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Regulation of Virulence of *Entamoeba histolytica*

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Abstract

Entamoeba histolytica is the third leading cause of parasitic mortality globally. *E. histolytica* infection is generally asymptomatic but the parasite has potent pathogenic potential. The origins, benefits and triggers of amebic virulence are complex. Amebic pathogenesis entails depletion of the host mucosal barrier, adherence to the colonic lumen, cytotoxicity and invasion of the colonic epithelium. Parasite damage results in colitis and in some cases, disseminated disease. Both host and parasite genotypes influence the development of disease, as do the regulatory responses they govern at the host-pathogen interface. Host environmental factors determine parasite transmission and shape the colonic microenvironment *E. histolytica* infects. Here we highlight research that illuminates novel links in host, parasite and environmental factors in the regulation of *E. histolytica* virulence.

Keywords

Virulence; microbiota; pathobiota; mucous; carbohydrate utilization

INTRODUCTION

Evolution of *E. histolytica* virulence

Entamoeba histolytica is an ancient obligate parasite of humans. The first documented case of amebiasis may be a Sanskrit description of bloody mucoid diarrhea from 3000 BCE (67). Today *E. histolytica* is estimated cause ~50 million cases of disease and 40,000–100,000 deaths annually (3, 56, 123). Evolutionary theories posit that obligate human parasites evolve toward commensalism but *E. histolytica* has maintained a potent capacity for virulence in comparison to the recently diverged avirulent *Entamoeba dispar*. The high frequency of chronic asymptomatic infection with *E. histolytica* and *E. dispar* indicates that virulence is dispensable for parasite survival, replication and transmission. In addition, virulence does not seem to confer a selective advantage in these processes. Similar levels of *E. histolytica* parasites were found in symptomatic and asymptomatic hosts, thus disease does not appear to enhance replicative capacity (58). Similarly, asymptomatic

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hosts passed high levels of infectious amebic cysts, apparently obviating virulence for transmission (84). *E. histolytica* virulence has been proposed to originate from coincidental selection by bacterial killing but coincidental selection does not explain why *E. dispar* which also kills and consumes bacteria is avirulent. Despite an uncertain evolutionary role, the mechanisms of *E. histolytica* virulence are increasingly understood. Virulence can also be a consequence of the immune responses in the host. *E. histolytica* has the capacity to cause serious infections in immunocompetent hosts but may be more severe in hosts with impaired immune function. *E. histolytica* virulence is not predictable and depends on multiple factors that determine the capacity of the parasite to damage its host.

Epidemiology of *E. histolytica*

E. histolytica is endemic to areas of Mexico, Central and South America, Asia, Africa, and the Pacific islands and where transmission occurs via fecally-contaminated food and water (reviewed in 8,9). *E. histolytica* is also common in parts of Asia and Australia in men who have sex with men (MSM) and can be transmitted sexually (reviewed in 10). A recent study in Japan detected antibodies to *E. histolytica* in 21% of 1303 HIV positive patients, 88% of *E. histolytica* infections were in MSM (138). HIV infection is associated with *Entamoeba* infection but HIV and AIDS do not appear to increase *E. histolytica* disease. *E. histolytica* infection extends beyond endemic regions as the third most common cause of chronic diarrhea in returning travelers (112).

Estimating the global burden of amebiasis is complicated by limited diagnosis capacities and surveillance in most endemic areas. In addition, there is extreme intra-host variability in incubation period and disease presentation. Only 10–20% of *E. histolytica* infections result in disease, which encompasses self-limiting colitis, invasive colitis, extra-intestinal infection and invasive organ abscess (56, 123). These clinical manifestations are not necessarily sequential and may appear after years of asymptomatic infection (56, 109, 123).

Multiple community based prospective studies in Bangladesh have revealed the consequences of *E. histolytica* infection in endemic regions, particularly for children (54, 55, 57, 90). In combination, these studies found that *E. histolytica* infection had a cumulative incidence of ~30% by 1 year of age reaching 60–90% by four years of age (54, 55, 57, 90). Despite the high prevalence of infection, only 3–10% of diarrheal episodes in this population were attributable to *E. histolytica* (55, 57, 125) (Figure 1). The case-control Global Enteric Multicenter Study (GEMS) found a similar incidence of *E. histolytica*-diarrhea in hospitalized children (2.0% in Mali and 3.4% in Bangladesh) though *E. histolytica* was the pathogen with the highest hazard ratio for death in the second year of life (71). Likewise, *E. histolytica* was significantly increased in cases of severe diarrhea relative to all diarrhea in Bangladesh (6.6% versus 10.3%) (125). Thus while symptomatic *E. histolytica* disease is rare it is associated with severe outcomes likely due to specific host vulnerabilities in combination with parasite virulence attributes. It is also worth noting that some children had no evidence of *E. histolytica* infection (seroconversion and/or PCR-detection in monthly stool samples) in the first two years of life.

Pathogenesis in the human host

The species name *histolytica* is derived from ancient Greek for tissue dissolving. Befitting its name, the parasite is capable of extensive tissue destruction. *E. histolytica* infection is established by parasite adherence to the colonic mucin layer. Trophozoites express a surface lectin with high affinity for galactose (Gal) and *N*-acetyl-D-galactosamine oligosaccharides (GalNAc) on host mucin and cells (reviewed in 22). Initial *E. histolytica* infection induces thickening of the mucosal layer, potentially due *E. histolytica* from contacting the intestinal epithelium. *E. histolytica* also produces a mucin secretagogue that stimulates goblet cell mucin secretion. Concerted glycosidase and protease activity can mediate degradation of the mucus polymer (Figure 1). In the absence of mucin, the amebic Gal/GalNAc lectin binds to Gal and GalNAc residues on the surface of exposed intestinal epithelial cells (IECs). Progressive disease is marked by mucin depletion, IEC flattening, and infiltrating neutrophils (104). In addition, *E. histolytica* secretory molecules disrupt tight junctions and intestinal ion transport provoking diarrhea. In accordance, *E. histolytica* pathology is significantly worse in the absence of mucin in the murine model of amebiasis. In MUC2 deficient mice, *E. histolytica* directly bound IECs causing greater pathology, barrier disruption, and secretory and pro-inflammatory responses (69). Amebic lesions in the intestinal epithelium can progress to necrotic flask-shaped ulcers containing trophozoites, bacteria and inflammatory cells. From these ulcers, trophozoites may invade the lamina propria and enter the bloodstream, often disseminating to the liver and causing amebic liver abscess (ALA) (37, 56). (Figure 1)

Differential virulence in *Entamoeba*

E. histolytica and *E. dispar* were classified as distinct species in 1993 based on the long-standing observation that *E. dispar* does not cause disease (31). This seperation redefined diagnostics, epidemiology and treatment of amebiasis (143). Since 1993, *E. dispar* has been infrequently detected in human colitis and ALA (142) and zenic *E. dispar* caused necrotic lesions in experimental ALA (32, 53). The free-living *Entamoeba moshkovskii* was initially believed to be avirulent (6) but was recently associated with human colitis and caused colitis in mice (119, 120). *Entamoeba bangladeshi* was discovered in cyst-containing diarrheal samples and may be a novel pathogenic species (114). In addition, differential virulence exists within *E. histolytica*- the Rahman strain was isolated from an asymptomatic individual and is considered avirulent. *In vitro* Rahman and *E. dispar* have reduced ability to ingest erythrocytes, damage colonic epithelia and cause ALA, relative to the virulent *E. histolytica* isolate HM1:IMSS. Infection with mixed *Entamoeba* species is also common (4, 143). Intra and interspecies competition for space and resources may increase parasite virulence. Co-infection could also be protective, however no cross-reactive immunity between *E. histolytica* and *E. moshkovskii* developed in mice (119).

Parasite factors that influence virulence

Differentiation

E. histolytica differentiates between an environmental cyst and replicative trophozoite. The chitin-rich cyst wall allows the parasite to survive outside the host and pass through the acidic stomach to establish infection. Excystation occurs in the small intestine and

each quadrinucleate cyst releases eight motile trophozoites, which establish infection in the colon. During infection trophozoites encyst and are excreted in the stool. The cues for excystation and encystation are unknown, and *in vitro* differentiation of *E. histolytica* remains elusive. The related *Entamoeba invadens* is a model for stage conversion. Dramatic transcriptional remodeling occurred during encystation and excystation of *E. invadens* (28, 34). Excystation triggered upregulation of carbohydrate metabolism (glycoside, hydrolases, hexokinases), protein synthesis, lipid biosynthesis and virulence (proteases, lectin) genes while encystation was marked by downregulation of metabolic processes and upregulation of genes for meiosis, chitin biosynthesis and phospholipase D (PLD) (28, 34). PLD is a lipid second messenger and inhibition of PLD activity blocked encystation of *E. invadens* (39). Differentiation is essential for transmission of *E. histolytica;* as such the environmental cues and parasite genes required for differentiation are ideal targets for transmission-blocking interventions.

