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REVIEW

Cell and Animal Models of Gastrointestinal Disease

Intestinal organoid coculture systems: current approaches, challenges, and future directions

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Abstract

The intestinal microenvironment represents a complex and dynamic ecosystem, comprising a diverse range of epithelial and nonepithelial cells, a protective mucus layer, and a diverse community of gut microbiota. Understanding the intricate interplay between these components is essential for uncovering the mechanisms underlying intestinal health and disease. The development of intestinal organoids, three-dimensional (3-D) mini-intestines that closely mimic the architecture, cellular diversity, and functionality of the intestine, offers a powerful platform for investigating different aspects of intestinal physiology and pathology. However, current intestinal organoid models, mainly adult stem cell-derived organoids, lack the nonepithelial and microbial components of the intestinal microenvironment. As such, several coculture systems have been developed to coculture intestinal organoids with other intestinal elements including microbes (bacteria and viruses) and immune, stromal, and neural cells. These coculture models allow researchers to recreate the complex intestinal environment and study the intricate cross talk between different components of the intestinal ecosystem under healthy and pathological conditions. Currently, there are several approaches and methodologies to establish intestinal organoid cocultures, and each approach has its own strengths and limitations. This review discusses the existing methods for coculturing intestinal organoids with different intestinal elements, focusing on the methodological approaches, strengths and limitations, and future directions.

coculture; immune cells; intestinal organoids; microbes; stromal cells

INTRODUCTION

The intestine exhibits a multifaceted and complex microenvironment characterized by epithelial lining comprising diverse types of epithelial cells with specialized functions (1) and is supported by an intricate network of stromal, immune, vascular, and neural cells (2). These components work together to maintain intestinal homeostasis and facilitate several physiological functions including nutrient absorption, maintenance of mucosal barrier integrity, and intestinal immune surveillance (3). In addition, the intestinal lumen is home to a vast and diverse community of microbes, collectively known as the gut microbiota. These microbes play a fundamental role in intestinal metabolic and immunological functions (4). The intestinal microenvironment, therefore, involves a dynamic interaction between the epithelial and nonepithelial cells and the microbiota. Understanding these interactions is vital for studying intestinal development, physiology, and pathophysiology of intestinal diseases.

The past decade has witnessed the emergence of intestinal organoids as a powerful tool for studying intestinal biology and physiology. Intestinal organoids are self-organized, three-dimensional (3-D) structures, "mini-guts," that faithfully recapitulate the architecture, cellular diversity, and physiology of the intestinal epithelium providing a physiologically relevant platform for investigating various aspects of intestinal epithelial structure and functions (5). The ability of intestinal organoids to model specific aspects of intestinal functions such as barrier function, nutrient absorption, and cell-cell interactions has made them a valuable tool for both basic and translational research (6, 7). However, current intestinal organoid models, particularly tissue-derived organoids, are limited in their utility as they only model the intestinal epithelial layer and lack other key nonepithelial components of the microenvironment such as the stromal

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and immune cells and the microbiome (5). To fully realize the potential of intestinal organoids, studies have aimed to establish intestinal organoid cocultures with microbes and other nonepithelial cells (8). Various intestinal organoid-microbe (bacteria and viruses) coculture systems have been developed, ranging from simple organoid-derived monolayer (ODM) cocultures to more sophisticated microfluidic intestine-on-chip systems (9). These systems enable the investigation of the mechanisms of microbial colonization, the impact of pathogenic and commensal microbes on the intestinal epithelium, and the epithelial immune response to microbial stimuli (10). In addition, organoids have been successfully cocultured with different types of immune cells to study mechanisms underlying immune tolerance and inflammation, with stromal cells to investigate stromal-epithelial interactions and to study their role in supporting epithelial cells and the pathogenesis of intestinal diseases, and with neural cells to study neural-epithelial interactions (2). There are also few studies that coculture intestinal organoids with more than one element e.g., immune cells and microbes or immune cells and fibroblasts, however, research in this field is still in its infancy.

The development of complex coculture systems will enable a more accurate representation of the in vivo interactions between the different components of the intestine. This will facilitate the study of cellular interactions in health and disease and will also allow for more accurate modeling of individual responses to various agents offering a valuable tool for drug screening and personalized medicine. This review, therefore, examines intestinal organoid coculture systems, highlighting the methodological approaches, key considerations, limitations, and future directions.

INTESTINAL ORGANOIDS: DEVELOPMENT AND APPLICATIONS

More than a decade ago, researchers from Hans Clevers laboratory published the first method for isolating adult intestinal stem cells (ISCs) and creating long-term 3-D cultures of mouse small intestine (11) and mouse and human colon (12, 13), which they termed "organoids." Established organoids develop polarized intestinal epithelium with all epithelial cell types (ISCs, enterocytes, goblet, enteroendocrine, tuft, and Paneth cells) (5). In these early studies, organoids were generated through the differentiation of adult stem cells (ASCs) isolated from the crypts of mouse and human intestine (12, 13). In addition to ASCs, organoids have also been developed from pluripotent stem cells (PSCs) including both embryonic stem cells (ESCs) and induced PSCs (iPSCs) (14).

