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ADDENDUM

The delicate balance between *Entamoeba histolytica*, mucus and microbiota

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ABSTRACT

Entamoeba histolytica (*Eh*) is a protozoan parasite of humans that colonizes the outer colonic mucus layer. Under conditions not fully understood, *Eh* breaches innate host defenses and invades the intestinal mucosa-causing amebic colitis and liver abscess. In asymptomatic infection, *Eh* interacts with and feeds on resident microbiota that forms biofilms on the outer colonic mucus layer. Despite the close association between *Eh* and commensal microbiota, we still lack basic knowledge on whether microbiota and/or their metabolites influence *Eh* virulence traits critical in disease pathogenesis. In the pathogenesis of intestinal amebiasis, *Eh* overcomes the protective mucus layer using a combination of mucinase/glycosidase and potent mucus secretagogue activity. In this addendum, we discuss the interconnected role of a healthy mucus barrier and the role commensal microbiota play in shaping innate host defense against *Eh*-induced pro-inflammatory and secretory responses critical in disease pathogenesis.

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Introduction

The intestinal epithelium constitutes a barrier between the luminal content and the lamina propria. This barrier is multilayered and regulates the flow of cells, gases, nutrients and other molecules between both compartments.[1] In the colon, the epithelial barrier is composed of three layers: a single layer of intestinal epithelial cells (IEC) on top of the lamina propria, the mucus layer in the middle and the microbiota and secreted products at the luminal side.[2] The colonic mucus layer can be divided into two compartments: right above the apical side of epithelial cells there is a condensed inner mucus layer which is devoid of bacteria,[3] while the superficial loose outer mucus layer is colonized by commensal microorganisms[4] (Figure 1a).

Microbiota refers to the community of microorganisms gathered together in a determined ecological niche. The human digestive tract harbors numerous, diverse and dynamic population of microorganisms, mostly bacteria, but also important numbers of protozoa, fungi and viruses. These microorganisms have been adapted to live on/in the mucus surface and in the intestinal lumen.[5] Intestinal microbiota is essential for correct nutrition, metabolism and immune function.[6,7] *E. histolytica* (*Eh*) is a human protozoa parasite and the causative agent of amebiasis presented as amebic colitis and amebic liver abscess. Of every 10 individual that are infected one will develop symptoms of the disease.[8] In symptomatic cases, the intensity of the disease varies from mild diarrhea to more intense manifestation characterized by anorexia, asthenia, abdominal pain, alterations in intestinal transit and mucoid diarrhea. If left untreated, intestinal obstruction, fever and vomit appear producing dehydration. When *Eh* overcomes the intestinal mucosa it causes necrosis extended to the submucosa and muscularis, producing the typical amebic "flask-shaped ulcer." Untreated mucosal ulceration could progress to peritonitis, sepsis and death.[8,9] Another species of *Entamoeba* morphologically indistinguishable from *Eh* called *E. dispar* also colonizes the colon. While nonpathogenic, recent studies suggest it could play a role in causing intestinal lesions. The only successful way to differentiate the species is by molecular methods such as PCR and antigen detection tests by ELISA.[10]

Eh have two stages in its life cycle: the trophozoite or vegetative form that colonizes the gut, and the cyst. In the trophozoite form, *Eh* inhabits the colon,