Genomic virulence

The *E. histolytica* genome is ~24 MB with large differences in gene content, copy number, ploidy and intergenic regions between isolates (46, 139). Structural and sequence variation has been associated with clinical outcome of *E. histolytica* infection (5, 7, 8, 43, 63, 140). Paired colon and liver isolates from ALA patients had different genotypes suggesting either mixed infections and/or genetic alterations during an infection leading to a subpopulation of invasive trophozoites (8). Sequencing of clinical isolates found that sequence diversity varies by gene and single nucleotide polymorphisms (SNPs) in *CYCLCIN2* and lectin genes were associated with disease (46, 139). The *E. histolytica* genome shows evidence of dynamic evolution marked by extensive recombination, rapid mutation, RNA silencing, and epigenetic silencing (129). *E. histolytica* retrotransposons (*LINES/SINES*) modify gene expression and structure. Retrotransposons were more highly expressed in virulent *E. histolytica* relative to non-virulent species and strains, indicating that rapid evolution occur during disease progression (80). Genomic evidence has indicated that that *E. histolytica* exchanges genetic information by homologous recombination. Recently, the first experimental demonstration of homologous recombination was reported and homologous recombination was induced by serum starvation (121). Increased characterization of circulating strains and sequential clinical isolates as patients progress from colonization to disease is needed to understand how genetic variation mediates *E. histolytica* virulence.

Metabolism and nutrient acquisition

E. histolytica has a unique reductive metabolism (77). *E. histolytica* lacks most amino acid biosynthetic pathways except those for serine and cysteine. Cysteine production may be conserved as a component of amebic oxidative stress resistance. *E. histolytica* also lacks purine, pyrimidine and thymidylate synthesis and utilizes salvage pathways. *E. histolytica* does not synthesize fatty acids but can synthesize a variety of phospholipids. *E. histolytica* imports galactose and glucose for fermentation in the anoxic colon and may also catabolize amino acids to produce ATP (9). *E. histolytica* possesses multiple amylases which can degrade starch (9). There is strong evidence for lateral gene transfer (LGT) from prokaryotes in the *E. histolytica* genome including genes for several glycosidases for the catabolism of fructose and galactose (77). Prokaryotic genes for carbohydrate transport and metabolism

may allow *E. histolytica* to exploit glycan degradation by its microbial co-inhabitants in the colon. *E. histolytica* relies on host, dietary and bacterial for essential nutrients including amino acids, nucleic acids, carbohydrates, lipids and vitamins. Axenic culture of *E. histolytica* required adapting phagocytic trophozoites to uptake of nutrients by pinocytosis in culture. Interestingly *E. dispar* has been more difficult to establish in axenic culture (26).

Pathogenic mechanisms of *E. histolytica*

Adherence

Surface molecules control adherence, signaling, ingestion and immune modulation at the host-parasite interface. Many amebic surface proteins have functional diversity in these processes including the heterotrimeric Gal/GalNAc lectin. The lectin is composed of heavy (HGL), light (LGL) and intermediate (IGL) subunits. The HGL contains the carbohydrate recognition domain (CRD), which strongly binds Gal and GalNAc residues on mucus and host cells (Figure 1). Inhibition of HGL via genetic silencing, neutralizing antibodies and excess ligands (including Gal and mucins) blocked adherence and killing of host cells *in vitro* (100). Additionally, anti-CRD-IgA was protective against re-infection in humans (57). The HGL subunit also contains an intracellular domain with homology to β-integrins and may have a functional role in signaling after CRD engagement. These observations in combination with the finding that secreted products or amebic lysates were not sufficient for cytolysis (85, 86) lead to the model of contact-dependent amebic cytotoxicity (Figure 2).

Contact-dependent cytotoxicity

Upon adherence *E. histolytica* has multiple cytotoxic effects including increased intracellular Ca^{2+}, reactive oxygen species (ROS) production, loss of membrane integrity, DNA fragmentation, phosphatidylserine (PS) exposure and caspase-3 activation (Figure 2) (reviewed in 26). Early experiments established a link between amebic cytotoxicity and amebic phagocytosis (95, 111).

Subsequent work established a model of sequential adherence, contact-dependent cytotoxicity and phagocytosis. *E. histolytica* preferentially ingests apoptotic cells *in vitro* via recognition of PS and collectins-though the molecular interactions were not defined until recently. Amebic calreticulin (CAL) was found to be the surface receptor for host C1q. Calreticulin was required for phagocytosis of apoptotic cells but did not mediate killing (133). The amebic kinase C2PK was recently found to bind PS and was required for formation of the phagocytic cup by recruiting amebic actin (122). Several amebic transmembrane kinases are also important for phagocytosis of apoptotic cells and may have additional roles in cytotoxicity. TMKB1–9 and TMK96 (PATMK) mediated adherence and phagocytosis of apoptotic cells *in vitro* and were required in murine colitis but not ALA (2, 15).

Trogocytosis

The paradigm of sequential adherence, cytotoxicity and ingestion was overturned by the recent discovery that trophozoites mainly ingest pieces of intact living cell in a process named trogocytosis. Trogocytosis is an active process that resembles phagocytosis in some

ways. Trogocytosis required adherence to the target cell, as well as C2PK. Trogocytosis also caused a rapid rise in intracellular Ca^{2+} and resulted in cell death but dead cells were not ingested (106). These similarities suggest that the relationship between amebic adherence, cytotoxicity and ingestion is much more dynamic than previously appreciated (Figure 2). Ingested erythrocytes are commonly observed in biopsies of amebiasis (104), thus contact-dependent trogocytosis and phagocytosis may both lead to tissue lysis at the intestinal epithelium.

Contact independent effects

Secreted amebic products can have contact independent effects on tight junction integrity and ion absorption. Amebic prostaglandin 2 (PGE2) bound IEC EP4 receptors and altered Claudin-4 localization diminishing tight junction integrity and increasing luminal Cl^- secretion (72). *E. histolytica* lysates contain a serotonin analog which inhibited cellular Na^+ and Cl^- absorption while stimulating Cl^- secretion in colonic tissue (85, 86). It is not known if *E. histolytica* serotonin is secreted. Accordingly intestinal expression of the Cl^-/HCO_3^- exchanger *SLC26A3*, and the *AQP8* aquaporin, were significantly upregulated during acute *E. histolytica* colitis in humans (98). (Figure 2).

Other mechanisms of damage appear to be contact-independent mediated by trophozoite dephosphorylated and degraded host tight junction zona occludens (ZO) (73), claudins and occluding (69, 72).

Proteases and hydrolases

Ingested material is degraded in the amebic phagolysosome. The bactericidal and digestive functions of the phagolysosome is mediated by proteases, lysozymes, glycosidases, cytolytic amoebapores and phospholipases (94, 118). Some degradative enzymes have been found to have dual functions in nutrient digestion and virulence. Notably, *E. histolytica* was recently found to have 6 surface-associated glycolytic enzymes (13). Surface glycolytic enzymes may degrade extracellular carbohydrates to forms that can be imported by the parasite. Surface glycosidases in other pathogenic parasites degrade host proteins and have roles in nutrient extraction, invasion and immune evasion (reviewed in 50). In addition, *E. histolytica* supernatants had glycosidase activity including β-N-acetyl-d-glucosaminidase, α-d-glucosidase β-d-galactosidase, β-l-fucosidase, and α-N-acetyl-d-galactosaminidase (88).

In addition to glycosidase activity, *E. histolytica* possesses an armamentarium of proteases. The cysteine proteases (CPs) have been of particular interest due to their *in vitro* activity against a variety of host molecules. Whether these activities are relevant at *in vivo* may depend on concentration, localization and pH in the colonic environment and remains to be determined. Inhibition of certain CPs either chemically or by gene silencing blocked *E. histolytica* adherence, cytotoxicity, and motility *in vitro* and *in vivo* assays (118). These experiments have particularly implicated CP5 in virulence. CP5 degraded mucin *in vitro* but CP-5 silenced parasites were still able to degrade mucin on colonic explants but were unable to invade the epithelium (11). The absence of a CP5 homolog in *E. dispar* further supports a role in invasion. *E. dispar* also lacks a CP1 homolog, the absence of CP1 and CP5 may

partially account for the dramatically lower overall protease activity relative to HM1:IMSS of *E. dispar* (140).

E. histolytica cysteine protease-binding proteins (CPBFs) were recently identified as regulators of protease activity and trafficking to phagosomes (40, 41, 92). CPBF1 bound the virulence-associated CP5 and was required for its activity (92). CPBF6 bound and trafficked α-amylase and γ-amylase to the amebic phagosomes (92). CPBF8 was required for localization of β-hexosaminidase and lysozyme to phagosomes. In addition silencing of *CPBF8* reduced cytotoxicity (78). The emerging dual roles of CPBFs in digestion in the phagosome and potentially virulence may impart functional flexibility of degradative enzymes depending on the available nutrient sources,

The recent characterization of the surface metalloprotease MSP-1 and the rhomboid intramembrane protease ROM1 has highlighted the importance of proteolysis in regulating adherence, phagocytosis and motility. Silencing of *MSP1* increased adherence to live and apoptotic cells but reduced motility, phagocytosis and cytotoxicity (126). Interestingly, ROM1 was only required for adherence to live cells-but was necessary for phagocytosis of live and apoptotic cells suggesting ROM1 has independent roles in these processes. ROM1 co-localized with the Gal/NAc lectin was reported to control shedding of host antibodies and complement (12). In both these studies however the phenotypes were apparently independent of alterations in Gal/GalNAc surface expression (57). The substrates of both MSP-1 and ROM1 are unknown. Potential candidates might be the serine, threonine, isoleucine-rich proteins (STIRPs) which were required for adherence and cytotoxicity in *E. histolytica* and are notably absent in *E. dispar* (81). Proteolytic mechanisms to rapidly regulate adherence may allow parasites to rapidly shift from an adherent phenotype during colonization to a motile invasive form in response to changing conditions. The activity, expression, disease phenotype and localization of amebic glycosidases and proteases and their chaperones are summarized in Table 1.

