. Intriguingly, we found that ammonium chloride treatment did not impact fluid-phase endocytosis (Fig 2.5C). Taken together with our observations on amebic trogocytosis and phagocytosis, these data suggest that amebic lysosomes might play an important role in the rapid degradation of ingested fragments or recycling of membranes and receptors required for continued amebic trogocytosis.

Discussion

For decades it was unclear how *E. histolytica* killed human cells. Recently we discovered that *E. histolytica* ingests fragments of live human cells through amebic trogocytosis, which leads to cell killing [30]. However, the mechanism of amebic trogocytosis is not well understood. Previous work has demonstrated that attachment of the parasite to the host cell is mediated largely by the parasite surface receptor Gal/GalNAc lectin and that an active parasite cytoskeleton is required for the ingestion of cell fragments [30]. Processes that are morphologically similar to amebic trogocytosis occur in other organisms, including humans and several eukaryotic parasites, but again the underlying mechanisms are poorly understood [36]. In the present study we demonstrated

that interfering with lysosomal acidification impairs amebic trogocytosis and cell killing, indicating a critical role for amebic lysosomes in both trogocytosis and cell killing.

It has been demonstrated that ingestion of host cell material via trogocytosis is the major mechanism of cell killing by *E. histolytica.* Previous work, that was done before the discovery of amebic trogocytosis, has shown that weak bases inhibit amebic killing of human cells [32]. Interestingly, studies comparing *E. histolytica* with the less pathogenic species *Entamoeba dispar* have noted that acidification of the phagosomes takes significantly longer in *E. dispar* and does not reach the same level of acidification, also indicating a possible role for lysosomes in the pathogenesis of amebiasis [125, 130]. Indeed, chloroquine, a lysosomotropic weak base that impairs lysosomal acidification [131], has been used to successfully treat amebic liver abscesses [132]. Our current findings shed new light on these observations by demonstrating that acid vesicle neutralization acts through the inhibition of trogocytosis and cell killing.

We have shown that interfering with acidification does not impact the initiation of amebic trogocytosis, but rather impairs continued ingestion and cell killing. There was no difference in the ability of untreated and acidification inhibitor-treated parasites to begin to ingest some human cell fragments, suggesting that the parasites were not simply impaired in their ability to attach to human cells (Fig 2.4B and C). This is consistent with previous work showing that treatment of parasites with moderate amounts of ammonium chloride for as long

as 48 hrs did not impact parasite attachment to mammalian cells or binding of colonic mucin [32, 133]. However, the amount of human material ingested by the acidification inhibitor-treated parasites was drastically reduced, indicating that the parasites' ability to continue ingesting human fragments was impaired. *E. histolytica* has been shown to acidify phagosomes within 2 minutes, suggesting that lysosomes are rapidly recruited and fuse with the phagosome [125]. Our data is consistent with the hypothesis that the inhibitor-treated parasites fail in this rapid acidification step, resulting in slowed or blocked ingestion. Continued ingestion of multiple human fragments appears to be required to kill human cells [30], thus these parasites are also impaired in their ability to kill human cells, as we observed (Fig 2.1D and E).

There are several possible roles that lysosomes might play in amebic trogocytosis: lysosomes might be required for efficient degradation of the ingested fragments, for rapid recycling of ingested receptors and membrane, or for the rapid formation of an acidified synapse at the site of ameba-host interaction. During amebic trogocytosis, parasites ingest numerous host cell fragments. In eukaryotes, lysosomes are crucial for the turnover of ingested material. Weak bases, such as ammonium chloride, raise the pH of amebic lysosomes, which would impair the function of pH-dependent lysosomal proteases [32]. Previous work has also shown that Concanamycin A-treated parasites failed to acidify their phagosomes to normal levels and were impaired in their ability to degrade phagocytosed *Leishmania* [125]. It is possible that lysosomes are crucial for continued amebic trogocytosis and cell killing because

they are required for efficient degradation of the ingested host fragments. Further study is needed to determine whether this is the case.

Our data show that interfering with acidification blocks receptor-dependent processes, both amebic trogocytosis and phagocytosis, but does not impair a receptor-independent process, fluid-phase endocytosis. These findings are consistent with previous reports that treatment of parasites with ammonium chloride decreased phagocytosis of bacteria and, similarly, treatment with the V-ATPase inhibitor, bafilomycin, decreased ingestion of both bacteria and human red blood cells [39]. Together, these data suggest that rapid recycling of membrane and receptors, facilitated by the amebic lysosomes, may be required for continued amebic trogocytosis and, thus cell killing.

It has also been suggested that amebic lysosomes may form an acidified synapse at the site of host cell attachment similar to the synapse created by mammalian osteoclasts, which secrete lysosomes into an acidified synapse during bone matrix degradation [134]. Live confocal microscopy and electron microscopy have both shown a massive accumulation of actin and exclusion of vesicles at the site of active host fragment ingestion, making this hypothesis less likely [30]. However, it is still possible that amebic lysosomes fuse at the ameba-host cell interface after attachment, but before active ingestion begins. Further study is needed to examine whether such a synapse is formed.

We acknowledge that, in addition to impairing lysosomal function, the acidification inhibitors used in this study are likely to impact other vesicles in the endocytic pathway. In this study we have used concanamycin A, which

specifically inhibits V-ATPases to block vesicle acidification. Studies in mammalian systems have shown that during endosome/phagosome maturation, vesicles accumulate V-ATPase complexes on their membranes and become progressively more acidic. V-ATPases are also involved in regulating the pH of sorting and recycling endosomes (review [135]). The impact of concanamycin A is likely to be most profound on the lysosomes, which have been shown in mammalian systems to have the greatest accumulation of V-ATPases, the lowest pH of vesicles in the cell, and a strong dependence on acidic pH for their function. Similarly, ammonium chloride is a membrane-permeable weak base and therefore may impact the acidification of other vesicles in the amebic endocytic pathway. However, ammonium chloride has been described as a lysosomotropic agent for its propensity to accumulate in lysosomes [136]. Like concanamycin A, ammonium chloride is likely to have the greatest impact on the lysosomes. In *Entamoeba histolytica,* it has been shown that exposure to as little as 10mM ammonium chloride for 20min increased acid vesicle pH to 6.11 [32]. While the endocytic pathway is an active area of study, we currently lack a well-defined set of markers to distinguish different vesicles within the endocytic pathway. Future discovery of such markers may allow for a further examination of the impact of these acidification inhibitors on specific vesicles within the endocytic pathway.

Tissue destruction is the hallmark of invasive *E. histolytica* infection and parasite cytotoxic activity is likely to drive tissue destruction. It has recently been discovered that human cell death occurs as a result of amebic trogocytosis. This

work sheds new light on an observation, first made 30 years ago, that weak bases inhibit amebic killing of human cells, by demonstrating that acid vesicle neutralization acts through the inhibition of trogocytosis. This work will contribute to a better understanding of the nature of trogocytosis as a fundamental biological process.

Methods

Cell Culture

Amebic trophozoites (HM1:IMSS) were cultured axenically at 35° C in TYI-S-33 as previously described [38]. Trophozoites were harvested during log-phase growth by centrifugation at 200 x *g* for 5 min at room temperature, followed by resuspension in M199s (medium 199 [Gibco] without phenol red and supplemented with 5.7mM cysteine [Sigma], 0.5% bovine serum albumin [Gemini] and 25mM HEPES [Sigma] at pH 6.8) [30].

Human Jurkat cells (Clone E6-1, ATCC) were grown at 37° C in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco), 10mM HEPES (Gibco), and 1mM sodium pyruvate (Gibco). Jurkat cultures were collected and enriched for viable cells as previously described [30].

Amebic Trogocytosis and Cell Killing Assay

Amebic trogocytosis by amebae was measured using imaging flow cytometry as described previously with some modifications [30]. Briefly, amebae were labeled

with 200nM CellTracker Green CMFDA (Invitrogen) in M199S for 10min at 37°C and then washed twice with M199S. Following CMFDA labeling, amebae were treated with pharmacological inhibitors or vehicle control for 1hr at 37° C. Amebae were treated either with ammonium chloride (Sigma) at 10mM, 50mM or 100mM or M199s as a control; or amebae were treated with concanamycin A (Sigma) at 10nM, 50nM, 100nM or an equal volume of DMSO (Molecular Probes) as a vehicle control. Jurkat cells were labeled with 5μM DiD (Assay Biotech) in M199S for 5 min at 37C, followed by 10 min at 4C, and then washed twice with M199S. Amebae pre-treated with ammonium chloride were co-incubated with labeled Jurkat cells at a ratio of 1:5, in biological duplicate at 37° C for 5 min, 20 min or 40 min in M199s containing ammonium chloride. Amebae pre-treated with concanamycin A or DMSO were washed twice with M199s, and then co-incubated with labeled Jurkat cells in biological duplicate at 37° C for 5 min, 20 min or 40 min in M199s. At the end of each time point, samples were immediately placed on ice and labeled with live/dead fixable violet (Invitrogen) at 1.6μl per ml for 30min in the dark. The cells were then fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, in the dark. Flow cytometry was performed using ImageStreamX Mark II (EMD Millipore). 10,000 events were collected for each sample and data were analyzed using IDEAS software (EMD Millipore).

Phagocytosis Assay

Jurkat cells were collected, washed with M199s and labeled with 5μM CMFDA

(Invitrogen) for 15min at 37° C as previously described [30]. Cells were then washed with M199s and necrotic cell death was induced by incubation of Jurkat cells at 55° C for 15min [38]. Amebae were collected, washed with M199s and treated with pharmacological inhibitors for 1hr at 37° C. Amebae were treated either with ammonium chloride (Sigma) at 10mM, 50mM or 100mM or M199s as a control; alternatively, amebae were treated with concanamycin A (Sigma) at 10nM, 50nM, 100nM or an equal volume of DMSO as a vehicle control. Amebae pre-treated with ammonium chloride were co-incubated with heat-killed Jurkat cells at a ratio of 1:5 in M199s containing ammonium chloride for 10 min at 37° C. Amebae pre-treated with concanamycin A or DMSO were washed with M199s, and then co-incubated with labeled Jurkat cells in M199s for 10 min at 37° C. After co-incubation all samples were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. All samples were washed with PBS, mounted with Vectashield H-1000 anti-fading agent, and imaged using Zeiss LSM software on a Zeiss LSM700 inverted confocal microscope equipped with a x63 apochromatic oil objective [30].Fields containing two or more amebae were imaged and amebae containing one or more human cells were scored phagocytosis positive. Results were expressed as percent phagocytosis, which is the number of ameba containing at least on Jurkat cell per slide divided by the total number of amebae imaged per slide.

Fluid-phase Endocytosis Assay

The assay was performed as described previously with some modifications [137]. Briefly, amebae were collected, washed with M199s and then treated with pharmacological inhibitors for 1hr at 37° C. Approximately $2.5x10^5$ amebae were incubated with 2mg/ml Rhodamine B dextran (10,000 MW, Invitrogen) for 40 min at 37° C. Amebae were then washed extensively with PBS and permeablized with 0.1% Triton-X 100. Samples were plated in quadruplicate on a 96-well black solid plate (Corning). Fluid-phase endocytosis assessed by measuring fluorescence intensity after excitation at 570nm using a fluorescence plate reader (BioTek).

Statistical Analysis

Data were analyzed using Prism 6 (GraphPad Software). *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 2.1

Acidification inhibitors decrease trogocytosis and cell killing. Amebae pre-treated with ammonium chloride for 1h were incubated with Jurkat cells for 5, 20 or 40 min in media containing ammonium chloride. Amebae pre-treated with concanamycin or vehicle for 1h were washed and incubated with Jurkat cells for 5, 20 or 40 min in media without inhibitor or vehicle. Afterwards all cells were stained with Live/Dead Violet on ice for 30 min and then fixed. Amebic trogocytosis and cell killing were analyzed using imaging flow cytometry. **(A)** Measurement of fragmentation of ingested material over time. **(B,C)** % High gate measures the percentage of events in the high gate, reflecting the quantity of fragments that have been ingested by the parasites **(D,E)** % Dead measures the percentage of host cells staining with Live/Dead Violet. Means and standard deviations are for biological duplicates (10,000 events/each). Data was analyzed by one-way ANOVA using Prism 6. *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

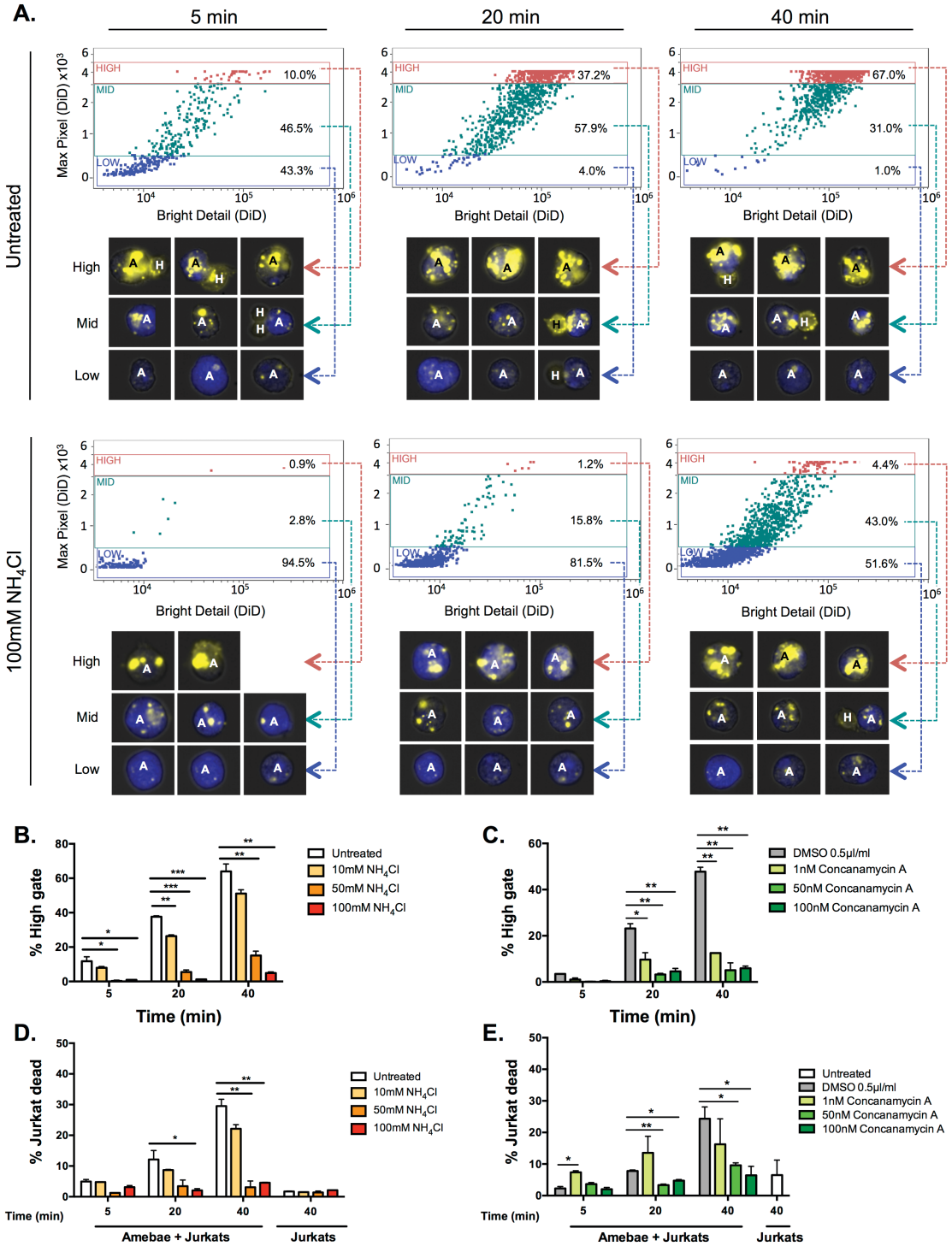

A.
5 min
20 min
40 min
Untreated
100mM NH4Cl
Max Pixel (DiD) x10³
Bright Detail (DiD)
HIGH
MID
LOW
10.0%
46.5%
43.3%
37.2%
57.9%
4.0%
67.0%
31.0%
1.0%
0.9%
2.8%
94.5%
1.2%
15.8%
81.5%
4.4%
43.0%
51.6%
High
Mid
Low
B.
% High gate
Time (min)
Untreated
10mM NH4Cl
50mM NH4Cl
100mM NH4Cl
C.
% High gate
Time (min)
DMSO 0.5µl/ml
1nM Concanamycin A
50nM Concanamycin A
100nM Concanamycin A
D.
% Jurkat dead
Time (min)
Amebae + Jurkats
Jurkats
E.
% Jurkat dead
Time (min)
Amebae + Jurkats
Jurkats

Figure 2.2.

Gating strategy for imaging flow cytometry. CMFDA-labeled amebae were incubated with DiD-labeled Jurkats; dead cells were stained with Live/Dead violet. Acquisition was performed on Amnis ImagestreamX Mark II flow cytometer. 10,000 events were collected. Analysis was performed using IDEAS software. **(1)** Focused events were selected using a gradient of brightfield. **(2)** Events gated in **(1)** were enriched for single events by removing compound events containing two or more cells not in contact. **(3)** Events gated in **(2)** were plotted based on aspect ratio and intensity of CMFDA staining to distinguish between single Jurkats, single amebae and clusters of amebae. **(4)** 'Single amebae' identified in **(3)** were assessed for the presence of human material, including fragments, whole human cells or both. Events gated as 'Jurkat positive' had some human material. **(5)** Events identified as 'Jurkat positive' were examined for internalization of the human material using the internalization score feature, which assesses the overlap between the DiD image and a mask based on the CMFDA image. Events gated as 'Internalization+' had internalized some human material **(6)**. To assess how much human material was internalized and whether the material was intact or fragmented, 'Internalization +' events gated in **(5)** were examined using the bright detail and max pixel intensity of DiD. Events were gated as 'Low,' 'Mid,' or 'High' based on these parameters. These three gates ('Low,' 'Mid,' 'High') were maintained in a constant position throughout all experiments. **(3a)** To assess viability of Jurkats events gated as 'single Jurkats'

in **(3)** were plotted based on granularity (side scatter, SCC) and intensity of Live/Dead Violet. **(3b)** To assess viability of amebae events gated as ‘single amebae’ in **(3)** were plotted based on granularity (side scatter, SCC) and the overlap of CMFDA and Live/Dead Violet.