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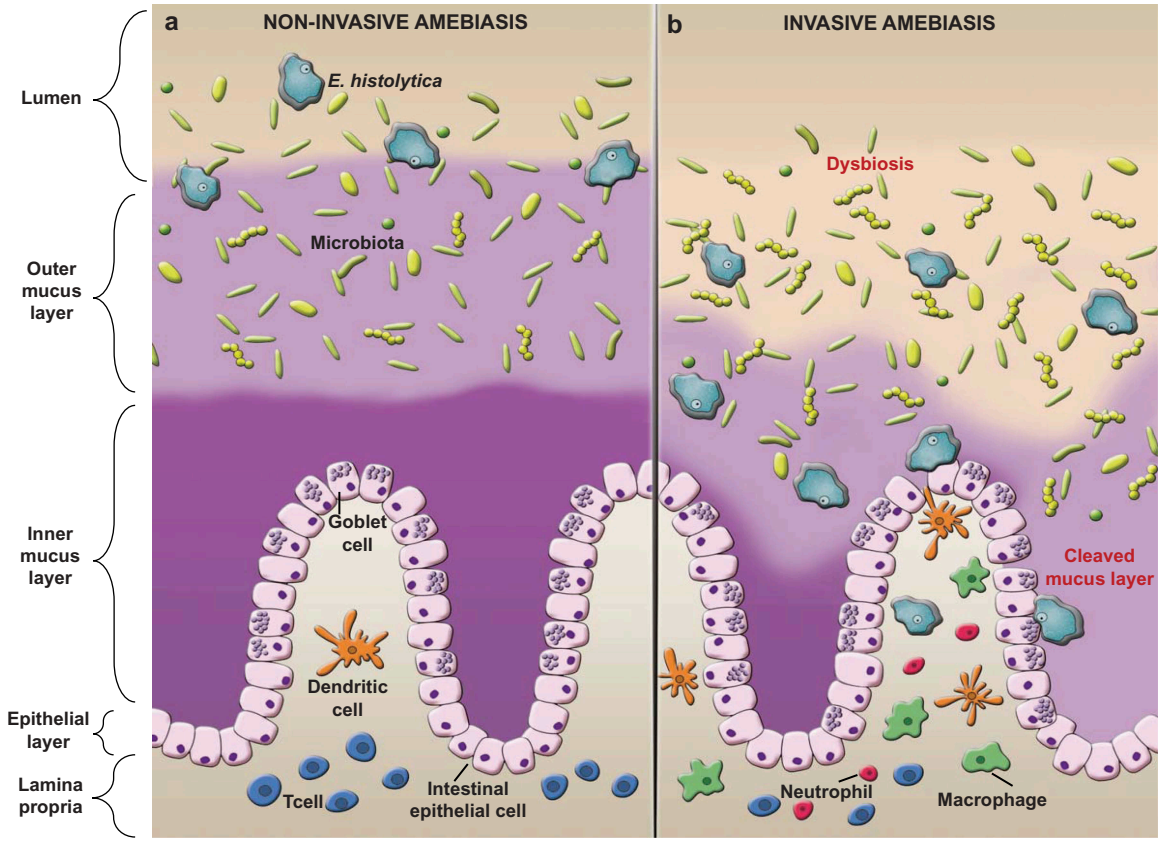

Figure 1. *Entamoeba histolytica* disrupts the mucus layer and cause invasive disease. (a) When *Eh* infects a healthy individual with an intact intestinal barrier, mucus and bacteria keep *Eh* in the outer mucus layer and away from the surface epithelium. Here, *Eh* feeds on bacteria, cell detritus and sugars from mucus establishing asymptomatic infections. (b) Under unknown conditions, *Eh* breaches innate host defenses by cleaving mucin glycans and MUC2 N terminal proteins with parasite glycosidase and cysteine proteases. *Eh*-induced mucus degradation compromises the epithelial barrier and alters microbial composition. *Eh* then contact epithelial cells via parasite Gal/GalNAc lectin and *Eh*CP-A5 to evoke mucus and water secretion and elicits acute pro-inflammatory responses in disease pathogenesis.

where it interacts with resident microbiota and the outer MUC2 mucus layer (Figure 1a). It is known that *Eh* requires commensal bacteria presence for its pathogenicity.[11] *Eh* feeds on microbiota or on bacteria-induced mucin glycans. To counteract this effect, goblet cells increase mucus release, keeping *Eh* away from the epithelial surface.[12,13] Increase mucus secretion could potentially promote differential bacterial colonization,[14] supporting the concept that interaction with microbiota-ameba-mucus could be critical in onset and progression of disease.

