ADDENDUM

Enterotoxigenic Escherichia coli is phagocytosed by macrophages underlying villus-like intestinal epithelial cells: Modeling ex vivo innate immune defenses of the human gut

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ABSTRACT

There is a paucity of information on diarrheagenic enterotoxigenic Escherichia coli (ETEC)’s interaction with innate immune cells, in part due to the lack of reliable models that recapitulate infection in the human gut. In a recent publication, we described the development of an ex vivo enteroid-macrophage co-culture model using human primary cells. We reported that macrophages residing underneath the epithelial monolayer acquired “resident macrophage” phenotype characterized by lower production of inflammatory cytokines and strong phagocytic activity. These macrophages extended projections across the epithelium, which captured ETEC applied to the apical side and reduced luminal bacterial load. Additional evidence presented in this addendum confirms these findings and further demonstrates that macrophage adaptation occurs regardless of the stage of differentiation of epithelial cells, and that ETEC uptake arises rapidly after infection. The enteroid-macrophage co-culture represents a novel and relevant tool to study host-cell interactions and pathogenesis of enteric infections in humans.

ETEC-induced intestinal epithelial cell dysfunction and innate immune activation

Enterotoxigenic Escherichia coli (ETEC) is highly prevalent in poor countries and in areas lacking adequate sanitary conditions. It is a leading cause of diarrhea in young children living in these endemic regions and the primary etiology of travelers’ diarrhea.\textsuperscript{1} ETEC infection manifests clinically by sudden watery diarrhea that lasts 3 to 4 days. Although generally self-limiting, ETEC diarrhea can lead to severe dehydration and death. Illness can resolve with rehydration and antibiotic treatment, although options have been limited due to increasing antibiotic-resistance.\textsuperscript{1} Since ETEC is a noninvasive bacterium, pathogenicity relies mainly on the secretion of enterotoxins: heat-labile toxin (LT), which has high homology with cholera toxin, and heat-stable toxin (ST), as well as a variety of colonization factors (CFs) which are fimbrial, fibrillar, or afimbrial structures on the bacterial surface.\textsuperscript{1,2} Beyond this broad understanding, precise mechanisms and molecules involved in ETEC pathogenesis remain to be elucidated. Studies have traditionally focused on roles of the CFs and enterotoxins. CFs are known to facilitate ETEC attachment to the small bowel, allowing ETEC LT and/or ST to be produced in close proximity to the intestinal epithelium. Once internalized through specific cell ligands, the toxins induce intracellular accumulation of cyclic AMP and GMP, respectively, which causes loss of electrolytes and water.\textsuperscript{2} More recently, additional factors such as the secreted EtpA adhesin molecule, the EtaA serine protease autotransporter (a member of the serine protease autotransporter of the Enterobacteriaceae [SPATE] protein family), and the secreted YgHJ metalloprotease have been implicated in virulence, by enhancing colonization through mucus degradation and bacterial...
attachment.\textsuperscript{3–5} Despite the progress made in delineating ETEC adherence and subsequent damage to intestinal epithelial cells, its interaction with the intestinal immune system in the early stages of infection, a critical step in determining host resistance, remains unknown. The fact that the enterotoxins cause disease by themselves and that ETEC has typically been regarded as a non-inflammatory pathogen might have shifted attention away from anti-microbial innate immune defenses. Meanwhile, mounting evidence from human and animal studies support the notion that ETEC infection is associated with mild intestinal inflammation and increased expression of innate immune genes in the gut mucosa.\textsuperscript{6–8} Leukocytes and lactoferrin as well as elevated levels of IL-8, IL-6, IL-1β, IFN-γ, and IL-1RA pro-inflammatory cytokines were found in fecal samples from children and adult travelers with ETEC diarrhea.\textsuperscript{6,9,10} Likewise, calprotectin was detected in stool of ETEC-infected piglets, indicating active recruitment of neutrophils to the intestinal lumen.\textsuperscript{11} In a mouse pulmonary model of ETEC infection, lung histopathology revealed a moderate to severe infiltration of neutrophils and macrophages in the alveoli.\textsuperscript{12} In addition, cytokines with pro-inflammatory effects, including IL-2, IL-12p40, IL-1β, IL-6, IFN-γ, and TNF-α have been observed in serum of ETEC-infected pigs.\textsuperscript{7} A study performed by Loos and colleagues examined early immune responses to ETEC in orally infected piglets and showed rapid (4h) upregulation of intestinal genes involved in innate immune function, which included IL-8, IL-1 and IL-17 cytokines, and MMP1 and MMP3 matrix metalloproteinases.\textsuperscript{8} The TLR4 and NF-kB signaling pathways are rapidly activated following ETEC infection, which likely contributes to the upregulation of these innate immune mediators.\textsuperscript{8,13,14} Together, these results provide strong evidence of active recruitment of phagocytic cells to the site of infection and local production of inflammatory mediators, and support the notion that innate immune components are activated and deployed in the wake of ETEC infection.

