GUT MICROBES
2019, VOL. 10, NO. 1, 100–104
https://doi.org/10.1080/19490976.2018.1479626

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ADDENDUM

Learning from the research on amebiasis and gut microbiome: Is stimulation by gut flora essential for effective neutrophil mediated protection from external pathogens?

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ABSTRACT

Amebiasis, caused by intestinal infection with *Entamoeba histolytica*, is one of the leading causes of parasite infection-related mortality and morbidity globally. Although its pathogenesis, including determinant factors of infection outcome, remains unclear, recent clinical data indicate that the gut microbiome plays a role in determining the severity of amebiasis. Recently, we investigated the effects of the gut microbiome on neutrophil mediated protection from *E. histolytica* infection using a mouse model. We identified that surface expression of CXCR2 on neutrophils was diminished in mice with dysbiosis, which resulted in decreased neutrophil recruitment to the infection site, allowing more aggressive intestinal tissue damage by *E. histolytica*. Our results indicated that oxidase activity during *E. histolytica* infection was also diminished after dysbiosis, consistent with the results from prior research. Thus, the gut microbiome plays an important role in regulating neutrophil phenotype when fighting against external pathogens.

ARTICLE HISTORY
Received 21 April 2018
Accepted 14 May 2018

KEYWORDS
gut microbiomes; *Entamoeba histolytica*; neutrophils; chemokine receptor; CXCR2; amebiasis

Severity of entamoeba histolytica infection and the gut microbiome in clinical studies

Amebiasis, caused by intestinal infection with *Entamoeba histolytica*, is one of the leading causes of parasite infection-related mortality and morbidity around the world.[1] Although disease severity ranges from self-limiting mild abdominal symptoms to life-threatening systemic disease, determinant factors of infection outcomes are still undefined.[2] Even in the same patient, invasive symptomatic disease can develop after long-term asymptomatic colonization,[3–5] and conversely, patients with amebic liver abscesses after medical treatment can be asymptomatic cyst passers.[6] These results suggest that not only are the genetics of both host and pathogen important for determining clinical symptoms of infected individuals, but also the gut environment surrounding *E. histolytica*. A group from India previously reported that the gut microbiome could be different between individuals who show different clinical forms of *E. histolytica* infection (e.g. asymptomatic infection vs colitis, colitis vs liver abscess).[7,8] Later, our group identified that the presence of one particular human commensal bacteria, *Prevotella copri*, in gut flora is associated with susceptibility of children to *E. histolytica* induced diarrheal disease in different geographic areas.[9,10] Old papers reported that gut bacteria, such as O55 *Escherichia coli* and *Shigella dysenteriae*, directly affect on the virulence of *E. histolytica* in vitro experiments.[11–13] Also, it was known that disease severity of autoimmune diseases, such as inflammatory bowel diseases and rheumatoid, are highly influenced by the gut microbiome.-[14–16] However, effect of gut microbiome on host immune response to external pathogen are rarely investigated in previous studies.

Roles of neutrophils in the severity of E. histolytica infection

Neutrophils have not only protective roles during *E. histolytica* infection but can also exacerbate disease (Figure 1). In the early phase of infection innate immune responses, especially neutrophil mediated reactions, play a pivotal role in protecting against *E. histolytica* invasion. This was shown in a previous

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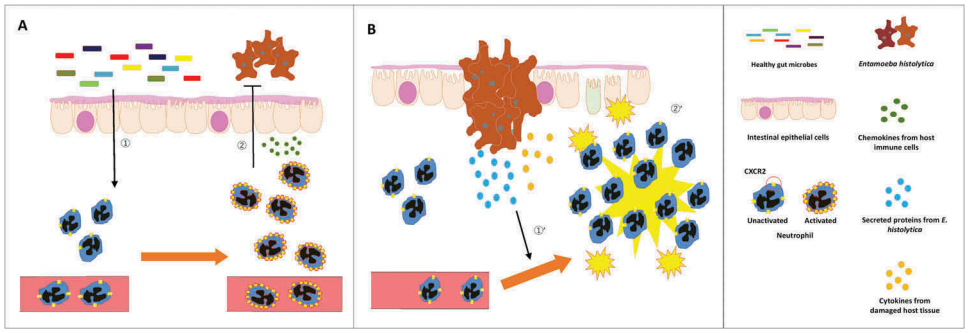

Figure 1. Protection from *E. histolytica* invasion by neutrophils activated by gut microbes at the early phase (A) and exacerbation of tissue damage by neutrophils excessively activated by *E. histolytica* secreted proteins at the later phase (B) of infection. (A) Neutrophils are continuously stimulated by host gut microbes in the resting state before infection (①). These neutrophils are potent in protecting host intestinal epithelial cells from *E. histolytica* invasion (②). **(B)** However, once *E. histolytica* invades the submucosa, neutrophils excessively activated by *E. histolytica* secreted proteins and host proinflammatory cytokines (①′) exacerbate tissue damage (②′).

in vivo experiment where either antibody deletion of neutrophils or disruption of neutrophil recruitment in knockout mice resulted in exacerbation of tissue invasion by *E. histolytica*.[17,18] In an *in vitro* study, it was shown that neutrophils activated by tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) have amebicidal activity through the release of reactive oxygen species (ROS).[19] Furthermore, in *in vivo* experiments, it was shown that administration of the proinflammatory cytokine interleukin (IL)-17 has a protective role against *E. histolytica* infection via the induction of innate immune responses in the gut.[20,21] However, once *E. histolytica* invades the submucosa, neutrophil mediated tissue damage contributes to the severity of *E. histolytica* infection. In a cohort study, it was reported that higher TNF-α production was shown to correlate with *E. histolytica* diarrheal disease in children.[22] Notably, in *in vivo* experiments, it was shown that the anti-inflammatory cytokine IL-10 is protective in *E. histolytica* infection by counteracting an exaggerated proinflammatory immune response by inhibiting the production of proinflammatory mediators, such as TNF-α.[23] Interestingly, *E. histolytica* secretes cytokine-like proteins such as prostagrandin-E2[24,25] and mammalian inflammatory factor during tissue invasion,[26,27] which induces excessive proinflammatory responses by neutrophils at the infection site. Thus, neutrophils play a critical role both in the protection against tissue invasion by *E. histolytica* and in the promotion of tissue damage by *E. histolytica*.