Mucus-bacteria interactions

The host-microbe interaction starts in the outer mucus layer. MUC2 O-glycosylated side-chains are rich in N-acetylglucosamine, galactose, N-acetylgalactosamine and fucose.[15] These saccharides residues are keys for commensals and pathogenic organisms binding through their lectins. Binding to the outer mucus layer expels pathogens and opportunistic organisms. Indigenous commensal bacteria harness this bond to have an ecological niche protected by mucus. Thus mucus plays a protective role in maintaining a healthy and stable microbial community, since commensals and pathogens are in constant competition for binding sites to the mucus layer.[16] Recent studies have showed that the O-glycan complex is highly conserved between individuals, suggesting a role in the selection of commensal microbiota providing binding sites to certain sugars.[17-18] This specificity suggests that intestinal mucin influences the composition of the microbiota community. Bacteria degrade mucus and use mucin glycans as an energy source, simultaneously, the released monosaccharaides are turn into SCFA and used as an energy source for intestinal epithelial cells.[19]

Butyrate and propionate induce MUC2 transcription via AP1 binding and histones acetylation and methylation on the MUC2 promoter.[20] Mucin is an important carbohydrate source, mainly in the distal colon where the abundance of fermentable carbohydrate substrates is low.[21] Therefore, bacterial stimulation of mucus secretion, as well as mucin degradation is part of the normal process of mucus turnover and contributes to maintain a functional protective barrier to potential hazards.

Mucus layer, a niche for *E. histolytica*

The first barrier *Eh* must overcome to invade the intestinal mucosa is the MUC2 mucin layers. *Eh* gains access to the intestinal cells by proteolysis of the mucin layer that covers the intestinal epithelium. This process is fundamental for the parasite to exert its pathogenic potential on host cells. *Eh* strongly binds to mucin via the Gal/GalNAc-lectin, which has high affinity for galactose and N-acetyl-D-galactosamine glycans present on the O-linked sugar side chains of the MUC2 molecule.[22] This interaction is essential for *Eh* colonization.

In disease pathogenesis, *Eh* cleaves the polysaccharides present in the outer structure of MUC2 utilizing various glycosidases, such as N-acetylgalactosamidase, N-acetyl-glucosaminidase,[23] β-galactosidase and β-N-acetyl-hexosaminidase.[24] Simultaneously, *Eh* proteases target the peptides in the poor glycosylated regions of the MUC2 C-terminus. *Eh* produces several enzymes with proteolytic activity, among them: cysteine proteases, serine proteases, metalloproteases and aspartic proteases.[25] Cysteine proteases have an important role in invasion and digestion of phagocytosed material, as they degrade different components of the extracellular matrix (fibronectin, laminin and collagen) among others.[26] Cysteine proteases are present in *Eh*-secreted components and have a crucial role in mucin degradation.[12] In addition to degrading MUC2 mucin, the parasite also evokes mucus hypersecretion by goblet cells. *Eh* is a potent mucus secretagogue that leads to goblet cells cavitation and mucus depletion. This is accomplished by cysteine protease-5 (*Eh*CP-A5) arginine-glycine-aspartate peptide (RGD) binding to αvβ3 integrin on goblet cells.[13] We have recently showed that in response to *Eh*, mucus secretion from goblet cells was modulated by a classical exocytosis manner mediated by the SNARE vesicle-associated membrane protein 8 (VAMP8) present in mucin granules,[27] advancing significantly our understanding on mucus secretion basally and in response to *Eh*. Recently, an interesting mechanism for host cell destruction by *Eh* has been described[28] called trogocytosis. This is characterized by *Eh* nibbling and ingesting pieces of epithelial cells consequently causing cell death.

Microbiota and ameba interactions

In the colon, *Eh* interacts with multiple components that can modulate the course of tissue invasion and destruction. Contact between *Eh* and resident microbiota constitutes the beginning of the first host-parasite interaction that could potentially initiate disease. *Eh* in the outer mucus layer[29] shares a niche rich in a diverse community of microbiota, directly interacting with them and benefiting from their presence. Colonic microbiota breaks down complex carbohydrates into glycans[19] that can serve as a nutrient source for *Eh*, at the same time, *Eh* can also feed on the resident microbiota. Infection of *Eh* in germ-free animals failed to induce disease, but *Eh* pathogenicity was re-established after inoculation with bacteria.[11] Similarly, *in vitro* cultures of *Eh* in axenic conditions decreased *Eh* virulence that was restored after inoculation and incubation with live bacteria.[30] More recently, it was shown that bacteria from the Enterobacteriacea family stimulated *Eh* gene response associated with high oxidative stress survival that was not observed when *Eh* was co-cultured with a probiotic strain.[31] The exact mechanism whereby the presence of enteric bacteria is required for *Eh* to express virulence-associated genes remains elusive, but it demonstrates the complex dynamism that exists between *Eh* and commensal organisms, and could potentially provide insights on explaining why only about 10% of infected individuals with *Eh* develop intestinal amebiasis.