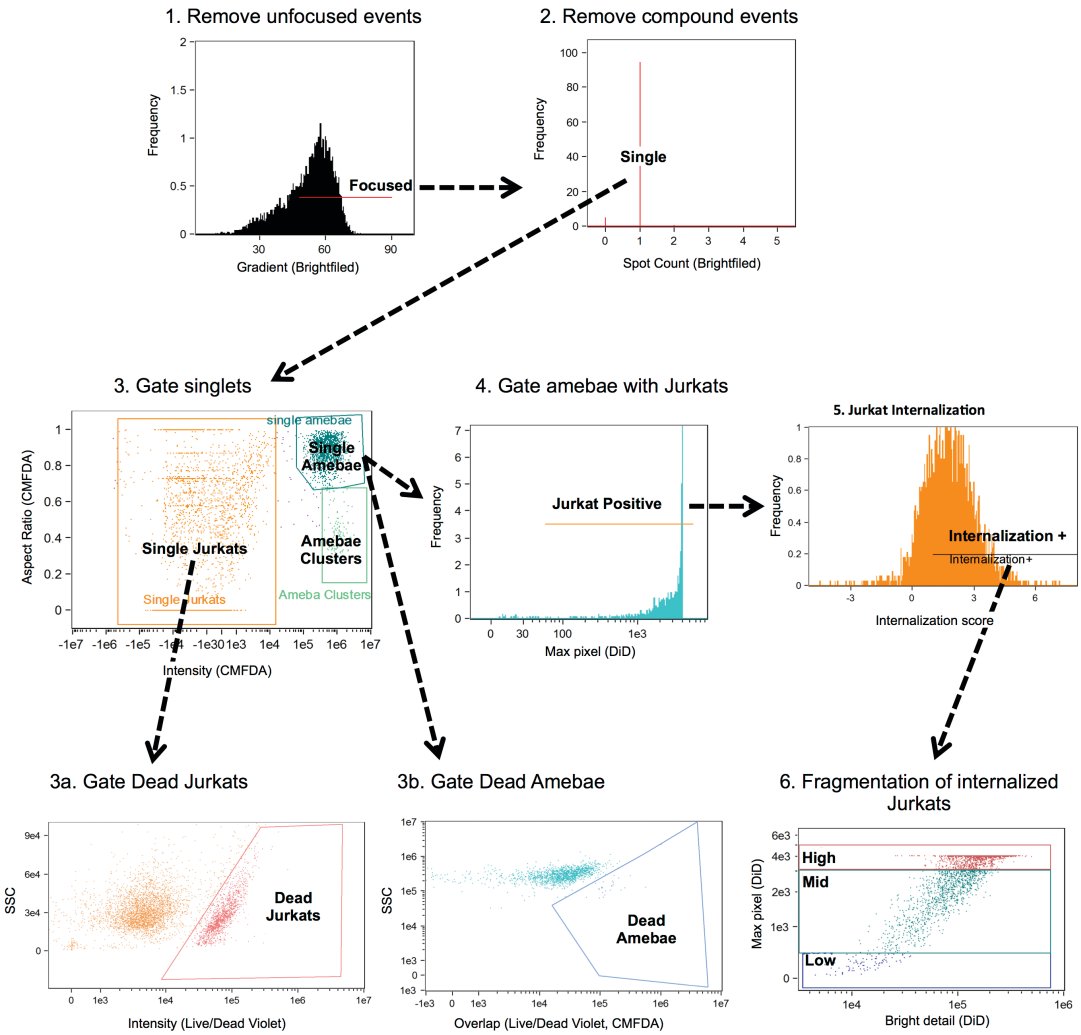
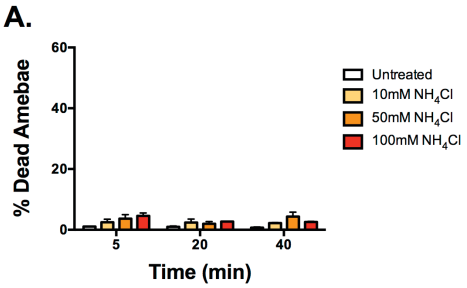

1. Remove unfocused events
Frequency
Focused
Gradient (Brightfiled)
2. Remove compound events
Frequency
Single
Spot Count (Brightfiled)
3. Gate singlets
single amebae
Single Amebae
Single Jurkats
Amebae Clusters
Ameba Clusters
Aspect Ratio (CMFDA)
Intensity (CMFDA)
4. Gate amebae with Jurkats
Jurkat Positive
Frequency
Max pixel (DiD)
5. Jurkat Internalization
Internalization +
Internalization+
Frequency
Internalization score
3a. Gate Dead Jurkats
Dead Jurkats
SSC
Intensity (Live/Dead Violet)
3b. Gate Dead Amebae
Dead Amebae
SSC
Overlap (Live/Dead Violet, CMFDA)
6. Fragmentation of internalized Jurkats
High
Mid
Low
Max pixel (DiD)
Bright detail (DiD)

Figure 2.3.

Acidification inhibitors do not cause parasite death. (A) Amebae pre-treated with ammonium chloride for 1h were incubated for 5, 20 or 40 min in media containing ammonium chloride. **(B)** Amebae pre-treated with concanamycin or vehicle for 1h were washed and incubated for 5, 20 or 40 min in media without inhibitor or vehicle. Afterwards all cells were stained with Live/Dead Violet on ice for 30 min and then fixed. Cell killing was analyzed using imaging flow cytometry. **(A,B)** % Dead Amebae measures the percentage of amebae staining with Live/Dead Violet. Data was analyzed by one-way ANOVA with multiple comparisons test using Prism 6.


A.
% Dead Amebae
60
40
20
0
5
20
40
Time (min)
Untreated
10mM NH4Cl
50mM NH4Cl
100mM NH4Cl

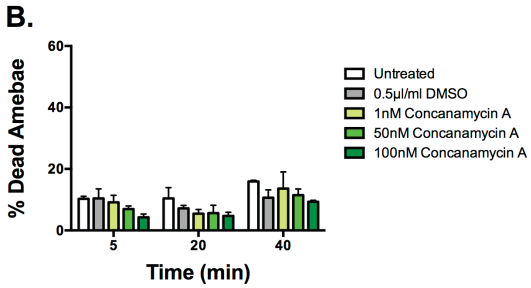

B.
% Dead Amebae
60
40
20
0
5
20
40
Time (min)
Untreated
0.5µl/ml DMSO
1nM Concanamycin A
50nM Concanamycin A
100nM Concanamycin A

Figure 2.4.

Acidification inhibitors do not inhibit initiation of amebic trogocytosis. Amebae were pretreated with inhibitor or vehicle for 1h. **(A,B)** Ammonium chloride-treated amebae were incubated with Jurkat cells in media containing ammonium chloride. **(C)** Amebae pre-treated with concanamycin were washed and incubated with Jurkat cells in media without inhibitor or vehicle. Amebic trogocytosis was analyzed using imaging flow cytometry. The % internalization reflects the percentage of amebae that have ingested any host material. Means and standard deviations are for biological duplicates (10,000 events/each). *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Data was analyzed by one-way ANOVA.

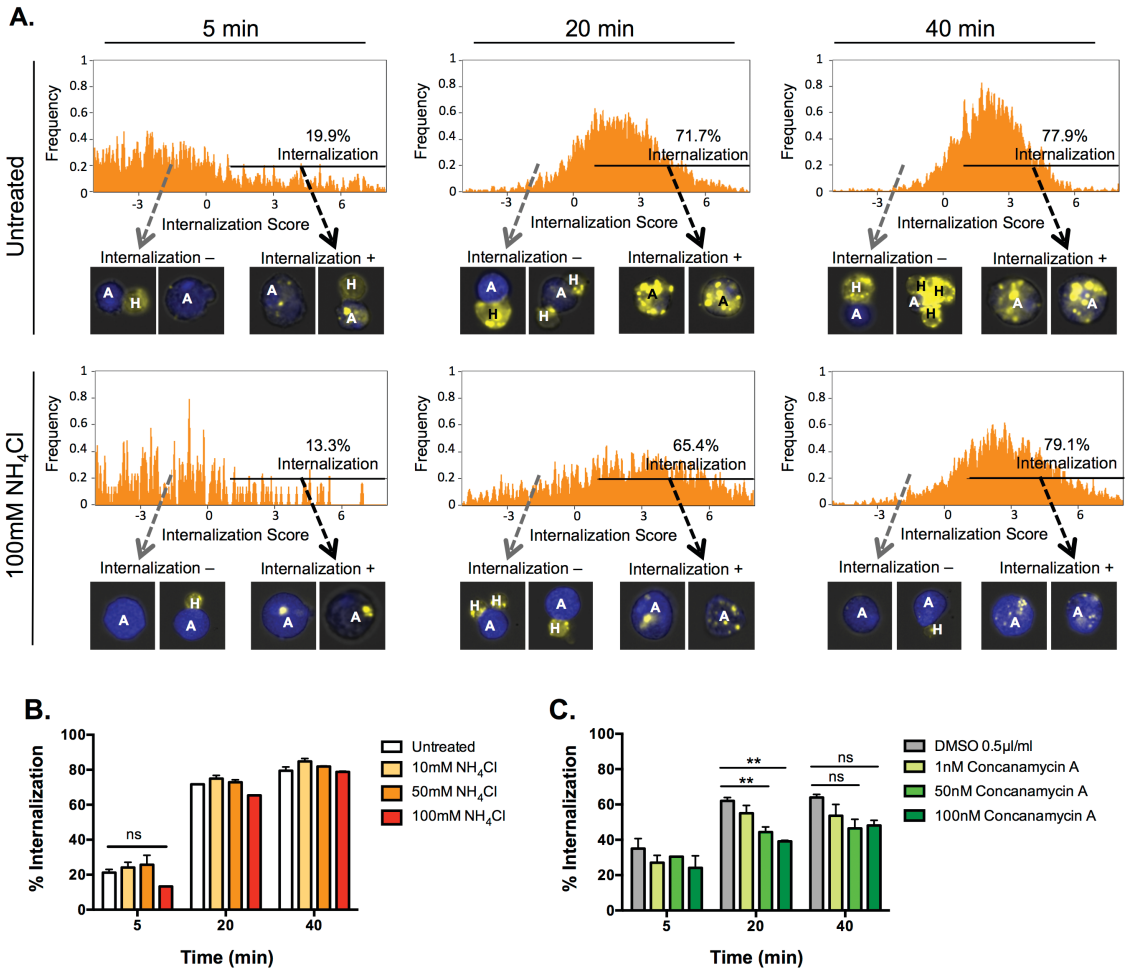
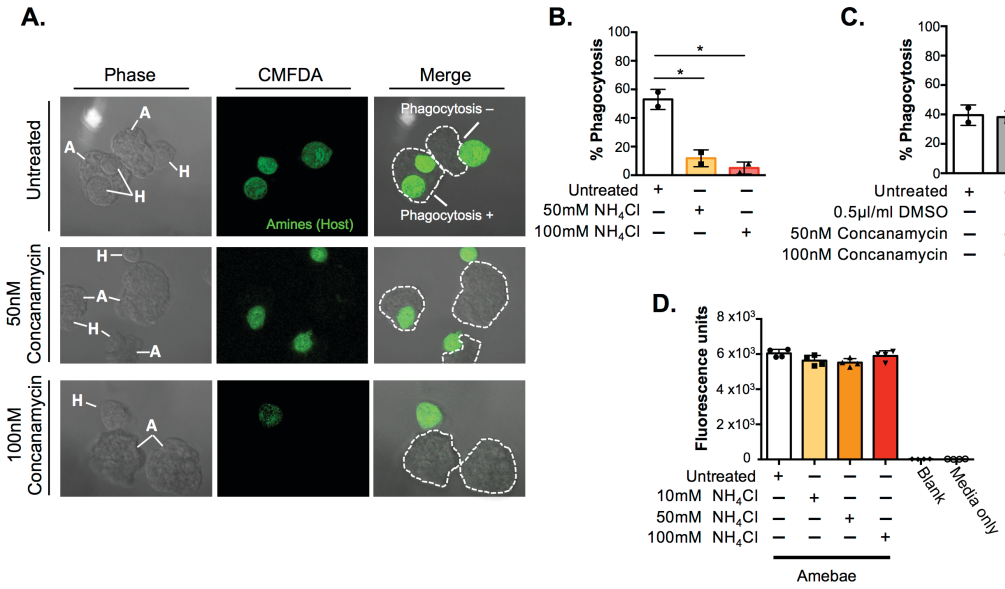

A.
5 min
20 min
40 min
Untreated
100mM NH4Cl
Frequency
19.9% Internalization
71.7% Internalization
77.9% Internalization
13.3% Internalization
65.4% Internalization
79.1% Internalization
Internalization Score
Internalization –
Internalization +
B.
% Internalization
Time (min)
ns
Untreated
10mM NH4Cl
50mM NH4Cl
100mM NH4Cl
C.
% Internalization
Time (min)
**
ns
DMSO 0.5µl/ml
1nM Concanamycin A
50nM Concanamycin A
100nM Concanamycin A

Figure 2.5.

Acidification inhibitors decrease phagocytosis, but not fluid-phase endocytosis. Amebae were co-incubated with heat-killed Jurkat cells for 10 min. After co-incubation all cells were fixed. Phagocytosis was analyzed using fluorescent confocal microscopy. **(A)** Confocal microscopy of unlabeled amebae and human Jurkat T-cells pre-labeled with CMFDA (green, labels amines). Top row, amebae with one or more human cells inside their outer membrane were scored phagocytosis positive. Middle and bottom row, representative images of amebae pre-incubated with concanamycin A. **(B)** Amebae pre-treated with ammonium chloride for 1h were incubated with heat-killed Jurkat cells for 10 min in media containing ammonium chloride. **(C)** Amebae pre-treated with concanamycin A for 1h were washed and incubated with heat-killed Jurkat cells for 10 min in media without inhibitor or vehicle. **(D)** Amebae pre-treated with ammonium chloride for 1h were incubated with RITC dextran for 40 min in media containing ammonium chloride, washed with PBS and then permeablized with 0.1% Triton-X 100. Fluid-phase endocytosis assessed using a fluorescence plate reader. Means and standard deviations are for biological duplicates **(B and C)** or technical quadruplicates **(D)**. *$p \leq 0.05$, ** $p \leq 0.01$. Data was analyzed by one-way ANOVA with Holm-Sildak's multiple comparisons test using Prism 6.


A.
Phase
CMFDA
Merge
Untreated
50nM Concanamycin
100nM Concanamycin
A
H
Amines (Host)
Phagocytosis –
Phagocytosis +
B.
% Phagocytosis
Untreated
50mM NH4Cl
100mM NH4Cl
C.
% Phagocytosis
Untreated
0.5µl/ml DMSO
50nM Concanamycin
100nM Concanamycin
D.
Fluorescence units
8 x10³
6 x10³
4 x10³
2 x10³
0
Untreated
10mM NH4Cl
50mM NH4Cl
100mM NH4Cl
Amebae
Blank
Media only

Chapter 3: Investigation of the function of amebic lysosomes in amebic trogocytosis

Introduction

Entamoeba histolytica is a protozoan parasite and the causative agent of amebiasis in humans. *E. histolytica* was named for it's potent ability to damage tissue (*histo* – tissue, *lytic* – dissolving) and invasive *E. histolytica* infection is characterized by tissue destruction, manifesting as massive intestinal ulceration or abscesses in other sites [138]. The parasite is highly cytotoxic to a wide range of human cells and parasite cytotoxicity is likely to drive tissue destruction. *E. histolytica* is able to kill human cells by ingesting fragments of the live human cell until the cell eventually dies, a process termed amebic trogocytosis [30]. This process begins with the attachment of the parasite to the host cell, mediated largely by the parasite surface protein Gal/GalNAc lectin [29-31]. Following attachment, the parasites ingest fragments of the host cell. These fragments can contain host cell membrane, cytoplasm, and mitochondria. The parasites continue ingesting fragments of the host cell until the host cell eventually dies. Previous work has shown that amebic trogocytosis is an active process, requiring a functional parasite actin cytoskeleton, signaling via phosphatidylinositide 3-kinase (PI3K) and C2-domain containing protein kinase (C2PK) and the AGC

family kinase EhAGCK1 [29-31, 139]. However, the mechanism of amebic trogocytosis is unclear.

Trogocytosis is generally defined as the transfer of fragments of cell material (including intact proteins but not whole cells), in contrast to phagocytosis, which involves the transfer of whole particles or cells. Trogocytosis requires close cell-cell contact, and the transfer of cell fragments occurs rapidly (within minutes) [36]. Processes that are morphologically similar to amebic trogocytosis have been observed in a number eukaryotes, ranging from other protozoa to mammals [36, 55-57]. Trogocytosis has been most extensively studied in human lymphocytes. Human lymphocytes, including T, B, natural killer, dendritic cells and macrophages undergo a process that has also been called trogocytosis [36]. Like amebic trogocytosis, human lymphocyte trogocytosis is a metabolically active process that requires signaling in the acceptor cell, and modulation of both the actin cytoskeleton and intracellular Ca^{2+} (minireview [50]). Phosphatidylinositide 3-kinase (PI3K) and the small GTPases TC21 and RhoG, and, have been identified as key players in T-cell trogocytosis [51]. Yet, trogocytosis remains poorly understood as a basic biological process.

We have recently discovered that interfering with lysosomal acidification significantly impairs amebic trogocytosis, phagocytosis and cell killing, suggesting a critical role for amebic lysosomes in all three processes. Based on these findings, we formulated three hypotheses for the role that lysosomes and acidic vesicles play in amebic trogocytosis: (1) the vesicles are essential for the efficient degradation of ingested fragments, (2) the vesicles are required for rapid

recycling of internalized receptors and membrane, and (3) the vesicles are necessary for the rapid formation of an acidified synapse at the site of ameba-host interaction.

During amebic trogocytosis, parasites ingest numerous human cell fragments. In eukaryotes, lysosomes are crucial for the turnover of ingested material. In our previous study, acidification inhibitors were used to interfere with amebic lysosomes [140]. These inhibitors have been shown raise the pH of amebic lysosomes, which would impair the function of pH-dependent lysosomal proteases [32, 125]. To test the hypothesis that lysosomes are crucial for continued amebic trogocytosis and cell killing because they are required for efficient degradation of the ingested host fragments, I examined the impact of cysteine protease activity on amebic trogocytosis and cell killing. Amebic cysteine proteases that localize to lysosomes and phagosomes are involved in the digestion of endocytosed material, including bacteria and host cells [76]. Using imaging flow cytometry to quantitatively assess the rates of trogocytosis and host cell killing, I found that inhibition of Cathepsin B-like cysteine proteases significantly decreased amebic trogocytosis and cell killing, indicating a crucial role for amebic lysosomes in these processes. Surprisingly, inhibition of Cathepsin B-like cysteine proteases had no impact on phagocytosis.

Our previous studies demonstrated that interfering with vesicle acidification blocked receptor-dependent processes, amebic trogocytosis and phagocytosis, but did not impair a receptor-independent process, fluid-phase endocytosis [140]. Based on these data, I hypothesized that rapid recycling of

membrane and receptors, facilitated by the amebic lysosomes, is required for continued amebic trogocytosis and, thus, cell killing. To investigate this hypothesis, I examined the impact of ammonium chloride, a weak base, on amebic trogocytosis and cell killing. Imaging flow cytometry was used to quantitatively assess the amount of Gal/GalNAc lectin on the surface of the parasite at rest and during amebic trogocytosis. I found that inhibition of lysosomal acidification did not significantly impact the amount of Gal/GalNAc lectin on the surface of the parasite at rest, but did significantly reduce the amount of surface Gal/GalNAc lectin during amebic trogocytosis. These data suggest that amebic lysosomes play an important role in the recycling of parasite surface receptors.