**Modeling gut microbial-host cell interaction in a human enteroid-macrophage co-culture**

In a recent paper published by our group, we described the development of an in vitro model consisting of human primary intestinal epithelium and macrophages to study early innate immune activation at the mucosal interface upon pathogen exposure (Fig. 1A).\textsuperscript{15} The model is built on 2-dimensional (2D) enteroid monolayers established from human intestinal crypt-derived stem cells. Significantly, the model recreates the “crypt-like” (non-differentiated) and “villus-like” (differentiated) cellular diversity of the human intestinal epithelium that harbors the major intestinal epithelial cell lineages (i.e. Lgr5\textsuperscript{+} stem cells, Paneth cells, enterocytes, goblet cells, and enteroendocrine cells) and mucus layer, as schematically represented in Figure 1A.\textsuperscript{15–18} Microbial-host cell interactions have traditionally been studied using transformed cell lines, yet their multiple genetic alterations and dysregulated function compromise the value and applicability of the results obtained. Further confounding factors are introduced by long-term cultures that fail to represent normal conditions. In preceding publications, our group described the successful establishment of enteroid and colonoid cultures from small intestinal and colonic tissue, respectively.\textsuperscript{15,19} It is worth emphasizing the practical advantages of enteroid monolayers, which unlike the 3D culture configuration (i.e. spheroids) maintained in Matrigel, facilitate controlled access to the apical epithelial cell surface for treatment, as it occurs in vivo. In the enteroid-macrophage co-culture model we recently established, the monocyte-derived macrophages were purposefully seeded on the basolateral side of the enterocytes to mimic their location in vivo (Fig. 1A).\textsuperscript{15} This configuration allows for epithelial cell-macrophage interactions and because cells reside in separate compartments, it also allows the interrogation of the polarity (i.e. apical or basolateral) of cellular responses. The majority of macrophages remains firmly adhered to the filter, although a small fraction detach during culture.

Different from traditional tissue culture systems, our enteroid-macrophage co-culture avoids macrophages being directly exposed to a high bacterial load, which may lead to events that bear little relevance to pathophysiological conditions.\textsuperscript{20} Instead, the sub-epithelial seeded macrophages more faithfully represent the shielding of non-activated phagocytic cells by the intestinal epithelial barrier in the human intestine.

In our recent publication, we demonstrated that macrophages could be engrafted underneath both non-differentiated and differentiated enteroid monolayers.\textsuperscript{15} We also showed that chemokines and cytokines known to play key roles in immune activation and maintenance of homeostasis in the gut mucosa, including TGF-β1, IL-8, IL-6, and IFN-γ, were
secreted by the “villus-like” enteroid-macrophage co-culture. Both macrophages and differentiated epithelial cells produced these cytokines when cultured alone and are therefore believed to contribute to the levels measured in the combined culture. It was clear, however, that macrophages were responsible for the higher basolateral levels of IL-8, IL-6, and IFN-γ, but not TGF-β1, produced by the ND enteroid-macrophage co-culture as compared to macrophages alone. Data correspond to mean ± SEM of multiple independent experiments; n = 6 for IL-8, n = 4 for TGF-β1, and n = 2 for IL-6 and IFN-γ. (C) ETEC are phagocytosed by human macrophages following overnight infection. DNA (bacterium and macrophage DNA), blue; ETEC, green; CD14 (macrophages), red. (D) Apical ETEC phagocytosed (yellow merge) by sub-epithelial macrophages extending dendrites (white arrowheads) across the epithelial cell monolayer. Actin (microvilli), white; nuclei, blue; ETEC, green; CD14 (macrophages), red; filter, dashed lines. E) ETEC CFU recovered from the apical compartment 30 min after infection was greatly reduced in the macrophage-enteroid co-culture as opposed to enteroid alone. Data represent mean ± SEM of N = 9 from 4 independent experiments. Methods and experimental conditions were the same as described in our previous publication.15

