

## REVIEW

## Creating a More Perfect Union: Modeling Intestinal Bacteria-Epithelial Interactions Using Organoids

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## SUMMARY

This review provides a step-by-step guide for investigating host-microbe interactions using organoids, covering the importance of organoid type selection, the advantages and disadvantages of different techniques to introduce microbes, and cutting-edge advances made to better mimic in vivo conditions.

Intestinal organoids have become indispensable tools for many gastrointestinal researchers, advancing their studies of nontransformed intestinal epithelial cells, and their roles in an array of diseases, including inflammatory bowel disease and colon cancer. In many cases, these diseases, as well as many enteric infections, reflect pathogenic interactions between bacteria and the gut epithelium. The complexity of studying this microbe-epithelial interface in vivo has led to significant focus on modeling this cross-talk using organoid models. Considering how quickly the organoid field is advancing, it can be difficult to keep up to date with the latest techniques, as well as their respective strengths and weaknesses. This review addresses the advantages of using organoids derived from adult stem cells and the recently identified differences that biopsy location and patient age can have on organoids and their interactions with microbes. Several approaches to introducing bacteria in a relevant (apical) manner (ie, microinjecting 3-dimensional spheroids, polarity-reversed organoids, and 2-dimensional monolayers) also are addressed, as are the key readouts that can be obtained using these models. Lastly, the potential for new approaches, such as air-liquid interface, to facilitate studying bacterial interactions with important but understudied epithelial subsets such as goblet cells and their products, is evaluated. (*Cell Mol Gastroenterol Hepatol* 2021;12:769-782; <https://doi.org/10.1016/j.jcmgh.2021.04.010>)

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Many aspects of gastrointestinal (GI) health, including intestinal development, nutrition, digestion, and healing, all are impacted positively by signals coming from the gut microbiome. Correspondingly, maladaptive interactions between the host and specific gut

microbes can lead to an array of infectious and idiopathic diseases including inflammatory bowel disease and colon cancer.<sup>1-3</sup> In most cases, these interactions reflect microbial contact with the intestinal epithelium. This single layer of cells, comprising an array of functionally distinct cellular subsets, acts as a protective barrier as well as a messenger, enabling cross-talk between luminal microbes and the underlying immune cells, nerves, and other cell types.<sup>4,5</sup>

Studying microbe-intestinal epithelial cell (IEC) interactions in vivo is highly complex, necessitating the use of reductionist co-culture systems that until recently focused on transformed cell lines. These cell models have expanded our understanding of microbe-IEC interactions, but their inability to reproduce the complex structure and functionality of the epithelium along the length of the GI tract has limited their relevance. Just over a decade ago, a revolution enabled the culturing and propagation of stem cells to form organ-specific cell types in 3-dimensional (3D) cultures, better known as organoids.<sup>6,7</sup> Since its emergence, intestinal organoid research has been a rapidly evolving field. Novel technologies and methodologies are being published daily, and many of these advances have been incorporated into modeling microbe-IEC interactions.

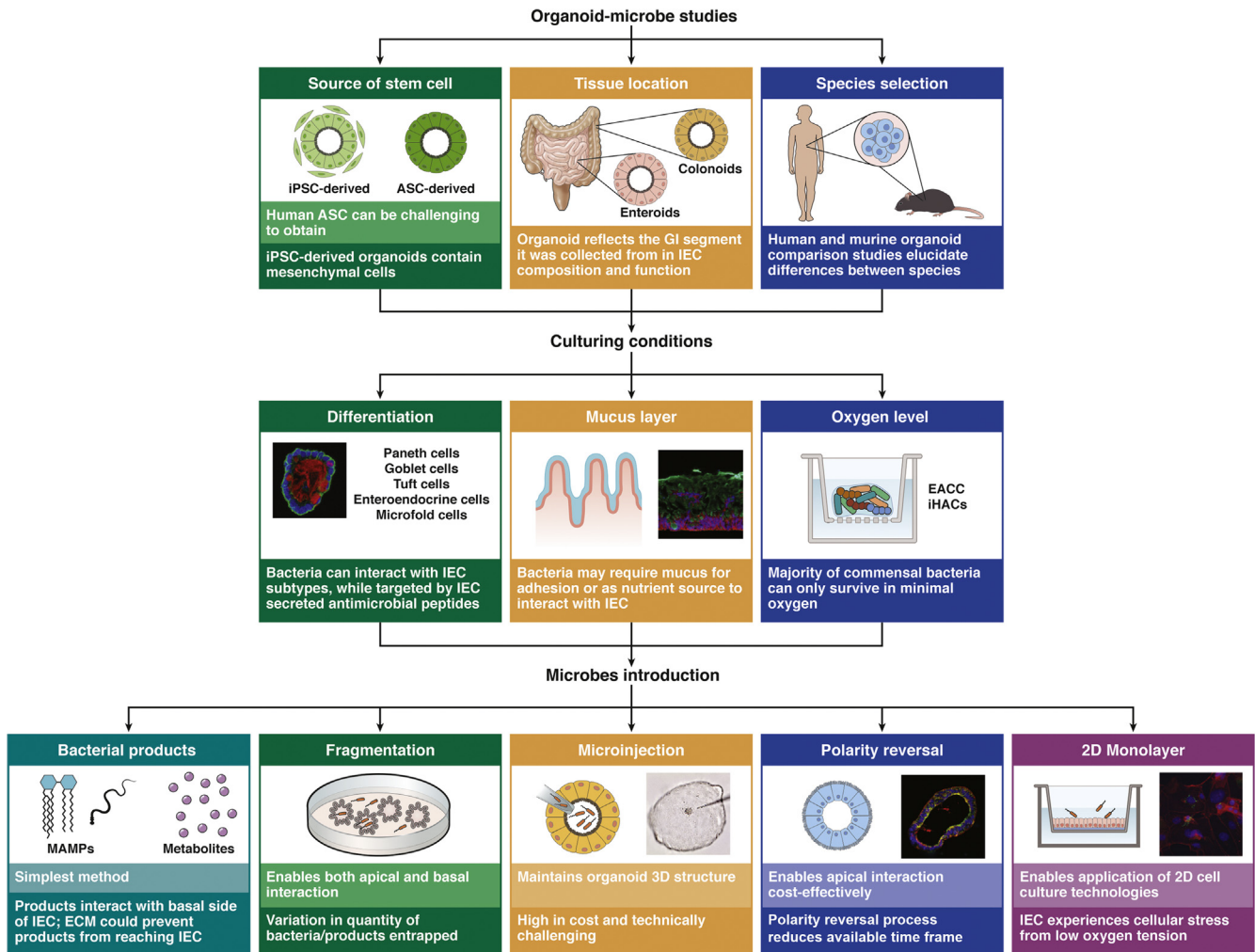
For this review, we have compiled these advances into a convenient guide focused on bacteria-host interactions within the GI tract because virus-organoid interactions recently were reviewed.<sup>8</sup> Criteria for choosing the proper sources of stem cells as well as the advantages and limitations of the different bacteria-organoid co-culture approaches are discussed. Moreover, options for better simulating the in vivo conditions of the GI tract, as well as modeling both pathogenic and commensal microbe interactions with organoids, are detailed. It is hoped that this

