Semin Immunopathol (2012) 34:771–785
DOI 10.1007/s00281-012-0349-1

REVIEW

The dynamic interdependence of amebiasis, innate immunity, and undernutrition

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Received: 17 April 2012 / Accepted: 21 September 2012 / Published online: 1 November 2012

Abstract *Entamoeba histolytica*, the protozoan parasite that causes amebic dysentery, greatly contributes to disease burden in the developing world. Efforts to exhaustively characterize the pathogenesis of amebiasis have increased our understanding of the dynamic host–parasite interaction and the process by which *E. histolytica* trophozoites transition from gut commensals to invaders of the intestinal epithelium. Mouse models of disease continue to be instrumental in this area. At the same time, large-scale studies in human populations have identified genetic and environmental factors that influence susceptibility to amebiasis. Nutritional status has long been known to globally influence immune function. So it is not surprising that undernutrition has emerged as a critical risk factor. A better understanding of how nutritional status affects immunity to *E. histolytica* will have dramatic implications in the development of novel treatments. Future work should continue to characterize the fascinating host–parasite arms race that occurs at each stage of infection.

Keywords *Entamoeba histolytica* · Amebiasis · Innate immunity · Undernutrition

Introduction

Entamoeba histolytica is a protozoan parasite endemic to much of the developing world. The WHO estimates that 100 million yearly *E. histolytica* infections result in approximately 100,000 deaths from dysentery, colitis, and extraintestinal diseases such as amebic liver abscess (ALA). Amebiasis spreads by ingestion of food and water contaminated with amebic cysts, which are resistant to the caustic environment of the stomach and pass unimpeded into the intestine. Excystation and colonization occur in the terminal ileum. *E. histolytica* typically establishes a commensal relationship with the host and cysts of nonpathogenic trophozoites are excreted, perpetuating the life cycle of the parasite. It is estimated that 90 % of cases are asymptomatic while 10 % of infections progress to symptomatic disease. A small but significant subpopulation of symptomatic individuals develops severe extraintestinal amebiasis, including amebic liver abscess [1, 2].

It has long been suspected but is now confirmed that undernutrition significantly increases susceptibility to *E. histolytica* infection. Undernutrition contributes to 2.2 million deaths and 21 % of disability-adjusted life years in children under 5. Estimates are that undernutrition and its associated diseases contribute to 35 % of child deaths and 11 % of the global disease burden [3]. Studies of amebiasis in human populations and mouse models have informed our knowledge of host factors that influence susceptibility to *E. histolytica* and parasite factors that may directly alter virulence. Adaptive immunity develops in continually exposed human populations and is believed to mainly result from production of neutralizing antibodies targeted to the Gal/GalNAc adherence lectin [4]. Secretory IgA against this lectin is associated with protection in humans [5] and has recently been shown to correlate with immunity in experimentally vaccinated baboons [6]. Studies targeting vaccine development in mice suggest that protective immunity is mediated by an interferon gamma (IFN-γ)-dependent T cell response during infection with *E. histolytica* trophozoites [7, 8]. As vaccine development is ongoing, this review focuses on the innate immune response during amebiasis. We

This article is a contribution to the special issue on Immunoparasitology - Guest Editor: Miguel Stadecker

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emphasize the importance of environmental factors that alter host resistance to amebic disease, in particular the emerging role of host nutritional status. We also explore recent work that has shed light on both host and parasite genetic factors important in the pathogenesis of *E. histolytica* infection.

Parasite

Known amebic virulence factors include the Gal/GalNAc adherence lectin, cysteine proteases (CPs), arginase, amebapores, alcohol dehydrogenase, peroxiredoxin, cyclooxygenase 2, and lipopeptidophosphoglycan (LPPG) [9, 10]. Studies of *E. histolytica* virulence continue to uncover new factors involved in pathogenesis and interaction with the host innate immune system and more are likely to emerge from further analysis of the parasite genome [11]. Characterized virulence factors of *E. histolytica* are often downregulated in nonpathogenic isolates [12, 13] or absent in the nonpathogenic amebic species *Entamoeba dispar* and *Entamoeba moshkovski* [14–16]

Recent work in *E. histolytica* genomics supports a role for the parasite genotype and genetic recombination in the progression of amebiasis. Ali et al. have found that trophozoites isolated from the gut and liver of patients with both intestinal and extraintestinal disease are genetically distinct from parasites in the stool [17]. One explanation is that genetically distinct subpopulations of virulent trophozoites exist in the gut. Alternatively, progression of amebic disease may correspond to genetic reorganization events such as recombination that promote an invasive phenotype. However, the host and parasite effectors driving these events at each stage of infection remain poorly defined. Developing more powerful tools to perform within-patient comparisons of amebic virulence at different stages of infection will greatly increase our understanding of this process. It also remains unclear whether regional genomic differences in *E. histolytica* strains affect pathogenicity.

Gilchrist et al. compellingly demonstrated that expression of virulence genes is regulated at the mRNA level in the first genome-wide analysis of the *E. histolytica* transcriptome in 2006. This study demonstrated that invasive trophozoites in mice significantly altered mRNA expression for genes with diverse roles in metabolism and virulence [18]. Work on the calcium-regulated transcription factor URE3BP also supports a crucial role for transcriptional regulation in amebic virulence. URE3BP was originally identified as a regulator of the Gal/GalNAc adherence lectin and ferredoxin. Later work identified a calcium-dependent phospholipid-associated peptide, ehC2A, which sequesters URE3BP to the plasma membrane in high calcium [19]. A comprehensive study of the role of URE3BP in amebic virulence has revealed that trophozoites expressing a dominant positive form of URE3BP are more pathogenic, forming dramatically larger lesions in the livers of challenged gerbils [20].

The potent cytotoxic effect that *E. histolytica* exerts on host cells is evidence that the parasite possesses a unique armamentarium of virulence factors. However, much work remains to be done to define parasite factors and how they interact with the host at each stage of disease. Further clarifying the mechanisms by which trophozoites transition from intestinal colonizers to invasive pathogens has potential for future anti-parasitic drug development.