Virulence regulation at the host-pathogen interface

Many *E. histolytica* virulence factors are genomically encoded and expressed in avirulent *Entamoeba* strains and species. Differences in gene content and expression that might mediate virulence have been extensively analyzed (49, 140). Overall, virulent HM1:IMSS has increased expression of adherence genes including *LGL1/5, STIRPs, SREHP*, and *KERP1* (127). *LGL3* is an exception with much higher expression in Rahman (140) but the significance of multiple copies of lectin genes is unclear, and complicated by the finding that *LGL2* and *LGL3* were downregulated during intestinal colonization of mice (48). Cysteine protease genes are differentially regulated in HM1:IMSS and Rahman. Regulation of specific CPs via expression and trafficking by CPBFs may control their degradative abilities. *CP5* was more highly expressed in HM1:IMSS relative to Rahman in a recent analysis (127). Previous work reported increased *CP4, CP6* and *CP1* and decreased *CP3*, *CP7* and *CP9* expression in HM1:IMSS relative to Rahman (140). Adaptation to the murine colon increased expression of *CP4*, *CP6*, and lysozyme (48). In interpreting these studies it is important to note that HM1:IMSS and Rahman were isolated over 30 years ago from different continents, therefore distinct expression profiles likely also reflect mutations and

epigenetic alterations from prolonged axenic *in vitro* growth that are unrelated to virulence capacity (49).

Metabolic flexibility

Analysis of *E. histolytica* adaptation from *in vitro* culture to murine colitis and human colon explants has shown that trophozoites rapidly adapt to massive changes in nutrient availability and oxidative stress (48, 127, 136). This adaptation does not accurately reflect adaptation to the colon during excystation; nonetheless adaptive capacity is critical for survival in the chaotic host environment. During murine colitis trophozoites initially downregulated glycolysis genes and induced lipase genes genes-likely in response to the scarcity of monosaccharaides in the colon relative to culture media (48). Adaptation to the mucosal surface of colonic explants induced genes involved in metabolism of complex carbohydrates and in glycolysis in HM1:ISS while Rahman expressed higher levels of lipases (127). HM1:IMSS rapidly bound and dissolved mucus on colonic explants, while Rahman and *E. dispar* bound but did not degrade mucus (10, 127). HM1:IMSS induced β-amylase expression during contact with colonic explants and silencing of β-amylase reduced mucin degradation (127). It has been proposed that the ability to catabolize host mucin as a carbon source may mediate invasive capacity (127).

The pathways of amebic mucin degradation have not been elucidated. The secreted glycosidase activity of *E. histolytica* is thought to mediate mucin degradation by exposing mucin peptides to amebic proteolysis (13, 76, 88). Trophozoites *in vitro* internalized and released host mucins without proteolytic degradation (23) though transcriptome studies have demonstrated that the regulation of metabolism genes is associated with mucin degradation (127)-thus the ability to sense nutrient sources and induce appropriate pathways may determine metabolic flexibility in the host environment.

Several lines of experimental evidence indicate that the ability to sense and respond to nutrient levels may mediate *E. histolytica* invasion. *E. histolytica* motility was highly increased by nutrient depletion and trophozoites were repelled by the byproducts of their own glycolysis-suggesting motility may be a competitive response (144). Increased motility was not observed upon starvation in *E. dispar*, suggesting this response may be associated with invasion (144). *E. histolytica* displays chemotactic movement toward serum, fibronectin and TNF-α, potentially directing trophozoites to the intestinal epithelium upon starvation (14). Further, glucose starvation dramatically increased *E. histolytica* motility, adherence and cytolysis *in vitro.* Low glucose activated the transcription factor URE3-BP and increased expression of *LGL1* (130). URE3-BP has previously been shown to regulate *HGL5* and ferredoxin during murine colitis and has been suggested to coordinate motility, adherence and oxidative stress resistance during infection (47, 48).

Stress resistance

G-protein signaling—In addition to surviving nutrient stress, adaptation is key for surviving other stresses in the mutable host environment. G-proteins signaling controls many processes including adherence, protease activity and phagocytosis and may mediate rapid adaptation in *E. histolytica* (reviewed in 95). *E. histolytica* encodes 8 putative ligand-

activated G-protein-coupled receptors (GPCRs) but only GPCR1 was expressed *in vitro* (103). GPCR-1 bound LPS in phagocytic cups (21) and was reported to bind RabB *in vitro*, which regulates amebic phagocytosis (61). *E. histolytica* also encodes the G-protein subunits Gα1 and Gβγ which interacted with RGS-RhoGEF. Overexpression of Gα1 increased trophozoite motility, adherence and cytotoxicity (17) while overexpression of RGS-RhoGEF had the opposite effect (16). The multiple effects of Gα1 signaling in virulence was attributed to their global regulation of parasite protease activity, but the mechanism remains to be defined (17). The finding that G-protein signaling was upregulated in HM1:IMSS relative to Rahman upon introduction to colonic explants further supports their role in mediating adaptation to host stress (127).

Oxidative stress—Trophozoites must detoxify reactive oxygen and nitrogen species (ROS/NOS) produced by infiltrating immune cells and during invasive disease beyond the anoxic colon. *E. histolytica* produces high levels of cysteine and numerous enzymes to combat oxidative stress which include: peroxiredoxin (PRX), superoxide dismutase (SOD), flavoprotein A, ferredoxin (FRX), thioredoxin(TRX) and TRX reductase (108, 136). TRX was crucial for buffering sensitive proteins during oxidative assault (117) and the amebicidal activities of metronidazole and auroanofin are mediated by disruption of TRX (29). The ability to survive oxidative stress is also associated with increased virulence. Oxidative stress induced upregulation of a stress-induced adhesion factor (SIAF) and a phospholipid transporting P-type ATPase/flippase (PTPA) which both have roles in adhesion and phagocytosis (108). Oxidative stress also induced metabolic alterations including glycerol and chitin biosynthesis potentially triggering encystation (64). Overall, HM1:IMSS responded more strongly to oxidative stress than either *E. dispar* or Rahman and surface localization of PRX in HM1:IMSS was associated with virulence (136).

Regulation of *E. histolytica* virulence by the colonic microbiota

Influence of enteric microbiota on *E. histolytica* virulence—The enteric microbiota is a nutrient source for *E. histolytica* and bacteria have long been recognized as a crucial determinant of the pathophysiology of *E. histolytica* infection (74). The microbiota is generally protective for enteric infection, however *E. histolytica* virulence seems to require the presence of other enteric organisms. In 1946 it was discovered that ameba caused similar colonic ulcers in symptomatic and asymptomatic individuals but symptomatic individuals had increased ulcer-associated bacteria suggesting that ulcerations in the absence of bacteria were not sufficient to cause disease (36). While provocative this result is likely confounded by infection with non-invasive *E. dispar*, which could not be distinguished from *E. histolytica*. Subsequent experiments found that germ-free animals were resistant to *E. histolytica* infection but the introduction of a single bacterial species restored amebic pathogenesis (101, 102). Axenization decreased parasite virulence (141) and incubation of axenic trophozoites with live bacteria increased virulence depending on the bacterial species (87). Incubation of *E histolytica* with enteropathogenic *E. coli* or Shigella increased adherence and cytotoxicity of *E. histolytica*, but had no effect on *E. dispar* (42). Conversely, incubation with specific *E. coli* strains decreased parasite virulence, and these effects have been attributed to regulation of the amebic lectin in response to distinct bacterial surface

lipopolysaccharides (87). These observations indicate the enteric microbiota likely regulate *E. histolytica* virulence during infection though this awaits confirmation *in vivo*.

***E. histolytica* perturbs the composition of the enteric microbiota—**There is some evidence that *E. histolytica* significantly alters the principal phyla of the host microbiota during disease. *E. histolytica*-induced dysbiosis was characterized by significantly less *Bacteroides, Clostridia*, *Lactobacillus*, *Campylobacter* and *Eubacterium* and significantly increased *Bifdobacterium* species (135). It will be interesting to determine if dysbiosis results from disruption of intestinal physiology by amebic pathology and peristalsis. *E. histolytica* could also directly induce dysbiosis through specific predation, lysis, or modulation of the host immune response. *In vitro* experiments have found that:

1. *E. histolytica* displayed preferential ingestion of some bacterial species (19, 87).
2. Cytolytic amoebapores had differential activity against certain bacterial species and eukaryotic cells *in vitro* (22).
3. *E. histolytica* induced and degraded colonic antimicrobial peptides but is resistant to their activity (27).

It remains to be seen if amebic dysbiosis is similar to dysbiosis induced by other enteric infections, as well as if asymptomatic *E. histolytica* infection results in comparable dysbiosis.