**Abbreviations used in this paper:** 2D, 2-dimensional; 3D, 3-dimensional; ALI, air-liquid interface; ASC, adult stem cell; EAEC, enteroaggregative *Escherichia coli*; ECM, extracellular matrix; GI, gastrointestinal; IEC, intestinal epithelial cell; iPSC, inducible pluripotent stem cell; LGR5, leucine-rich repeat-containing G-protein-coupled receptor 5; LPS, lipopolysaccharide; NOD2, nucleotide-binding oligomerization domain-containing protein 2; SCFA, short-chain fatty acid; TLR, Toll-like receptor.

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**Figure 1.** Brief guide on designing bacteria–IEC interaction experiments using organoids. Organoids need to be generated from the most relevant (and obtainable) stem cell source, GI region, and appropriate host. Before introducing the microbe, organoids also need to model specific *in vivo* intestinal epithelium conditions such as undergoing differentiation to form specific IEC subtypes, a functional mucus layer, and a gradient oxygen level. These conditions facilitate the addition, adhesion, and replication of microbes *in vitro*, which can be modeled in various ways, each having its advantages and disadvantages. Staining of images in order of appearance: differentiation: blue, DAPI; red, MUC2; green, E-cadherin; mucus layer: blue, DAPI; red, E-cadherin; green, AAL; polarity reversal: blue, DAPI; red, villin; green, E-cadherin; 2D monolayer: blue, DAPI; red, actin; green, GFP *S Typhimurium*. DAPI, 4',6-diamidino-2-phenylindole; EACC, Enteroid-anaerobe co-culture system; GFP, Green fluorescent protein; iHAC, intestinal hemi-anaerobic co-culture system.

guide (Figure 1) will benefit researchers seeking to enter the field as well as experienced organoid researchers looking to optimize their current model systems.

## Choosing Your Stem Cells: Pluripotent or Adult?

The first and perhaps most important choice for researchers is whether they should use inducible pluripotent stem cells (iPSCs) or adult stem cells (ASCs) to generate intestinal organoids. Their creation and maintenance require distinct protocols, in concert with their structures and functions being fundamentally different.

iPSCs can be induced to differentiate and form organoids through the manipulation of complex developmental cues

that include key interactions with extracellular matrix (ECM).<sup>9</sup> The main advantage of working with iPSCs is the ability to generate organoids without the need for primary tissues, because obtaining human tissue biopsy specimens can be logistically challenging for many researchers. Despite their convenience, because iPSC-organoids are derived using developmental cues, they remain in a more fetal-like state and often are missing key features of a mature epithelium.<sup>10</sup> Similarly, because the phenotype of these organoids is described as being less organ-specific, they may not exactly mimic the epithelium of a particular intestinal region.<sup>10</sup> Most iPSC-derived organoids also contain mesenchymal cells as a by-product of their differentiation,<sup>9</sup> although recent methodologic advancements can generate organoids free of mesenchymal cells.<sup>11</sup> Notably, the presence of

mesenchymal cells can be beneficial because they supply cues for IEC development and maintenance, yet their presence can hinder studies in which IEC-intrinsic responses are desired.

In contrast, ASCs are isolated from host tissues and, as a result, organoids derived from ASCs can provide the cellular diversity that is biologically representative of the originating tissue. In 2009, Hans Clevers and his group<sup>6</sup> isolated leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5)+ stem cells from intestinal crypts, culturing the cells in a complex ECM (Matrigel, Corning, #356255) with a precise supplementation of growth factors that enabled long-term propagation. ASC-derived small intestinal (enteroids) and large intestinal (colonoids) organoids were shown to mature into fully functional crypts and differentiated epithelium that provided a snapshot of the gut epithelium in vivo.<sup>12</sup> Moreover, ASC organoids do not contain the mesenchymal cells found in iPSC organoids. Although obtaining the tissues necessary for human ASC organoids can be challenging, biobanks that serve as sources for tissues and organoids, while also providing donor information (tissue site, age, sex, disease), are growing in number and accessibility. Consequently, to study microbe interactions with the gut epithelium, ASC-derived organoids appear more relevant and convenient than iPSC-derived organoids and thus are the focus of this review.