To generate PSC-derived organoids, ESCs or iPSCs undergo a stepwise differentiation process to endoderm, hindgut spheroids, and then 3-D organoids (15). Given the pluripotent nature of ESCs and iPSCs, the organoids develop a polarized intestinal epithelium surrounded by a supporting mesenchyme, containing immature smooth muscle cells, subepithelial myofibroblasts, and fibroblasts. These organoids could also develop neuronal and vascular endothelial cells (14, 16). As such, these organoids have more complex cellular structures as compared with ASC-derived organoids making them suitable models for studying intestinal development, intestinal disease modeling, and regenerative medicine (17). Conversely, due to the multipotent nature of ASCs, these cells differentiate into epithelial cells without mesenchyme and hence ASC-derived organoids have a less complex structure. However, they closely resemble adult intestinal epithelium structure, maturity, and functionality and can be derived from any region of the small intestine (enteroids) or colon (colonoids) (18). In addition, these organoids display high genetic fidelity with donor tissue making them a better model for translational research in the field of drug discovery and precision medicine (16).

To create 3-D organoids, hindgut spheroids or intestinal crypts are embedded in a basement membrane extract (BME), e.g., Matrigel that helps induce the polarization of the intestinal epithelium to form a lumen-enclosed structure with apical and basal surfaces (11). The enclosed lumen structure restricts direct access to the apical epithelium in the intact 3-D organoid. To expose the apical surface, 3-D organoids can be sheared into small fragments and then reseeded in BME. Alternatively, a more recent approach involves eversion of epithelial polarity by culturing established 3-D organoids in suspension instead of BME domes (19, 20). This spontaneously reverses organoid polarity so that the apical surface is facing outward while at the same time maintaining its 3-D structure. Another approach for culturing organoids with easier access to both apical and basal surfaces is the development of ODMs. In this method, 3-D organoids are dissociated into single cells and seeded in precoated culture transwell inserts or cell culture plates. The epithelial cells then polarize to form a two-dimensional (2-D) epithelial monolayer with apical and basal surfaces (21). Different approaches have their advantages and limitations and are selected based on research questions to be explored.

Generally, intestinal organoids mimic intestinal structure and function (6), therefore, offering a multicellular and physiologically relevant model that can be used for many applications including studying intestinal biology, modeling intestinal diseases, and as tools for personalized medicine and drug screening (7). However, organoid models still have several limitations that need to be addressed. First, the heterogeneity in organoid size and degree of differentiation could create variability between experimental groups. In addition, these organoids do not fully capture cellular complexity and interactions within the intestinal microenvironment and lack luminal flow and peristaltic movement. Hence, establishing organoid coculture systems (8) and using engineering-based approaches such as developing microfluidic intestine-on-chip devices (22) could be used to address current limitations. Several approaches have been used to incorporate nonepithelial components into existing organoid models and to construct multicellular and more complex systems. This includes combining cells as a cellular mixture, microinjection, generation of apical-out organoids or ODMs, developing multilayer cultures, or using microfluidic chips (Fig. 1). The various methods used for coculturing different intestinal components with organoids are discussed in the following sections.

Organoid-Bacteria Coculture

One of the major components of the intestinal environment is the gut microbiome that refers to the trillions of microbes, primarily bacteria, that reside in the luminal