Both differentiated and non-differentiated epithelium cultures, the IL-8, IL-6, and IFN-γ produced were released primarily to the apical side, as depicted by the higher amounts contained in the apical culture supernatant (Fig. 1B). The fact that cytokines produced by intestinal epithelial cells are mainly secreted towards the lumen as opposed to the basolateral side where immune cells reside in vivo, was intriguing. The apical production of immune mediators has been also observed in epithelium from other organs such as lung and uterus and may hint to the migration of immune cells beyond the intestinal barrier, which would depend on cytokine signals for stimulation.21–23
We have also shown in our previous paper that macrophages enhanced the epithelial barrier function, evidenced by increased electrical resistance and increased enterocyte height suggesting a novel paracrine role for macrophages in intestinal epithelial differentiation.15

**Macrophages in the intestinal enteroid co-culture model have reduced pro-inflammatory function**

Macrophages comprise a heterogeneous population. Various distinct phenotypes have been defined through *in vivo* and *in vitro* monocyte differentiation studies.24,25 Mature macrophages originate from circulating monocytes and can be activated by discrete stimuli to become M1, which are pro-inflammatory and drive T helper (Th) 1 type responses, or M2, which are anti-inflammatory and promote tissue repair and drive Th2 responses. The gut lamina propria harbors a phenotypically and functionally distinct population known as “resident” macrophages. Although derived from blood monocytes, unlike M1 macrophages, human intestinal resident macrophages display profound inflammatory anergy and maintain homeostasis.26 Despite the downregulation of pro-inflammatory cytokine production, resident macrophages have high phagocytic capacity (for luminal bacteria, non-harmful foreign molecules and cellular debris) as well as bactericidal activity.26–29 During the development and optimization of the enteroid-macrophage co-culture model, mature (not activated) macrophages, also known as M0, were added to the enteroid monolayer. This non-activated phenotype was selected to ascertain discernible changes (i.e. activation towards a resident, M1, or M2 phenotype) prompted by molecules expressed, released, and/or induced by the intestinal epithelium. In our recent publication, we showed that macrophages engrafted into the differentiated “villus-like” enteroid monolayers (for as little as 24h) exhibited a marked reduction in their capacity to produce chemokines and pro-inflammatory cytokines IL-8, IL-6, and IFN-γ.15 In contrast, the levels of TGF-β1, a strong promoter of homeostasis,30 remained unchanged. The same was observed in the additional experiments described in this addendum using co-cultures containing non-differentiated “crypt-like” enteroids (Fig. 1B). Unlike the other cytokines, which were released apically by the epithelial cells (regardless of differentiation), TGF-β1 was mainly secreted to the basolateral side. The direct exposure of macrophages to the enterocyte-released TGF-β1 in the basolateral compartment could explain their acquisition of a resident phenotype.26,28 It would be interesting to test whether enteroid-derived TGF-β1 induces macrophage differentiation and function. In this addendum, we confirm our observations suggesting that monocyte-derived macrophages acquire a resident “tolerogenic” function when cultured with differentiated epithelial cell monolayers, this time demonstrating that the same cell phenotypic change occurs when M0 macrophages are cultured in the presence of “crypt-like” intestinal epithelial cells (Fig. 1B). Further analysis of cell surface marker expression and cytokine expression in both conditions would allow establishing the identity and exact phenotype of these enteroid-interacting macrophages.