## Introducing Bacteria to the Organoid System: Choosing the Best Technique

Exploring the interactions that occur between billions of luminal bacteria and the intestinal epithelium is key to understanding gut function under homeostatic and disease states. As shown with gnotobiotic mice, in the absence of a microbiota, the gut epithelium undergoes abnormal development, increased metabolic stress and defects in barrier function, and mucin secretion.<sup>13,14</sup> In contrast, enteric bacterial pathogens often directly infect IECs, causing physiological dysfunction as well as inflammatory responses that potentially contribute to many chronic diseases.<sup>15</sup> Thus, it is fundamental to select an appropriate method to introduce the microbe of interest, largely keeping faithful to the in vivo interactions, while also considering cost and time.

### Adding Bacterial Products to the Media

A healthy intestinal epithelium normally undergoes very limited direct interactions with the gut microbiota. Instead, bacterial products and metabolites diffuse out of the intestinal lumen to the mucosal surface where IECs sense bacterial-derived, microbe-associated molecular patterns or metabolites such as short-chain fatty acids (SCFAs). This can lead to differential gene expression and phenotypic changes in IEC stemness and their immune functions.<sup>16</sup> With this in mind, ideally, organoids should undergo chronic, low-level stimulation with bacterial products to be more reflective of the in vivo intestinal epithelium.

Various strategies have been used to study the effects of commensal-derived products on IEC homeostasis such as the direct supplementation of SCFA to organoid media.

Through transcriptomic analysis, this showed that butyrate, propionate, or SCFA mixtures enhanced IEC turnover and differentiation, in concert with more budding and reduced stem cell numbers by reducing histone deacetylase 3 activity.<sup>17,18</sup> This effect was reversed by adding the bacterial metabolites phytate and inositol trisphosphate to the organoids, which stimulate histone deacetylase 3 activity.<sup>19</sup> In both human and murine enteroids, butyrate promoted the production of retinoic acid, a critical signaling molecule for IEC development, homeostasis, and immunity.<sup>20,21</sup> Microbe-associated molecular patterns also were found to have equally crucial roles in maintaining homeostasis, as was shown with SCFAs. For example, lipopolysaccharide (LPS) isolated from commensal bacteria activated Toll-like receptor 4 (TLR4) in colonoids, leading to inhibition of Lgr5+ stem cell-based IEC proliferation.<sup>22</sup>

In addition, products from bacterial pathogens can be used in place of live bacteria to study their involvement in IEC pathophysiology and innate immune responses, especially with secreted toxins and effectors that can act on IECs without the pathogen itself needing close contact. For example, *Clostridium difficile*, as an obligate anaerobe, can be difficult to culture. Researchers instead added the *C. difficile* toxin A (TcdA) and toxin B (TcdB) to colonoids, finding they directly interact with the Wingless/Integrated (Wnt)-receptor Frizzled, as well as triggering caspase 3/7-induced apoptosis, while other forms of cell death were dispensable for protection against these toxins.<sup>23,24</sup> Thus, bacterial products can partially replicate the effects of live bacteria, although adding these factors to intact 3D organoids (basolateral side of IECs) may not mimic their actions in vivo because bacteria and their products normally contact the apical surface of IECs.<sup>16</sup> In other instances, it still is essential to work with live bacteria, especially in the case of those pathogens that cause disease through direct interactions with epithelial surfaces, such as by invading or adhering to IECs.

### Fragmentation

Fragmentation involves recovering organoids from ECM, then mechanically disrupting them into fragments to be mixed and incubated with bacteria or bacterial products, before reseeding them back into the ECM. As a result, these fragments reassemble into 3D organoids with the introduced bacteria/products entrapped within their lumens,<sup>25</sup> thereby permitting bacterial interactions with both the apical and basal sides of IECs. A study introducing muramyl dipeptide via organoid fragmentation showed the cytoprotective effects of muramyl dipeptide because it activated the nucleotide-binding oligomerization domain-containing protein (NOD) 2-receptor pathway as well as autophagy to protect Lgr5+ stem cells from oxidative stress, thereby protecting crypt homeostasis.<sup>26</sup> Similarly, enteroid fragmentation and consequent proteomic analysis were used to show that infection with the pathogen *Listeria monocytogenes* altered innate host defenses, including the complement and coagulation cascades, Hypoxia-inducible factor-

1 signaling, ferroptosis, and NOD-like receptor signaling.<sup>27</sup> Although clearly this method can be used to study both homeostatic and pathogenic bacteria–organoid interactions, there are several limitations, including variation in the quantity of bacteria/products entrapped per organoid and the fact that this approach leads to bacterial interactions with both the apical and basolateral sides of IECs, hence the physiological relevance could be questioned.