Regulation of *E. histolytica* virulence by the microbiota—The composition of the intestinal microbiota is mechanistically linked to the nutritional and immune status of the host. In *E. histolytica* endemic areas infants are also chronically infected with multiple enteric pathogens, termed the pathobiota (115, 125). Experimentally, pretreatment of intestinal epithelial cells with enteropathogenic bacteria prior to *E. histolytica* infection increased inflammatory cytokine production, decreased epithelial barrier integrity and resulted in enhanced trophozoite adherence and subsequent cytotoxicity (42, 43). During human infection the enteric pathobiota may also lead to greater inflammation and amebic tissue damage by enhancement of the inflammatory response, decreased barrier function and specific modulation of the amebic lectin. The composition of the enteric microbiota controlled susceptibility to infectious colitis in mice via modulation of intestinal ion channel genes including *SLC26A3* (45) and aquaporin activity (128). Both *SLC26A3* and *AQP8* aquaporin were significantly upregulated during acute *E. histolytica* colitis in humans (98) further implicating specific microbiota in mediating host susceptibility.

E. histolytica depends on host and microbial nutrients to survive. The microbiota produce glycosidases that degrade complex polysaccharides into forms available for host absorption (96). Microbial glycosidase activity determines levels of free colonic carbohydrates (the glycobiome). The microbiota-dependent glycobiome has an emerging role in regulating the virulence of enteric pathogens (39). The FusKR signaling pathway is a novel fucose-responsive regulator of virulence genes in Enterohemorrhagic *Escherichia coli* (EHEC) (97). *Clostridium difficile* disease was mediated by sialic acid levels *in vivo*, while *Salmonella typimurium* virulence depended on both fucose and sialic acid *in vivo* (93). The finding that glucose starvation enhanced *E. histolytica* virulence, motility and lectin expression by

URE-3BP(130), a transcription factor previously linked to virulence capacity (47) suggests similar mechanisms could exist in *E. histolytica* (Figure 3).

Host factors that influence *E. histolytica* virulence regulation

Immune response and immune evasion

Cell Mediated—The immune response is a critical mediator of amebic virulence, however *E. histolytica* infects immunocompetent hosts. Disease seems to be enhanced by the immunodeficiency of malnutrition while defects in T-cell immunity in HIV/AIDS does not seem to mediate increased disease. Another mystery is the particular disposition to ALA in men, despite equivalent susceptibility to infection. The mucin barrier is the first layer of defense blocking trophozoite adherence and cytotoxicity to the intestinal epithelium (24). In the absence of mucin, trophozoites contact the intestinal epithelium (Figure 1). IECs recognized the CRD of the Gal/GalNAc lectin via TLR-2/4 and activated NF-κB leading to the production of inflammatory cytokines including IL-8, IL-6, IL-12, IL-1β, IFN-γ and TNF-α (10, 43). *In vivo* neutrophils predominated in amebic lesions while macrophages were infrequent (37). *In vitro* activated neutrophils and macrophages have amebicidal activity however *E. histolytica* displayed reciprocal killing (44). Clearance of infection was associated with IFN-γ, while IL-4 and TNF-α are correlated with disease (60, 78, 99, 116). IFN-γ production by peripheral mononuclear cells (PMNs) significantly correlated with protection from future *E. histolytica* disease in children (59). In vaccinated mice protection required IFN-γ-producing CD4+ T-cells and IL-17-producing CD8+ T-cells (51).

The predominance of ALA in men may also be due to IFN-γ. In experimental ALA, protection was mediated by IFN-γ from natural killer T-cells (NKT) while TNF-α producing macrophages increased tissue damage (60, 78). Female mouse NKTs produced more IFN-γ, in a testosterone dependent fashion, mediating ALA protection (79). Experimental ALA introduces trophozoites directly into the liver and does not model upstream immune responses prior to invasive disease in humans. Nonetheless, human and experimental studies have indicated that impaired cell-mediated immunity can worsen host damage by *E. histolytica*. *In vitro* evidence indicates trophozoites are capable suppressing cell mediated immunity by: killing immune cells (107) proteolytic cleavage of pro-IL-1 β (causing activation) and IL-18 (causing degradation) (118). In addition, *E. histolytica* secrets PGE2 which downregulated induced IL-8, decreased macrophage MHC II expression and may inhibit T-cell activation and oxidative capacity (137).

Adaptive Immunity—Adaptive immunity is also protective against *E. histolytica* as previous infection and vaccination reduced susceptibility to subsequent infections in mice (51, 120). In humans, protection from reinfection is associated with the sIgA response (1, 54, 55, 57). Conversely serum antibodies were associated with increased frequency and severity of amebic disease, though the reason for this is unclear (20,12). *In vitro* antibodies bound *E. histolytica* and blocked attachment to host cells and molecules (75). Surface-bound antibodies also activated the complement membrane attack complex (MAC) (20). *E. histolytica* evaded antibody-mediated defenses by rapidly shedding bound antibodies in

a Gal/GalNAc lectin cap (25). The Gal/GalNAc lectin also inhibited MAC formation on trophozoites (20) and *E. histolytica* CPs degraded the complement factors C3a and C5a (110) and host IgA and IgG (68, 131).

Nutrition

Malnutrition causes immunodeficiency and increased susceptibility to *E. histolytica* (reviewed in 92). Children with *E. histolytica* diarrhea are significantly more likely to be malnourished or stunted (91). In addition, malnourished children had three times more *E. histolytica*-associated diarrheal episodes than children without malnutrition (89). Malnutrition was specifically and significantly associated with *E. histolytica* compared to all enteric infections (89). The nutritional cytokine leptin is a critical link between nutritional status and immunity (38). Reduced leptin signaling due to a leptin receptor (LEPR) polymorphism (Q223R) is associated with increased susceptibility to *E. histolytica* diarrhea in children and ALA in adults (33). Leptin-deficient mice (52) and LEPR Q223R (82) mice are more susceptible to *E. histolytica* infection. Infection of intestinal epithelial LEPR-knockout mice and *in vitro* studies demonstrated that protection was dependent on leptin activation of STAT3 in intestinal epithelial cells (52, 83). Mice lacking LEPR at the intestinal epithelium had similar body weight, food intake, fecal microbiota and antimicrobial peptide expression (105) indicating that protection is mediated by specific leptin-regulated immune mechanisms which are known to include prevention of apoptosis, increased mucin secretion and enhanced intestinal cell repair and proliferation (35, 113, 124). There is emerging evidence that *E. histolytica* infection may cause nutritional deficits. *E. histolytica* infection is associated with intestinal inflammation, mucosal disruption, diminished barrier integrity, ion secretion and dysbiosis, with potentially compounding effects on the nutritional status of the infected host (62). *E. histolytica* intestinal damage, in particular chronic inflammation and mucus depletion may be triggers for environmental enteropathy further impairing nutrient absorption (70) (Figure 4). Breast-fed infants are at lower risk of *E. histolytica* infections. It was recently shown that human milk oligosaccharides as well as synthetic galacto-oligosaccharides protected human IECs from amebic cytotoxicity *in vitro*. Galacto-oligosaccharides are stable, inexpensive and commonly added to infant formula thus these results have implications for nutritional interventions for *E. histolytica* (66).

Conclusion

E. histolytica virulence depends on a complex interaction of parasite, host and environmental factors, When *E. histolytica* progresses to virulence the destruction of colonic environment can lead to degradation of protective mucus, disrupted epithelial barrier function, deregulated ion transport, local and systemic inflammation, impaired nutrient absorption, and disruption of the microbiota. The host processes altered by *E. histolytica* virulence are inherently and reciprocally linked and infection has severe impacts in vulnerable hosts. This capacity is unique to *E. histolytica* and while many of the mechanisms for virulence are defined the benefits for *E. histolytica* survival relative to closely related avirulent strains and species are not understood. A salient feature of *E. histolytica* is the capacity to sense and adapt to diverse host nutrient sources and stresses.

The multifunctional Gal/GalNAc lectin is critical for adherence in the host colon and may also mediate downstream signaling upon ligand binding. Engagement of lectin by distinct host glycans may prime parasite gene expression for survival in a particular host niche. Mucin binding may induce a phagocytic, mucus dwelling lifestyle where host mucin and microbiota are the primary nutrient sources. Upon mucin depletion, parasites may sense carbohydrate scarcity and display enhanced motility and directional migration to the epithelium. Engagement of cell-associated glycans may lead to priming of an invasive phenotype that prepares the parasite for oxidative stress and nutrient extraction from host cells. The recent discovery of trogocytosis suggests that *E. histolytica* does not ingest intact living cells-but ingests cellular pieces leading to cell death, thus cytotoxicity may be collateral damage from nutrient extraction from host IECs. It is known that an appropriate cell mediated immune response can lead to clearance of *E. histolytica*, while an inappropriate response can increase tissue damage, allowing parasites to invade the lamina propria. Colonization may be specifically regulated to allow parasites to compete in dense colonic microbiota. Thus far, research has focused defining the events that are associated with pathogenesis. Further attention will be required to understand parasite persistence and colonization—which have developed through co-evolution within the human host. The multilayered effects of diet, nutrition, microbiota, immunity and glycobiome may create a colonic environment that induces *E. histolytica* virulence due to mucus, nutrient and immune depletion. Host nutrition, microbiota and immunity exist in a delicate network that is critical for overall host health. Disruption of these intrinsically linked processes by *E. histolytica* may have compounding effects for host susceptibility to disease and for parasite virulence regulation.