Environment

Diarrheal diseases like amebiasis are endemic to the developing world where a number of environmental factors including limited access to clean food and water, and exposure to other enteropathogens contribute to increased risk of infection [1, 3, 16, 21–23]. We are now in the 14th year of tracking the medical history of a cohort of children living in Mirpur, an urban slum of Dhaka, Bangladesh where *E. histolytica* infection is one of the major causes of diarrheal illness. Some of the major findings from this population are summarized in Table 1. A picture has begun to emerge in which infant undernutrition and stunting predisposes young children to amebiasis, which in turn exacerbates preexisting nutritional imbalance by altering gut function.

Undernutrition increases the risk of diarrhea and its associated mortality [24]. Undernourished children have higher rates of infection by protozoan parasites particularly *E. histolytica* and *Cryptosporidium* relative to well-nourished controls [25]. A recent study of infants from Mirpur in the first year of life by Mondal et al. showed that undernutrition and stunting at 12 months of age is significantly associated with nutritional status at birth, duration and severity of diarrheal episodes, and diminished intestinal barrier function [26]. While this association is quite clear, the complex etiology is not yet fully understood.

Protein energy malnutrition is the most common cause of human secondary immunodeficiency, which is particularly problematic for diarrheal diseases that further diminish nutrient uptake from the gastrointestinal tract [27–30]. Acute starvation initiates energy-saving mechanisms, shunting power away from immune function. Chronic nutritional deprivation leads to severe immune deficits exemplified by the dramatic thymic atrophy observed in cadavers of undernourished individuals [31, 32]. Complement deficits, reduced IgA production and specificity, altered gut barrier function due to restructured microvilli, impaired phagocytic capacity, and diminished oxidative burst have all been described as consequences of energy deficit in the immune

Table 1 Summary of studies from Mirpur amebiasis birth cohort

Author	Date	Study	Major findings
Haque et al.	2001	Measured stool IgA anti-lectin and association with incidence of *Entamoeba histolytica* diarrhea	Mucousal IgA antibodies raised against the amebic GalNAc-lectic are protective against *E. histolytica*. Corroborated in 2006
Haque et al.	2002	Anti-lectic serum IgG	Isolated trophozoites were genetically diverse
		Anti-lectic fecal IgA	Lack of serum IgG against the GalNAc lectic linked to resistance
		PCR analysis by isolated trophozoites	86 % reduction in new infection of 12 months in children who developed IgA anti-lectin
Haque et al.	2003	Diagnostic tools to measure causes of diarrheal episodes	Well-nourished children had fewer episodes of diarrheal overall
			Most frequent causes of diarrhea included enterotoxigenic *Escherichia coli*, *Shigella*, *Rotavirus*, *Giardia lamblia*, *Cryptosporidium parvum*, and *E. histolytica*
			E. histolytica contributed to overall morbidity from diarrheal illness
Duggal et al.	2004	Genotype leukocyte antigen class II alleles	Children with DQB1*0601/DRB1*1501 haplotype were 10.1 times more resistant to *E. histolytica* and less likely to be sero-positive for anti-lectin IgG
			HLA class II-restricted immune responses protective against *E. histolytica* infection
Tarleton et al.	2006	Verbal and nonverbal test for cognitive ability	Cognitive score negatively associated with stunting in school-age children
			E. histolytica diarrhea was not independently associated with cognitive deficits when the study was control with social factors
Mondal et al.	2006	Weight for age *Z*-score	*E. histolytica*-associated diarrheal illness was negatively associated with growth of preschool children as measured by WHO WAZ and HAZ
		Heigth for age Z-score Stool antigen diagnostics for *Cryptosporidium* and *E. histolytica*	*Cryptosporidium* and *Giardia* diarrheal episodes not associated with growth
		Microscopic diagnosics for *Giardia*	
Haque et al.	2007	IFN-γ production by amebic antigen stimulated PBMCs	Above-average secretion of IFN-γ by PBMCs associated with longer survival without *E. histolytica* diarrheal episodes
			Adjusted for stunting, the increased risk remained mildly significant
			Concluded that IFN-γ production linked to nutritional status is protective
Mondal et al.	2009	Analyzed association between *E. histolytica* diarrhea and undaernutrition	Enterotoxigenic *E. coli*, *Cryptosporidium*, and *E. histolytica* infection more common in malnourished children
			Malnutrition contributes to susceptibility in a subset of enteric infections
Peterson et al.	2010	TNF-α production by amebic antigen stimulated PBMCs	High level of TNF-α production associated with increase risk of the first and recurrent *E. histolytica* diarrheal episodes
		Microarray analysis of whole blood and colon biopsy samples from patients in acute and convalescent stages of amebiasis	Symptomatic infection was associated with significantly higher production of TNF-α protein and mildly elevated TNF-α message levels
Duggal et al.	2011	Measured genetic variants in leptin and the leptin receptor and investigated their association with amebiasis	Childern homozygous for a SNP encoding a single amino acid substitution in the leptin receptor (Q223R) were found to be nearly four times more likely to have an infection compared with those homozygous for the wild-type allele
			Adult ALA patients were less likely to carry the protective glutamine allele
			Mice carrying the protective allele were significantly more resistant to experimental amebiasis
Peterson et al.	2011	Microarray analysis of intestinal biopsies in acute and convalescent amebiasis	REG1A and REG1B were highly upregulated in human biopsies from patients with acute versus convalescent amebiasis
		RT-qPCR for REG1A/1B	Result confirmed by RT-qPCR
		Challenged REG1 knockout mice with *E. histolytica* trophozoites	REG1 knockout mice were significantly more susceptible to experimental amebiasis

Table 1 (continued)

Author	Date	Study	Major findings
Mondal et al.	2012	Analysis of association between malnutrition, socioeconomic state, immune factor and amebiasis in the first year of life	Children malnourished at birth were significantly more susceptible to *E. histolytica*, *Cryptosporidium*, and ETEC infections Malnutrition in 12 months is predicted by prolonged diarrheal episodes, intestinal barrier dysfunction, maternal education, and family income Concluded that malnutrition at birth predisposes children to amebiasis which alters intestinal barrier function and influences nutritional status at 12 months

Here, we describe major findings from the long-term prospective study of a population living in Mirpur, an urban slum of Dhaka, Bangladesh. Children were observed every other day. Diarrheal stools were tested for *E. histolytica* using antigen detection and PCR. Blood was drawn every 4 months for detection of serum antibodies and cytokine response, and monthly stool samples were obtained to measure secretory immunoglobulin A response. Anthropometrics were assessed every 4 months

system (reviewed in [33]). Global suppression and dysregulation of immune function associated with acute and chronic undernutrition may be the most important independent predictor of *E. histolytica* diarrhea in the first year of life.