### Microinjection Into Organoid Lumens

Another way to introduce bacteria/products into enclosed 3D organoids is by microinjecting them into the organoid lumen. This method maintains the 3D organoid structure while ensuring contact between the bacteria and the physiologically relevant apical side of the epithelium.<sup>28</sup> There are several studies outlining the standardization and optimization of this technique, including methods for calculating optimal injection volumes,<sup>29,30</sup> the use of fluorescent bacteria to aid injection visualization and assessment,<sup>31,32</sup> repeated microinjections,<sup>33</sup> and even the construction of a high-throughput microinjection platform.<sup>34</sup>

Both commensal and pathogenic bacteria have been microinjected into organoids. Notably, when a mixture of commensal bacteria from stool filtrates was microinjected into the hypoxic lumens of colonoids, they maintained their species diversity for more than 96 hours.<sup>34</sup> Moreover, the growth of the anaerobe *Bifidobacterium adolescentis* was sustained for at least 72 hours after microinjection, emphasizing the advantage of the hypoxic organoid lumen for maintaining commensal diversity and growth.<sup>34</sup> For pathogens, *Salmonella enterica* serovar Typhimurium was observed to stimulate Paneth cell granule secretion (including  $\alpha$ -defensins) in enteroids, but only when administered on the apical side, underscoring the importance of proper microbe orientation when studying pathogenesis.<sup>29</sup> Another study investigated the effects of long-term, repeated microinjection of genotoxic *Escherichia coli*, and found that the injected enteroids acquired mutational changes comparable with the signatures of colorectal cancer, highlighting the carcinogenic effect of this *E. coli*.<sup>33</sup> Taken together, the microinjection approach provides advantages over other methods by allowing researchers to study both commensal and pathogen interactions with IECs in an integral 3D structure. Other advantages include the ability to inject a precise dose of bacteria, as well as providing hypoxic conditions, and longer experimental time frames that enable the study of organoid proliferation and the emergence of IEC subtypes. However, microinjection can cause structural damage to organoids, while the high cost of the microinjection equipment and the technical challenges of this approach are not ideal for beginners, or for high-throughput applications.

### Organoid Polarity Reversal

A recently described technique reverses the polarity of 3D organoids so that their apical side faces outward. Achieved by removing ECM proteins from the culture system,

mature organoids are grown in suspension for 3 days to allow the organoids to turn inside out, with the final inverted organoids showing proper epithelial barrier integrity and nutrient uptake.

Once achieved, this approach bypasses the need for microinjection because microbes can be simply added to the culture media to directly interact with the organoid's (now outward facing) apical surface. In the initial study describing this approach, researchers used 2 invasive pathogens, *S. Typhimurium* and *L. monocytogenes* to infect apical-out, basal-out, or mixed apical-/basal-out enteroids, and found that *S. Typhimurium* preferentially infected the apical surface whereas *L. monocytogenes* preferred the basal surface of the enteroids. These findings reinforced the concept that these 2 enteric pathogens use distinct strategies to invade a polarized epithelium.<sup>35</sup> Similarly, enteroaggregative *E. coli* (EAEC) was found to preferentially attach to the apical side of mucin-secreting goblet cells on apical-out organoids.<sup>36</sup> Thus, this technique models microbe interactions with the apical surface of IECs using a 3D structure and consequently facilitates the collection and assessment of chemokines and other secretions released from the apical side of IECs into the media during these interactions. A major limitation, however, is that organoids require approximately 3 days in suspension before they undergo reversal, thus reducing available experimental time frames. Other technical challenges include increased cell death and the fact that reversed organoids often adhere to each other owing to the lack of ECM. Moreover, because reversed organoids are grown in suspension, the difficulty of changing media can lead to unrestrained bacterial growth as well as the accumulation of toxins that could impact organoid viability negatively. These issues pose obstacles for researchers to design long-term, microbe–organoid experiments, making studies focused on homeostatic host–microbe interactions difficult to address using this approach.

### 2-Dimensional Monolayers and Air Liquid Interface

The most widely adapted method of introducing bacteria/products involves disrupting 3D organoids to allow IECs to form 2-dimensional (2D) monolayers. Sacrificing 3D structure, 2D organoid-derived monolayers provide a simplified yet relevant model that allows the application of many conventional cell culture techniques to evaluate IEC functionality, such as tight junctions, cell differentiation, and immune responses.<sup>37</sup> Using Transwells to separate the apical and basal compartment furthers IEC differentiation<sup>12</sup> and has the potential to incorporate additional host factors such as mesenchymal cells or immune cells to better mimic the *in vivo* environment. Upon apical infection with pathogenic *L. monocytogenes*, *Shigella flexneri*, or *S. enterica* Typhi, transepithelial electrical resistance and fluorescein isothiocyanate–dextran assays showed increased permeability in organoid monolayers. Infections also were accompanied by increased secretion (both basolateral and apical) of interleukin 8, along with other proinflammatory



responses unique to each pathogen.<sup>38–40</sup> Similarly, another study added flagellin to a colonoid-derived monolayer, and observed TLR5 activation promoted interleukin 8 secretion, which was amplified by an endoplasmic reticulum stress response.<sup>41</sup> In contrast, the addition of other pathogenic bacterial products including lipoteichoic acid, as well as LPS, to enteroid-derived monolayers did not trigger IEC innate immune responses.<sup>42</sup>

A recent study showed an unfortunate limitation of the current 2D monolayer system: long-term culturing of organoids as monolayers generated low oxygen tension, leading to cellular stress, reduced differentiation, and a lack of secretory cells.<sup>43</sup> Notably, these effects could be reversed by using air-liquid interface (ALI) culturing, where the apical side of the monolayer is exposed to air while the basal side remains submerged in media. ALI was found to reduce oxygen stress, thereby re-establishing healthy and differentiated monolayers with homeodomain-only protein homeobox (Hox+) quiescent stem cells.<sup>43,44</sup>

Taken together, 2D organoid-derived monolayers provide the simplest approach to study microbe-IEC interactions and offer the potential to measure the apical release of host protective factors such as mucins. Nonetheless, this method requires a large number of IECs for the initial setup and may not accurately depict IEC proliferation, migration, and death. With the development of more technologies geared toward 3D applications, such as light-sheet and 2-photon microscopy,<sup>45</sup> as well as microfluidic chip technology that permits the morphogenesis of tube-shaped intestinal structures with an intact lumen and the addition of 3D vascular structures,<sup>46–48</sup> the field eventually could create a niche that integrates both 2D and 3D technologies. This would yield improved and more relevant *in vitro* models to further enhance host-microbe interaction studies.