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Acronyms and Definitions

Zenic	co-culture with one or more unidentified organisms
Axenic	a pure culture of single species
Pathogenic potential	the ability to cause disease in a given environment
Trogocytosis	contact-dependent amebic ingestion of pieces of living cells (from the Greek, trogo-nibble)
Microbiota	the collection of microbes colonizing a host
Microbiome	The collection of genes of the microbiota
Pathobiota	The collection of pathogens in a host
Glycobiome	the glycan composition of a host niche
ALA	Amebic liver abscess

IEC	intestinal epithelial cell
Gal/GalNAc	galactose/N-acetyl-D-galactosamine
PRX	peroredoxin
TRX	thioredoxin
ROS	reactive oxygen species
CAL	amebic calreticulin
CP	cysteine protease
PS	phosphatidylserine

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Summary points: Virulence regulation of *E. histolytica* by the enteric microbiota

Direct effects

- The microbiota and associated metabolome provide essential nutrient sources for *E. histolytica* that are required for **survival** in the colon.
- The levels of specific microbial products or species regulate **virulence** of *E. histolytica* parasites via regulation of virulence factors involved in binding, ingestion and killing of bacterial species.

Indirect effects

- Dysbiosis alters host nutritional status and immune function that correlate with susceptibility to *E. histolytica* including lower IFN-γ production
- Pathobiota induce an inflammatory cascade, barrier dysfunction and nutrient malabsorption, which increase susceptibility to *E. histolytica* infection.
- *E. histolytica* depletion of host mucous eliminates the spatial separation between the microbiota/pathobiota and the intestinal epithelium aggravating inflammation and associated amebic tissue damage during *E. histolytica* infection.
- *E. histolytica* degradation of host antimicrobial peptides compromises intestinal immune homeostatic interactions
- *E. histolytica* alteration of the host microbiota. Thee microbiota controls:
  - Host nutrient availability and absorption
  - Mucins level and integrity
  - Leptin production
  - Competitive exclusion of pathogens
  - Intestinal barrier function and ion transport

Future issues: Which unanswered questions are most important for improving human health?

Parasite factors

1. What is the mechanism of carbohydrate sensing in *E. histolytica*? What are the roles of specific *E. histolytica* glycosidases in virulence? What is the function of secreted and surface-associated glycosidases?

Host factors

2. Do genetic (Q223R), immune (IgG, IFN-γ), specific co-infections, or other biomarkers predict which *E. histolytica* infected individuals will develop disease? Why are serum antibodies to *E. histolytica* correlated with disease?
3. Why is *E. histolytica* specifically associated with human malnutrition? Does *E. histolytica* infection cause malnutrition and environmental enteropathy?

Parasite Environment

4. How does *E. histolytica* impact the composition of the enteric microbiota and regulation of the microbiome? Are there differences between colonization and disease? What are the implications of *E. histolytica* infection in infants on the maturing microbiota?

5. Do specific species of the host microbiota and/or pathobiota alter *E. histolytica* virulence?

6. Do differences in the glycobiome due to dietary intake, mucin level, and microbial glycosidases regulate virulence in *E. histolytica*?

7. Which interventions will be most effective to limit *E. histolytica* mortality and morbidity? Can *E. histolytica* be eradicated?

    a. Improved sanitation

    b. Increased diagnosis and treatment of asymptomatic infection

    c. Screening for individuals likely to develop invasive disease (IgG?)

    d. Development and implementation of a vaccine

    e. Nutritional therapy. Specific carbohydrates?

Sidebar: Disentangling the virulence networks of *Entamoeba*

Entamoeba must dynamically sense, respond and exploit host and microbiota compounds to acquire nutrients, evade immunity and survive in the host. The integration of existing *E. histolytica* genomic, transcriptional, biochemical and proteomic datasets into interacting pathways may illuminate novel parasite responses associated with virulence. These pathways can also be compared to datasets from *E. dispar* and *E. histolytica* Rahman to highlight global responses that are specific to invasion. As analytical and experimental tools improve it will be possible to incorporate ‘omics’ data from the infecting parasite, infected host and colonic environment (including microbiota, microbiome, pathobiota, pathbiome and colonic metabolome). Response-related networks of parasite and host data will be insightful into the dynamic regulation of *E. histolytica* virulence in susceptible and resistant hosts. The potential applications of large datasets include: the identification of host susceptibility biomarkers, understanding the relationship of enteric co-infections and the discovery of novel parasite virulence pathways induced *in vivo.* Integrating and incorporating diverse host/pathogen data could direct targeted host and pathogen therapeutics interventions and permit extrapolative prediction of the functions of hypothetical and unknown *Entamoeba* genes that may be important in virulence.

Sidebar: Measuring *E. histolytica* virulence

A brief overview of common methods for assaying virulence phenotypes of *E. histolytica.*

In vitro

In vitro assays using cultured cells can be useful for measuring virulence traits including adherence, cytotoxicity, protease activity, monolayer destruction, motility, phagocytosis and trogocytosis. It is important to note that some cell types may not reflect the cells encountered by *E. histolytica* during natural infection. In addition, the linked nature of these phenotypes can make it difficult to ascribe a specific functionality *in vitro.*

Ex vivo

In human colon explant *E. histolytica* degrades colonic mucus, migrates along collagen networks, degrades ECM, kills host cells and invades tissue. In addition, colonic explants produced a potent inflammatory response. This technique, in combination with imagining and transcriptome studies has provided key insights into *E. histolytica*'s invasion and virulence potential of human tissue. However, the explant model is limited to understanding early stages of infection.

In vivo

E. histolytica naturally infects humans and some other primates and the development of animal models that reproduce natural infection has been problematic. A variety of animals have been used including dogs, kittens, primates, rabbits, guinea pigs, hamsters and gerbils. Mouse strains display differential susceptibility to amebic colitis. Natural resistance in animals has been informative to understanding protection in humans and pointed to immune cells (mainly neutrophils), pro-inflammatory cytokine production and mucin content as mediators of innate protection. Experimental studies of invasion beyond the intestine have focused on ALA in susceptible hamsters and gerbils, which develop hepatic lesions. Encystation of *E. histolytica in vitro* has not been successful, thus both colitis and ALA models rely on direct introduction of trophozoites into the colon or liver. To date an animal model that captures the complete cycle of natural *E. histolytica* infection does not exist. Reviewed in (132).

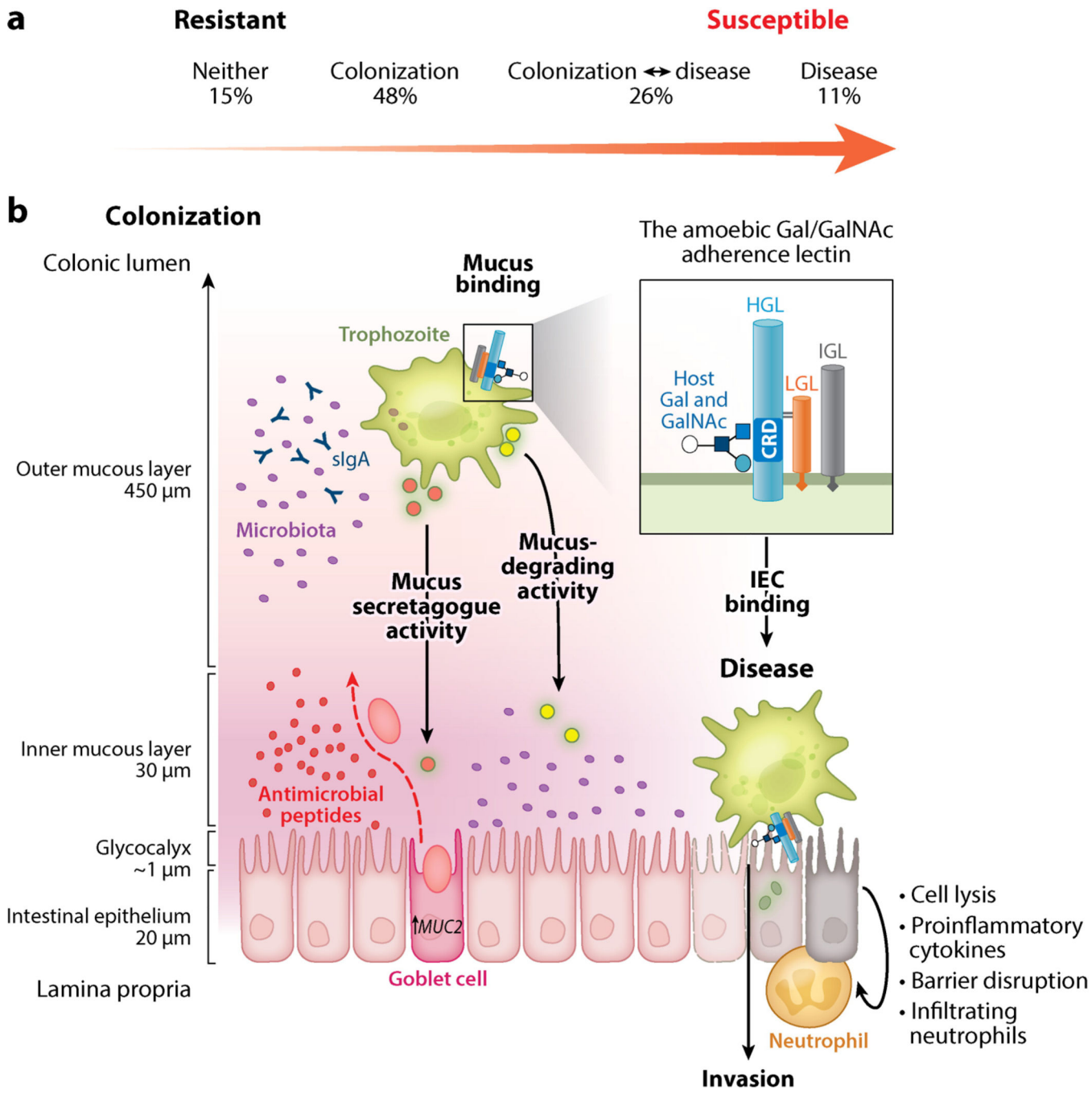

Figure 1.