Effects of the gut microbiome on neutrophil activation in a mouse model

We recently reported results from *in vivo* experiments using a mouse model of intestinal *E. histolytica* infection, which proposed a mechanism whereby gut microbiome-activated neutrophils protect host tissue from the invasion of external pathogens.[28] In this paper, we first induced "dysbiosis" by the administration of an antibiotic cocktail, which is commonly used in studies for deleting normal gut flora.[29–34] Thereafter, *E. histolytica* was directly injected into the cecum for challenge, then we assessed tissue invasion by *E. histolytica* and immune responses using cecal tissue from pretreated "dysbiosis" mice. As expected, damage to intestinal epithelial cells by *E. histolytica* was more severe, and *E. histolytica* clearance was delayed. In contrast, both reactive oxygen species activity, as assessed by fecal levels of lipocalin-2 and tissue myeloperoxidase (MPO) activity, and the number of neutrophils at the infection site, assessed by the number of $Ly6G^{hi}$ and $SiglecF^{-}$ granulocytes in the cecum, were diminished in antibiotic pretreated "dysbiosis" mice, although proinflammatory cytokines (IL-1β) and chemokines (CXCL2/macrophage inflammatory protein (MIP)-2 and CXCL1/keratinocyte chemoattractant (KC)) from tissue macrophages were more highly elevated in these mice. These results strongly suggested that neutrophils in mice with dysbiosis have a lower potency of amebicidal activities, both qualitatively and

quantitatively. In fact, we demonstrated that chemokine receptors on the surface of neutrophils were diminished after antibiotic induced dysbiosis, and that amebic colitis was more severe after blockade of CXCR2 by neutralizing antibody before *E. histolytica* challenge. Interestingly, the impact of CXCR2 blockade on tissue MPO activity was less profound than that seen by antibiotic pretreatment, despite the lower number of neutrophils at the site of infection after CXCR2 blocking. Furthermore, histopathological damage by *E. histolytica*-induced colitis was also less severe than that seen after antibiotic pretreatment. We concluded that the low expression of CXCR2 was an important determining factor for the higher susceptibility to *E. histolytica* infection in antibiotic pretreated "dysbiosis" mice, but not the sole explanation.

Phenotypic regulation of neutrophils (mainly activation) by the gut microbiome in an autoimmune disease model was already described prior to our report.[35] The authors showed that the severity of sickle cell disease is relieved under antibiotic induced dysbiosis, due to fewer activated "aged neutrophils" which are characterized by the surface markers $CD62L^{low}$ and $CXCR4^{hi}$. They confirmed that the activated phenotype of neutrophils in sickle cell disease, as shown by aged neutrophils, have increased efficacy in attaching to red blood cells in *in vitro* experiments. They have also shown that there is a trend for higher expression of CXCR2 on aged neutrophils in an earlier paper.[36] These results from previous papers are consistent with our results seen in phenotypic changes of neutrophils in *E. histolytica* infection. Furthermore, our report is the first showing that neutrophil activation by the gut microbiome contributes to the protection from infectious diseases.

Future perspectives

Previous works, including our recent paper, strongly suggest that it is possible to induce a protective neutrophil response to infectious diseases via the gut microbiome, possibly with probiotics. However, detailed molecular mechanisms for neutrophil activation by the gut microbiome or its derived molecules remain to be determined at present. Moreover, previous papers using human neutrophils show inconsistent results. The expression of chemokine receptors on neutrophils and monocytes of humans was downregulated by Toll-like receptor (TLR)-2 and -4 receptor agonists in *in vitro* experiments,[37,38] whereas lipopolysaccharides (LPS) directly enhance amoebicidal activity of human neutrophils.[39] Moreover, the species of commensal bacteria present in humans are different from those in mice. Therefore, in future studies, the molecular mechanisms of neutrophil activation by the gut microbiome should be investigated using human neutrophils and microbes from the human gut. Second, as described above, neutrophil mediated immune responses contribute not only to protection from *E. histolytica* invasion but also to the exacerbation of tissue damage by *E. histolytica* at the later phase of infection. Thus, investigations revealing the detailed mechanism of neutrophil activation in different situations will provide future directions for novel therapies based on the induction of neutrophil mediated protection in infectious diseases.

Acknowledgments

We thank Simon Teteris, PhD, from the Edanz Group (www.edanzediting.com/ac), for editing the English text of a draft of this manuscript.

Funding

This work was supported by the Emerging/Re-emerging Infectious Diseases Project of Japan from the Japan Agency for Medical Research and Development (AMED), a Grant for National Center for Global Health and Medicine (29-2013), and NIH grant R01 AI026649. The funders had no role in study design, data collection and interpretation.

Competing interests

The authors report no conflicts of interest.

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