**ETEC is captured by sub-epithelial resident macrophages**

Human monocyte-derived macrophages phagocytose ETEC *in vitro* (Fig. 1C). How human intestinal macrophages interact with ETEC is not known. It has been shown in mice that intestinal resident macrophages can sample and process luminal antigens and organisms, and stimulate an adaptive immune response in collaboration with dendritic cells.27,31,32 In our recent paper, we showed that human macrophages engrafted with enteroid monolayers develop projections (i.e. transepithelial dendrites), which extended across the intestinal epithelium without disturbing the barrier integrity.15 This was the first demonstration of macrophage luminal sampling in a model of human primary cells. Interestingly, when ETEC was applied to the apical surface of the enteroid monolayer, the number of macrophage extensions moving through the monolayer toward the bacteria markedly increased. Macrophage transepithelial dendrites in contact with luminal bacteria were visualized by immunofluorescent confocal microscopy (Fig. 1D). The number of apical colony-forming units (CFU) in ETEC-exposed co-culture was significantly reduced following overnight infection, which reflects successful macrophage sampling and phagocytic activity. The strong phagocytic activity of the macrophages cultured with epithelial cells, along with the downregulation of pro-inflammatory cytokines, such as IL-8, IL-
6, and IFN-γ, further support their resident functional profile.

In this addendum, we confirm the phenotypic adaptation and ETEC phagocytic activity of the co-cultured macrophages and further characterize this process by showing that it occurs very quickly, as revealed by a remarkable reduction of CFU as early as 30 min post-infection (Fig. 1E). The luminal ETEC sampling by sub-epithelial human macrophages in the co-culture model is in agreement with finding of intracellular bacilli in murine lung macrophages following pulmonary ETEC challenge. The molecular factors that either directly or indirectly (through epithelial cells) triggered and enhanced macrophage transepithelial projections are not known. These findings are intriguing and open an entire new avenue of investigation. Recent studies have shown that Pet toxin, a SPATE produced by enteroaggregative E. coli (EAEC), stimulates motility and cytokine expression by macrophages. SPATEs form a large family of many related proteins among the pathogenic E. coli strains; it would be worth investigating whether similar factors are produced by ETEC to trigger an intestinal macrophage response.

Potential role of macrophages during human ETEC infection

ETEC is an extracellular organism that (in normal conditions) attaches but is not expected to translocate across the gut mucosal barrier. However, results reported in our previous publication and in this addendum demonstrate that the bacterium is sensed and taken up by human macrophages attached to the opposite side of the intestinal epithelium through transepithelial projections (Fig. 1D and E). These macrophages underwent adaptation and functional phenotypic changes triggered by epithelial cells. This unique ETEC-macrophage interaction is important on several levels: 1) upon microbial sensing, macrophages become activated and produce mediators that will attract other phagocytic cells, 2) being professional antigen presenting cells, together with dendritic cells, macrophages can present antigens to stimulate (or re-stimulate) T cells; antigen-loaded apoptotic macrophages (as well as released antigens) allow for cross-presentation by professional phagocytic cells and stimulation of B and T cells, and 3) macrophages could provide a means of microbial or antigenic translocation. Although the fate of the organisms within the macrophages was not evaluated in our study, one possible outcome is active intracellular killing, which is consistent with the prompt CFU reduction observed in our study and the heightened bactericidal activity ascribed to resident macrophages. The resulting ETEC antigens could be presented or cross-presented by macrophages and dendritic cells, resulting in T and B cell activation and induction of adaptive immunity. Alternatively, ETEC may simply translocate across the monolayer via macrophages; extracellular bacterial pathogens such as E. coli K1 and Enterococcus faecalis have been shown not only to survive but also replicate in the harsh intracellular macrophage environment. Unlike ETEC, however, these strains can produce a systemic infection believed to stem from bacteria that replicate within phagocytes and spread to distal sites. The fact that ETEC does not become systemic means that some sort of mechanism allows for its clearance in the intestine. Whether this is effectively and uniquely performed by macrophages or involves additional cells and immune components, needs to be determined.