E. histolytica virulence depends on a dynamic interaction in the infected host. (a) Continuum of E. histolytica disease in a natural population. Analysis of diarrheal and monthly surveillance stool samples for E. histolytica detected four possible outcomes in the first two years of life: (1) no evidence of infection, (2) colonization with no E. histolytica–associated diarrhea, (3) diarrhea with prior asymptomatic colonization and/or subsequent asymptomatic persistence, or (4) E. histolytica–associated diarrhea with no previous colonization. This pattern reinforces the importance of both parasite and host

factors in the outcome of an E. histolytica infection. (b) To establish infection, E. histolytica must bind and adhere in the host colon. Adherence is mediated by an amoebic lectin with a carbohydrate-recognition domain (CRD) that binds galactose (Gal) and N-acetyl-d-galactosamine (GalNAc) on host glycoconjugates with high affinity. The Gal/GalNAc lectin is composed of heavy (HGL), intermediate (IGL), and light (LGL) subunits. The CRD is located on the HGL, which also contains a putative intracellular signaling domain. HGL forms a disulfide bond with LGL. The HGL-LGL heterodimer can associate with the glycosylphosphatidylinositol-anchored IGL, but this subunit does not have a well-defined function (inset). Colonic mucin forms a dense polymeric gel over the intestinal epithelium, which trophozoites bind to with high affinity. Trophozoites also induce mucin secretion by goblet cells. In colonization, mucin binding mediates attachment and provides a nutrient source for E. histolytica. Mucin polymers may be degraded by amoebic proteases and glycosidases for nutrients, and the mucosal microbiota provides a nutrient source via amoebic phagocytosis. The transition from colonization to disease is marked by destruction of the mucin barrier. Mucus depletion may result from enhanced amoebic degradation and/or depletion of mucin stores by continual secretion during chronic infection. Other factors including coinfections, host diet, and disruption of the microbiota can also mediate mucus depletion. Mucus depletion exposes the intestinal epithelium to E. histolytica trophozoites. The amoebic lectin CRD binds to Gal and GalNAc on exposed intestinal epithelial cells (IECs) and the cell-associated glycocalyx. Adherence to IECs results in amoebic cytotoxicity and the release of proinflammatory molecules. Abbreviation: sIgA, secretory immunoglobulin A.

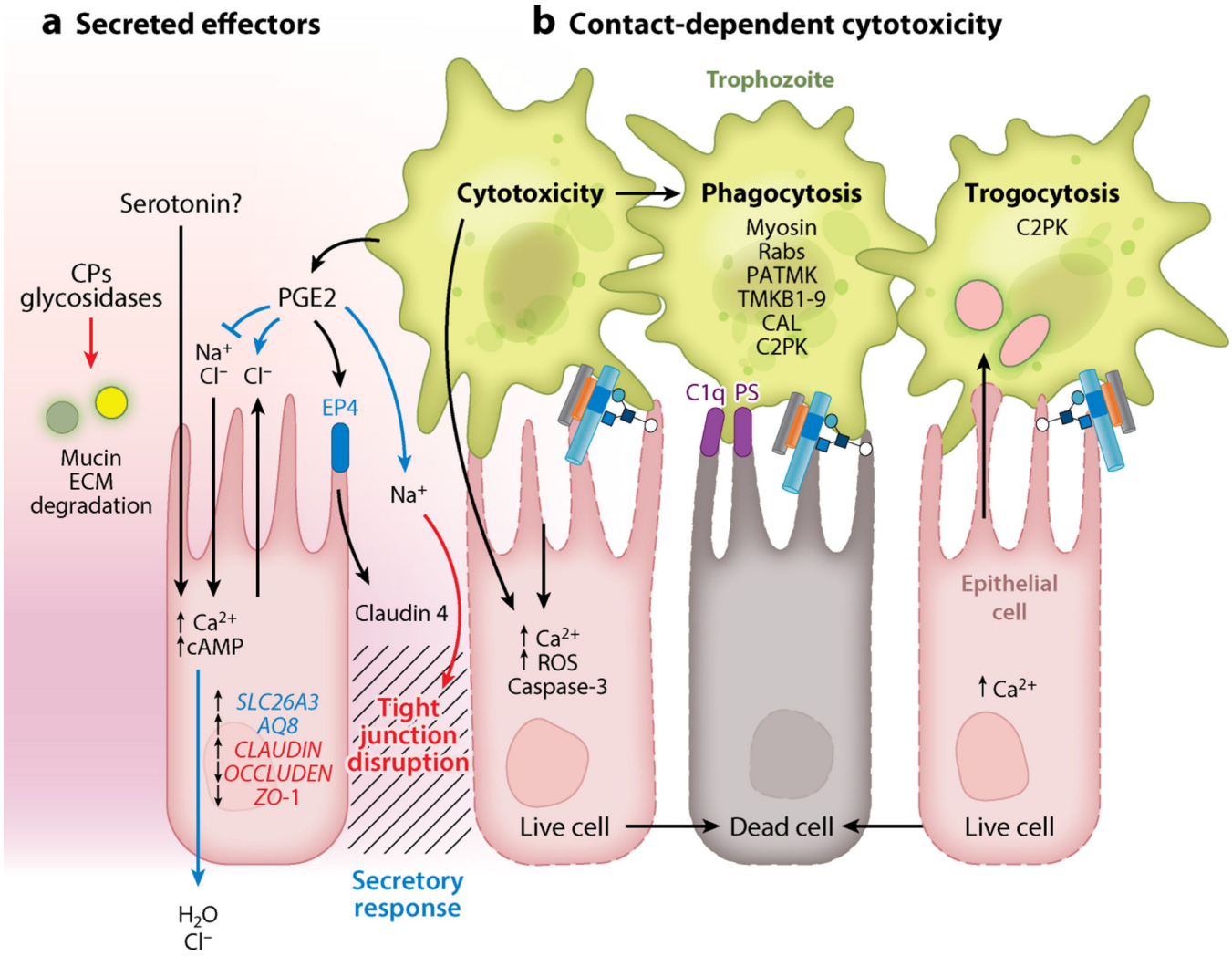

Figure 2.

Pathology at the intestinal epithelium. (a) Physiological mediators of E. histolytica diarrhea. Secreted amoebic effectors have contact-independent physiological effects on intestinal epithelial cells. (1) Proteases and glycosidases degrade mucus and extracellular matrix (ECM) proteins. (2) Amoebic PG2 disrupts barrier function by binding to host EP4, leading to altered expression and localization of tight junction proteins including zona occludens proteins and claudins. (3) PG2 also increased secretion and disrupted ion gradients, leading to decreased cellular Na2+ absorption and increased Na2+ and Cl− secretion at the apical surface. Amoebic serotonin is present in amoebic lysates, but it is not known if it is secreted. Serotonin elevates intracellular Ca2+ and cAMP, leading to increased H2O and Cl− secretion at the serosal surface. Disruption of barrier function further disrupts ion gradients at the intestinal epithelium, and these effects are likely the physiological mediators of amoebic diarrhea (secretory response, blue; tight junction disruption, red). (b) In vitro trophozoites must adhere to target cells to induce death. Contact-dependent killing can be mediated by amoebic activation of host caspase-3 through an undefined mechanism and a rapid apoptotic-like death, preceded by elevated intracellular

Ca2+ and reactive oxygen species. E. histolytica phagocytosis is initiated by exposed C1q and phosphatidylserine (PS) on apoptotic cells, which are bound by amoebic calreticulin (CAL) and C2K, respectively. Amoebic kinases PATMK, TMKB1–9, and TMK39 are also involved in phagocytosis. Phagosome formation requires vesicular trafficking and cytoskeletal rearrangement controlled by G-proteins and amoebic myosin. Trogocytosis is a distinct contact-dependent mechanism of amoebic cytotoxicity. In trogocytosis, trophozoites actively ingest pieces of living cells, resulting in membrane disruption and rapid target cell death. Trogocytosis also requires amoebic C2PK and leads to increased intracellular Ca2+ prior to cell death; however, in trogocytosis E. histolytica does not ingest cells after killing. Other abbreviations: CP, cysteine protease; PGE2, prostaglandin 2; ROS, reactive oxygen species.