E. histolytica disturbs microbiota and promotes translocation

The recent growth in molecular and bioinformatics techniques has made possible significant advances in the identification, classification and characterization of organisms that make up the microbiota. By using these techniques and under the premise that certain bacteria have been associated to different pathologies,

several studies have been carried out to propose that specific bacteria could be relevant in the development of amebiasis. Recent studies from endemic areas have shown that *Eh* infection alters resident bacterial composition (Figure 1b). A study in Northern India linked *Eh*-positive patients with a dysbiotic state, characterized by a decrease in *Bacteroides, Clostridium coccoides, C. leptum, Lactobacillus, Campylobacter* and *Eubacterium* with a corresponding increase in *Bifidobacterium* species.[14] Another study in the Cameroon showed that individuals positive for *Eh* infection presented with an augmented number of bacterial species (alpha diversity) and a decrease in inter-individual variation (beta diversity). This study also linked *Eh* presence with an increase in Clostridiales and Ruminococcaceae and a corresponding decrease in *Prevotella copri* and *Fusobacteria*.[32] A longitudinal study performed in children from a rural zone in Bangladesh associated parasite-induced diarrhea with an expansion of *Prevotella copri*,[33] a bacteria that has been associated with intestinal inflammation. A different study done in patients with amebic liver abscess (ALA) could not relate specific bacteria to ALA incidence; however, most of the ALA patients were co-infected with different bacteria, and particularly presented with a high abundance of *Klebsiella*.[34] The fact that *Eh* induces the production of antimicrobial peptides but are resistant to their cytopathic effect[35] could explain the alteration in microbial composition observed in *Eh* infections. All these association studies came to the same conclusion: it is not known if the observed dysbiosis was a cause or an effect of *Eh* infection. Recently, a metagenomics study on the *vitro* association between *Eh* and enteric bacteria showed that *Eh* preferentially phagocytose *Lactobacillus ruminus, Faecalibacterium prausnitzii, Bifidobacterium longum* and *B. ruminantium*. These findings suggest that *Eh* preferentially phagocytose commensal bacteria that are part of the healthy microbiota that have important beneficial effects in the host.[36] We have previously shown that *Eh* alters tight junction proteins and affect epithelial barrier permeability[37] and promoted the translocation of intestinal bacteria into mucosal surfaces as well as dissemination to other organs.[38] Numerous factors, such as the glycobiome (the microbiota carbohydrate breakdown), the dynamics of opportunistic pathogens under a dysbiotic state and the host immune responses against *Eh* infection, and their effects on microbial composition, among others, need to be considered to clarify this complex relationship.