It has been suggested that amebic lysosomes may form an acidified synapse at the site of host cell attachment similar to the synapse created by mammalian osteoclasts [134]. To explore the hypothesis that amebic lysosomes form an acidified synapse at the ameba-host cell interface I sought to localize amebic lysosomes during trogocytosis using live confocal microscopy. I observed that amebic lysosomes are highly dynamic organelles, moving rapidly throughout the cell. In general, amebic lysosomes appeared randomly dispersed throughout the cell during amebic trogocytosis, however in a few rare images, the lysosomes appeared to polarize towards the site of ameba-host attachment. Unfortunately, I found that using our current microscope it is not technically feasible to conclusively establish the existence of an acidified synapse, due to the rapid and dynamic movement of the amebic lysosomes.

Results

E-64d inhibits amebic cysteine proteases in a dose-dependent manner.

The *E. histolytica* genome encodes more than 50 cysteine proteases (CPs) [74, 75]. Under culture conditions, however, the majority (>95%) of cysteine protease activity is attributed to four CPs: EhCP1, EhCP2, EhCP5, and EhCP7 [74-76]. These four cysteine proteases are structurally similar to human Cathepsin-L and appear to have human Cathepsin-B like activity [77]. The role of amebic cysteine proteases in tissue invasion and destruction has been extensively studied. Indeed, secreted cysteine proteases have been shown to degrade colonic mucus and the extracellular matrix (ECM) [82-85]. However, amebic cysteine proteases are also found intracellularly. Cathepsin-like amebic cysteine proteases are targeted to lysosomes and phagosomes where they digest material, including phagocytosed bacteria and host cells [76].

Previous work has shown that treatment with E-64 or its derivatives, E-64c and E-64d, decreases amebic cysteine protease activity [83, 141]. E-64, a secondary metabolite isolated from *Aspergillus japonicus*, functions as a potent and irreversible cysteine protease inhibitor [142]. E-64c, a synthetic analog of E-64, is less hydrophilic than E-64 but still poorly cell-permeable. E-64d is a synthetic cell-permeable ethyl ester of E-64c. E-64d is hydrolyzed by intracellular esterases to E-64c [142]. Treatment with E-64, E-64c, and E-64d have each been shown to impair destruction of a host cell monolayer *in vitro*, a proxy for tissue disruption [84, 86, 88, 143].

To determine whether amebic cysteine proteases are required for amebic trogocytosis and cell killing, I attempted to inhibit amebic cysteine proteases. However, there is no consensus in the *Entamoeba* literature as to the use of E-64, E-64c or E-64d. The amount of drug used varied widely from as little as 100nM E-64 in one study to as much as 200μM E-64 in another [39, 88]. The length of time that amebae were incubated with the drug also differed, ranging from 20min to 4h [83, 144]. Therefore, it was necessary to first determine the optimal drug concentration and treatment time necessary to inhibit amebic cysteine proteases. E-64d was used, because it is cell permeable, in contrast to E-64 and E-64c, which are very poorly cell permeable and therefore require endocytosis for cell entry.

Amebae were treated with 1μM to 100μM E-64d or vehicle (DMSO) alone at 37°C for 1h. Following this incubation, the parasites were washed extensively to remove free inhibitor and lysed. The parasite lysates were then assayed for their ability to degrade a mammalian Cathepsin B substrate. I found that E-64d reduced amebic cysteine protease activity in a dose-dependent manner (Fig. 3.1A). To confirm that cysteine protease inhibition was irreversible following the inhibitor washout, parasites were treated with 1-100μM E-64d or vehicle (DMSO) alone at 37°C for 1h, then washed extensively to remove free inhibitor and incubated in media for a further 40min at 37°C. The parasites were lysed and the lysates assayed for their ability to degrade the mammalian Cathepsin B substrate. I found that the inhibitory effect of E-64d was maintained following washout, with 97.1% inhibition of amebic cathepsin B-like activity at 40min after

washout of 100μM E-64d (Fig. 3.1B). Taken together, these data indicated that treatment with 100μM E-64d for 1h is sufficient to inhibit amebic Cathepsin B-like cysteine proteases and this effect is maintained for at least 40min following the removal of the inhibitor.

E-64d decreases amebic trogocytosis and cell killing.

I have previously shown that impairing vesicle acidification using either ammonium chloride, a weak base, or concanamycin A, a V-ATPase inhibitor, drastically decreases amebic trogocytosis and cell killing [140]. Interfering with vesicle acidification likely impairs vesicular enzymes that function at low pH, including cysteine proteases such as amebic EhCP1 (reviewed in [145]). To determine whether amebic cysteine proteases are required for continued amebic trogocytosis and cell killing, I inhibited amebic cysteine proteases using E-64d. CMFDA-labeled parasites were treated with 100μM E-64d for 1 h. The parasites were then washed extensively to remove free inhibitor, and co-incubated with DiD-labeled human Jurkat T-cells or media alone for 5, 20 or 40 min. Thus only the amebae, not the Jurkat T-cells were exposed to E-64d. After co-incubation of the parasites with the human cells, all cells were stained with the permeability dye Live/Dead Violet and fixed. I then assessed trogocytosis and cell killing using imaging flow cytometry (Fig. 3.2). To quantify trogocytosis, I measured fragmentation of the human material within the amebae, as previously described by Ralston et al. (Fig 3.2A, Fig 2.2 shows the full gating strategy).

As expected, 100μM E-64d treatment dramatically reduced amebic cysteine protease activity (Fig 3.2E). In control amebae the percentage of amebae that ingested a high number of human fragments increased over time from 6.0% at 5 min to 40.9% at 40 min. However, the inhibitor-treated parasites showed significantly less ingestion, with only 20.6% of parasites treated with 100μM E-64d ingesting a high number of human fragments after 40 min (Fig 3.2A and 3.2B). Treatment with E-64d also decreased cell killing (Fig 3.2C). This finding is consistent with an earlier report that pre-treatment of amebae with 50μM E-64d for 4h significantly reduced cell killing [144]. Treatment with E-64d alone did not directly cause amebic death (Fig 3.2D). Human cells were not exposed to E-64d in this assay. Together, these data suggest an important role for amebic cysteine proteases in amebic trogocytosis and cell killing.

E64-d does not impair phagocytosis.

Amebic trogocytosis (ingestion of cell fragments) shares many similarities with another endocytic process, phagocytosis (ingestion of whole cells). Both amebic trogocytosis and phagocytosis are receptor-dependent processes, requiring attachment to a target cell via the amebic Gal/GalNAc lectin [30, 31]. These processes also require amebic actin rearrangement and PI3K signaling [30, 38, 39]. Thus far, no pathways unique to trogocytosis have been discovered in any organism, therefore, I assessed the impact of cysteine protease inhibition on phagocytosis. *E. histolytica* has been shown to preferentially phagocytose (ingest whole) dead human cells and trogocytose (ingest fragments) live human

cells [30]. To determine whether cysteine protease inhibition impairs phagocytosis, CMFDA-labeled parasites were treated with 100μM E-64d for 1h at 37°C, washed extensively, and co-incubated with DiD-labeled heat-killed human Jurkat T-cells for 5 min, 20 min, or 40 min (Fig 3.3A). Following co-incubation, all cells were fixed and phagocytosis was assessed using imaging flow cytometry. Surprisingly, I found that treatment with 100μM E-64d did not impair phagocytosis when measured as the percentage of amebae that had phagocytosed ≥1 Jurkat T-cell (Fig 3.3B) or when measured as the number of ingested Jurkat T-cells per ameba (Fig 3.3C). From these data I conclude that amebic Cathepsin B-like cysteine proteases are required for efficient amebic trogocytosis and cell killing, but not phagocytosis.

Treatment with the acidification inhibitor ammonium chloride results in decreased parasite surface lectin during amebic trogocytosis.

It has been shown that parasite attachment to host cells via the parasite Gal/GalNAc lectin is required for amebic trogocytosis and host cell killing [29-31]. As amebae ingest host cell fragments, amebic plasma membrane is internalized such that ingested fragments are surrounded by an ameba-derived membrane [30]. Thus, surface Gal/GalNAc lectin is continually internalized during trogocytosis. I hypothesized that rapid and efficient recycling of both membrane and surface receptors, such as the Gal/GalNAc lectin, are likely to be crucial for continued trogocytosis.

To test this hypothesis, I assessed the amount of lectin on the surface of acidification inhibitor-treated parasites at rest and during amebic trogocytosis. CMFDA-labeled amebae were treated with ammonium chloride for 1h. Parasites were incubated with or without DiD-labeled human Jurkat-T cells for 5, 20 or 40 min in media containing ammonium chloride. Previous studies have shown that *E. histolytica* is able to rapidly re-acidify lysosomes within minutes of ammonium chloride removal, therefore ammonium chloride was maintained in the media throughout the incubation with Jurkat-T cells [32]. Following co-incubation of amebae and Jurkat-T cells, all cells were stained with the cell permeability dye Live/Dead Violet and then fixed. After fixation, amebae were stained for surface Gal/GalNAc lectin using an anti-Gal/GalNAc lectin antibody (H84) and fluorescent secondary (Fig 3.4A). As expected, treatment with ammonium chloride decreased both amebic trogocytosis and cell killing (Fig 3.4B and C). The amount of surface Gal/GalNAc lectin was quantified using imaging flow cytometry. I found that there was no difference in surface lectin between inhibitor-treated and control parasites at steady state (Fig 3.4E). In contrast, I found that inhibitor-treated parasites had modestly decreased levels of the amebic surface Gal/GalNAc lectin following amebic trogocytosis (Fig 3.4D).

Amebic lysosomes do not consistently polarize to the site of ameba-host attachment.

It has been shown that impairing vesicle acidification substantially decreases amebic trogocytosis and cell killing, suggesting a crucial role for

amebic acidic vesicles in trogocytosis and cell killing [32, 140]. However, the precise role of amebic vesicles in trogocytosis is unclear. It has been suggested in the literature that amebic lysosomes may form an acidified synapse at the site of host cell attachment similar to the synapse created by mammalian osteoclasts, which secrete the contents of lysosomes to form an acidified synapse during bone matrix degradation [134]. Live confocal microscopy and electron microscopy both show a massive accumulation of actin and exclusion of vesicles at the site of active host fragment ingestion during trogocytosis [30]. However, it is possible that amebic lysosomes fuse at the ameba-host cell interface before active ingestion begins. To investigate this hypothesis, I first sought to establish whether amebic lysosomes are specifically recruited to the site of ameba-host interaction. *E. histolytica* lacks orthologs of common mammalian lysosomal markers, such as lysosome-associated membrane glycoproteins (LAMPs) and ameba-specific lysosomal markers, thus I labeled amebic lysosomes with Lysosensor, an acidotropic dye that accumulates in acidic organelles, likely as a result of protonation. Lysosensor-labeled amebae were co-incubated with DiD-labeled Jurkat-T cells and the localization of amebic lysosomes during trogocytosis was observed using live confocal microscopy. I observed that the amebic lysosomes were highly dynamic organelles, trafficking rapidly throughout the parasite (Fig 3.5C). The rapid movement of both the amebae and amebic vesicles confounded efforts to track the movement the amebic lysosomes before and during trogocytosis. I found that the amebic lysosomes were generally randomly dispersed throughout the parasite during trogocytosis (Fig 3.5A). In

rare cases, amebic lysosomes appeared to cluster close to the site of ameba-host attachment and to co-localize with ingested bites (Fig 3.5B). It is possible that these rare events are indicative of the formation of an acidified synapse at the site of ameba-host interaction, however it is also possible that the events observed in Fig 3.5B may result from random chance. In particular, the rapid movement of both the amebae and amebic vesicles confounded efforts to track movement the amebic lysosomes before and during trogocytosis. Thus, from these observations it is not possible to definitively establish the existence of an acidified synapse.

Discussion

Trogocytosis, the ingestion of cell fragments (but not whole cells), has been observed in a wide range of eukaryotes from protozoa to mammals. Yet, it remains poorly understood as a basic biological process. *E. histolytica* is able to kill human cells by ingesting fragments of the live human cell until the cell eventually dies, a process termed amebic trogocytosis [30]. As described in Chapter 2, we have recently discovered that amebic lysosomes play a critical role in both amebic trogocytosis and cell killing [140]. However, the precise role that amebic lysosomes play amebic trogocytosis is still unclear. In this study, I present evidence that amebic lysosomes may play a multifactorial role in trogocytosis, with functions in both the efficient degradation of ingested material and the rapid recycling of amebic surface receptors.

Amebic cysteine proteases that localize to lysosomes and phagosomes are involved in the digestion of material, including phagocytosed bacteria and host cells [76]. In this chapter, I have shown that interfering with amebic cysteine proteases using the irreversible cysteine protease inhibitor E-64d impairs amebic trogocytosis and cell killing (Fig 3.2). This is consistent with previous work showing that prolonged treatment of parasites with moderate amounts of E-64d significantly reduced cell killing [144]. *E. histolytica* has been shown to acidify phagosomes within 2 minutes, suggesting that lysosomes are rapidly recruited and fuse with the phagosome [125]. My data is consistent with the hypothesis that the inhibitor-treated parasites fail to efficiently degrade material in the phagolysosome, resulting in slowed or blocked ingestion. Continued ingestion of multiple human fragments appears to be required to kill human cells [30], thus these parasites are also impaired in their ability to kill human cells (Fig 3.2B).

Treatment with E-64d resulted in approximately a 50% decrease in amebic trogocytosis compared to the control. In contrast, my previous work using two different acidification inhibitors resulted in >90% decrease in amebic trogocytosis [140]. The difference in the degree of inhibition of trogocytosis in these two studies may be attributable to the efficiency of the inhibition of lysosomal function by these two distinct approaches. Multiple amebic lysosomal functions are expected to be impaired by increased lysosomal pH, as most lysosomal functions are likely dependent on a low pH environment. In contrast, E-64d is a specific inhibitor of a single class of proteases, the cysteine proteases. Importantly, however, these two distinct approaches both reduce amebic

trogocytosis and cell killing. Taken together, these findings suggest that lysosomal functions, in addition to protease activity, may be required for efficient trogocytosis. The rapid recycling of amebic surface receptors may be one such activity.

Previously, we found that interfering with vesicle acidification blocked amebic trogocytosis and phagocytosis, both receptor-dependent processes, but did not impair fluid-phase endocytosis, a receptor-independent process [140]. It is possible that inhibition of lysosomal acidification results in the inhibition of recycling of the amebic surface receptors required for trogocytosis, such as the Gal/GalNAc lectin [30, 64]. In this chapter, I observed that that, at rest, parasites treated with the acidification inhibitor ammonium chloride had similar levels of surface amebic Gal/GalNAc lectin to untreated parasites (Fig 3.4E). In contrast, there was a significant decrease in the amount of Gal/GalNAc lectin on the surface of inhibitor-treated parasite compared to control parasites following trogocytosis (Fig 3.4D). This suggests that rapid recycling of amebic surface receptors, facilitated by the amebic lysosomes, may be required for continued amebic trogocytosis and, thus cell killing. The magnitude of the decrease in surface lectin on the inhibitor-treated parasites was less than the decrease in amebic trogocytosis that we observed using the same inhibitor (Fig 3.4B, Fig 2.1). Taken together with my findings on the requirement for amebic cysteine proteases in amebic trogocytosis, these data suggest that amebic lysosomes play an important role in both the efficient degradation of ingested material and the rapid recycling of amebic surface receptors.

It has been suggested in the literature that amebic lysosomes may form an acidified synapse at the site of host cell attachment [134]. I have observed that amebic lysosomes are highly dynamic organelles that generally appeared to be randomly dispersed throughout the parasite during trogocytosis (Fig 3.5A). This finding is consistent with previous live confocal microscopy and electron microscopy experiments, which have shown a massive accumulation of actin and the exclusion of vesicles at the site of active host fragment ingestion [30]. However, in rare cases, amebic lysosomes appeared to cluster close to the ameba-host interface and to co-localize with ingested fragments (Fig 3.5B). It is possible that these rare events are attributable to the formation of an acidified synapse at the site of ameba-host interaction, but it is also possible that the events observed in Fig 3.5B result from random chance. Therefore, further study using a more advanced microscope is needed to examine whether such a synapse is formed.

Amebic trogocytosis (ingestion of cell fragments) shares many similarities with another endocytic process, phagocytosis (ingestion of whole cells). However, amebic trogocytosis likely involves a distinct endocytic pathway, requiring trogocytosis-specific signaling and effectors. In Chapter 2, we demonstrated that interfering with vesicle acidification blocks both amebic trogocytosis of live human cells and phagocytosis of dead human cells. In contrast, I found that cysteine protease inhibition significantly reduced amebic trogocytosis of live human cells (Fig 3.2) but did not impair phagocytosis of dead human cells (Fig 3.3). These findings suggest that amebic cysteine proteases

play a crucial and distinct role in amebic trogocytosis. There are several possible roles that cysteine proteases might play in amebic trogocytosis: amebic cysteine proteases may be required to activate trogocytosis-specific receptors or to expose ligands for trogocytosis-specific signaling. It is also possible that trogosomes (vesicles containing ingested fragments) mature more quickly than phagosomes containing dead human cells, therefore trogocytosis is more sensitive to protease inhibition than phagocytosis.

Mammalian cysteine cathepsins have been shown to catalyze the activating cleavage of endolysomal receptors, such as Toll-like receptor 9 (TLR9), which senses unmethylated DNA [146, 147]. Interestingly, it has been observed that treatment of murine macrophages with acidification inhibitors including Bafilomycin A1 (a V-ATPase inhibitor related to Concanamycin A) impaired signaling of TLR9 and another endolysosomal receptor, TLR7. In contrast, cysteine protease inhibition only impaired signaling of TLR9 [146]. Thus it is possible that treatment with acidification inhibitors impairs signaling of endolysosomal receptors involved in both trogocytosis and phagocytosis, while cysteine protease inhibition could impact a trogocytosis-specific receptor.

It has been shown that *Staphylococcus aureus*-induced TLR activation is enhanced following phagosomal maturation and digestion of the bacteria by lysosomal hydrolases, suggesting that there is a pool of TLR ligands that becomes accessible only after digestion of the bacteria [148-150]. It is possible that cysteine protease degradation can release ligands that were previously obscured, activating a trogocytosis-specific signaling pathway.

It has been shown that mammalian phagocytes acidify phagosomes and endosomes at different rates depending on the route of ingestion and the target. Work with murine macrophages has shown that endocytic vesicles containing small particles rapidly fused with lysosomes, while larger particles remained in early phagosomes for longer periods of time [151]. It has also been observed that macrophage phagosomes containing unopsonized apoptotic cells acidified significantly faster than phagosomes containing IgG-opsonized live target cells [152]. It is possible that that trogosomes mature more rapidly than phagosomes, and thus rapid and efficient degradation of ingested material may be more important in amebic trogocytosis than phagocytosis.

Methods

Cell Culture

Amebic trophozoites (HM1:IMSS) were cultured axenically at 35°C in TYI-S-33 as previously described [38]. Trophozoites were harvested during log-phase growth by centrifugation at 200 x *g* for 5 min at room temperature, followed by resuspension in M199s (medium 199 [Gibco] without phenol red and supplemented with 5.7mM cysteine [Sigma], 0.5% bovine serum albumin [Gemini] and 25mM HEPES [Sigma] at pH 6.8) [30].