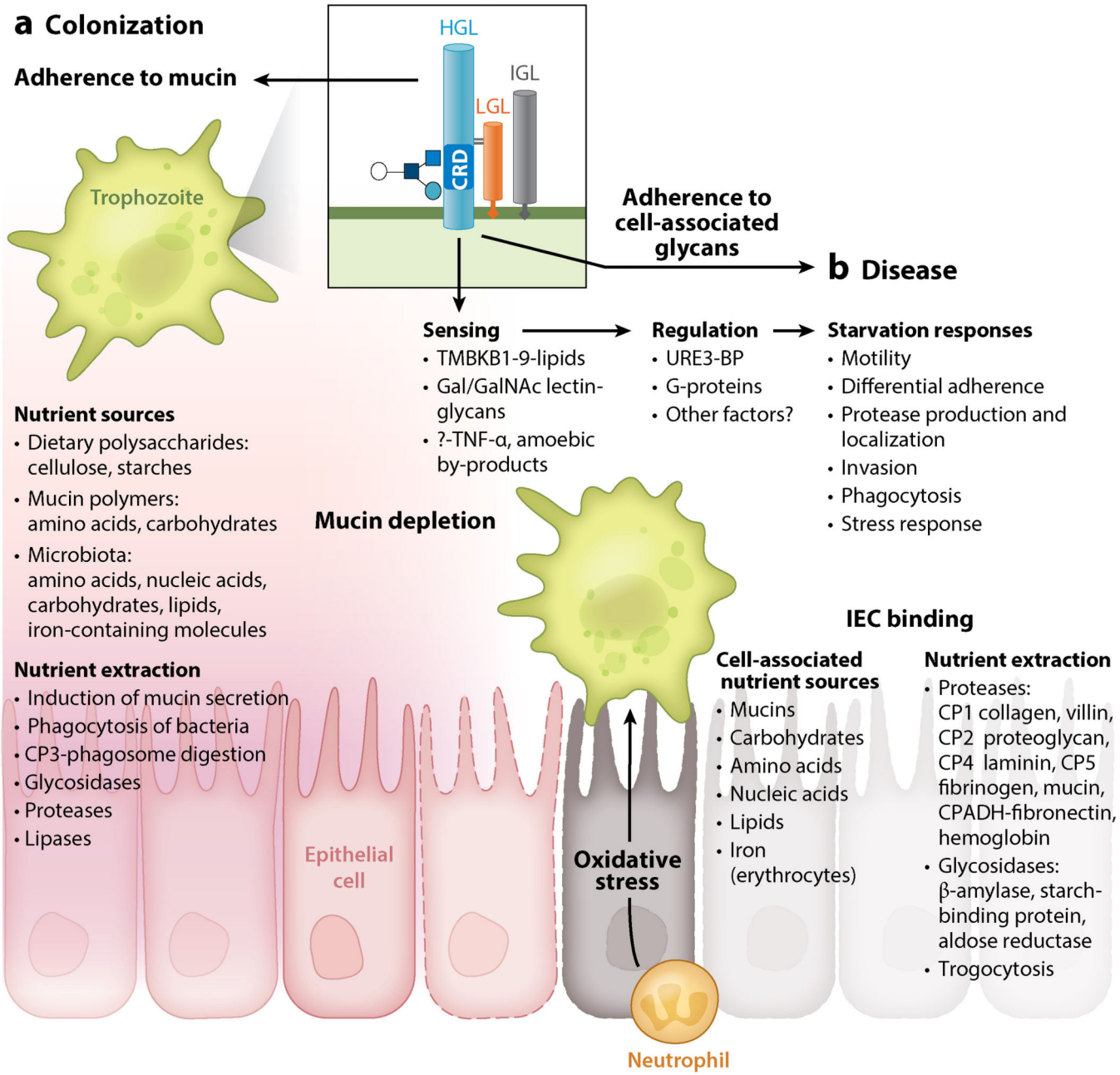

Figure 3.

E. histolytica adaptive ability mediates nutrient extraction and survival in the fluctuating colonic environment. (a) Colonic nutrient sources include dietary polysaccharides, microbiota, and human cellular molecules including mucin. Microbial glycosidases degrade complex polysaccharides into fatty acids absorbed by intestinal epithelial cells (IECs) and sugars for their own metabolism. An intact mucous layer provides plentiful nutrients from mucin and microbiota, inducing a parasite program for colonization. (b) Upon mucus depletion, nutrient starvation induces virulence and activates the transcriptional regulator URE3-BP. Starvation responses include decreased adherence and enhanced motility and oxidative stress resistance. Upon adherence to host cells, virulence factors are induced to

enable extraction of cell-associated nutrients. Abbreviations: CP, cysteine protease; CRD, carbohydrate-recognition domain; Gal, galactose; GalNAc, N-acetyl-d-galactosamine; HGL, heavy lectin subunit; IGL, intermediate lectin subunit; LGL, light lectin subunit.

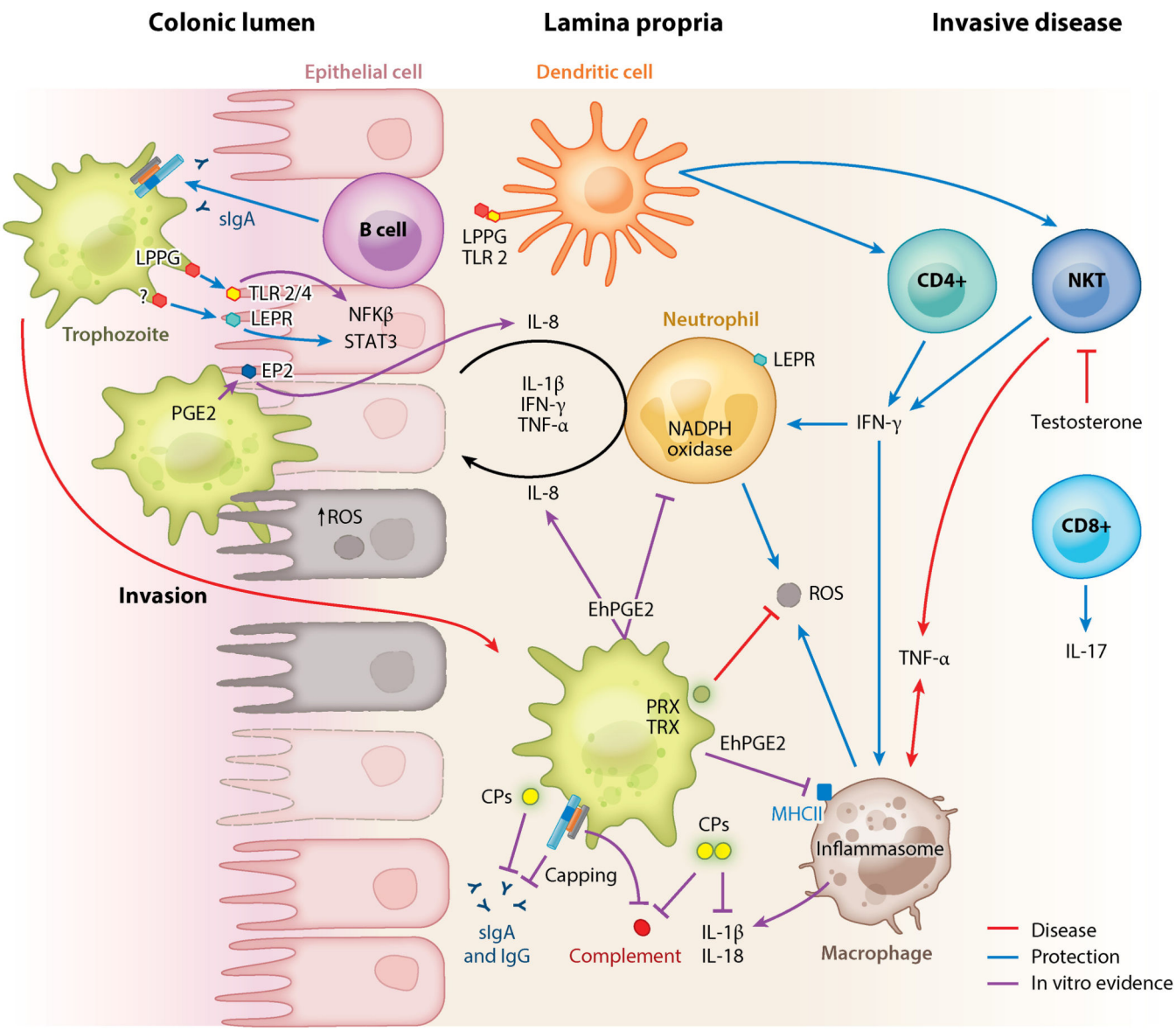

Figure 4.