## Modeling Age and Site Specifications of the Intestinal Epithelium

Many microbes require specific structural and functional features to interact with their host epithelium, which could be impacted if the *in vitro* organoid model does not recreate relevant *in vivo* conditions. This can involve mimicking an appropriate developmental stage, a specific GI region or IEC subtype, the mucus layer, and proper oxygen levels.

### Development and Organization

A large gap exists in our understanding of how the human intestine functions in early life, especially how its development is impacted by microbiota colonization. Early colonization with pathogenic microbes or impaired maturation could lead to profound developmental defects and disorders such as necrotizing enterocolitis.<sup>49</sup> Organoids offer the potential to model some of these processes and improve our understanding of neonatal intestinal diseases.

The neonatal intestinal epithelium undergoes distinctive developmental changes when it is exposed to different microbial populations. A murine neonatal enteroid study suggests that IEC maturation to the adult state depends on

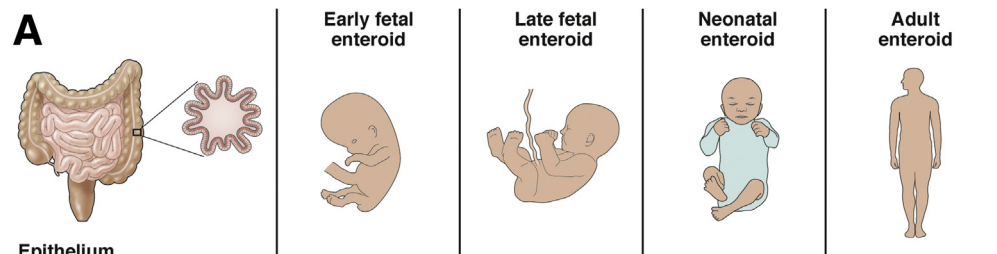
intrinsic factors, largely independent of the microbiota, when the enteroids are maintained in culture over 4 weeks.<sup>50</sup> In contrast, a separate study showed that commensal metabolites isolated from baby fecal filtrates up-regulated growth pathways in murine enteroids, facilitating stem cell proliferation.<sup>51</sup> In comparison with human adult enteroids, human fetal enteroids show reduced markers of IEC maturation, as well as altered immune responses and barrier function. Only late-stage fetal enteroids are responsive to LPS and heat-killed bacteria, whereas early stage fetal enteroids remain unresponsive when compared with adult enteroids.<sup>52</sup> Furthermore, fetal IEC immune responses are attenuated when in the presence of indole-3-lactic acid derived from *Bifidobacterium longum infantis*, a metabolite found in breastmilk.<sup>53</sup> These studies highlight the differential responses between immature and mature IECs and emphasize the need for further studies on the role of microbes in IEC development (Figure 2).

As the GI tract matures, it differentiates into segments with unique functions, cell populations, and microbiota composition. Consequently, organoids isolated from each GI region (enteroids, colonoids, and gasteroids) show corresponding differences in IEC composition, function, and architecture, which also can be species specific.<sup>12,54,55</sup> For example, enteroids can contain Paneth cells, which are not found in colonoids, and Paneth cells, both *in vivo* and *in vitro*, secrete antimicrobial peptides that can curb *S. Typhimurium* growth.<sup>32,56,57</sup> Likewise, the enteric pathogen EAEC preferentially interacts with organoids derived from certain GI regions, with site-specific adherence patterns to duodenal and jejunal enteroids, and much less adherence to ileal enteroids or colonoids. The inflammatory responses from each segment also varied, although it seemed largely dependent on the level of EAEC infection.<sup>36,58</sup> Moreover, human and murine organoids from different GI regions express varying levels of pattern recognition receptors, and thus their responses to bacterial stimulation differ. Although organoids from all sites express TLR4, only murine gasteroids and colonoids respond to LPS owing to their expression of the accessory protein CD14, whereas human organoids from other regions of the GI tract were completely unresponsive owing to their lack of CD14.<sup>55,59</sup> These results highlight the importance of selecting organoids originating from the appropriate GI segment and species when studying specific bacteria-organoid interactions because fundamental differences exist between human and murine organoids, as well as between those collected from different GI segments. These differences likely will lead to varying degrees of bacterial adherence and growth, while also causing discrepancies in host responses (Figure 2).