Human Jurkat T cells (Clone E6-1, ATCC) were grown at 37° C in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco), 10mM HEPES

(Gibco), and 1mM sodium pyruvate (Gibco). Jurkat cultures were collected and enriched for viable cells as previously described [30].

Amebic Trogocytosis and Cell Killing Assay

Amebic trogocytosis by amebae was measured using imaging flow cytometry as described previously with some modifications [30, 140]. Briefly, amebae were labeled with 200nM CellTracker Green CMFDA (5-chloromethylfluorescein diacetate,

Invitrogen) in M199S for 10min at 37°C and then washed twice with M199S. Following CMFDA labeling, amebae were treated with pharmacological inhibitors or vehicle control for 1hr at 37° C. Amebae were treated either with ammonium chloride (Sigma) at 10mM, 50mM or 100mM or M199s as a control; or amebae were treated with E-64d (EST, EMD-Millipore) at 100μM or an equal volume of DMSO (Molecular Probes) as a vehicle control. Jurkat cells were labeled with 5μM DiD (DiIC_{18}(5)-DS, Molecular Probes) in M199S for 5 min at 37°C, followed by 10 min at 4C, and then washed twice with M199S. Amebae pre-treated with ammonium chloride were co-incubated with labeled Jurkat cells at a ratio of 1:5, in biological duplicate at 37°C for 5 min, 20 min or 40 min in M199s containing ammonium chloride. Amebae pre-treated with E-64d or DMSO were washed twice with M199s, and then co-incubated with labeled Jurkat cells in biological duplicate at 37° C for 5 min, 20 min or 40 min in M199s. At the end of each time point, samples were immediately placed on ice and labeled with live/dead fixable violet (Invitrogen) at 1.6μl per ml for 30min in the dark. The cells were then fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, in the dark.

For surface Gal/GalNAc lectin staining, fixed cells were washed with cold PBS and incubated with the H85 anti-Gal/GalNAc lectin monoclonal antibody at 8μg/ml in PBS for 45 min at 4C, followed by goat anti-mouse Alexa Flour 546 (Invitrogen) at 10μg/ml in PBS for 45 min at 4C. Flow cytometry was performed using ImageStreamX Mark II (EMD Millipore). 10,000 events were collected for each sample and data were analyzed using IDEAS software (EMD Millipore). Example images in Fig 3.2 have been cropped to minimize blank space.

Phagocytosis Assay

Jurkat cells were collected, washed with M199s and labeled with 5μM DiD (Molecular Probes) for 5 min at 37C, followed by 10 min at 4C, and then washed twice with M199S. Jurkat cells were then incubated at 55° C for 15min to induce necrotic cell death [38]. Amebae were collected, washed with M199s, labeled with 200nM CMFDA in M199S for 10min at 37°C and then washed twice with M199S. Amebae were then treated with 100μM E-64d or an equal volume of DMSO as a vehicle control for 1hr at 37° C. Following this incubation, amebae were washed with M199s, and co-incubated with DiD-labeled heat-killed Jurkat cells in M199s for 5, 20, or 40 min at 37°C. After co-incubation, all samples were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. Flow cytometry was performed using ImageStreamX Mark II (EMD Millipore). 10,000 events were collected for each sample and data were analyzed using IDEAS software (EMD Millipore). Focused events were selected using a gradient of brightfield. Then focused events were enriched for single events by removing compound events containing two or more cells not in contact. Single events were

plotted based on aspect ratio and intensity of CMFDA staining to distinguish between single Jurkats, single amebae and clusters of amebae. ‘Single amebae’ were plotted based on area and intensity of DiD staining to distinguish between ‘phagocytosis+’ amebae (containing ≥1 Jurkat cells) and ‘phagocytosis-’ (containing no Jurkat cells). ‘% Phagocytosis+ amebae’ was calculated by dividing the number of ‘phagocytosis+ amebae’ by the number of ‘single amebae’ and multiplying by 100. The number of ingested Jurkats per ameba in the first 100 ‘phagocytosis+’ images was counted manually. Example images in Fig 3.3 have been cropped to minimize blank space.

Cathepsin B activity assay

Cathepsin B activity was assessed using a fluorometric Cathepsin B Activity assay kit (abcam) according to the manufacturer’s directions with some modifications. Briefly, amebae were collected, washed with M199s and treated with E-64d or an equal volume of DMSO (vehicle) for 1hr at 37°C. amebae were then washed M199s and resuspended at $2x10^5$ cells/ml. 500µl aliquots were either immediately flash frozen in liquid nitrogen or incubated in M199s for 40min at 37°C and then flash frozen in liquid nitrogen and stored at -80°C. For cell lysis, samples were thawed on ice, washed with cold PBS and resuspended in 50µl of chilled CB Cell lysis buffer (abcam), and incubated on ice for 15 min. Samples were then centrifuged for 5 min at 4°C at maximum speed, the supernatant was transferred to a clean tube and lysates were stored at -80°C. For the Cathepsin B activity assay, sample lysates were thawed on ice, and an aliquot was diluted 1:100. Reaction wells were set up in a black, clear-bottom 96-well plate (Corning)

as follows: experimental wells contained 50µl of the diluted sample lysate; negative control wells contained 50µl of the diluted DMSO-treated sample lysate and 2µl CB inhibitor (Z-Phe-Phe-FMK, abcam); background control wells contained 50µl CB Cell lysis buffer. 50µl CB Reaction Buffer (abcam) and 2µl of 10mM CB Substrate AC-RR-AFC (200µM final concentration, abcam) was added to each well. The plate was incubated at 37°C for 15 min protected from light. Fluorescence output was measured at Ex/Em = 400/505nm on a fluorescence plate reader (BioTek).

Live confocal imaging of amebic lysosomes

Amebae were labeled with 1µM Lysosensor Green DND-189 (Molecular Probes) in M199S for 1h at 37°C and then washed twice with M199S. Jurkat cells were labeled with 5µM DiD ($DiIC_{18}(5)$-DS, Molecular Probes) in M199S for 5 min at 37°C, followed by 10 min at 4C, and then washed twice with M199S. Lysosensor-labeled amebae were co-incubated with DiD-labeled Jurkat-T cells at a ratio of 1:5. Cells were immediately imaged in glass bottom 35-mmculture dishes (Mattek), fully filled with M199S, using Zeiss LSM software on a Zeiss LSM700 inverted confocal microscope equipped with a 63x apochromatic oil objective and a heated stage.

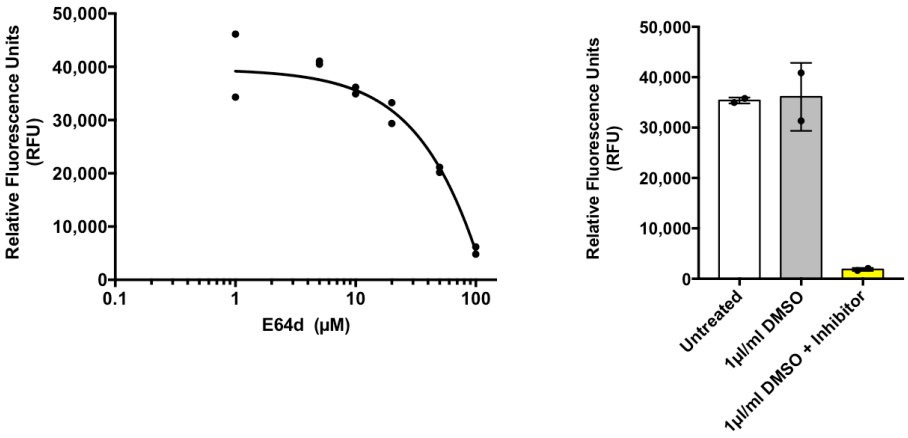
Statistical Analysis

Data were analyzed using Prism 6 (GraphPad Software). *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 3.1

E64-d inhibits amebic cysteine proteases in a dose-dependent manner. Amebae pre-treated with media (untreated), vehicle (1µl/ml DMSO) or E-64d (1-100µM) for 1h were either (**A**) flash frozen and lysed or (**B**) washed, incubated in media for 40min, then flash frozen, and lysed. Z-Phe-Phe-FMK (irreversible cathepsin B and L inhibitor) was added to an aliquot of vehicle-treated cell lysate as a negative control. All samples were assayed for their ability to degrade AC-RR-AFC (mammalian Cathepsin B substrate) using a fluorescence plate reader. Means and standard deviations represent biological duplicates. Curve fitting was done using non-linear regression in Prism 6.

A.


Relative Fluorescence Units (RFU)
50,000
40,000
30,000
20,000
10,000
0
0.1
1
10
100
E64d (μM)
Relative Fluorescence Units (RFU)
50,000
40,000
30,000
20,000
10,000
0
Untreated
1μl/ml DMSO
1μl/ml DMSO + Inhibitor

B.

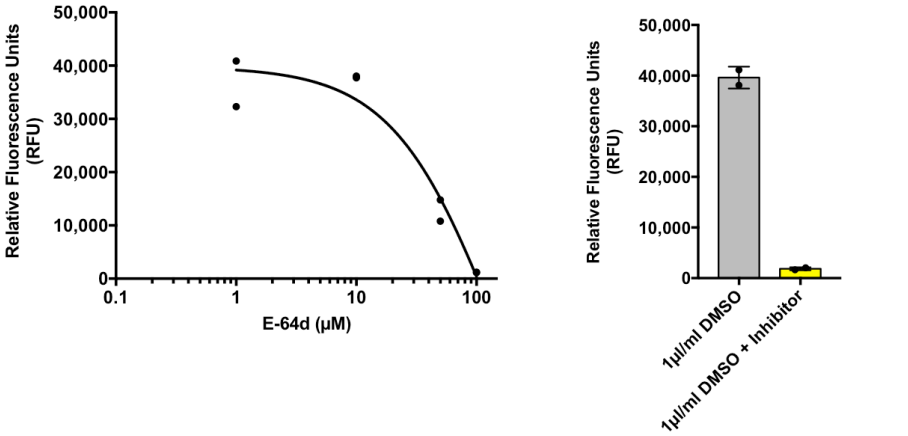

Relative Fluorescence Units (RFU)
50,000
40,000
30,000
20,000
10,000
0
0.1
1
10
100
E-64d (μM)
Relative Fluorescence Units (RFU)
50,000
40,000
30,000
20,000
10,000
0
1μl/ml DMSO
1μl/ml DMSO + Inhibitor

Figure 3.2

E-64d treatment decreases amebic trogocytosis and cell killing. Amebae labeled with CMFDA (blue, amines) were pre-treated with 100μM E-64d or 1μl/ml DMSO (vehicle) for 1h, washed and incubated with DiD-labeled (yellow, membrane) Jurkat-T cells for 5, 20 or 40 min at 37°C in media without inhibitor or vehicle. Afterwards all cells were stained with Live/Dead Violet on ice for 30 min and then fixed. Amebic trogocytosis and cell killing were analyzed using imaging flow cytometry. **(A)** Measurement of fragmentation of ingested material over time. Representative images are shown for each gate. **(B)** % High gate measures the percentage of events in the high gate, reflecting the quantity of fragments that have been ingested by the parasites **(C)** % Jurkat dead measures the percentage of host cells staining with Live/Dead Violet. **(D)** % ameba dead measures the percentage of amebae staining with Live/Dead Violet. **(E)** Amebae pre-treated with 100μM E64-d for 1h were, washed, incubated in media without inhibitor or vehicle at 37°C for 1h and then flash frozen. Samples were then thawed on ice and lysed. Lysates were assayed for cysteine protease activity using a Cathepsin B activity kit. Means and standard deviations are for biological duplicates. Data shown is representative of 5 independent experiments. Data was analyzed by student's t-test using Prism 6. *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

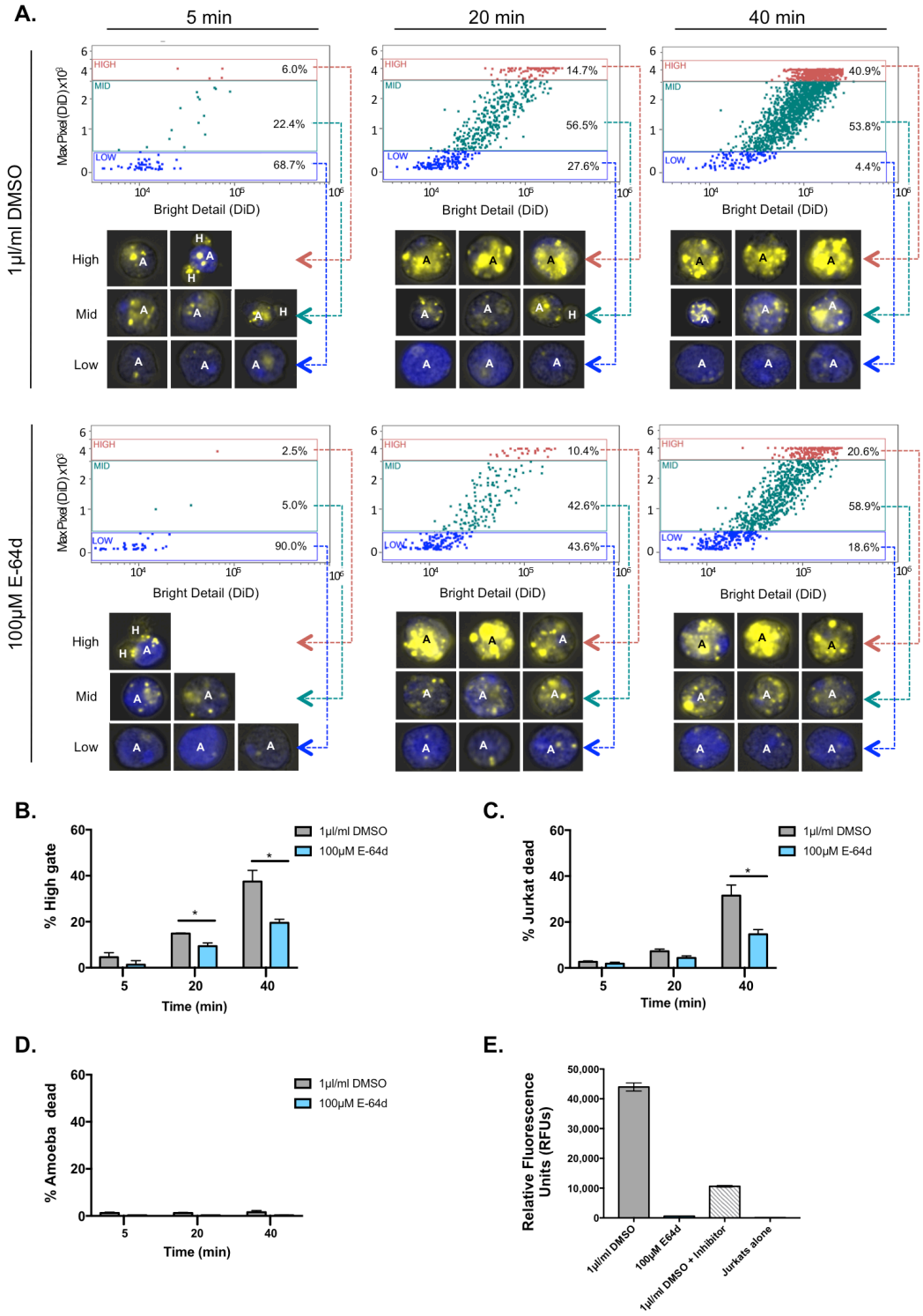

A.
5 min
20 min
40 min
1µl/ml DMSO
100µM E-64d
MaxPixel(DiD)x10³
Bright Detail (DiD)
HIGH
MID
LOW
6.0%
22.4%
68.7%
14.7%
56.5%
27.6%
40.9%
53.8%
4.4%
2.5%
5.0%
90.0%
10.4%
42.6%
43.6%
20.6%
58.9%
18.6%
High
Mid
Low
B.
% High gate
Time (min)
1µl/ml DMSO
100µM E-64d
C.
% Jurkat dead
D.
% Amoeba dead
E.
Relative Fluorescence Units (RFUs)
1µl/ml DMSO
100µM E64d
1µl/ml DMSO + Inhibitor
Jurkats alone

Figure 3.3

E-64d treatment does not affect phagocytosis. CMFDA-labeled (blue, amines) amebae pre-treated with 100μM E-64d or 1μl/ml DMSO (vehicle) for 1h were washed and incubated with heat-killed DiD-labeled (orange, membrane) Jurkat-T cells for 5, 20 or 40 min at 37°C in media without inhibitor or vehicle. After co-incubation all cells were fixed. Phagocytosis was analyzed using imaging flow cytometry. **(A)** Measurement of phagocytosis over time. Representative images are shown for each population. **(B)** % Phagocytosis measures the percentage of single amebae that ingested one or more Jurkat-T cells. **(C)** The number of ingested Jurkats per ameba was counted manually (100 images/sample). Means and standard deviations are for biological duplicates. Data shown is representative of 3 independent experiments. Data was analyzed by student's t-test using Prism 6. *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