Reciprocally, *E. histolytica* diarrheal episodes increase the risk of becoming undernourished [23, 26, 34, 35]. Children with *E. histolytica*-associated diarrhea are nearly three times more likely to be clinically undernourished and are at dramatically increased risk of stunting [34].

Childhood undernutrition and diarrhea are inextricably linked with profound consequences for treatment outcomes in the developing world. Intestinal inflammation resulting from repeated exposure to enteric pathogens leads to malabsorption of critical nutrients, while secondary immunodeficiency related to compromised nutritional status makes children under 5 particularly vulnerable to the devastating cycle of undernutrition, disease, and poverty. To break this cycle, a holistic approach is needed. Treatment strategies to target *E. histolytica* diarrhea in the context of an undernourished patient must be designed. More broadly, nutritional supplementation should be enriched to provide essential nutrients affected by repeated exposure to enteropathogens and such supplements could become a routine part of treatment. Finally, successful vaccines may utilize adjuvants that stimulate innate factors deficient in undernourished individuals. While therapeutic and nutritional interventions are critical, human genetic factors that affect innate resistance to *E. histolytica* infection further complicate the interaction between nutritional status and parasitic disease.

Host

Natural immunity in humans

Mounting evidence suggests that host genetic factors influence frequency and severity of *E. histolytica* diarrheal episodes. The HLA class II allele DQB1*0601 and the haplotype DQB1*0601/DRB1*1501 are associated with drastically decreased rates of *E. histolytica* infection [36]. More recently, a 12-year study of the Mirpur cohort has shown that a single amino acid replacement (Q223R) in the extracellular domain of the leptin receptor encoded by a SNP is associated with a nearly fourfold increase in susceptibility to infection by *E. histolytica* as well as an overall decrease in time to infection. The effects of the mutation were confirmed in a population of adult male ALA patients, where homozygosity for the allele encoding arginine at position 223 was associated with twofold increased susceptibility to extraintestinal disease. These observations highlight the importance of leptin signaling and point to a specific mutation that increases susceptibility to *E. histolytica*, decreases time to infection, and increases the likelihood of developing extraintestinal amebiasis [37].

It is clear that the host genetic background influences susceptibility to amebiasis. However, environmental factors complicate the picture by simultaneously affecting the phenotype. It is important to delineate primary resistance determined by genetics and secondary resistance determined by nutritional status and other environmental factors. It is critical to identify more host genetic factors that affect *E. histolytica* infection. These studies will not only increase our understanding of the pathogenesis of amebiasis but also aid in targeting therapies to at-risk populations.

Cytokines

TNF-α

TNF-α is a cytokine with diverse roles in immunity. Depending on the factors recruited to ligand-bound TNFR1/2, TNF-α can stimulate growth and differentiation through MAPK, survival and inflammation through NFκB, or cell death through caspase activation [38]. The amebic lectin induces TNF-α production by macrophages [39] and

TNF-α acts as a potent chemoattractant for *E. histolytica* [40]; however, the role of TNF-α in amebiasis is not entirely clear.

In vitro, TNF-α enhances killing of *E. histolytica* trophozoites by neutrophils and macrophages [41, 42]. In vivo, TNF-α seems to exacerbate disease. Blocking TNF-α production in the human xenograph SCID mouse model of amebiasis reduces inflammation and intestinal damage [43]. This finding was recently extended to human populations where TNF-α production by amebic antigen stimulated PBMCs correlated with increased susceptibility to *E. histolytica* diarrhea [44]. It has been suggested that IFN-γ and prostaglandin E2 modulate TNF-α production during infection [45]. Thus, a complex array of factors likely determines whether TNF-α has a protective or destructive role during amebic infection.

IFN-γ

Analysis of cytokine profiles in the Mirpur study population has also shown that above average production of IFN-γ but not IL-5 by amebic antigen stimulated PBMCs was associated with favorable outcomes and decreased incidence of *E. histolytica* infection. When controlled for nutritional status, the observation remained only mildly significant suggesting that IFN-γ production was dampened in malnourished subjects [46]. The importance of IFN-γ has been recapitulated in murine vaccination studies, which have demonstrated that IFN-γ is a significant correlate of protection for *E. histolytica* [7, 8].

IL-10

IL-10-deficient mice are more susceptible to amebic infection [47], and undernutrition modifies IL-10 production [48] perhaps contributing to increased susceptibility of undernourished individuals to *E. histolytica* diarrhea. Overall, IL-10 is likely to play a role in connecting nutritional status to amebiasis.

Leptin

The adipocytokine leptin links nutrition to immunity. Immune functions are impaired and leptin levels low in undernourished individuals [49]. However, the role of leptin in starvation-induced immune dysregulation has not been fully elucidated. Data from human studies into the effects of leptin deficiency on immunity in malnourished children are contradictory [50, 51]. Nonetheless, mice and humans with impaired leptin signaling exhibit compromised immune function and are more susceptible to many infections, including amebiasis [52, 53]

Leptin signals through the long form of its receptor via the receptor-associated Janus kinase 2 (JAK2). JAK2 phosphorylates intracellular tyrosine residues Y985, Y1077, and Y1138, which recruit SHP2, STAT5, and STAT3, respectively. SHP2 positively regulates the ERK/c-fos pathway [54]. Activated STAT3 upregulates SOCS3, which serves as both a feedback inhibitor of leptin signaling and a repressor of apoptotic pathways. Leptin receptor-mediated SOCS3 signaling also regulates a number of proinflammatory pathways [55]

Leptin signaling plays a crucial and well-characterized role in a number of immune pathways that are important for protection against amebiasis. Leptin enhances CCL chemokine secretion in murine macrophages [56], which promotes chemotaxis of monocytes. Leptin also acts directly to mobilize monocyte populations via leptin-dependent PI3K/Akt activation [57]. In leptin and leptin receptor-deficient mice, macrophages exhibit phenotypic variations like diminished phagocytic capacity and lowered cytokine production [58, 59].