### IEC Subtypes

Modeling host-microbe interactions reflective of the *in vivo* situation demands the development of organoid systems containing mature and functional IECs, including the enrichment of IEC subtypes to investigate more specific interactions.<sup>60</sup> Unfortunately, many widely used protocols

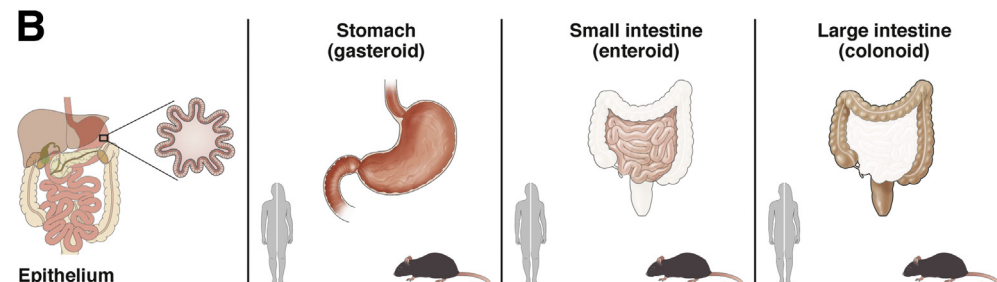
**A**



Epithelium	Early fetal enteroid	Late fetal enteroid	Neonatal enteroid	Adult enteroid
Maturation <sup>1</sup>	-	+	N/A	+++
Response to cytokine	-	++++	N/A	+
Response to LPS	+	++++	N/A	-
Response to SCFA (indole) <sup>1</sup>	++	+++	N/A	-

<sup>1</sup>Experiments done on mouse enteroids

**B**



Epithelium	Stomach (gasteroid)		Small intestine (enteroid)		Large intestine (colonoid)	
Response to LPS	-	+++	-	+	-	++
Response to FlIC	+++	-	++	+	+++	+
Response to Pam3CSK4	+	-	-	++	-	+++
EAEC adherence	N/A	N/A	+++	N/A	+	N/A

**Figure 2. Maturation, function, location, and species specificity of intestinal organoids and bacterial interactions.** (A) Schematic representation of human IEC maturation and function of intestinal organoids relative to developmental stages. (B) Schematic representation of organoid bacterial interactions relative to species and GI locations. Number of (+) signs indicate the degree of the response, (-) negative signs indicate no response, and N/A indicates no data available. FlIC, flagellin; Pam3CSK4, Pam3CysSerLys4.

generate organoids containing only semidifferentiated IECs that lack rarer IEC subtypes.<sup>54,56</sup> The incorporation of differentiation factors such as DAPT, a Notch signaling inhibitor, and CHIR99021, a Wnt signaling potentiator in organoid media, promotes differentiation and enriches for Paneth cells within enteroids.<sup>61–64</sup> Supplementation with stem cell niche factors insulin growth factor-1 and fibroblast growth factor also can preserve the in vivo cellular diversity and self-renewing capacity of intestinal crypts.<sup>65</sup>

Specific cell-type-enriched organoids have been used when modeling enteric pathogens that target specific IEC subtypes. For instance, supplementing enteroids with tumor necrosis factor  $\alpha$  and receptor activator of nuclear factor- $\kappa$ B ligand (NF $\kappa$ B) enriches for M cells, and such enteroids were used previously to confirm that *S flexneri* use M cells as portals for invasive entry.<sup>39</sup> In addition to Paneth cells<sup>61,66,67</sup> and M cells,<sup>39,68–70</sup> researchers successfully have enriched organoids for goblet cells,<sup>62,66,67</sup> tuft cells,<sup>71</sup> and enteroendocrine cells.<sup>72,73</sup> Together, these enriched organoids provide useful models when investigating cell-

type-specific interactions with bacteria. Nonetheless, it is important to consider whether a phenotype observed in these enriched organoids may be an indirect effect of their specialized media because many of the growth factors and inhibitors used to manipulate IEC differentiation also may affect other signaling pathways.<sup>67</sup> In addition, enriched organoids develop crypt-like structures leading to reduced lumen volume, consequently affecting the ability to introduce microbes, particularly through microinjection, because this procedure is largely dependent on the volume of bacterial culture delivered into the organoid lumen.

### Modeling the Mucus Layer

The intestinal mucus layer is a dynamic and highly complex matrix that physically separates intestinal luminal contents from the underlying epithelium.<sup>74</sup> It plays an active role in controlling microbial interactions with IECs because its heavily glycosylated mucin proteins are critical for bacterial adhesion and also act as a nutrient source for

**Table 1.** Overview of Techniques Used to Evaluate Goblet Cell and Mucus Function

Techniques	Used on	Compatible organoid method	Goblet cell properties/mucus functions	Reference
Immunofluorescent staining for mucus composition	In vitro ALI monolayer; in vivo mouse colon staining	2D monolayer; 3D organoids	Expression of mucus protein components MUC2, MUC5AC, TFF3, AGR2, RELM $\beta$ , FCGBP, CLCA1, and ZG16	75,90
Isotope label of mucin	In vivo and ex vivo mouse colon and mucus	2D monolayer	Mucin synthesis and production after $^3\text{H}$ -glucosamine injection	91
GalNAz label of mucin (Click-iT)	In vivo mouse small intestine	2D monolayer; 3D organoids	Mucin synthesis and production by labeling mucin glycoproteins	92–94
Secretagogue stimulation	Ex vivo mouse colon	2D monolayer; 3D organoids	Mucin secretion and goblet cell maturity stimulated by acetylcholine or analogs	95,96
Activated charcoal and micropipettor	Ex vivo mouse colon	2D monolayer	Mucus thickness measured by adding charcoal particles on mucus apical surface	75,95–97
Fluorescent beads	In vitro monolayer; ex vivo mouse intestine	2D monolayer	Mucus thickness measured by distance of beads to epithelium	90,94,98
Penetrable beads	In vitro monolayer; ex vivo mouse intestine	2D monolayer	Mucus permeability to bacteria-sized fluorescent beads	75,95,96
LC-MSMS	In vivo mouse colon mucus	2D monolayer	Proteomic analysis of the mucus layer	75,94,98