Microbiota role for an appropriate immune response against *E. histolytica*

As mentioned above, several studies have shown the importance of commensal microbiota in *Eh* virulence. We have recently demonstrated that a dysbiotic state renders the host hyper responsive for increased pro-inflammatory cytokine and chemokine production with hyper secretory responses toward *Eh*[38] (Figure 2a,b). These finding are of great relevance, as individuals with dysbiosis (either by disease, antibiotics or due to a poor diet) that are infected with *Eh* are at high risk to develop severe intestinal amebiasis associated with acute inflammation compared to individuals with a healthy microbiota. Within the clinical setting, this is pertinent, since amebiasis is endemic in developing countries and in sectors of the population (children, elderly or those immunosuppressed) in which they usually administer significant amounts of antibiotics, even without the need for a medical prescription. In this same study, we also demonstrated using germ-free mice that in the absence of commensal microbiota, the normal host immune responses toward *Eh* infection are severely impaired (Figure 2c). These results could be due to a combination of factors such as: (1) naivety of the germ-free mice immune system,[39] caused by the absence of microbiota, and (2) the biochemical nature of Muc2 is different in germ-free mice. These mice posses a thinner and penetrable Muc2 layer,[40] and their O-glycan monomers are shorter than wildtype,[41] thus modifying *Eh*-mucus interactions. With the absence of bacteria in the lumen from which to feed, *Eh* could be forced to approach mucosal epithelial cells in search for alternative energy sources, explaining why *Eh* are in close contact with the epithelium in infected germ-free mice (Figure 2c). Of extreme relevance was that *Eh*-induced inflammation provoked dysregulation of the transcription factor for secretory goblet cells *Math1*, through a mechanism that was microbiota dependent. These results lead us to hypothesize that *Eh* could manipulate innate host defense by modulating goblet cell differentiation to decrease mucus production, which in turn, enabling *Eh* to reach the intestinal epithelium. To date, there is