Leptin signals secretion of proinflammatory cytokines TNF-α and IL-6 [60] and may regulate the monocytic oxidative burst [61]. Stimulation with leptin upregulates production of interleukin 1 receptor antagonist [62] and IFN-γ inducible protein [63]. Overall, leptin contributes to a Th1 proinflammatory immune response, which is crucial for clearance of trophozoites during amebiasis. Leptin receptor null mice (db^{-}/db^{-}) exhibit a skewed Th2 cytokine profile and diminished T cell proliferation [64], which correlate with increased susceptibility to *E. histolytica*.

Proinflammatory chemokines and cytokines IL-6, IL1-β, and CXCL1 are upregulated in response to leptin [65], and leptin concentrations spike during gut inflammation [66]. The primary action of leptin in intestinal epithelial cells (IECs) appears to be mediated through STAT3, a transcription factor known to regulate epithelial homeostasis during infection and inflammation [67, 68]. Pathogenic strains of bacteria in mice activate STAT3 in colonic epithelial cells, initiating a protective Th17 mucosal immune response, characterized by secretion of IL-17. TH-17 response stimulates production of IL-6 and IL-8, potent mediators of inflammation and neutrophil chemotaxis, respectively [69].

Leptin stimulates anti-apoptotic and proliferative pathways that promote barrier function and wound healing in epithelial layers [66, 69–73]. Leptin signaling is anti-apoptotic in a variety of cell types [74]. Dendritic cells from *db^{-}/db^{-} mice are more prone to apoptosis potentially as a result of decreased PI3K/Akt, STAT3, and IκB-α signaling [75, 76]. Increased caspase-3 expression is observed in colonic epithelial cells of db^{-}/db^{-} mice [69].*

The pleiotropic role of leptin in immune function and energy balance supports the hypothesis that leptin is critical to an appropriate immune response in enteric diseases like amebiasis and may explain mechanistically the strong association between undernutrition and amebic disease. Recent studies in humans and mice have revealed the importance of leptin signaling in protection against *E. histolytica*.

Guo et al. have shown that ob^{-}/ob^{-} and db^{-}/db^{-} mice are more susceptible to amebic infection than are wild-type controls. Moreover, leptin signaling in the intestinal epithelium alone was sufficient to confer wild-type resistance. The site of leptin-mediated resistance to *E. histolytica* was determined using tissue specific deletions of the leptin receptor in *E. histolytica* resistant C57B2/6 mice. Deletion of the leptin receptor in the intestinal epithelium was sufficient to render mice susceptible to amebic infection. Expression of a leptin receptor deficient in STAT5 signaling also rescued the susceptible phenotype. However, complementation with receptors deficient in SHP-2 or STAT3 did not restore resistance, implicating STAT3 and SHP2 but not STAT5 in leptin-mediated resistance to amebiasis [52].

To further understand leptin signaling in host defense, we developed an assay to measure amebic cytotoxicity in single cells. Physiological concentrations of leptin were protective against amebic cytotoxicity in cells that endogenously or exogenously expressed leptin receptor. In HEK293T cells exogenously expressing leptin receptor signaling mutants, only cells deficient in the leptin-dependent STAT3 signaling pathway lost leptin-mediated resistance to amebic challenge while SHP2 and STAT5 mutants remained resistant.

The Q223R mutation in the leptin receptor also increased amebic cytotoxicity in vitro, and cells expressing the Q223 form of the leptin receptor displayed significantly higher levels of activated STAT3 in response to leptin [77]. In combination, these results imply that leptin-dependent STAT3 activation mediates direct cellular resistance to amebic cytotoxicity while leptin-activated SHP-2 signaling may be important in coordinating the global immune response to amebiasis. It is important to emphasize that this mechanism was elucidated in murine and in vitro models of amebic disease and has yet to be extensively validated in humans.

Innate cell-mediated immunity to *E. histolytica*

Intestinal immunity

The intestinal epithelium is essential for protection against amebiasis. Approximately 90 % of *E. histolytica* infections are commensal, suggesting that the gut is highly effective at limiting progression to symptomatic disease [78]. The protective role of the epithelium in amebiasis is mediated by the mucousal layer, which prevents ameba from adhering to IECs. IECs and tight gap junctions provide a second physical barrier to invasion. As these barriers begin to fail, epithelial cells recruit and activate other effectors of innate immunity through chemokine and cytokine release. Resident microbes may also affect *E. histolytica* virulence and intestinal inflammation. A simplified model of intestinal invasion by *E. histolytica* trophozoites is depicted in Fig. 1.

Mucousal layer

Mucus offers the first layer of protection against invading trophozoites in the intestine. Goblet cells secrete mucins, which solubilize to form a gelatinous barrier between the lumen and epithelium forming a barrier against enteric pathogens. Studies by Chadee et al. have shown that secreted colonic mucins are critical for gastric mucosal repair [79] and resistance to bacterial colitis [80]. The process by which invasive *E. histolytica* trophozoites penetrate the protective mucous barrier to contact and kill sub-mucousal epithelial cells is well understood. The Gal/GalNac adherence lectin of *E. histolytica* adheres to glycosylated components of the mucousal matrix [81]. Next, parasitic cysteine proteases cleave mucins at sites of low glycosylation. Degrading mucins and other components of the mucous layer allows ameba to bind sub-mucousal cells, induce cell death, and penetrate the epithelium [82].

Recent work in *MUC2* knockout mice has highlighted the specific importance of mucins as protective molecules against *E. histolytica*. Bergstrom et al. demonstrated that MUC2 deficient mice challenged with *E. histolytica* displayed an acute proinflammatory response, characterized by increased gross pathology, luminal TNF-α and IFN-γ, and altered expression of gap junction proteins [80]. Results from Chadee et al. recently recapitulated this finding in colonic loops from *MUC2*-deficient mice and have gone on to show that trophozoites also require MUC2 to invade the epithelium. *E. histolytica* appears to induce higher *MUC2* expression and trophozoites become trapped in the mucous coating of the colonic epithelium presumably before invading.