AGR2, anterior gradient 2; CLCA1, chloride channel regulator, calcium-activated-1; FCGBP, IgGFc-binding protein; GalNAz, N-azidoacetylgalactosamine; LC-MSMS, liquid chromatography with tandem mass spectrometry; MUC2, mucin 2; MUC5AC, mucin5AC; RELM $\beta$ , resistin-like molecule  $\beta$ ; TFF3, Trefoil Factor 3; ZG16, zymogen granule membrane protein 16.

commensals and pathogens alike.<sup>13,75</sup> Thus, as we develop more accurate host–bacterial organoid models that include different IEC subtypes, it is equally important to develop functional goblet cells able to generate a mucus layer.

Many studies remove Wnt ligands from the media to induce organoid-derived monolayers to produce a mucus layer. In a recent study, human colonoid monolayers formed a mucus layer 5 days after Wnt removal and, interestingly, the bacterial pathogen enterohemorrhagic *E coli* (EHEC) preferred infecting these monolayers over undifferentiated monolayers, suggesting a role for mucus as an anchor or nutrient source for EHEC.<sup>76</sup> Similarly, mucus-producing, differentiated, colonoid monolayers were used to define the role of an EAEC-produced serine protease, showing that its ability to degrade organoid-produced mucus improved bacterial penetration and colonization of the underlying IECs.<sup>77</sup> Mucus-producing, enteroid-derived monolayers also have been used to model *C difficile*–induced enteritis and confirmed a protective role for mucus (Muc2) because the addition of Muc2 delayed *C difficile*–derived toxins from reaching the monolayers.<sup>78</sup> These results highlight that an intact mucus layer can alter bacterial pathogenesis in vitro and thus its addition may aid in understanding complex enteric pathogen–host interactions.

Additional techniques now are being used to enrich for mucus in organoid models. One method mimics commensal cues via the addition of SCFAs. SCFAs epigenetically modify the enteroids, leading to concentration-dependent increases in the expression of goblet cell markers, as well as common mucus constituents and antimicrobial peptides.<sup>18</sup> Notably, it remains unclear if the mucus produced by these monolayers truly reflects the in vivo mucus layer. Mucus should be largely impenetrable to commensal bacteria, show a complex glycosylation pattern, as well as contain an array of mucus-associated proteins as shown in Table 1. Moreover, the mucus layer should sustain its own renewal by tightly regulating its production, secretion, and degradation. Thus, it is crucial to validate the composition (glycosylation, proteins) and functionality (permeability, secretion, production) of mucus layers produced in vitro. Currently, there are few studies in which organoids are characterized for the presence and function of goblet cells or a mucus layer, however, several methods used in other systems may be applicable and are summarized in Table 1.

### Oxygen Levels and Anaerobic Bacteria

The majority of intestinal commensal bacteria are facultative or obligate anaerobes that can survive only in environments containing minimal oxygen.<sup>79,80</sup> Correspondingly, the colonic lumen is hypoxic under healthy conditions.<sup>81</sup> In contrast, most researchers perform their bacteria-organoid studies using ambient incubator conditions of 18%–21% O<sub>2</sub>.<sup>82</sup> O<sub>2</sub> levels are affected further by factors such as ALI and ECM density,<sup>44</sup> resulting in oxygen gradients that are not representative of in vivo conditions.<sup>81</sup> Thus, researchers need to develop systems that create a dynamic oxygen gradient from the apical to the basolateral sides of IEC monolayers, for example, growing cultures in

modified anaerobic chambers. The enteroid–anaerobe co-culture system (EACC), as well as the intestinal hemianaerobic co-culture system (iHAC), recently were developed to establish a physiological hypoxia gradient for tissue culture.<sup>83,84</sup> Both systems deliver oxygen to the basolateral side of the monolayer, with IECs consuming the O<sub>2</sub>, preventing it from reaching the apical side, rendering the apical region hypoxic. Notably, this O<sub>2</sub> gradient promotes increased IEC barrier integrity and facilitates the survival of obligate anaerobes such as *Bacteroides thetaiotaomicron* and *Blautia* species for up to 24 hours. *B adolescentis*, *Bacteroides fragilis*, *Clostridium butyricum*, and *Akkermansia muciniphila* also can survive for 48 hours in this system, driving IECs toward a more mature and immunotolerant state.<sup>83,84</sup> These 2 robust models re-create the oxygen gradient seen in vivo to address interactions between anaerobic microbes and gut epithelium, and define the extent to which disrupting this system can result in dysbiosis and intestinal inflammatory disorders.<sup>79,81,85</sup>

### IEC Intrinsic Immune Responses to Bacteria Using Organoids

Aside from their other functions, IECs serve as a central hub in mucosal innate immunity, expressing an array of innate receptors that sense various microbial ligands from the gut lumen and respond through the release of antimicrobial peptides and cytokines. IEC responses often shape subsequent immune responses from underlying immune cells.<sup>55,59</sup> Interestingly, many recent studies have shown that the innate immune responses of ASC-derived organoids to commensal and pathogenic bacteria are very different from those obtained using cancer cell lines.