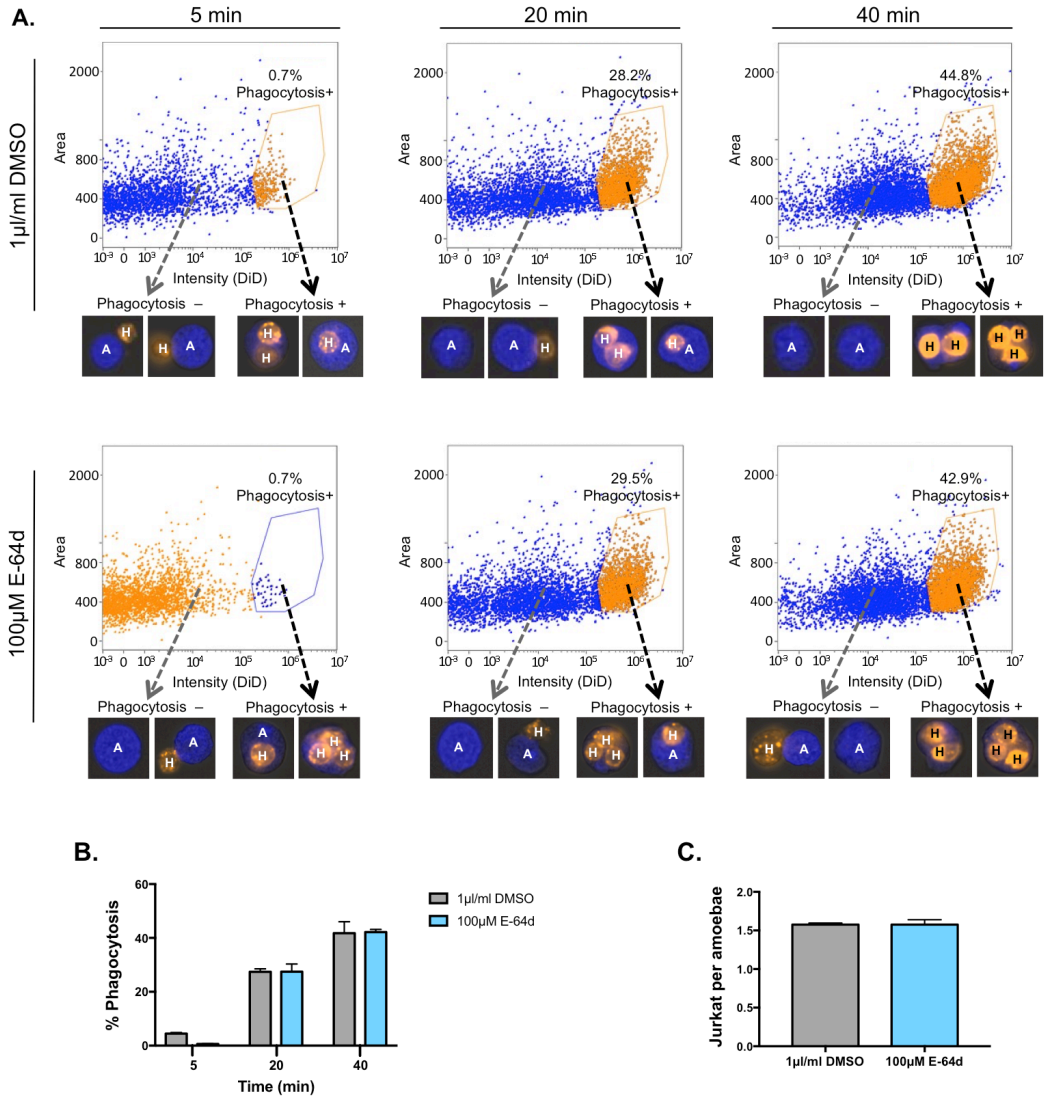

A.
5 min
20 min
40 min
1µl/ml DMSO
100µM E-64d
0.7% Phagocytosis+
28.2% Phagocytosis+
44.8% Phagocytosis+
0.7% Phagocytosis+
29.5% Phagocytosis+
42.9% Phagocytosis+
Area
Intensity (DiD)
Phagocytosis –
Phagocytosis +
B.
% Phagocytosis
Time (min)
1µl/ml DMSO
100µM E-64d
C.
Jurkat per amoebae
1µl/ml DMSO
100µM E-64d

Figure 3.4

Ammonium chloride decreases parasite surface lectin during trogocytosis. **(A-D)** Amebae pre-treated with ammonium chloride for 1h were incubated with Jurkat cells in media containing ammonium chloride for 5, 20 or 40 min. **(A)** Representative image of an ameba stained with CMFDA (blue, amines) and anti-lectin antibody (yellow, surface lectin) that has ingested multiple DiD-labeled Jurkat cell fragments (orange, membrane). The ameba was alive before fixation and therefore does not stain with Live/Dead violet (violet, cell permeability). **(B)** % High gate measures the percentage of events in the high gate, reflecting the quantity of fragments that have been ingested by the parasites **(C)** % Jurkat dead measures the percentage of host cells staining with Live/Dead Violet. **(D) ‘**Mean intensity (anti-lectin)’ measures the mean intensity of anti-lectin staining per ameba, reflecting the amount of surface lectin on the parasites. **(E)** Amebae pre-treated with ammonium chloride for 1h were incubated in media containing ammonium chloride for 5, 20 or 40 min (without Jurkat T-cells). Afterwards all cells were stained with Live/Dead Violet on ice for 30min and then fixed. Amebae were then incubated with an anti-Gal/GalNAc lectin mouse monoclonal antibody, followed by a goat anti mouse IgG-Alexa 546 conjugate. Surface lectin was assessed using imaging flow cytometry. **‘**Mean intensity (anti-lectin)’ measures the mean intensity of anti-lectin staining per ameba, reflecting the amount of surface lectin on the parasites. Data shown is representative of 3 independent experiments. *$p \leq 0.05$, ** $p \leq 0.01$. Data was analyzed by one-way ANOVA with Holm-Sildak’s multiple comparisons test using Prism 6.

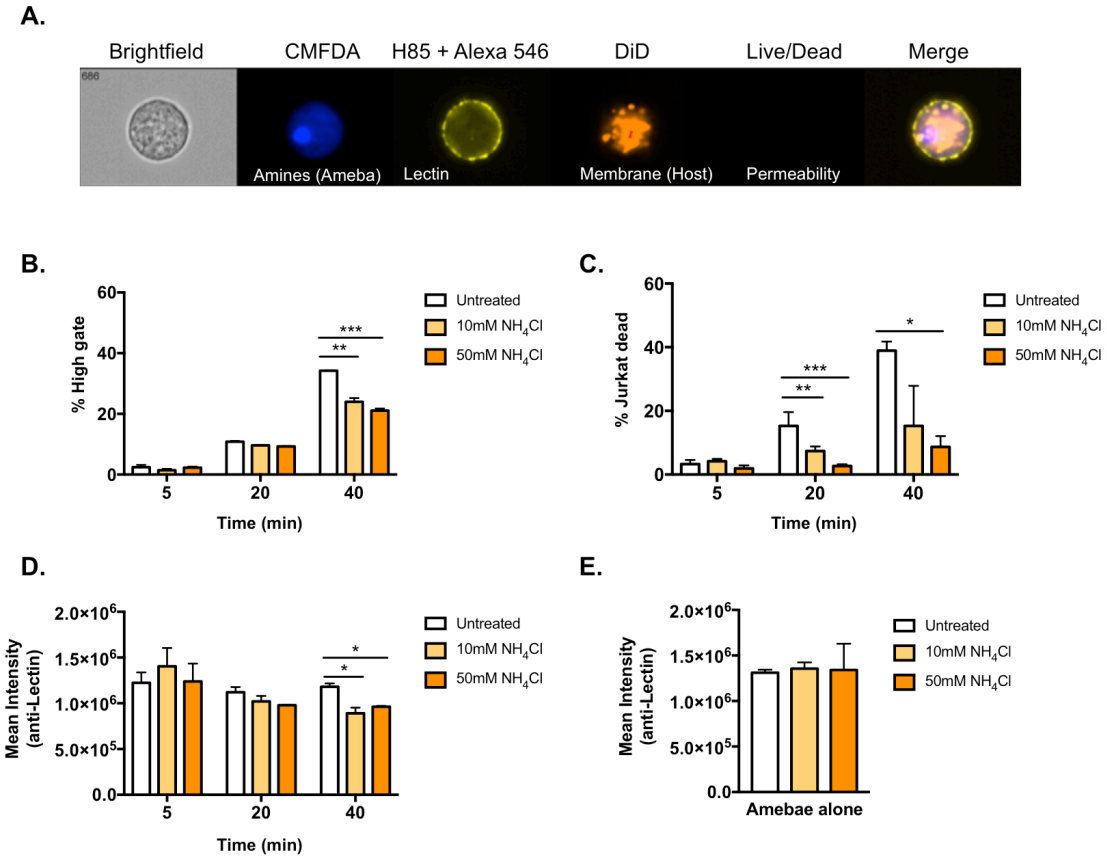
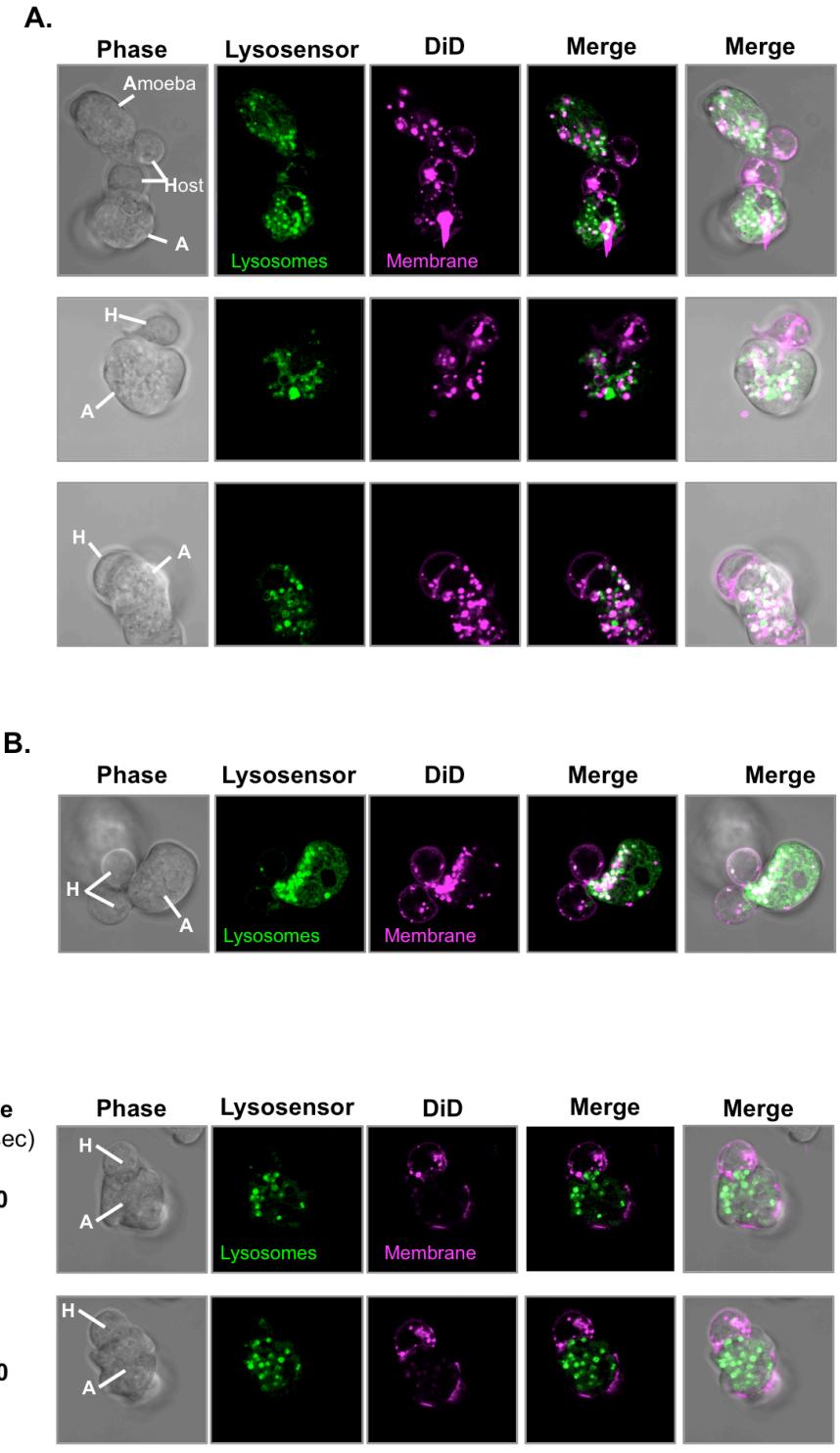

A.
Brightfield
CMFDA
H85 + Alexa 546
DiD
Live/Dead
Merge
Amines (Ameba)
Lectin
Membrane (Host)
Permeability
B.
% High gate
Time (min)
C.
% Jurkat dead
Time (min)
D.
Mean Intensity (anti-Lectin)
Time (min)
E.
Mean Intensity (anti-Lectin)
Amebae alone
Untreated
10mM NH4Cl
50mM NH4Cl

Figure 3.5

Amebic lysosomes do not consistently polarize towards the site of ameba-host interaction. Confocal microscopy of amebae labeled with Lysosensor (green, lysosomes) were co-incubated with Jurkat-T cells labeled with DiD (purple, membrane). **(A)** Representative images of amebae during amebic trogocytosis. **(B)** Rare image in which amebic lysosomes appear to polarize during amebic trogocytosis. **(C)** Representative time lapse of ameba during amebic trogocytosis.


A.
Phase
Lysosensor
DiD
Merge
Merge
Amoeba
Host
A
Lysosomes
Membrane
H
A
H
A
B.
Phase
Lysosensor
DiD
Merge
Merge
H
A
Lysosomes
Membrane
C.
Time
(min:sec)
Phase
Lysosensor
DiD
Merge
Merge
0:00
H
A
Lysosomes
Membrane
0:40
H
A

Chapter 4: Analysis of the role of EhRab7B in lysosomal function and amebic trogocytosis

Introduction

As discussed in Chapter 2, my previous data indicate that amebic lysosomes play a critical role in both trogocytosis and cell killing [140]. This work shed new light on an observation, first made 30 years ago, that weak bases inhibit amebic killing of human cells, demonstrating that acid vesicle neutralization acts through the inhibition of trogocytosis [32]. The work described in Chapter 2, as well as prior work on the role of amebic lysosomes in cell killing, involved the use of pharmacological inhibitors. A caveat to this approach is that inhibitors may have off-target effects, which could complicate the interpretation of the results. In the work discussed in Chapter 2, I used two different pharmacological inhibitors of lysosomal acidification with different mechanisms of action to minimize the likelihood that my findings would be confounded by off-target effects. However, the risk of off-target effects cannot be entirely eliminated, therefore I aimed to complement my work by using a genetic approach to interfere with the function of amebic lysosomes.

Despite the fact that amebic lysosomes were first identified as early as 1970, the organelle remains under study [153]. The amebic Rab GTPase EhRab7B has been localized to both phagosomes and lysosomes [72, 119]. Rab

GTPases are small GTPases that regulate the formation, trafficking and fusion of vesicles and thus play a crucial role in all endocytic processes. In mammals, the Rab GTPase Rab7 has been localized to late endosomes, lysosomes and autophagosomes (reviewed in [105]). Human Rab7 on late endosomes/lysosomes has been shown to recruit two effectors, Rab-interacting lysosomal protein (RIPL), which recruits dynein, and oxysterol binding protein-like 1A (OSBPL1A), which is required for dynein motor activity [101, 102]. Thus Rab7 is implicated in the microtubule-dependent translocation of late endosomes/lysosomes. GTP-bound mammalian Rab7 is crucial for recruiting the retromer complex, thus implicating Rab7 in retromer-mediated endosomal sorting [116].

The *E. histolytica* genome encodes a large array of Rab GTPases: more than 100 Rabs have been identified in *E. histolytica*, compared to 66 encoded in the human genome and 11 identified in *S. cerevisiae* [106-108]. Importantly, the *E. histolytica* genome encodes 9 Rab7 isoforms (Rab7A-I), while mammals typically encode one or two Rab7 isoforms (Rab7A and Rab7B) [72]. EhRab7A, B, C, D, and E have been associated with the amebic phagosome, but only two of these EhRabs, EhRab7A and EhRab7B have been studied in detail [117-119]. These two EhRab7s appear to have distinct functions. EhRab7A has been localized to both the phagosome and the prephagososmal vacuole (PPV), a structure that forms upon the initiation of phagocytosis and is believed to serve as a hub for the activation and storage of digestive proteins before transport to the phagosome [120, 121]. Work on EhRab7A has also suggested that it plays

an important role in trafficking proteins from the PPV and phagosomes to the Golgi apparatus. Similar to human Rab7A, EhRab7B has been localized to phagosomes and lysosomes [72, 119]. Previous work has shown that constitutive expression of a mutated GTP-bound form (H69L) of EhRab7B (EhRab7B-GTP-HA) results in moderately impaired phagocytosis, phagosome acidification and degradation of phagocytosed cells, suggesting that EhRab7B may play a role in trafficking of lysosomes or fusion of lysosomes with the phagosome [72].

Based on previous work, I hypothesized that interfering with EhRab7B would impair lysosomal trafficking and/or fusion and therefore inhibit amebic trogocytosis and cell killing. To interfere with the function of EhRab7B I constitutively expressed a mutant EhRab7B protein using the EhRab7B-GTP-HA construct developed by Saito-Nakano et al. [72] and a mutant EhRab7B protein using the EhRab7B-GDP-HA construct, that I developed. The EhRab7B-GTP-HA construct is expected to produce a constitutively active mutant protein, while the EhRab7B-GDP-HA construct is expected to produce a constitutively inactive mutant protein. Parasites were also transfected with an EhRab7B-wt-HA construct, which expresses wild-type EhRab7B with an HA epitope tag, and an empty vector construct, as a control. Western blot analysis confirmed that the EhRab7B-wt-HA, and EhRab7B-GTP-HA proteins were expressed at roughly equivalent levels, while the EhRab7B-GDP-HA protein was expressed at a significantly lower level in two independent transfections. Using imaging flow cytometry to quantitatively assess the rates of trogocytosis and host cell killing, I found no impact on amebic trogocytosis, phagocytosis or cell killing following

transfection with the empty vector, EhRab7B-wt-HA, EhRab7B-GTP-HA or EhRab7B-GDP-HA constructs.

Results

EhRab7B-GDP-HA is expressed at a dramatically lower level than either EhRab7B-GTP-HA or EhRab7B-wt-HA.

Amebic EhRab7B exhibits 51% sequence identity and 68% sequence similarity with human Rab7A (Fig 4.1). The putative amebic GTP-binding sequences are identical to the human GTP-binding sequences, with the exception of a histidine at position 69 (glutamine in humans) and an asparagine at position 129 (isoleucine in humans) (Fig 4.1).

There is currently no gene knockdown system for *E. histolytica.* Indeed efforts to establish a knockdown system have been complicated by the finding that *E. histolytica* has variable ploidy, ranging from 1n to 8n depending on life cycle stage and culture conditions [6, 7]. Therefore, I took advantage of the fact that Rabs act as molecular switches, cycling between an active, GTP-bound form and an inactive, GDP-bound form. I transfected parasites with plasmids to express wild-type EhRab7B with an amino terminal 3HA-tag (Rab7B-wt-HA), an EhRab7b H69L mutant with an amino terminal 3HA-tag (Rab7B-GTP-HA), an EhRab7b T24N mutant with an amino terminal 3HA-tag (Rab7B-GDP-HA) or the parent plasmid (empty vector), which lacks both the EhRab7B gene and the 3HA-tag. The constructs are constitutively expressed under the control of the cysteine synthase promoter, a strong amebic promoter. The EhRab7B-wt-HA

and EhRab7B-GTP-HA plasmids were both developed by Saito-Nakano et al. [72]. The EhRab7B-GTP-HA construct contains a single point mutation (A → T) in the GTP-binding sequence resulting in an amino acid change at position 69 from histidine to leucine (Fig 4.2A). In other organisms, a mutation to leucine at this position has been shown to impair GTP hydrolysis, thus locking the Rab in the GTP-bound 'active' confirmation [154, 155]. Generally, this results in a neutral or positive phenotype. Unexpectedly, Saito-Nakano et al. reported that the EhRab7B-GTP-HA mutant had a dominant-negative phenotype [72]. I constructed the EhRab7B-GDP-HA plasmid by PCR-mediated mutagenesis using EhRab7B-wt-HA as a template. The EhRab7B-GDP-HA construct contains a single point mutation (C → A) in the GTP-binding sequence resulting in an amino acid change at position 24 from threonine to asparagine (Fig 4.2A). In other organisms, this mutation has been shown to impair GTP binding, thus locking the Rab in the 'inactive' confirmation [154, 155]. Generally, this results in a dominant-negative phenotype. All plasmids were transfected in duplicate and transfected parasites were selected with 6μg/ml geneticin.