E. histolytica cysteine protease 5 (CP5) directly cleaves MUC2. Expression of this protease likely allows the parasite to overcome the protective mucous barrier [83]. When CP5 is silenced, trophozoites fail to penetrate the colonic lamina propria and do not induce proinflammatory cytokine secretion in ex vivo models of amebic colonic invasion [84]. These results suggest that disassociation of *E. histolytica* from the colonic epithelium by mucous, prevents a damaging inflammatory response.

Microbiome

The microbes that colonize the shallow section of the mucosal layer of the intestinal epithelium may improve barrier function by inhibiting adherence of pathogenic species and competing for nutrients. *Entamoeba* are naturally colonized by bacteria, which affect parasite virulence [85]. The axenization of *Entamoeba* alters the surface antigens of the parasite [86]. As trophozoites feed primarily on resident gut flora, it is plausible that differences in host microbial ecology contribute to the initiation of a virulent phenotype.

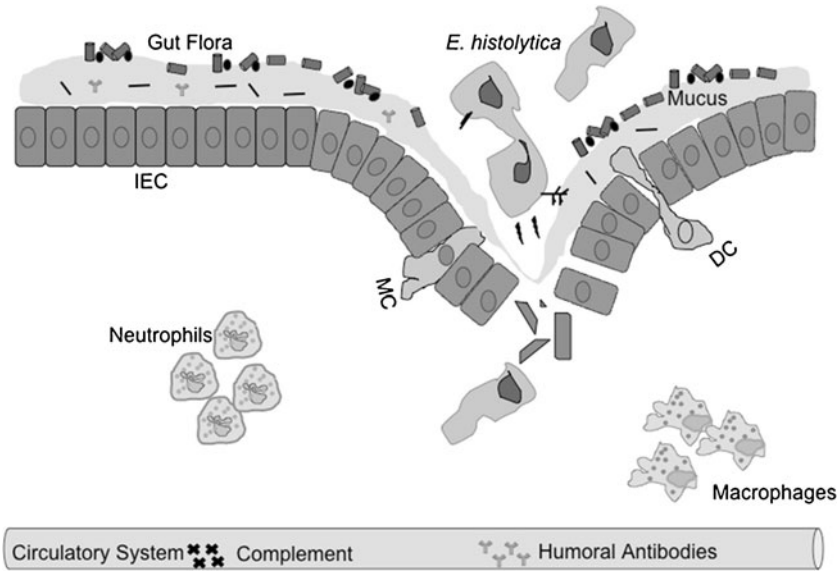

Fig. 1 Intestinal immunity. *E. histolytica* trophozoites encounter a layered host defense as they invade the intestine. Intestinal epithelial cells (*IECs*) direct an inflammatory response to invasive ameba by recruiting neutrophils, macrophages, and other immune cells. Trophozoites that survive may progress to the circulatory system where they encounter humoral antibodies and must evade complement-mediated lysis

Already, reduction in *lactobacillus* species has been observed in ALA patients [87].

Numerous studies have shown that microbial composition is linked to nutritional status and its composition can alter nutrient absorption and bioavailability [88–91]. Nutrient deprivation alters host susceptibility but may also contribute to virulence of the parasite. Several micro- and macronutrients have been shown to influence *E. histolytica* virulence including calcium via regulation of URE3BP [19], glucose [92], cholesterol [93], and iron [94].

The emerging field of microbial metagenomics is likely to continue to reveal an important role for the gut microbiome in the pathogenesis of *E. histolytica* via modulation of the immune response and virulence of the parasite itself.

Antimicrobial compounds

Secreted immunoglobulins, defensins, and cathalecidins reinforce mucousal barrier function, actively preventing pathogenic colonization by ameba. Cathelicidins, antimicrobial peptides resident in the gut mucosa, are important mediators of mammalian innate immunity and while *E. histolytica* trophozoites activate cathelicidin production in both human and mouse intestinal epithelial layers, they are resistant to their cytolytic effect. Amebic cysteine proteases cleave the cathelicidins LL-37 and CRAMP but the fragments retain bactericidal activity [95].

Four regenerating gene (REG) C-type lectins have been characterized in humans. Overall, REGs function to induce cellular proliferation and inhibit apoptosis. REGIα seems to have a physiological role in the gastric mucosa and REGs are found throughout the gastrointestinal system. *REGIα*, *REGIβ*, and *REGIII* mRNAs are overexpressed in the colon in inflammatory bowel disease, Crohn's disease, and ulcerative colitis [96]. Microarray analysis of intestinal biopsies from patients in early and recovery stages of amebiasis has shown that *REGIα* and *REGIβ* are highly upregulated during acute infection relative to convalescence and immunohistochemical studies confirm this result [97]. Thus, REGs likely have a functional role in regeneration of the intestinal epithelium after amebic disease. The recently discovered antimicrobial function of REGIII [98] suggests that REG proteins may have dual functions in intestinal barrier reinforcement and amebic killing [99].

Intestinal epithelial cells

IECs recognize multiple amebic antigens and direct the immune response in the acute phase of amebiasis. It is generally thought that the epithelium downregulates pattern recognition receptors (PRR) to prevent chronic inflammation in response to resident microbial antigens [9]. Interestingly, stimulation of IEC layers with the adherence lectin of *E. histolytica* increases expression of TLR2, which recognizes amebic LPPG and activates NFκB-regulated secretion of proinflammatory cytokines from human monocytes, macrophages, and dendritic cells [100]. In vitro, when stimulated with amebic antigens, IECs secrete TNF-α, IL-6, GROa, and GM-CSF [101]. *E. histolytica* DNA has been shown to activate TLR9 [100] which recognizes unmethylated CpG dinucleotides [101]. Additionally, CP5 binds to α(V)β(3) integrin on Caco-2 colonic cells via the RGB motif and stimulates an NFκB-mediated proinflammatory responses via PI3K/Akt [102, 103]. In the SCID mouse–human xenograft model of ALA, amebic challenge increased IL-8 and IL-1β secretion presumably promoting acute neutrophilic infiltration [104]. IEC death itself may recruit and stimulate immune cells. It has long been thought that contact-

dependent killing by trophozoites was canonically apoptotic. Amebic cytotoxicity induces membrane blebbing, TUNEL positivity, surface exposure of phosphatidylserine, and caspase activation. Furthermore, caspase inhibition and overexpression of anti-apoptotic effectors like Bcl-2 are protective in mouse models of disease [105]. Pyropototic and necrotic death are increasingly recognized as important for amebic cytotoxicity as host cells experience a rapid loss of membrane integrity not consistent with canonical apoptosis. These latter death pathways may contribute more potently to inflammation in the intestine through release of host cellular components.