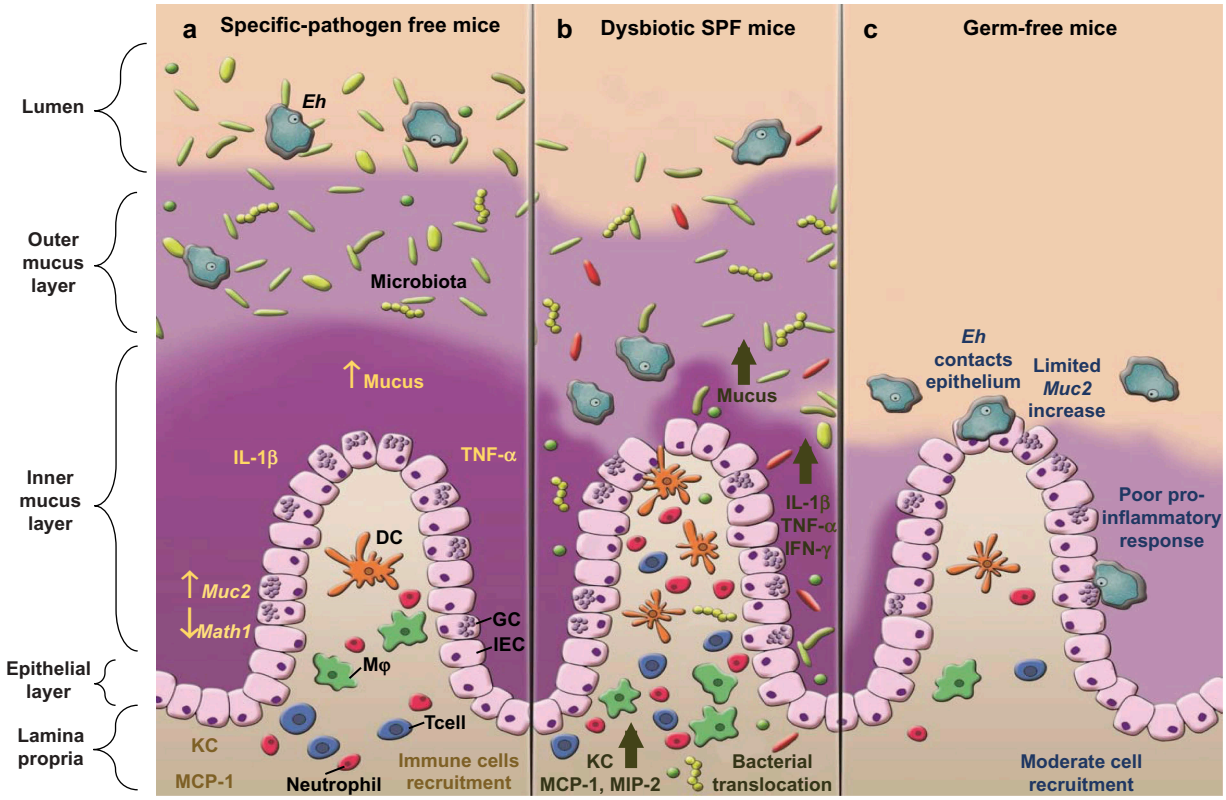

Figure 2. Host response to *E. histolytica* is enhanced when intestinal barriers are disrupted. (a) In specific-pathogen free mice (SPF), with a functional intestinal barrier, the presence of *Eh* evokes an inflammatory response characterized with increase mucus secretion, pro-inflammatory cytokines IL-1β and TNF-α and chemokines KC and MCP-1 with corresponding immune cells recruitment. There is also a reduction of *Math1* transcription factor and an increase in *Muc2* expression. (b) Under antibiotic-induced dysbiosis, *Eh* evokes robust mucus secretion, increased pro-inflammatory and chemokines that promoted bacterial translocation. (c) In germ-free mice, *Math1* and *Muc2* transcript, as well as pro-inflammatory cytokine expression are unaltered. Due to a thinner and biochemically altered mucus layer, *Eh* readily contact and disrupt the intestinal epithelium. GC: goblet cell, IEC: intestinal epithelial cell, M□: macrophage, DC: dendritic cells.

no concrete information on the effect of Math1 on inflammatory-related infectious processes. Certainly, more research must be done to understand the role of this secretory cell transcription factor in the maintenance of homeostasis. Interestingly, this phenomenon also promoted bacterial translocation in the proximal colon and the ileum. Our results showed that *Eh*-induced inflammation in the proximal colon promoted bacterial translocation and disrupted goblet cell lineage. Further investigation will be needed to better understand the connection between commensal microbiota and the immune response to this parasite. Intestinal bacteria can affect the outcome of parasitic helminths infections (reviewed in[42]) as well as those associated with some parasitic protozoa (reviewed in[43]). Studies done in germ free mice have showed that microbiota is important for protozoa to mount a characteristic immune response with *Giardia duodenalis*,[44] *Leishmania amazonensis*[45] and *Schistosoma mansoni* infection.[46] Interestingly this is not the case for *Trypanosoma cruzi* infection,[47] where germ-free mice presented with a worse disease outcome. Parasitic infections in germ-free mice are in its infancy and more studies will be needed to delineate the role play by intestinal bacteria in shaping innate and systemic innate host defenses and the role specific bacterium play in educating the immune system.

Concluding remarks and future directions

Undoubtedly, there is a complex *Eh*-mucus-microbiota relationship. Being able to glimpse and accurately understand the dynamism and the way in which these entities are related is of utmost importance to advance our understanding on the pathogenesis of intestinal amebiasis. Our recent work reveals an interconnection among these three entities, demonstrating that *Eh* in a dysbiotic environment

exacerbates not only the immune response but also mucus and water secretion. We also reported that *Eh* decreased Math1 transcription which was required for goblet cell differentiation.[48] These findings require further investigation to unravel the mechanism by which *Eh* targets secretory lineage maturation. Our study is the first to characterize the host response to *Eh* in germ-free mice. An important finding was that despite the closeness of *Eh* to epithelial cells, the typical pro-inflammatory and secretory response to *Eh* was absent in these mice. These findings reinforce the notion that microbiota plays a critical role in educating and shaping innate host defenses at mucosal surfaces. The importance of intestinal microbiota in maintaining homeostasis is evident and new important roles are continually attributed to it. New and modern molecular techniques will allow us to interrogate its composition, ecology and metabolism in future studies. Being able to understand the relationship and interaction that this has with a parasite of global importance would be highly relevant to treat and manage, not only amebiasis, but other parasitic problems in developing countries. A recent study[49] aimed to identify human protozoa using metagenomics found that co-infection of different protozoa species, mostly commensals, is fairly common. Interestingly, this study was not able to identify a single positive sample for *Eh*, either because the individuals were reported as healthy, and/or because of the challenge in extracting genetic material from *Eh* cyst. This study reinforces the importance of recognizing commensal protozoa as part of our microbiota.[50] This is critical for understanding the dynamics and interactions between different communities that make up the intestinal microbiota. Our studies are just beginning to understand the complex interconnected relationship between *Eh*, mucus and bacteria; however, an explanation of the mechanisms underlying this phenomenon requires further investigation.

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