Aside from the TLRs and NOD receptors mentioned earlier, IECs also use intrinsic inflammasomes to sense and respond to invading pathogens. In vivo activation of inflammasomes during *S Typhimurium* infection selectively expels infected IECs into the intestinal lumen.<sup>86</sup> Recently, we confirmed these responses reflect IEC intrinsic inflammasomes, by infecting murine colonoid-derived monolayers with *S Typhimurium*. It was notable that the monolayers did not express the same levels of inflammatory caspases as in vivo until they were treated with interferon (IFN)- $\gamma$ , which helped IECs reach a state of heightened immune activation. This led the treated IECs to express the same inflammatory caspases as their in vivo counterparts, and efficiently extrude infected cells.<sup>87</sup> In a separate study, we also identified differences in the preferential activation of these inflammatory caspases between human and murine enteroids upon their infection with *S Typhimurium*.<sup>88</sup> Because inflammasomes are expressed by many cell types such as macrophages and neutrophils, studying enteroids helps clarify the role of IEC intrinsic signaling and allows side-by-side comparison between human beings and mice. This further emphasizes the importance of choosing the appropriate species and stimulation when using organoids to study host–microbe interactions because findings made with cells from 1 species or microbe may not prove relevant under other conditions. Moreover, it highlights that to model the in vivo epithelium,



**Table 2.** List of Organoid–Immune Cell Co-culture Studies

Co-culture	Organoid type	Immune cell used	Microbe	Technique	Findings	Reference
Organoids and immune cells	Human fetal enteroids, colonoids	Fetal CD4 <sup>+</sup> Tem cells	NA	Mix in Matrigel	Low numbers of CD4 <sup>+</sup> Tem cells support stem cell growth, while high numbers impair IEC development via TNF- $\alpha$	99
	Murine enteroids	Th1, Th2, Th17, iTreg cells	NA	In media; mix in Matrigel	MHCII on Lgr5 <sup>+</sup> ISC interact with Th and Treg cells, each producing different cytokines to regulate ISC renewal and differentiation	100
	Murine enteroids	ILC3	NA	Mix in Matrigel	ILC3 produces IL22 to promote ISC proliferation (via STAT3 signaling) after tissue damage	101
	Murine enteroids	ILC2-enriched MLN cells	NA	Transwell	IL33 induces ILC2 to produce IL13, a cytokine that enhances IEC differentiation to goblet cells	102
	Murine enteroids	IEL	NA	Mix in Matrigel	IELs propagate and could migrate along the basolateral surface of IEC into and out of the enteroids	103
	Murine enteroids	CD4 <sup>+</sup> , CD8 <sup>+</sup> T cells	NA	Mix in Matrigel	T cells can be co-cultured long term with enteroids and acquire IEL-like migration and expression patterns	104
	Mouse and human organoids	T cells, DCs	NA	Mix in Matrigel	Dysregulated T-cell activation from bone marrow transplant leads to increased IFN $\gamma$ production and ISC injury	105
Organoids, immune cells, and microbes	Human enteroid monolayer	Human PBMC-derived macrophages	EPEC, ETEC	Transwell	Macrophages project through Transwell filter to interact and phagocytose ETEC and EPEC found on the apical side of the enteroid monolayers	106
	Human enteroid monolayer	PBMC	<i>C. difficile</i> toxin A, <i>E. coli</i> O6	Transwell (ALI)	Mucus layer prevented toxin-mediated junction disruptions and bacteria-induced cytokine secretions from PBMCs	90

DC, dendritic cell; EPEC, Enteropathogenic *E. coli*; ETEC, Enterotoxigenic *E. coli*; IEL, intraepithelial lymphocyte; IFN, interferon; IL, interleukin; ILC, innate lymphoid cell; ISC, intestinal stem cell; iTreg, induced T regulatory cells; MHCII, Major histocompatibility complex II; MLN, mesenteric lymph node; PBMC, peripheral blood mononuclear cell; STAT3, Signal transducer and activator transcription 3; Tem, T-effector memory cells; Th, T helper cells; TNF, tumor necrosis factor.

baseline IEC responses should be assessed in the context of integrating commensal microbes,<sup>42,55,59</sup> as well as immune cells to organoid models, a subject that was reviewed recently and is summarized in Table 2.<sup>89</sup>

## Conclusions

Taken together, it is clear that intestinal organoids offer impressive potential to accurately model in vivo bacteria-IEC interactions. Many variables need to be taken into consideration when using organoids: the stem cell origin, the tissue location, the culturing method, not to mention all the complexities regarding the heterogeneity of human samples such as sex, age, ethnicity, and disease-related status. Although new organoid models have tackled several of these issues, including improving our ability to introduce microbes, stimulants, or immune cells, as well as produce mucus and control O<sub>2</sub> levels, there are still several physiologically relevant factors that are missing, such as mechanical forces from luminal flow and peristalsis. Consequently, it is essential to continue exploring how to generate more relevant features of the native gut epithelium in organoids, to better define their interactions with gut microbes. Another pressing issue is to develop methods to generate organoids on a larger scale in a more reproducible, accessible, and inexpensive fashion. Acknowledging the impressive advances already made, organoids will be a key translational platform to discovering personalized treatments unique to individual patients.

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Xiao Han reviewed the concept and design, performed the literature review, drafted the manuscript, and approved the final manuscript; Matthias Mslati, Emily Davies, and Yan Chen performed the literature review, drafted the manuscript, and approved the final manuscript; Joannie M. Allaire was responsible for study supervision, literature review, critical revision of the manuscript for important intellectual content, and approved the final manuscript; and Bruce A. Vallance was responsible for study supervision, critical revision of the manuscript for important intellectual content, and approved the final manuscript.

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The authors disclose no conflicts.

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