Amebic invasion through the IEC layer and into the lamina propria may occur passively through small gaps resulting from inflammation [101]. Active penetration is likely mediated by amebic proteases that cleave gap junctions, facilitating transepithelial movement [14]. Interestingly, *E. histolytica* has adapted to dampen the NFκB-mediated inflammatory response of IECs via induction of heat shock protein 27 (hsp27), which suppresses NFκB transcriptional regulation [106].

These observations suggest that the intestinal epithelium is crucial barrier to infection by *E. histolytica*. Pathogenic ameba must breach the mucousal matrix to access IECs, whose PRRs recognize and respond to amebic invasion. At the onset of acute amebic colitis, ulcers form and inflammatory signals from IECs recruit effectors of innate immunity to the site of danger while initiating an adaptive response. The degree of immune activation in the intestinal epithelium likely determines the progression and severity of amebiasis as much of the resultant tissue damage is mediated by the host inflammatory response.

Post-epithelial immunity

Neutrophils The primary role of neutrophils in innate immunity is to destroy and engulf pathogens in affected tissues. Neutrophil extracellular NADPH oxidase generates reactive oxygen species that are toxic to invading pathogens. Neutrophils also express surface receptors for opsonins that facilitate phagocytosis of pathogens marked by other branches of the immune system for destruction [115]. Neutrophils also exude extracellular traps (NETs) composed of chromatin and serine proteases that ensnare and neutralize pathogens [116]. A role for NETs in the pathogenesis of amebiasis has yet to be characterized.

Trophozoites that breach the epithelial barrier elicit a rapid infiltration of neutrophils to the site of infection in early stages of intestinal disease [107, 108]. IECs secrete IL-8, a potent neutrophil chemoattractant, when exposed to *E. histolytica* [109]. Activation and translocation may also occur in response to anaphylotoxins C5a and C3a of the complement system though amebic proteases cleave and inactivate both effectors [110]. It is generally accepted that neutrophils are responsible for much of the tissue damage associated with amebic colitis. While neutrophils do possess amebacidal activity, which likely aids in pathogen clearance for asymptomatic infections, virulent trophozoites have adapted effective mechanisms of resistance and counterattack. Neutrophils in culture amplify the cytopathogenicity of *E. histoltyica* trophozoites on epithelial cells [111] likely due to neutrophil degranulation of toxic cellular components [112].

Leptin deficiency and genetic differences in leptin signaling pathways may also affect neutrophil response to amebic invasion. Neutrophils express a short form of the leptin receptor that is sufficient for leptin signaling, which inhibits apoptotic pathways and acts as a chemoattractant [54, 113], and may modulate oxidative capacity in neutrophils during infection [114].

There is some evidence from mouse and in vitro models of amebiasis that neutrophils play a protective role early in invasion [105, 117, 118]. Neutrophil depletion in the SCID mouse model exacerbates intestinal disease and liver abscess formation [105, 117, 118]. However, the GR-1 antibodies used in these studies may also deplete monocytes and eosinophils. In early stages of hepatic amebiasis, neutrophils are the major effectors of the acute inflammatory response, [119] but can be both protective [120] and destructive [111].

Neutrophils primed with IFN-γ, TNF-α, or LPS do exhibit amebicidal activity [112]. However, *E. histolytica* has proven to be far more effective at killing neutrophils, with more virulent strains killing at a ratio of 1 trophozoite to 3,000 neutrophils [112]. *E. histolytica* trophozoites induce neutrophil apoptosis directly by engaging with integrins and by activating NADPH oxidase [121, 122]. Invasive amebic strains are resistant to the respiratory burst as an amebic peroxidoredoxin protects trophozoites from reactive oxygen species [121, 122]. Neutrophil corpses are quickly phagocytosed by trophozoites [112]. Figure 2 summarizes some of what is known about the role of neutrophils in host defense against *E. histolytica*.

Macrophages Macrophages play important roles in the pathogenesis of both intestinal and hepatic amebiasis. Macrophages are activated by *E. histolytica* trophozoites and are amebicidal when stimulated with CSF, IFN-γ, and TNF-α [112]. The amebic surface component LPPG activates a classical inflammatory macrophage response upon TLR 2/6 and TLR 4 binding [123]. *E. histolytica* DNA also promotes macrophage activation by TLR9 binding [104]. Furthermore, macrophages upregulate TLR2 in response to the amebic galactose-specific lectin [104]. Macrophage amebicidal activity is mediated by nitric oxide synthase (NOS) [124]. NO inhibits important amebic virulence factors including the cysteine proteases and alcohol dehydrogenase 2 [125].

Invasive ameba are able to regulate macrophage responses in a number of ways. Analogous to their interaction with neutrophils, trophozoites inhibit the respiratory burst of