Immune regulators of E. histolytica virulence. The mucin barrier, mucosal sIgA to the carbohydrate-recognition domain of the galactose (Gal)/N-acetyl-d-galactosamine (GalNAc) lectin, and leptin signaling via a leptin receptor (LEPR) at the epithelium are critical determinates of protection in the colonic lumen. The Gal/GalNAc lectin activates Toll-like receptors (TLRs) on intestinal epithelial cells (IECs), leading to IL-8 secretion and neutrophil recruitment. IEC damage from amoebic cytotoxicity further induces secretion of proinflammatory mediators and disrupts tight junctions, enhancing neutrophil infiltration. Neutrophil reactive oxygen species (ROS) can kill trophozoites; however, trophozoites also kill immune cells. ROS can also exacerbate host tissue damage. Trophozoite peroxiredoxin (PRX) and thioredoxin (TRX) detoxify ROS. Amoebic PG2 can suppress ROS production and impair major histocompatibility complex II (MHCII) expression on macrophages, inhibiting their antigen-presenting ability. Dendritic cells (DCs) in the lamina propria also

act as antigen-presenting cells and recognize amoebic LPPG via TLR-2. DCs can activate natural killer and CD+ T cells. In invasive disease IFN-γ-producing natural killer T cells (NKTs) are associated with production. TNF-α production by NKTs and macrophages is associated with increased disease severity. The Gal/GalNAc lectin activates the NLRP3 inflammasome and secretion of IL-1β and IL-18 in macrophages in vitro, though it is not known if this is protective or deleterious. Amoebic proteases cleave complement, IgA, IgG, pro-IL-1β, and IL-18. The Gal/GalNAc lectin inhibits formation of complement membrane attack complex (MAC) and can mediate the capping and shedding of bound antibodies. Processes associated with protection in vivo (animals and/or humans) are indicated with blue arrows; processes associated with disease in vivo are shown in red. Purple lines indicate in vitro evidence. Other abbreviations: CP, cysteine protease; PGE2, prostaglandin 2; sIgA, secretory immunoglobulin A.

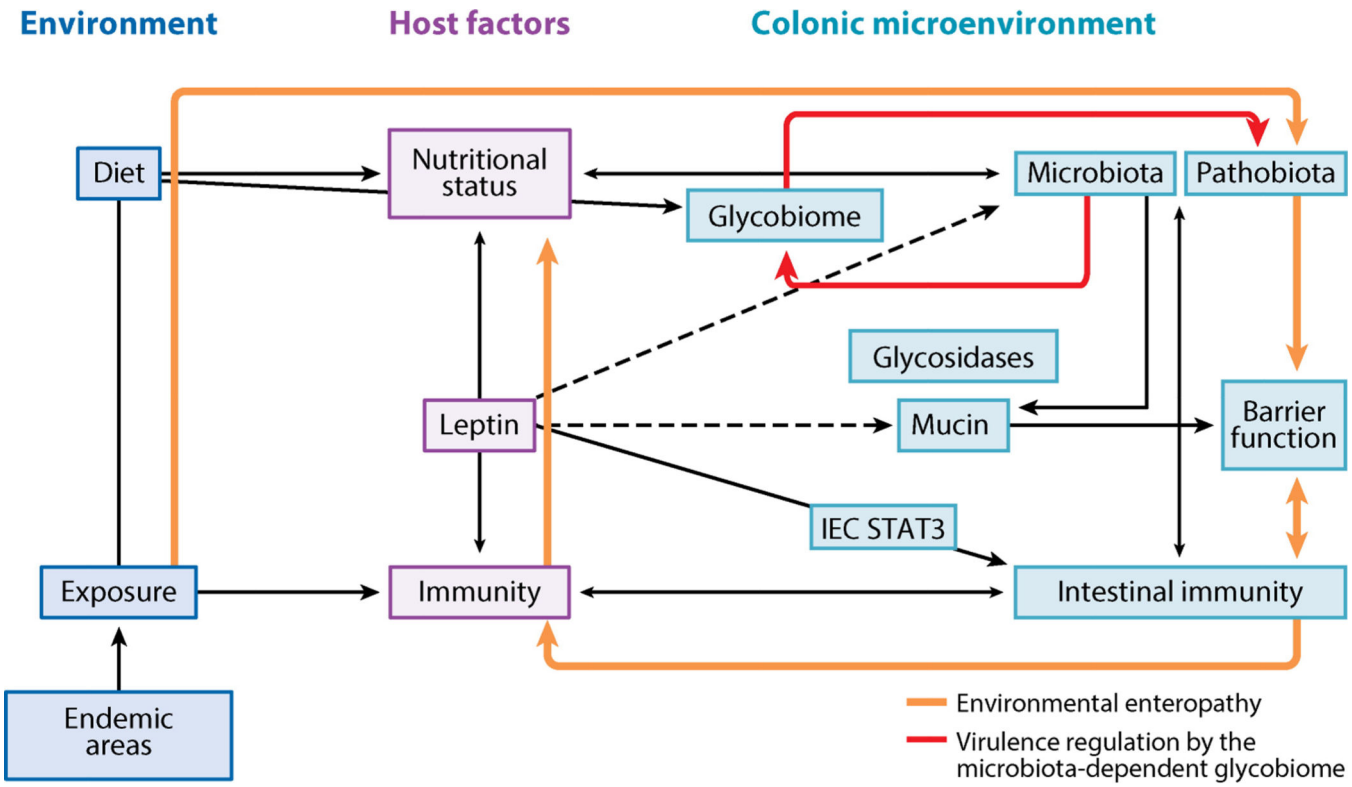

Figure 5.

The vicious cycle of enteric infection and malnutrition. E. histolytica cysts enter the host via fecally contaminated food and water. In areas where E. histolytica infection is endemic, amoebic infection is pervasive and accompanied by multiple other enteric pathogens. The linked immune and nutritional status of the host determines whether infections will be resolved or established in the host intestine. Leptin levels regulate the immunodeficiency of malnutrition, and reduced leptin increases susceptibility to E. histolytica and other pathogens. Leptin signaling is critical for protection from E. histolytica at the intestinal epithelium. In the absence of immune clearance or treatment, E. histolytica and other enteric pathogens establish chronic infection as part of the pathobiota. The pathobiota causes chronic intestinal inflammation and mucus depletion. Chronic inflammatory responses disrupt the absorptive and barrier functions of the intestine, worsening malnutrition and leading to environmental enteropathy. E. histolytica causes dysbiosis with potential consequences for host nutrition and immunity, as the microbiota mediates intestinal immune homeostasis and nutrient extraction. The microbiota stimulates antimicrobial peptide and mucin production by intestinal epithelial cells (IECs), leading to exclusion of pathogens. Microbial metabolism of dietary and host-derived carbohydrates is essential for host nutrient absorption and for microbial metabolism and leads to competitive exclusion of some enteric pathogens. Microbial metabolism of complex polysaccharides in the colon produces short-chain fatty acids and oligosaccharides, which are critical for host nutrition. E. histolytica encodes sugar transporters and glycosidase genes from prokaryotes, indicating that E. histolytica is capable of exploiting free sugars produced by microbial metabolism. The

microbially derived glycobiome is also an emerging regulator of enteric pathogen virulence. Dashed lines are hypothetical.

Table 1:

Activity, expression and localization of proteases

Name	Substrate	Virulence phenotypes and expression	Localization	Ref
CP1	Collagen pro-IL-1β IL-1β(active) villin C3C3b (active)	Upregulated in murine colitis, ALA, HM1:IMSS v Rahman. Upregulated by mucin. KD did not prevent monolayer destruction. Absent in *Ed.*	Surface Phagosome	(30, 48, 118, 145)
CP2	Proteoglycan	Upregulated by mucin. Overexpression increased monolayer destruction but KD did not prevent monolayer destruction.	Membrane-associated Phagosome	(30, 118)
CP3	Nutrients in phagosome	Upregulated in Rahman v. HM1:IMSS. present in *Ed*	Cytoplasmic Phagosome	(94, 127, 140)
CP4	C3 IgA Lamanin Pro-IL-18 (degrades)	Upregulated in murine colitis and experimental ALA. KD blocked ALA, Chemical inhibition blocked murine colitis upregulated in HM1:IMSS v Rahman. Induced by cell contact, mucin.	Secreted Nuclear Phagosome	(30, 48, 118)
CP5	IgG Mucin BSA Integrin binding	KD prevents lamina propria invasion in colonic explants and monolayer destruction. Overexpression increased monolayer destruction but KD did not prevent monolayer destruction. Upregulated in HM1:IMSS and ALA isolates. Upregulated by mucin. Absent in *Ed*	Surface Phagosome	(30, 48, 118)
CPADH (CP112 +ADH112)	Collagen Fibronectin, Hemoglobin Integrin binding	Antibodies block adherence, phagocytosis, monolayer destruction and ALA.	Cytoplasmic vesicles Plasma- membrane Secreted	(118)
CPBF1	Binds CP5	Required for CP5 activity	ER Phagosomes	(92)
CPBF6	Binds α-amylase and γ-amylase	Transports α-amylase and γ-amylase to phagosome	Lysosomes Phagosomes	(41)
CPBF8	Binds β-hexosaminidase and lysozymes	KD decreased digestion of bacteria in phagosomes and cytotoxicity	Lysosomes Phagosomes	(40)
MSP-1	Metalloprotease Homology to leishmanolysin	KD increased adherence / reduced motility, phagocytosis and cytotoxicity. Upregulated in HM1:IMSS v. Rahman. Absent in *Ed*	Surface	(126, 127)
ROM1	Intramembrane protease, binds HGL	KD reduced adherence/cytotoxicity	Surface Cap Vesicles	(12)
β-amylase	Starch	Upregulated in HM1:IMSS v. Rahman. Induced by explant contact. KD reduced mucin degradation.	Surface, Cytoplasmic vesicles	(127)
Lysozyme	Nutrient degradation	Upregulated in murine colitis. Upregulated in Rahman v. HM1:IMSS.	Surface, Phagosome	(48, 127)

KD-knockdown, *Ed-E. dispar*