The expression of the HA-tagged constructs was assessed by western blot analysis. After probing with an anti-HA epitope tag antibody, I found that both the EhRab7B-wt-HA and EhRab7B-GTP-HA transfectants expressed a ~27kDa HA-tagged protein, corresponding to the expected size of the HA-tagged EhRab7B (Fig 4.B, lanes 2-5) [72]. Both transfectants, in duplicate, appeared to express the HA-tagged protein at approximately equivalent levels. Initially, the EhRab7B-GDP-HA transfectants did not appear to express an HA-tagged, as no

band was observed on the blot (Fig 4.B, lanes 6 and 7). However, a longer exposure of the blot confirmed that the duplicate EhRab7B-GDP-HA transfectants expressed a ~27kDa HA-tagged protein, albeit at a much lower level than either the EhRab7B-wt-HA transfectants or the EhRab7B-GTP-HA transfectants (Fig 4.2C, lanes 6 and 7). These results were further confirmed by imaging flow cytometry. Transfectants were permeablized with 0.2% Tween-20 and stained with an anti-HA antibody. I found that both the EhRab7B-wt-HA and EhRab7B-GTP-HA transfectants stained for an HA-tagged protein with equivalent intensity (Fig 4.2D). In contrast, the EhRab7B-GDP-HA transfectant stained for an HA-tagged protein at a much lower intensity, only 21% the intensity of the EhRab7B-wt-HA transfectants (Fig 4.2D). These findings suggests that expression of the EhRab7B-GDP-HA construct may be deleterious to the amebae. As expected, empty vector transfectants did not express an HA-tagged protein (Fig 4.2 C, lane 8). A recombinant *E. coli* protein containing an HA-tag was used as an antibody control (Fig 4.2 C, lane 9). Unfortunately, there is no available antibody against EhRab7B, therefore it was not possible to assess the relative expression of the HA-tagged proteins relative to the native protein.

Expression of EhRab7B-GTP-HA or EhRab7B-GDP-HA had no detectable impact on amebic trogocytosis and cell killing.

To assess the impact of the expression of the mutant EhRab7B proteins on amebic trogocytosis and cell killing, transfected parasites were labeled with CMFDA and then co-incubated with DiD-labeled human Jurkat T-cells for 5, 20 or

40 min. Following co-incubation of the parasites with the human cells, all cells were stained with the permeability dye Live/Dead Violet and fixed. I then assessed trogocytosis and cell killing using imaging flow cytometry. To quantify trogocytosis, I measured fragmentation of the human material within the amebae, as previously described by Ralston et al. (Fig 4.3A, Fig 2.2 shows the full gating strategy).

In empty vector transfectants the percentage of amebae that ingested a high number of human fragments increased over time from an average of 4.4% at 5 min to 33.2% at 40 min. The EhRab7B-wt-HA transfectants showed similar rates of ingestion, with an average of 29.4% of parasites ingesting a high number of human fragments after 40 min (Fig 4.3A). Contrary to our expectations, the EhRab7B-GTP-HA and EhRab7B-GDP-HA transfectants also showed comparable rates of ingestion, with an average of 30.1% and 29.8% of parasites ingesting a high number of human fragments after 40 min, respectively (Fig 4.3A). All four transfectants also showed similar rates of cell killing (Fig 4.3B). Together, these data suggest that neither the EhRab7B-GTP-HA transfectants nor the EhRab7B-GDP-HA transfectants have a defect in trogocytosis or cell killing.

Expression of EhRab7B-GTP-HA or EhRab7B-GDP-HA had no detectable impact on phagocytosis.

Amebic trogocytosis (ingestion of cell fragments) shares many similarities with phagocytosis (ingestion of whole cells). *E. histolytica* has been shown to

preferentially phagocytose (ingest whole) dead human cells and trogocytose (ingest fragments) live human cells [30]. To determine whether expression of the EhRab7B-GTP-HA or the EhRab7B-GDP-HA protein impaired phagocytosis, transfected parasites were co-incubated with heat-killed human Jurkat T-cells for 5 min, 20 min, or 40 min. Following co-incubation, all cells were fixed and phagocytosis was assessed using imaging flow cytometry (Fig 4.4A). I found that expression of the EhRab7B-GTP-HA construct did not impair phagocytosis when measured as the percentage of amebae that had phagocytosed ≥1 Jurkat T-cell (Fig 4.4B) or when measured as of the amount of ingested host material (Fig 4.4C). Similarly, I found that expression of the EhRab7B-GDP-HA construct did not impair phagocytosis (Fig 4.4B and C).

Discussion

In Chapter 2, my data suggest that amebic lysosomes play a critical role in both trogocytosis and cell killing [140]. Both the work described in Chapter 2 and the prior work on the role of amebic lysosomes in cell killing involved the use of pharmacological inhibitors [32]. Therefore, I aimed to complement my work in Chapter 2 by using a genetic approach to impair amebic lysosomal function. In the present study, I found that expression of mutant EhRab7B proteins did not impair amebic trogocytosis, cell killing, or phagocytosis.

EhRab7B is believed to play a role in trafficking of lysosomes or fusion of lysosomes with the phagosome [72]. Therefore, I endeavored to interfere with lysosomal function by disrupting EhRab7B. Parasites were transfected in

duplicate with one of two mutant EhRab7B constructs: the EhRab7B-GTP-HA construct expressed a mutant EhRab7B predicted to be locked in the 'active' conformation, while the EhRab7B-GDP-HA construct expressed a mutant EhRab7B predicted to be locked in the 'inactive' conformation. Parasites were also transfected with an EhRab7B-wt-HA construct expressing an HA-tagged EhRab7B protein and an empty vector construct as controls. I have shown that transfection with EhRab7B-GTP-HA or EhRab7B-GDP-HA had no detectable impact on amebic trogocytosis or cell killing (Fig 4.3). These results were unexpected because EhRab7B-GDP-HA was expressed at significantly lower levels than either EhRab7B-GTP-HA or EhRab7B-wt-HA, suggesting that expression of the mutant EhRab7B-GDP-HA protein had a negative impact on the amebae. Therefore, I also assessed the impact of EhRab7B-GTP-HA and EhRab7B-GDP-HA expression on phagocytosis of heat-killed Jurkats and found no discernable impact (Fig 4.4). This is consistent with previous work using a related EhRab7B-GTP fusion construct that showed that expression of the mutant protein had no impact on phagocytosis of yeast compared to expression of an EhRab7B-wt fusion protein [72].

There are several possible reasons that expression of the mutant EhRab7B proteins did not impact amebic trogocytosis or phagocytosis. As mentioned previously, *E. histolytica* expresses 9 different EhRab7 isotypes, thus it is possible that one or more of the 8 other EhRab7 isotypes may compensate for EhRab7B in the parasites expressing a mutant EhRab7B protein. It was not possible to assess the relative expression of the mutant EhRab7B proteins

relative to the native protein. Therefore it is also likely that the mutant proteins were not expressed at sufficiently high levels to significantly impair lysosomal function. Further study, potentially using inducible EhRab7B mutant constructs, may provide additional insight into the role of EhRab7B in lysosomal function.

Methods

Cell Culture

Amebic trophozoites (HM1:IMSS) were cultured axenically at 35° C in TYI-S-33 as previously described [38]. Trophozoites were harvested during log-phase growth by centrifugation at 200 x *g* for 5 min at room temperature, followed by resuspension in M199s (medium 199 [Gibco] without phenol red and supplemented with 5.7mM cysteine [Sigma], 0.5% bovine serum albumin [Gemini] and 25mM HEPES [Sigma] at pH 6.8) [30].

Human Jurkat T cells (Clone E6-1, ATCC) were grown at 37°C in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco), 10mM HEPES (Gibco), and 1mM sodium pyruvate (Gibco). Jurkat cultures were collected and enriched for viable cells as previously described [30].

Cloning and Transfection

Plasmids to express wild-type *Eh*Rab7B with a 5' 3HA-tag (Rab7B-wt-HA) and *Eh*Rab7b H69L mutant with a 5' 3HA-tag (Rab7B-GTP-HA) were a kind gift of Tomoyoshi Nozaki [72]. Plasmid to express *Eh*Rab7B T24N was constructed

using PCR-mediated mutagenesis (QuikChange Lightning, Agilent) usingEhRab7B-wt-HA as a template. Parent plasmid lacking both the 3HA-tag and gene insert (empty vector) was a gift of Mayuresh Abhyankar.

Transfections were performed in duplicate as previously described with some modifications [156]. Briefly, 20µg of plasmid in 1.8ml CryoTube vial (Thermo Scientific) was diluted in 200µl M199-LHA (Medium 199 supplemented with 5.7mM cysteine, 25mM HEPES, and 0.6mM ascorbic acid [Sigma] at pH 6.8). 30µl Attractene (Qiagen) was added to each tube. Tubes were incubated at room temperature to allow for transfection complex formation. Heat-inactivated adult bovine serum was added to the remaining M199-LHA to a final concentration of 15%. Amebae were collected and suspended in M199-LHA with serum at $2.5x10^5$ cells/ml. $4.5x10^5$ amebae were added to each transfection tube. Each plasmid was transfected in quadruplicate tubes. The tubes were mixed by inversion and incubated horizontally for 3h at 37°C. Contents of two duplicate transfection tubes were added to warm TYI-S-33 in a single $25cm^2$ tissue culture flask and incubated overnight at 37°C, resulting in 2 flasks per plasmid. After 24h incubation, 3µg/ml Geneticin (G418, Gibco) was added for selection. After 1-2 weeks selection was increased to 6µg/ml Geneticin.

Protein preparation and Western blotting

2x Laemmli sample buffer (Biorad) with 710mM ß-mercaptoethanol (Sigma) was heated to 75°C. Amebae were collected at 4°C and washed with cold PBS (Gibco). Samples were then pelleted at 4°C, placed on ice and the supernatant was removed. 0.1 volumes 10x Protease Inhibitor Cocktail (Sigma)

was added to hot 2x Laemmli sample buffer and sample was immediately resuspended in 2x Laemmli sample buffer with protease inhibitors at $1x10^6$ cells/ml and boiled for 2 min at 95°C. Samples were immediately run on a 12% SDS-PAGE gel. 2µl of *E. coli* whole cell lysate containing a 41 kDa recombinant protein with an N-terminal HA epitope tag (abcam) was also loaded run on the gel. The gel was transferred to a nitrocellulose membrane and treated with BLOT-FastStain (G-Biosciences) per the manufacturer's directions. Blots were then imaged and the BLOT-FastStain reagent was removed by shaking in warm deionized water for 10min, per the manufacturer's directions. Blots were washed extensively with deionized water, and blocked with 5% blocking solution (PBS supplemented with 5% bovine serum albumin, 5% milk and 0.05% Tween-20 [Sigma]), shaking, for 1h at room temperature. Blots were incubated with anti-HA.11 epitope tag monoclonal antibody (clone 16B12, Biolegend) at 1:1000 in blocking solution, shaking, for 1h. Blots were then washed in PBS-T (PBS with 0.05% Tween-20) and incubated with goat anti-mouse IgG HRP (Thermo Fischer Scientific) at 1:5000 in blocking solution, shaking for 1h. Blots were again washed with PBS-T and exposed to film (Kodak).

Amebic Trogocytosis and Cell Killing Assay

Amebic trogocytosis by amebae was measured using imaging flow cytometry as described previously with some modifications [30, 140]. Briefly, amebae were labeled with 200nM CellTracker Green CMFDA (Invitrogen) in M199S for 10min at 37°C and then washed twice with M199S. Jurkat cells were labeled with 5µM DiD ($DiIC_{18}(5)$-DS, Molecular Probes) in M199S for 5 min at

37C, followed by 10 min at 4C, and then washed twice with M199S. CFMDA-labeled amebae were co-incubated with DiD-labeled Jurkat cells at a ratio of 1:5, in biological duplicate at 37°C for 5 min, 20 min or 40 min. At the end of each time point, samples were immediately placed on ice and labeled with live/dead fixable violet (Invitrogen) at 1.6µl per ml for 30min in the dark. The cells were then fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, in the dark.

For internal HA-staining, fixed cells were washed with cold PBS, treated with cold 100mM glycine to quench free aldehydes, and permeablized in PBS supplemented with 0.2% Tween-20 for 1 min. Cells were blocked for 1h in blocking solution (0.2% Tween-20 supplemented with 5% bovine serum albumin). Cells were then washed with 0.2% Tween and incubated with an anti-HA.11 epitope tag monoclonal antibody (clone 16B12, Biolegend) at 1:1000 in blocking solution for 45min at 4°C, followed by goat anti-mouse Alexa Flour 546 (Invitrogen) at 10µg/ml in blocking solution for 45 min at 4C. Cells were resuspended in PBS and flow cytometry was performed using ImageStreamX Mark II (EMD Millipore). 10,000 events were collected for each sample and data were analyzed using IDEAS software (EMD Millipore).

Phagocytosis Assay

Jurkat cells were collected, washed with M199s and labeled with 5µM DiD (Molecular Probes) for 5 min at 37C, followed by 10 min at 4°C, and then washed

twice with M199S. Jurkat cells were then incubated at 55°C for 15min to induce necrotic cell death [38]. Amebae were collected, labeled with 200nM CMFDA in M199S for 10min at 37°C and washed twice with M199S. Amebae were then co-incubated with DiD-labeled heat-killed Jurkat cells in M199s for 5, 20, or 40 min at 37°C. After co-incubation, all samples were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. Flow cytometry was performed using ImageStreamX Mark II (EMD Millipore). 10,000 events were collected for each sample and data were analyzed using IDEAS software (EMD Millipore).

Statistical Analysis

Data were analyzed using Prism 6 (GraphPad Software). *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 4.1

Comparison of amino acid sequences of *E. histolytica EhRab7B* and human Rab7A. Sequence alignment of EhRab7B and *H. sapiens* Rab7A. Sequences were aligned by BLASTP 2.7.1+ (National Center for Biotechnology Information, NCBI). Amino acid residues that are identical between the two sequences are recorded in the center line. Amino acid similarity is indicated by '**+**' in the center line. The human GTP-binding sequences are indicated by blue boxes. The mutation nucleotides of the EhRab7B-GTP construct and the EhRab7B-GDP construct are indicated by a purple '★' and a red '★' respectively.

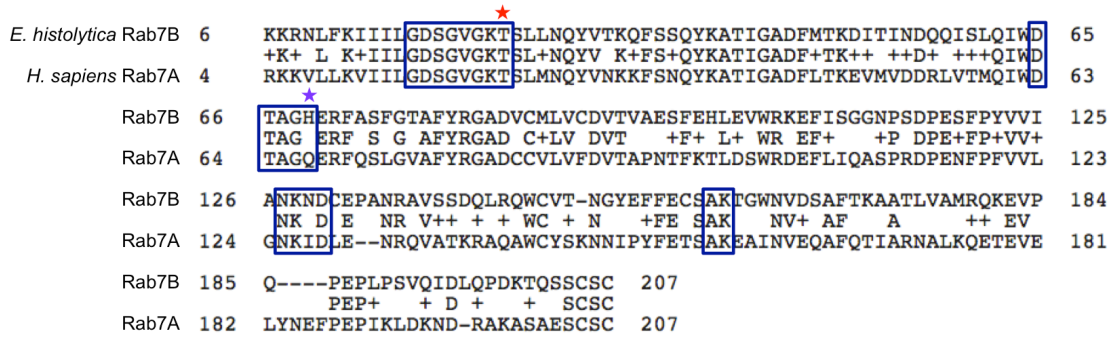
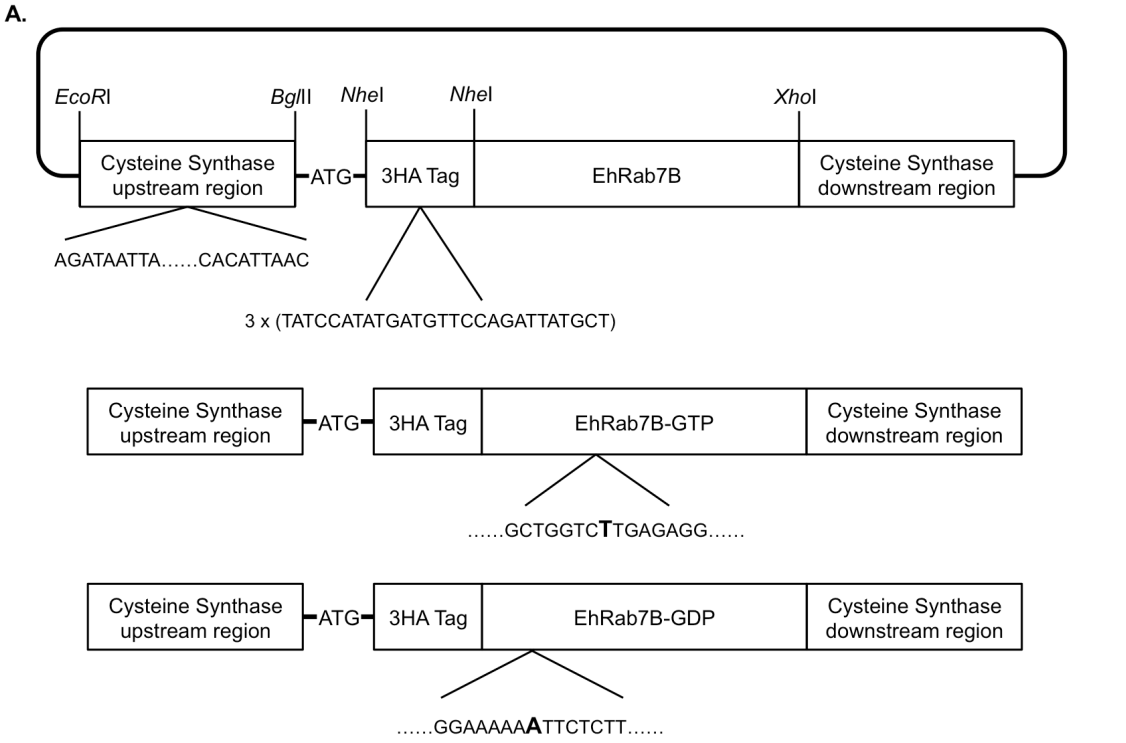

E. histolytica Rab7B 6 KKRNLFKIIILGDSGVGKTSLLNQYVTKQFSSQYKATIGADFMTKDITINDQQISLQIWD 65
+K+ L K+IILGDSGVGKTSL+NQYV K+FS+QYKATIGADF+TK++ ++D+ +++QIWD
H. sapiens Rab7A 4 RKKVLLKVIILGDSGVGKTSLMNQYVNKKFSNQYKATIGADFLTKEVMVDDRLVTMQIWD 63
Rab7B 66 TAGHERFASFGTAFYRGADVCMLVCDVTVAESFEHLEVWRKEFISGGNPSDPESFPYVVI 125
TAG ERF S G AFYRGAD C+LV DVT +F+ L+ WR EF+ +P DPE+FP+VV+
Rab7A 64 TAGQERFQSLGVAFYRGADCCVLVFDVTAPNTFKTLDSWRDEFLIQASPRDPENFPFVVL 123
Rab7B 126 ANKNDCEPANRAVSSDQLRQWCVT-NGYEFFECSAKTGWNVDSAFTKAATLVAMRQKEVP 184
NK D E NR V++ + + WC + N +FE SAK NV+ AF A ++ EV
Rab7A 124 GNKIDLE--NRQVATKRAQAWCYSKNNIPYFETSAKEAINVEQAFQTIARNALKQETEVE 181
Rab7B 185 Q----PEPLPSVQIDLQPDKTQSSCSC 207
PEP+ + D + + SCSC
Rab7A 182 LYNEFPEPIKLDKND-RAKASAESCSC 207

Figure 4.2

Construction and expression of HA-tagged EhRab7B, EhRab7B-GTP and EhRab7B-GDP. (A) Schematic representation of the plasmids. Plasmids to express EhRab7B-HA-GTP and EhRab7B-HA-GDP are derived from the plasmid to express HA-tagged wild type EhRab7B. Nucleotide sequences of selected regions are shown. Mutated nucleotides are highlighted in **bold**. **(B and C)** Western blot analysis of lysates from EhRab7B-HA-wt, EhRab7B-HA-GTP and EhRab7B-HA-GDP transfectants using BLOT-FastStain protein stain (top blot) or anti-HA antibody (bottom blot). Blot probed with anti-HA antibody (bottom blot) was exposed to film for **(B)** 30 sec or **(C)** 5 min. **(D)** Amebae were fixed, permeablized with 0.2% Tween-20, and incubated with an anti-HA.11 epitope tag monoclonal antibody. HA-staining was assessed by flow cytometry. 'Mean Intensity (anti-HA)' measures the mean intensity of HA staining per single amebae. Data was analyzed by one-way ANOVA with multiple comparisons using Prism 6. **** $p \leq 0.0001$.