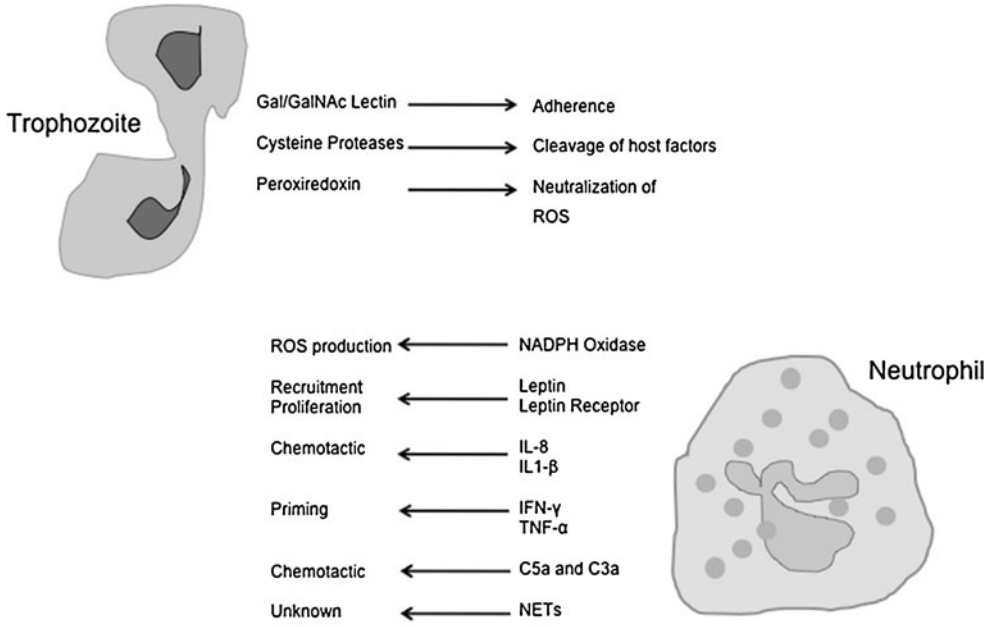

Fig. 2 Neutrophils recruited to the site of *E. histolytica* infection target pathogens by secreting NADPH oxidase, an enzyme that catalyzes the production of reactive oxygen species. Interleukins, interferons, and tumor necrosis factor alpha prime and recruit more immune cells. The parasite responds with peroxiredoxin and cysteine proteases, which cleave and neutralize host factors to make way for lectin-mediated adherence and effectors of amebic cytotoxicity

macrophages. With macrophages, this function depends on an amebic arginase that competitively converts L-arginine, a substrate of macrophage NOS, to L-ornithine [126]. Ameba also modulate macrophage cytokine secretion [127, 128]. Production of TNF-α is blocked by amebic degradation of c-fos and TNF-α transcripts [129]. *E. histolytica* produces a cyclooxygenase 2, the prostaglandin product of which has host-immunomodulatory function [130, 131]. Prostaglandin triggers a cAMP spike in macrophages. This signal activates PKA and IL-8 secretion [131]. PKA inhibits expression of Th1 cytokines and PKC-mediated NO synthesis [130]. Finally, the monocyte locomotion inhibitory factor (MLIF) of *E. histolytica* has been implicated in altering macrophage function. MLIF inhibits NO and proinflammatory cytokine production while stimulating the secretion of IL-10, an important anti-inflammatory effector [132, 133].

Macrophages represent a secondary line of defense after the massive infiltration of neutrophils during acute hepatic amebiasis. NOS is protective and its liver specific deletion increases severity of ALA in infected mice [124]. IFN-γ is also important for macrophage-mediated protection against trophozoites. Blocking IFN-γ receptors increases susceptibility to ALA in mice [134]. Classically activated macrophages are effective at clearing trophozoites and even alternative activation can promote wound repair and recruitment of adaptive immune cells [135]. *E. histolytica* suppresses both activated classes of macrophages, undermining cell-mediated immunity and leading to establishment of chronic ALA [127]. Figure 3 summarizes some of what is known about the role of macrophages in host defense against *E. histolytica*.

Natural killer and natural killer T cells Natural killer cells do not require priming to be functionally cytotoxic against pathogens. Their canonically myopic "killer" function has been challenged in recent years as more studies elucidate diverse roles for NK cells in immune regulation [136]. Activated NK cells secrete IFN-γ and TNF-α, important factors in host-immunity to amebiasis. NK cells infiltrate the liver during the acute phase of experimental ALA in mouse models [137]. Virulent trophozoites stimulate NK cell cytotoxicity more effectively than do non-pathogenic strains [138]. *E. histolytica* may regulate NK cells through CP-mediated cleavage of C5a, an anaphylotoxin known to activate NK cells during sepsis [139].

NKT cells express markers that delineate NK cells but co-express αβ TCR. They differ from most T-cell populations in that they recognize antigens presented by CD1d instead of MHC associated antigens. Invariant NKT (iNKT) cells are a subset of NKT cells that express invariant α-TCR and produce large amounts of pro- and anti-inflammatory cytokines upon activation. iNKT cells are activated to produce IFN-γ but not IL-4 in a TLR/CD1d-dependent manner [140]. Studies in mouse models of ALA suggest that clearance of trophozoites during early stages of hepatic colonization is dependent on NKT cell activity. Mice lacking NKT cells are highly susceptible to ALA [141]. Clearance was associated with elevated IFN-γ production.

Like neutrophils, NKT cells likely have a context-dependent effect on disease progression. Their response to amebic antigens may contribute to polarizing the immune response toward the Th1 phenotype thought to be protective in amebiasis. In terms of ALA, after the acute phase of hepatic infiltration, particularly resistant trophozoites survive and expand. At this stage, iNKT cells may increase tissue damage by propagating deleterious inflammatory signals and stimulating neutrophil chemotaxis.