In addition to queries about the pathogen, there are many unanswered questions about the fate of the infected macrophages. It has been suggested that ETEC infection can kill murine macrophage cell lines; however, in contrast to other diarrheagenic E. coli, ETEC failed to induce markers of apoptosis (i.e. DNA fragmentation and TUNEL staining), leaving unknown the mechanism of death involved. While we did not observe significant death of ETEC-exposed macrophages in co-culture, a more comprehensive analysis of macrophage response to ETEC is warranted.

Conceivably, apoptotic macrophages could be captured by other macrophages or dendritic cells and microbial antigens cross-presented for activation of T cells. Apoptotic bodies could also prime B cells. This mechanism would explain the induction of systemic (even protective) immunity by a non-invasive pathogen. Indeed, ETEC-exposed individuals (also animals) develop strong adaptive immunity consisting of serum antibodies, CD4⁺ T cells expressing gut-homing markers, and IgA B memory cells. LT and CF antibodies are believed to contribute to protection according to human challenge studies. Furthermore, increased levels of IL-4 and IL-8 but not TNF-α, IL-6, and IFN-γ have been associated
with reduced severity of disease, suggesting a protective role of Th2 type cytokines. The involvement of mucosal Th2 type cells (e.g. M2 macrophages, type 2 innate lymphoid cells, B cells) and molecules (e.g. IL-4, IL-5, IgA) during ETEC infection could be easily interrogated in a “crypt-like” or “villus-like” enteroid co-culture model as the one described herein.

**Interrogating enteric infection and immunity ex vivo in a enteroid-immune model**

Human enteroids or colonoids have been used successfully to study interactions of enteric pathogens with the human epithelial cell barrier; the gut-recreated complexity of the system is such that it allows us to dissect steps and cells involved in pathogenesis, including effects on enterocyte microvilli, tight junctional regulation, and ion/nutrient transporter activity that may be correlated to disease severity. The macrophage-enteroid co-culture model developed by our group provides a new tool to study host cell and pathogen interactions with innate immune cells. Although our work thus far has been focused on macrophages, we are currently expanding the system to include other innate phagocytic cell types (i.e. polymorphonuclear neutrophils and dendritic cells). Further elaborated enteroid co-cultures possibly containing B and T lymphocytes will be forthcoming. Distinct immune cell phenotypes could be incorporated one at a time or together to address specific questions in a reductionist model manner easy to manipulate and reproduce. Such models will generate mechanistic insight into how immune cells interact and respond to enteric pathogens. In addition, the co-culture systems provide the opportunity to interrogate the mutual dependency of the intestinal epithelium and immune cells for survival, differentiation, and function using primary human cells arranged in a configuration that resembles the human gut in vivo. In our studies, the intestinal epithelial monolayer finely modulated macrophage phenotype and function, and in turn, these “intestine-adapted” macrophages supported the epithelial cell barrier. Saha and colleagues demonstrated that murine macrophages secrete Wnt ligands necessary to regenerate the intestinal epithelium following radiation injury. The fascinating intercell coordination that takes place in the human gut has yet to be unraveled.

As observed for several other enteric pathogens, the host secretor status plays a role in susceptibility to ETEC infection and illness. We are currently addressing this question using enteroid-immune co-cultures which, unlike *in vitro* models using immortalized cell lines, allow for an in-depth analysis of individual biological diversity (secretors vs. non-secretors).

In conclusion, we have demonstrated that human monocyte-derived macrophages co-cultured with primary epithelial cell monolayers acquire a resident functional phenotype. These macrophages sense ETEC on apical side of the epithelial cells, develop transepithelial projections and quickly phagocyte the organism, greatly reducing the luminal bacterial load. The model is novel and relevant to the study of pathogenesis and host-cell interactions, and will advance our understanding of enteric infections in humans.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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**Author contributions**

G.N. and M.D. performed experiments; NC.Z. obtained microscopy images; G.N. and MF.P. wrote the manuscript; all authors contributed ideas, reviewed, and edited the manuscript.

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