A.
EcoRI
BglII
NheI
NheI
XhoI
Cysteine Synthase upstream region
ATG
3HA Tag
EhRab7B
Cysteine Synthase downstream region
AGATAATTA……CACATTAAC
3 x (TATCCATATGATGTTCCAGATTATGCT)
Cysteine Synthase upstream region
ATG
3HA Tag
EhRab7B-GTP
Cysteine Synthase downstream region
……GCTGGTCTTGAGAGG……
Cysteine Synthase upstream region
ATG
3HA Tag
EhRab7B-GDP
Cysteine Synthase downstream region
……GGAAAAAATTCTCTT……

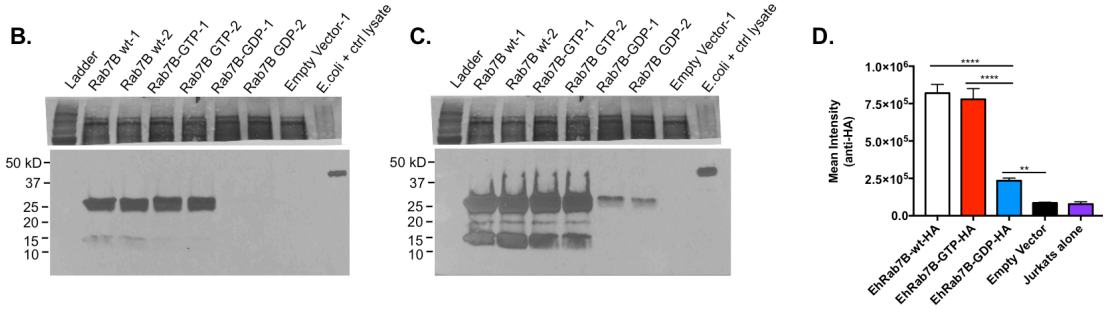

B.
Ladder
Rab7B wt-1
Rab7B wt-2
Rab7B-GTP-1
Rab7B GTP-2
Rab7B-GDP-1
Rab7B GDP-2
Empty Vector-1
E.coli + ctrl lysate
50 kD
37
25
20
15
10
C.
Ladder
Rab7B wt-1
Rab7B wt-2
Rab7B-GTP-1
Rab7B GTP-2
Rab7B-GDP-1
Rab7B GDP-2
Empty Vector-1
E.coli + ctrl lysate
50 kD
37
25
20
15
10
D.
Mean Intensity (anti-HA)
1.0×10^6
7.5×10^5
5.0×10^5
2.5×10^5
0.0
****
****
**
EhRab7B-wt-HA
EhRab7B-GTP-HA
EhRab7B-GDP-HA
Empty Vector
Jurkats alone

Figure 4.3

Expression of EhRab7B-HA-GTP and EhRab7B-HA-GDP do not impair amebic trogocytosis or cell killing. Amebae were incubated with Jurkat-T cells for 5, 20 or 40 min at 37°C. Afterwards all cells were stained with Live/Dead Violet on ice for 30 min and then fixed. Amebic trogocytosis and cell killing were analyzed using imaging flow cytometry. **(A)** % High gate measures the percentage of events in the high gate, reflecting the quantity of fragments that have been ingested by the parasites **(B)** % Jurkat dead measures the percentage of host cells staining with Live/Dead Violet. Data shown is representative of 5 independent experiments. Data was analyzed by one-way ANOVA with multiple comparisons test using Prism 6.

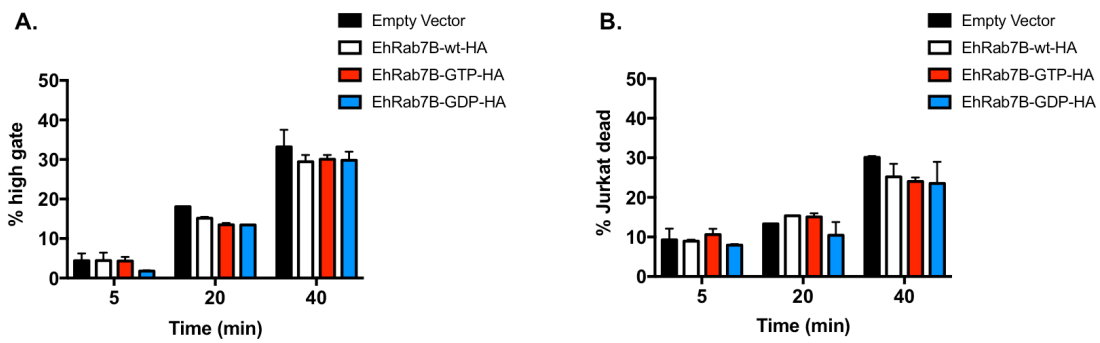
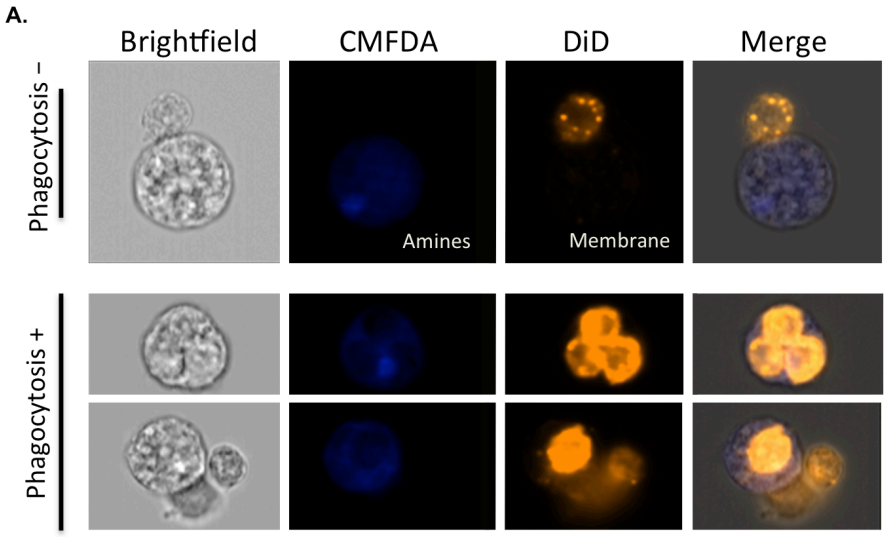
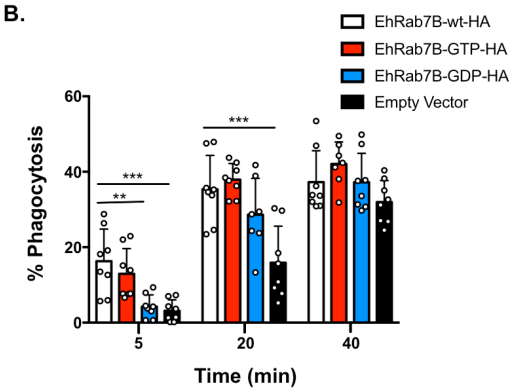
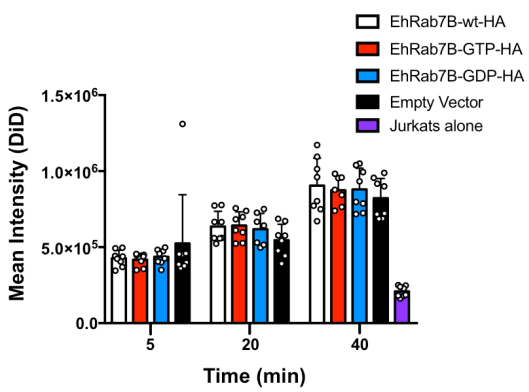

A.
Empty Vector
EhRab7B-wt-HA
EhRab7B-GTP-HA
EhRab7B-GDP-HA
% high gate
50
40
30
20
10
0
5
20
40
Time (min)
B.
Empty Vector
EhRab7B-wt-HA
EhRab7B-GTP-HA
EhRab7B-GDP-HA
% Jurkat dead
50
40
30
20
10
0
5
20
40
Time (min)

Figure 4.4

Expression of EhRab7B-HA-GTP and EhRab7B-HA-GDP do not impair phagocytosis. (A-C) Amebae were incubated with heat-killed Jurkat cells for 5, 20 or 40 min at 37°C. After co-incubation all cells were fixed. Phagocytosis was analyzed using imaging flow cytometry. **(A)** Imaging flow cytometry of amebae pre-labeled with CMFDA (blue, labels amines) and Jurkat T-cells pre-labeled with DiD (orange, labels membrane). Single amebae were gated for phagocytosis based on ameba area and intensity of DiD (Jurkat) staining. Top row, amebae alone or with a human cell outside the amebic plasma membrane were gated phagocytosis negative. Middle and bottom row, representative images of amebae with one or more human cells inside their outer membrane that were gated phagocytosis positive. **(B) '**% Phagocytosis' measures the percentage of single amebae that ingested one or more dead Jurkat-T cells. **(C) '**Mean intensity DiD' measures the mean intensity of DiD staining per amebae, a rough measure of the amount of ingested host material. Data shown combined from four independent experiments **(B,C).** Data was analyzed by one-way ANOVA with multiple comparisons test using Prism 6. *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.


A.
Brightfield
CMFDA
DiD
Merge
Phagocytosis –
Phagocytosis +
Amines
Membrane


B.
EhRab7B-wt-HA
EhRab7B-GTP-HA
EhRab7B-GDP-HA
Empty Vector
% Phagocytosis
Time (min)
***
**
***


C.
EhRab7B-wt-HA
EhRab7B-GTP-HA
EhRab7B-GDP-HA
Empty Vector
Jurkats alone
Mean Intensity (DiD)
Time (min)

Chapter 5: Conclusions and Future Directions

Amebic lysosomes play a crucial role in amebic trogocytosis.

Tissue destruction is the hallmark of invasive *E. histolytica* infection, manifesting as massive intestinal ulceration or abscesses in other sites. *E. histolytica* is highly cytotoxic to a wide range of human cells, and parasite cytotoxic activity is likely to drive tissue destruction. The parasite is able to kill human cells by ingesting pieces of live human cells until the human cell eventually dies, a process termed amoebic trogocytosis [30]. However, we lack an understanding of the mechanism of amebic trogocytosis and cell killing.

Trogocytosis has been observed in a range of eukaryotes, from protozoa to mammals. As yet, there have been relatively few studies on trogocytosis – a PubMed search for the term ‘trogocytosis’ returned only 165 results as of January 2018 – and there have been even fewer studies investigating the underlying mechanism of trogocytosis. Trogocytosis has been most extensively studied in human lymphocytes. Lymphocyte trogocytosis typically involves the transfer of plasma membrane fragments (containing membrane and surface proteins) from an antigen-presenting cell to a lymphocyte [36, 40]. For instance, it has been shown that T-cells can trogocytose peptide-MHC complexes from antigen presenting cells and present those complexes to other T-cells [43]. In

NK-cells, it has been shown that trogocytosis is a metabolically active process which requires signaling in the acceptor cell, and modulation of both the actin cytoskeleton and intracellular Ca2+ [157]. Phosphatidylinositide 3-kinase (PI3K) and the small GTPases TC21 and RhoG, have been identified as important effectors in T-cell trogocytosis [51]. However, the precise mechanism of trogocytosis in lymphocytes remains unclear.

As the general mechanism of trogocytosis is poorly understood, previous work on amebic trogocytosis has investigated whether effectors with a known role in phagocytosis also play a role in trogocytosis. Thus it has been shown that both amebic trogocytosis and phagocytosis are receptor-dependent processes, requiring attachment to a target cell via the amebic Gal/GalNAc lectin [30, 31]. Both processes also require amebic actin rearrangement and signaling via PI3K and *E. histolytica* C2-domain-containing protein kinase (EhC2PK) [30, 38, 39].

Early research on *Entamoeba histolytica* indicated that inhibiting amebic lysosomal acidification impaired amebic killing of human cells [32]. Interestingly, studies comparing *E. histolytica* with the less-pathogenic species *Entamoeba dispar* have found that phagosomes in *E. histolytica* were acidified much more quickly and reached a lower pH than phagosomes in *E. dispar*, suggesting a role for lysosomes in the pathogenesis of amebiasis [125]. Previous work has demonstrated that *E. histolytica* is able to kill human cells through amebic trogocytosis. Therefore, we hypothesized that amebic lysosomes are crucial for continued amebic trogocytosis and cell killing.

This hypothesis is supported by our findings that disruption of lysosomal function using two independent acidification inhibitors dramatically decreased amebic trogocytosis and cell killing [140]. We observed that interfering with lysosomal acidification did not impact the initiation of amebic trogocytosis, but rather impaired continued ingestion and cell killing. This suggests that amebic lysosomes are not required for the initial attachment of amebae to human cells. *E. histolytica* has been shown to acidify phagosomes within 2 minutes, suggesting that lysosomes are rapidly recruited and fuse with the phagosome [125]. Thus, our data are consistent with the hypothesis that the inhibitor-treated parasites fail to rapidly acidify the trogosome (vesicle containing an ingested fragment), resulting in slowed or blocked ingestion.

We hypothesize that amebic lysosomes are crucial for amebic trogocytosis and cell killing because they are required for both efficient degradation of ingested material and for the rapid recycling of internalized amebic surface receptors. This hypothesis is supported by our finding that disruption of lysosomal digestion via inhibition of amebic cysteine proteases significantly reduced both amebic trogocytosis and cell killing. Additional evidence comes from our observation that interfering with vesicle acidification blocked amebic trogocytosis and phagocytosis, both receptor-dependent processes, but did not impair fluid-phase endocytosis, a receptor-independent process, as well as our observation that there was a significant decrease in the amount of Gal/GalNAc lectin on the surface of inhibitor-treated parasite compared to control parasites following trogocytosis.

It is possible that amebic lysosomes may be required to form an acidified synapse at the site of host cell attachment [134]. In general, I observed that amebic lysosomes appeared to be randomly dispersed throughout the parasite during trogocytosis. In rare cases, however, amebic lysosomes appeared to cluster close to the ameba-host interface and to co-localize with ingested fragments. It is possible that these rare events are attributable to the formation of an acidified synapse at the site of ameba-host interaction, but it is also possible that the events result from random chance. Unfortunately, we found that our current microscopy equipment was insufficient to capture the highly dynamic movement of the amebic lysosomes during trogocytosis. Therefore, further study using a more advanced microscope is needed to examine whether such a synapse is formed.

We aimed to confirm our findings by interfering with amebic lysosomal function through a genetic approach. Parasites were transfected to express mutant EhRab7B proteins, with the aim of impairing lysosomal trafficking and/or the fusion of lysosomes with the trogosome. Unfortunately, we found that while the mutant EhRab7B proteins were expressed, their expression had no impact on amebic trogocytosis, cell killing or phagocytosis. It is possible that one of the eight other EhRab7s may be able to compensate for EhRab7B. It is also possible that the mutant EhRab7B proteins were not expressed at sufficiently high levels, as we were unable to assess the expression levels of mutant EhRab7B proteins relative to the native protein. Future work could take advantage of inducible

expression systems to provide additional insight into the role of EhRab7B in lysosomal function.

Amebic lysosomes may have different functions in amebic trogocytosis and phagocytosis.

Amebic trogocytosis (ingestion of cell fragments) shares many similarities with another endocytic process, phagocytosis (ingestion of whole cells), thus an important question is whether amebic trogocytosis is mechanistically distinct from phagocytosis (Fig. 5.1) [30]. Murine dendritic cells have been shown to segregate microbial and apoptotic cells into distinct phagosomes [158]. Similarly, human neutrophils were shown to phagocytose zymosan (derived from yeast cell walls) and antibody-coated beads in different ways, and at different rates [159]. These studies suggest that different targets may be phagocytosed via different pathways. Studies have also indicated that target size, shape, and rigidity can influence the rate and mode of target internalization as well as the subsequent pathway of the ingested target (reviewed in [150]). A study investigating the binding and uptake of beads of various shapes and sizes found that both bead size and shape impact actin recruitment in murine macrophages [160]. It has been shown that murine macrophages preferentially ingest rigid targets rather than less rigid targets [161]. Notably, the surfaces of bacteria, yeast and other microbes with cells walls are more rigid than mammalian cells [161]. Several studies have suggested that the engagement of different receptors or a single

receptor by different ligands can result in differing phagocytic pathways [152, 158, 162, 163]. Taken together, these findings suggest that trogocytosis likely involves a distinct set of effectors and signaling pathways.

Thus far, no pathways unique to trogocytosis have been discovered in any organism. During ingestion of Chinese hamster ovary cells (CHO cells) and human red blood cells (RBCs) it has been observed that a ‘tunnel’ of CHO or RBC material stretched into the parasite, referred to as ‘suction’ or ‘microphagocytosis’ [164-166]. Similar ‘tunnels’ have also been observed during amebic trogocytosis [30]. Increasing the rigidity of RBCs by exposure to increasing concentrations of fixatives reduced ‘microphagocytosis’ suggesting that that target cell deformability impacts the mechanism of ingestion [164]. It has also been observed that *E. histolytica* will preferentially phagocytose (ingest whole) dead human cells and trogocytose (ingest fragments) live human cells [30]. Intriguingly, it has recently been reported that the amebic kinase AGC family kinase 1 (EhAGCK1) appears to localize differently during trogocytosis of CHO cells compared with ingestion of human RBCs [139]. Together, these findings suggest that that there may be pathways that are unique to amebic trogocytosis.