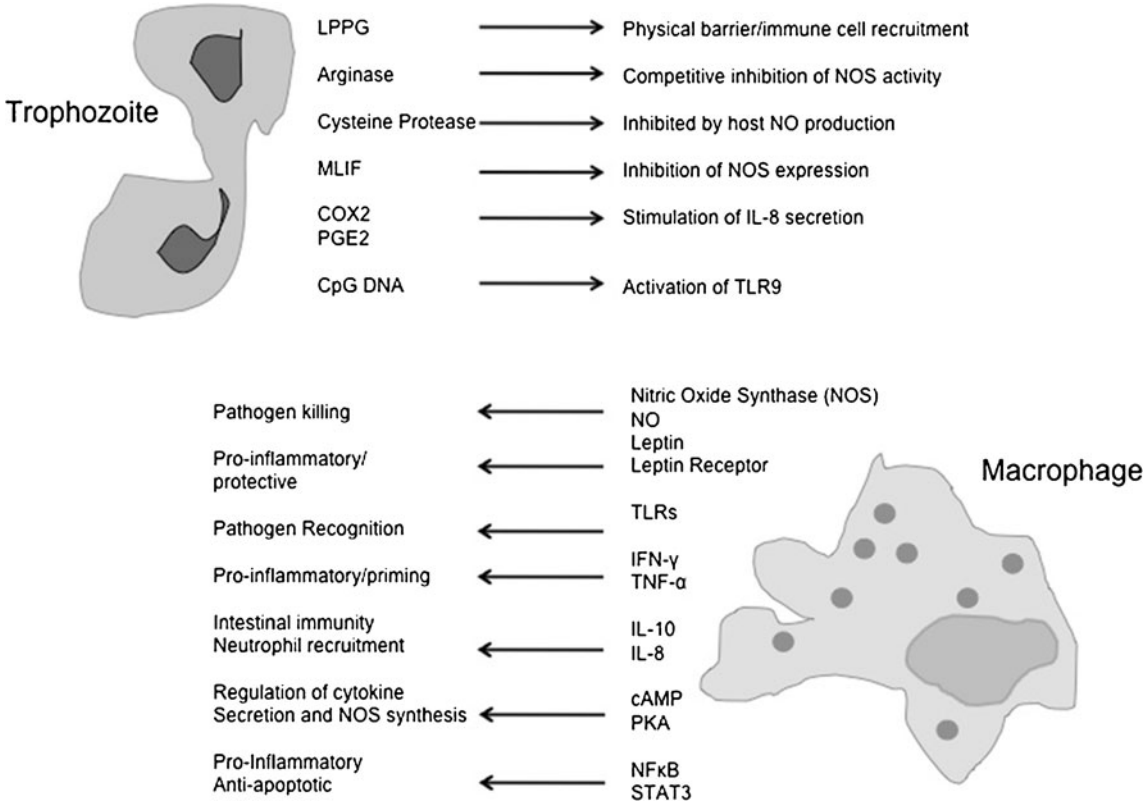

Fig. 3 Macrophages recruited to the site of *E. histolytica* infection are classically activated by interferon gamma and other cytokines. They produce NO to kill pathogens and secrete IL-10 and IL-8, promoting gut barrier function and neutrophil recruitment, respectively. The parasitic arginase competitively inhibits and the MLIF alters expression of host nitrous oxide synthase. *E. histolytica* CpG DNA activates TLR9

Complement The pathogenesis of extraintestinal amebiasis depends on evasion of humoral innate immunity by *E. histolytica* and both alternative and classical branches of the complement system are involved. All complement pathways converge to produce a pathogen-associated C3-convertase that cleaves C3 into the opsonin C3b and the inflammatory anaphylotoxin C3a. The proteolytic cascade culminates in pathogen lysis via deposition and polymerization of pore-forming membrane attack complexes (MACs) on opsonized invaders [142].

E. histolytica can activate both the alternative branch and the serum-antibody-dependent classical branch of the complement cascade [143, 144]. A neutral *E. histolytica* cysteine protease cleaves C3 to an active isoform of C3b [144]. In trophozoites that are susceptible to complement, C3b, a cleavage product of amebic CP, successfully recruits terminal effectors of the cascade resulting in MAC formation and parasite lysis [145]. Trophozoites isolated from asymptomatic cases are readily lysed in a complement-dependent manner, but pathogenic trophozoites from patients with amebic liver abscess or invasive colitis are resistant to complement-mediated killing [146]. These studies suggest that complement is an important barrier against invasion by *E. histolytica*, presumably contributing to the low incidence of extraintestinal disease. Furthermore, amebic resistance to complement-mediated killing represents an adaptation unique to trophozoites that cause extraintestinal disease.

Host cells prevent their own complement-mediated lysis by expressing surface CD59. Expression of CD59 inhibits opsonization and MAC formation on host cells [147]. The Gal/GalNac lectin of *E. histolytica* is a multisubunit GPI-anchored complex essential for parasite recognition of host surfaces. Braga et al. showed that this lectin contains a CD59-like region that inhibits MAC formation and protects the parasite from complement-mediated lysis [148]. In agreement with these results, global inhibition of GPI anchor formation leaves previously resistant *E. histolytica* trophozoites susceptible to complement-mediated lysis [149]. Other CD59-like amebic proteins may also contribute to resistance. Ventura et al. recently identified a novel 21 kDa amebic surface protein that reacts with human anti-CD59 [150]. However, its functionality as an inhibitor of MAC formation and its molecular identity have yet to be elucidated.

Amebic cysteine proteases also play a role in complement evasion. The surface EhCP implicated in AP activation by C3 cleavage [144] also cleaves and inactivates the inflammatory anaphylotoxins C3a and C5a [110]. Thus CPs appears to play opposing roles in evasion of host immunity by *E. histolytica*, on the one hand activating complement and on the other suppressing numerous inflammatory pathways downstream

of C3a and C5a. A third mechanism of complement resistance may involve enrichment of lypophosphoglycan (LPG) in the outer leaflet of the *E. hystolytica* plasma membrane. While LPG is produced in the pathogenic *E. histolytica*, significant levels are not observed in the non-pathogenic strain *E. dispar* [151]. LPG protects *Leishmania* from complement-mediated lysis by impeding the deposition of proteolytic complexes and preventing MAC formation [152]. *E. histolytica* trophozoites may employ a similar strategy.

ALA is approximately five times more prevalent in men than in women, suggesting a sexual dimorphism in susceptibility to extraintestinal amebiasis [153]. Differences in the cytopathogenic effects of complement on *E. histolytica* between the two sexes have been implicated [9]. Nonimmune female serum kills trophozoites more effectively than does nonimmune male serum [154]. However, it is unlikely that complement is solely responsible for the difference in susceptibility to extraintestinal disease. Interestingly, the sexual dimorphism observed in humans was corroborated in mouse studies [134]. Female B6 mice were more effective than males at clearing invasive trophozoites from the liver in a SCID mouse model of ALA [141]. Clearance was associated with a rapid NKT cell response and high IFN-γ production [141]. Male mice cleared infections less effectively, IFN-γ production was less robust, and significant IL-4 production was observed.

Conclusions

Generations of research have provided a tremendous body of knowledge about host immunity to amebiasis. Recent work has highlighted the importance of the parasite genome and transcriptome in progression to a virulent phenotype. Significant progress has also been made in vaccine and drug development and we now have a better understanding of environmental factors that contribute to amebiasis. The complexity of the interaction between *E. histolytica* trophozoites and host-innate immunity continues to make identification of factors that influence susceptibility to infection a fascinating challenge. Effective approaches have united powerful techniques in population genetics, immunology, and epidemiology to probe the full complexity of the relationship. Using work in human populations to inform mouse and in vitro studies of amebiasis has been particularly effective, allowing us to focus on host and parasite factors proven to effect immunity in humans.

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