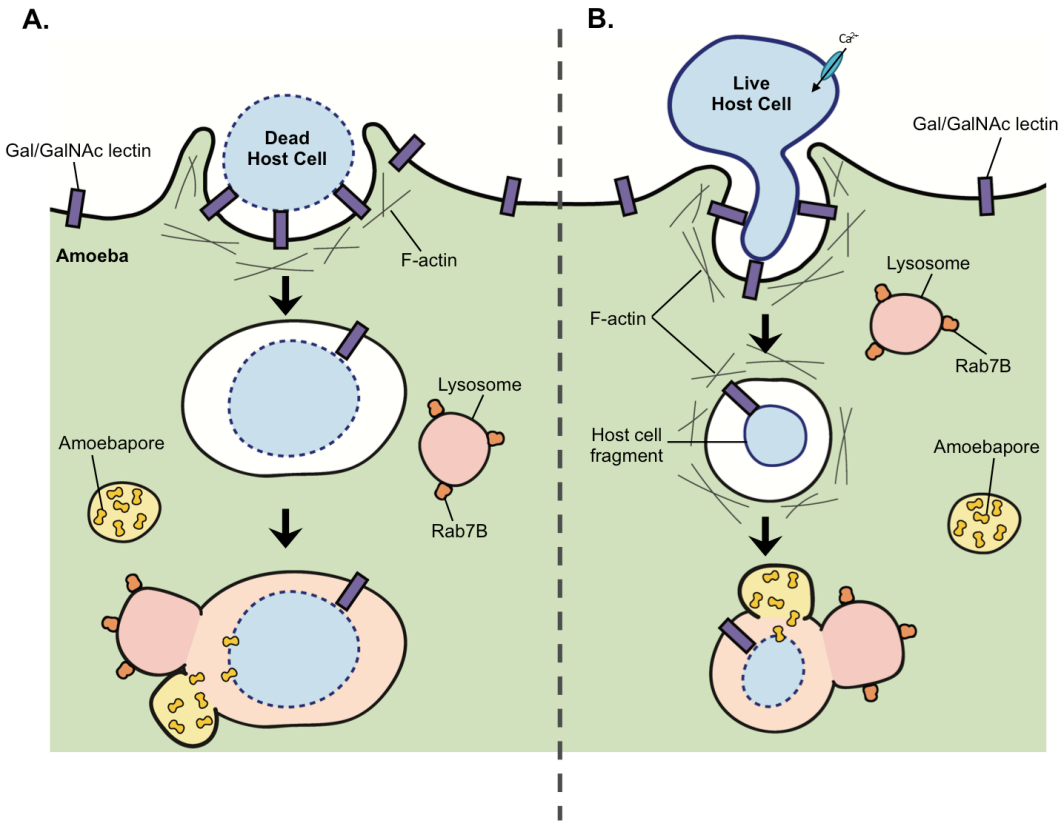
In Chapter 2, we report that impairing lysosomal function through the inhibition of lysosomal acidification dramatically reduced both amebic trogocytosis and phagocytosis, suggesting that some lysosomal functions are required for both processes. Most lysosomal functions are expected to be impaired by increases in lysosomal pH, as the majority of these functions are likely dependent on a low pH environment. In contrast, when we found that

impairing lysosomal digestion through the inhibition of amebic cysteine proteases significantly reduced amebic trogocytosis, but did not impact phagocytosis, suggesting that there may be distinct lysosomal functions required for amebic trogocytosis.

There are several possible roles that lysosomal cysteine proteases might play in amebic trogocytosis, which are discussed in detail in Chapter 3 (see 'Discussion' section). Briefly, amebic cysteine proteases may be required to activate trogocytosis-specific receptors or to expose ligands for trogocytosis-specific signaling. It is also possible that trogosomes mature more quickly than phagosomes, and thus trogocytosis is more sensitive to protease inhibition than phagocytosis. These possibilities offer interesting avenues for further investigation of the pathways and effectors unique to amebic trogocytosis.

Figure 5.1

Model for Phagocytosis and Amebic Trogocytosis. (**A)** Phagocytosis begins with attachment of the ameba (green) to the dead host cell (blue) via the parasite surface receptor Gal/GalNAc lectin (purple). Following attachment, the parasite ingests the whole target cell, a process that requires actin rearrangement. The phagosome then undergoes maturation and eventually fuses with Rab7B-associated lysosomes. The phagosome can also fuse with vesicles containing amoebapores, amebic pore-forming proteins which likely aid in breakdown of the phagosome contents. **(B)** Similar to phagocytosis, amebic trogocytosis begins with attachment of the ameba (green) to the host cell (blue) via Gal/GalNAc lectin. Following attachment, the parasites ingest fragments of the host cell, a process that also requires actin rearrangement. The ingested fragments appear to be surrounded by two membranes, an outer membrane derived from the amebic plasma membrane and an inner membrane derived from the host plasma membrane. After the initiation of trogocytosis, a rapid rise in intracellular Ca^{2+} is observed in the host cell. The trogosome then undergoes maturation and eventually fuses with Rab7B-associated lysosomes. The trogosome can also fuse with vesicles containing amoebapores, which likely aid in breakdown of the phagosome contents.


A.
B.
Gal/GalNAc lectin
Dead
Host Cell
Amoeba
F-actin
Lysosome
Amoebapore
Rab7B
Live
Host Cell
Ca2+
Gal/GalNAc lectin
Lysosome
F-actin
Rab7B
Host cell
fragment
Amoebapore

Appendix: Exploring the role of amoebapores in amebic trogocytosis and cell killing

Introduction

Amoebapores are a family of three pore-forming proteins: Amoebapore A (ApA), Amoebapore B (ApB) and Amoebapore C (ApC). Amoebapores are believed to play a central role in host cell killing by *E. histolytica*. The proteins have sequence similarity to pore-forming proteins produced by mammalian lymphocytes [66]. Purified amoebapores are toxic to both eukaryotic cells and bacterial cytoplasts, however high concentrations are required to kill eukaryotic cells and the dying cells do not exhibit the same features as cells directly killed by the amebae [67, 68]. ApA is the most highly expressed and well-studied of the three amoebapores. There is no gene knock out system for *E. histolytica.* It was found that epigenetic gene silencing of ApA resulted in decreased cytotoxicity [69]. However, the silencing approach that was used affected chromatin structure and altered the expression of many genes [70]. Therefore, it is not clear if the phenotype is due to silencing of ApA.

It has long been thought that amoebapores are secreted from the parasite and kill host cells by directly modifying their membranes. However, secretion has not been experimentally demonstrated, nor have any of the amoebapores been demonstrated to traffic to host cell membranes. In fact, amoebapores absolutely

require low pH (~pH 5.2) for pore-forming activity [71]. Additional studies have shown that amoebapores localize to lysosomes and co-localize with ingested bacteria [72, 73]. Indeed, it was noted that ApA-silenced parasites degraded ingested bacteria less efficiently [73]. Together, the existing data are not sufficient to support the proposed role of the amoebapores as secreted cell killing effectors. Taken together, these findings suggest that amoebapores function as pore-forming proteins in the amebic lysosome, rather than as extracellular effectors. I hypothesize that amoebapores act in cell killing by aiding in the processing of trogocytosed host material.

Results

Construction of plasmids to silence ApA, ApB, ApC and ApA-ApB-ApC in tandem.

To assess whether amoebapores function in trogocytosis, I first targeted Amoebapore A (ApA), since previous studies employing epigenetic gene silencing demonstrated that silencing of ApA is sufficient to reduce cell killing [69]. I also targeted Amoebapore B (ApB) and Amoebapore C (ApC) for silencing. However, previous studies on the silencing of ApA were complicated by off-target silencing of additional genes [69, 167]. It is also possible that the parasite might compensate to some extent for a knockdown of one amoebapore by increasing expression of one or both of the other amoebapores. Therefore, I also targeted all three amoebapores for simultaneous knockdown. I used an RNAi-based plasmid silencing system, which harnesses the endogenous RNAi

machinery of *E. histolytica* (Fig. A.1A) [168]. The plasmid contains a 'trigger' gene that is endogenously silenced. Insertion of a gene of interest downstream of the 'trigger' causes silencing of the gene of interest, due to RNA-dependent RNA polymerase activity, which results in spreading of gene silencing [168]. I inserted ApA, ApB, ApC or all three Ap genes (ApA-ApB-ApC) into the silencing plasmid developed by Morf et al. (Fig. A.1B).

Preliminary results suggest that transfection with ApA silencing plasmid impairs amebic trogocytosis.

I transfected parasites with the ApA silencing vector or the empty vector as a control, in duplicate. I attempted to assess ApA silencing using Western blot analysis, however, I was unable to detect ApA in empty vector parasites using an anti-ApA antibody. I also attempted to assess ApA silencing using qRT-PCR, however the RNA isolated from the transfected parasites was of insufficient quality. To assess the impact of transfection with the ApA silencing plasmid on amebic trogocytosis and cell killing, CMFDA-labeled transfected parasites were co-incubated with DiD-labeled human Jurkat T-cells or media alone for 5, 20 or 40 min. Following co-incubation of the parasites with the human cells, all cells were stained with the permeability dye Live/Dead Violet and fixed. I then assessed trogocytosis and cell killing using imaging flow cytometry. To quantify trogocytosis, I measured fragmentation of the human material within the amebae, as previously described by Ralston et al. (Fig A.3, Fig 2.2 shows the full gating strategy). Intriguingly, one of the two ApA transfectants showed a significant

decrease in trogocytosis and a trend towards decreased cell killing compared to empty vector control. This suggests that transfection with the ApA silencing plasmid may result in decreased amebic trogocytosis and cell killing. Additional work is needed to determine whether this is the case.

Future Directions

Additional work is needed to determine whether amoebapores play a role in amebic trogocytosis. Plasmids to silencing ApA, ApB, ApC or all three amoebapores in tandem (ApA-ApB-ApC) have been constructed and preliminary results are encouraging that ApA may have a role in trogocytosis. Bracha et al. previously reported that parasites with a 60% decrease in ApA expression by western blotting showed a significant decrease in cell killing [69]. In future work, knockdown efficacy should be assessed at the protein level for ApA using improved protein preparation protocols. Unfortunately, antibodies are only available for ApA [169]. mRNA levels of all three amoebapores should be monitored by real-time RT-PCR to assess knockdown efficacy and to monitor for off-target effects of silencing individual amoebapores. Parasites transfected with ApA, ApB, ApC or ApA-ApB-ApC should be assessed for their ability to trogocytose and kill human cells. To further control for off-target effects, knockdown mutants could be complemented with a knockdown-resistant re-coded copy of the silenced gene(s).

Methods

Cell Culture

Amebic trophozoites (HM1:IMSS) were cultured axenically at 35° C in TYI-S-33 as previously described [38]. Trophozoites were harvested during log-phase growth by centrifugation at 200 x *g* for 5 min at room temperature, followed by resuspension in M199s (medium 199 [Gibco] without phenol red and supplemented with 5.7mM cysteine [Sigma], 0.5% bovine serum albumin [Gemini] and 25mM HEPES [Sigma] at pH 6.8) [30].

Human Jurkat T cells (Clone E6-1, ATCC) were grown at 37°C in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco), 10mM HEPES (Gibco), and 1mM sodium pyruvate (Gibco). Jurkat cultures were collected and enriched for viable cells as previously described [30].

Cloning and Transfection

The gene silencing plasmid pKT-Rom was a kind gift of Upinder Singh [168]. Plasmids to silence ApA, ApB, ApC and ApA-ApB-ApC were constructed using pKT-Rom as a template. The full-length ApA, ApB or ApC nucleotide sequence was inserted into pKT-Rom at using the SmaI and XhoI restriction sites. Numerous attempts to insert ApA, ApB and ApC in tandem into pKT-Rom failed. Therefore a construct containing the full-length sequences of ApA, ApB and ApC in tandem was obtained from IDT. This construct contains a single

nucleotide deletion at position 777. The ApA-ApB-ApC was subsequently inserted into pKT-Rom using the SmaI and XhoI restriction sites.

Transfections were performed in duplicate as previously described with some modifications [156]. Briefly, 20µg of plasmid in 1.8ml CryoTube vial (Thermo Scientific) was diluted in 200µl M199-LHA (Medium 199 supplemented with 5.7mM cysteine, 25mM HEPES, and 0.6mM ascorbic acid [Sigma] at pH 6.8). 30µl Attractene (Qiagen) was added to each tube. Tubes were incubated at room temperature to allow for transfection complex formation. Heat-inactivated adult bovine serum was added to the remaining M199-LHA to a final concentration of 15%. Amebae were collected and suspended in M199-LHA with serum at $2.5x10^5$ cells/ml. $4.5x10^5$ amebae were added to each transfection tube. Each plasmid was transfected in quadruplicate tubes. The tubes were mixed by inversion and incubated horizontally for 3h at 37°C. Contents of two duplicate transfection tubes were added to warm TYI-S-33 in a single $25cm^2$ tissue culture flask and incubated overnight at 37°C, resulting in 2 flasks per plasmid. After 24h incubation, 3µg/ml Geneticin (G418, Gibco) was added for selection.

Protein preparation and Western blotting

Amebae were collected at 4°C and washed with cold PBS (Gibco). Samples were then pelleted at 4°C, placed on ice and resuspended in Lysis buffer (50mM Tris-Cl pH 8.3, 150mM NaCl, 1% Nonidet P40, 1x protease inhibitor cocktail [Sigma]) at $1x10^4$ cells/µl. 0.25 volumes of 4x Laemmli sample buffer was added and samples were boiled for 5 min at 95°C. Samples were allowed to cool to room temperature, spun at 12,000xg for 1min to pellet cell

debris, and the supernatant was run on a 12% SDS-PAGE gel. The gel was transferred to a nitrocellulose membrane and blocked with 5% blocking solution (PBS supplemented with 5% bovine serum albumin, 5% milk and 0.05% Tween-20 [Sigma]), shaking, for 1h at room temperature. Blots were incubated with anti-ApA antibody at 1:500 or anti-URE3-BP monoclonal antibody (4D6) at 10µg/ml in blocking solution, shaking, for 1h. Blots were then washed in PBS-T (PBS with 0.05% Tween-20) and incubated with goat anti-mouse IgG HRP (Thermo Fischer Scientific) at 1:5000 in blocking solution, shaking for 1h. Blots were again washed with PBS-T and exposed to film (Kodak).

Amebic Trogocytosis and Cell Killing Assay

Amebic trogocytosis by amebae was measured using imaging flow cytometry as described previously with some modifications [30, 140]. Briefly, amebae were labeled with 200nM CellTracker Green CMFDA (Invitrogen) in M199S for 10min at 37°C and then washed twice with M199S. Jurkat cells were labeled with 5µM DiD ($DiIC_{18}(5)$-DS, Molecular Probes) in M199S for 5 min at 37C, followed by 10 min at 4C, and then washed twice with M199S. CFMDA-labeled amebae were co-incubated with DiD-labeled Jurkat cells at a ratio of 1:5, in biological duplicate at 37°C for 5 min, 20 min or 40 min. At the end of each time point, samples were immediately placed on ice and labeled with live/dead fixable violet (Invitrogen) at 1.6µl per ml for 30min in the dark. The cells were then fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, in the dark.

Statistical Analysis

Data were analyzed using Prism 6 (GraphPad Software). *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure A.1

Silencing system developed by Morf et al. (A) Insertion of ApA gene downstream of the 'trigger' gene results in the generation of small RNAs against ApA, and silencing of ApA. **(B)** Schematic of ApA-ApB-ApC. Image adapted from Morf et al. 2013.

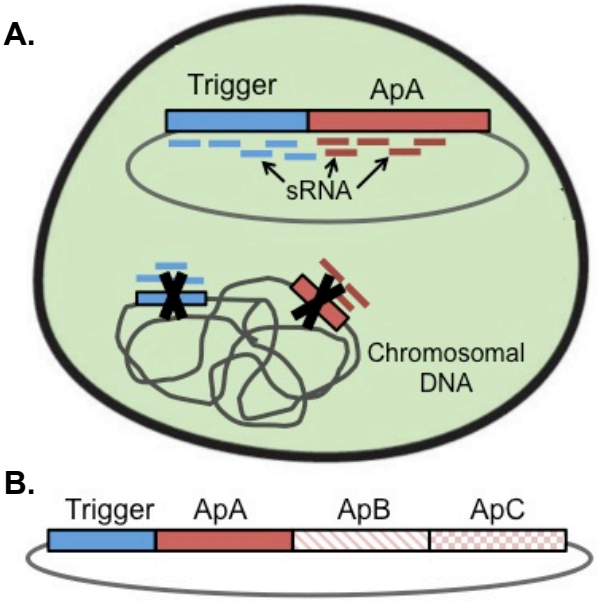
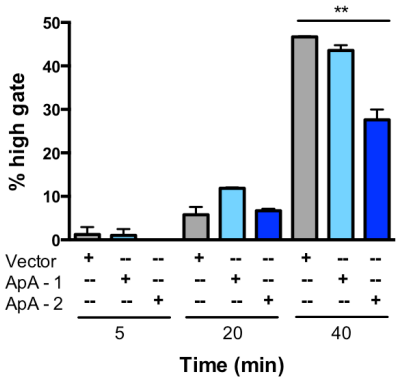
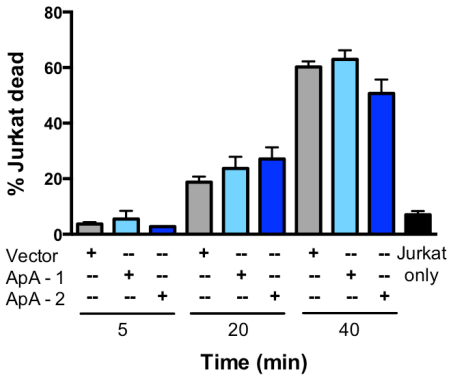

A.
Trigger
ApA
sRNA
Chromosomal
DNA
B.
Trigger
ApA
ApB
ApC

Figure A.2

Transfection with ApA silencing plasmid decreases amebic trogocytosis in one of two transfectants. Amebae transfected with the ApA silencing plasmid in two independent transfections (ApA-1 or ApA-2) or amebae transfected with an vector control were incubated with Jurkat-T cells for 5, 20 or 40 min at 37°C. Afterwards all cells were stained with Live/Dead Violet on ice for 30 min and then fixed. Amebic trogocytosis and cell killing were analyzed using imaging flow cytometry. **(A)** % High gate measures the percentage of events in the high gate, reflecting the quantity of fragments that have been ingested by the parasites **(B)** % Jurkat dead measures the percentage of host cells staining with Live/Dead Violet. Data shown is representative of 1 experiment. Data was analyzed by one-way ANOVA with multiple comparisons test using Prism 6. *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.


**
50
40
30
20
10
0
% high gate
Vector + -- -- + -- -- + -- --
ApA - 1 -- + -- -- + -- -- + --
ApA - 2 -- -- + -- -- + -- -- +
5
20
40
Time (min)


80
60
40
20
0
% Jurkat dead
Vector + -- -- + -- -- + -- -- Jurkat
ApA - 1 -- + -- -- + -- -- + -- only
ApA - 2 -- -- + -- -- + -- -- +
5
20
40
Time (min)

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