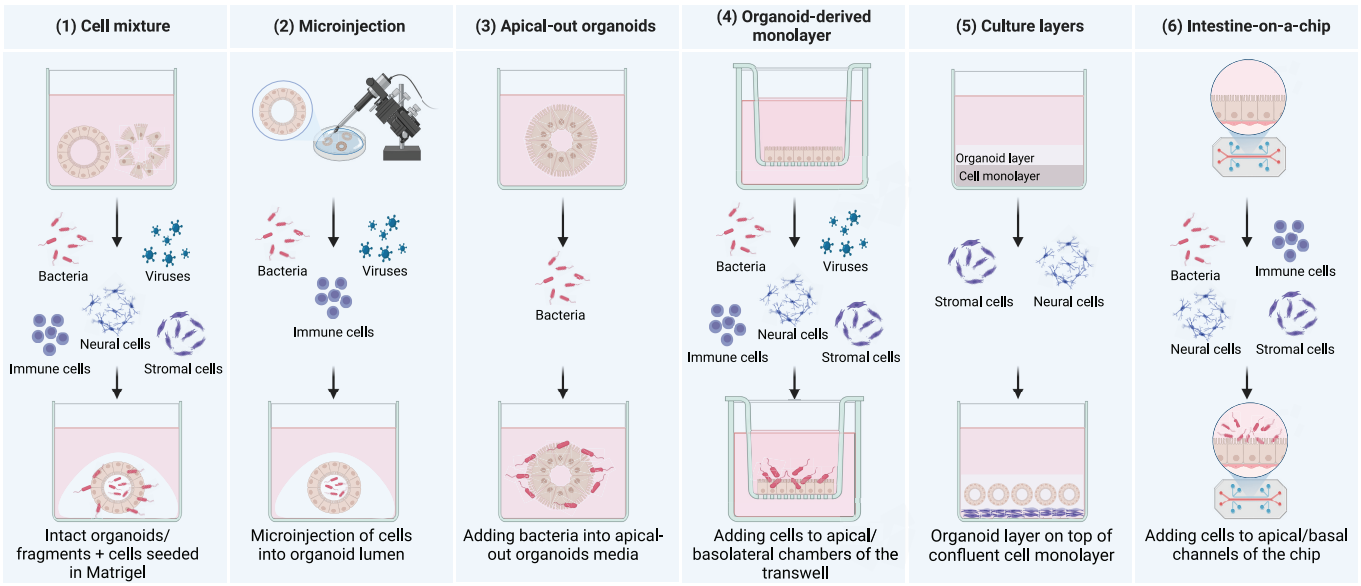

Figure 1. Summary of the major approaches for intestinal organoid cocultures. 1) Cell mixture method involves mixing intact organoids or organoid fragments with nonepithelial cells (bacteria, viruses, immune, stromal, and neural cells). Nonepithelial cells are suspended in the culture media while intact organoids/fragments are seeded in Matrigel. 2) Microinjection is used to deliver the nonepithelial cells (bacteria, viruses, or immune cells) into the lumen of three-dimensional (3-D) organoids. 3) Nonepithelial cells (bacteria) are added directly to the media of 3-D apical-out organoids. All components remain in suspension culture. 4) Nonepithelial cells (bacteria, viruses, immune, stromal, or neural cells) are added to the apical or basolateral chambers of the transwell system. The nonepithelial cells are isolated in their respective chambers and only those cells in the apical chamber directly contact the organoid monolayer. 5) A 3-D organoid layer is cultured on the top of a confluent monolayer of nonepithelial cells (stromal or neural cells). 6) Nonepithelial cells (bacteria, immune, stromal, or neural cells) are added to the apical or basal channels of the intestine-on-chip device. (Note: bacteria and stromal cells are used to illustrate the final coculture setup.) Created in BioRender. Al-qadami, G. (2024) BioRender.com/f19e653.

epithelium of the intestine. In addition to the indigenous microbiota, bacteria can be transferred into the intestine from the external environment such as bacteria associated with intestinal infections. As such, bacteria cocultured with intestinal organoids could be used to study the role of gut commensals on intestinal health and to model intestinal infection. To develop coculture models, methods such as incubation with intact organoids or sheared organoid cells, ODMs, microinjection, and apical-out organoids have been used (Fig. 1).

One of the first methods used to coculture bacteria with organoids is the infection of organoids via the cell mixture approach. This technique involves establishing a 3-D organoid culture in BME. Once ready for infection, BME is dissolved to release the organoids. In some studies, the bacterial inoculum is added directly to the media of intact 3-D organoids (19, 23, 24), and in others, the organoids are first mechanically sheared before the bacteria suspension is added to the organoid fragments (25–28). Once incubated for a specific period, organoids are reseeded in fresh BME and incubated until analysis (25–30). Several studies have used this method to coculture organoids with *Salmonella Typhimurium* (*S. Typhimurium*) (19, 23, 25), *Listeria monocytogenes* (*L. monocytogenes*) (19, 26, 28–30), *Lactobacillus reuteri D8* (*L. reuteri D8*) (24), *Citrobacter rodentium* (*C. rodentium)* (24), *Lactobacillus acidophilus* (*L. acidophilus)* (25), *pks+ Escherichia coli* (*E. coli)* (27) and *Mycobacterium avium paratuberculosis (MAP)* (31) (Table 1). Although this method involves loss of the organoid 3-D structure, it offers a scalable approach and allows more homogeneous exposure of organoid cells to microbes. However, exposure to the apical or basal surfaces cannot be controlled as both surfaces will be in contact with bacteria in the media. Furthermore, due to exposure to oxygen in the media, this method has been mainly used for coculturing facultative anaerobes like *S. Typhimurium*. Although, in one study, the anaerobic gut microbe *L. reuteri* was incubated with 3-D organoids for 48 h, however the authors did not assess the viability of the bacteria postincubation (24). This is critical to ensuring the interaction of epithelial cells with live bacteria mimics in vivo interactions.

ODM is another method for coculturing microbes with intestinal organoids. Due to its simplicity, it has been widely used to study enteric pathogens such as *E. coli* (27, 32, 33, 38, 40, 41, 43–47, 49, 50), *Shigella flexneri* (*S. flexneri)* (34–36, 44), *S. Typhi and Typhimurium* (37, 42, 44, 48, 51), oral pathobionts *Fusobacterium nucleatum* (*F. nucleatum*) (40), members of the gut microbiota such as *Bifidobacterium adolescentis*, *Bacteroides fragilis (B. fragilis)*, *Clostridium butyricum*, *Akkermansia muciniphila* (39), *Enterococcus faecalis V583*, and *Bacteroides thetaiotaomicron* (*B. thetaiotaomicron)* (50), probiotic bacteria *L. reuteri NPL-88* (50) and *E. coli Nissle* (48), and the bovine pathogen *MAP* (31) (Table 1). In this approach, confluent and differentiated monolayers are infected by adding bacteria to the apical surface of the culture systems (33, 34, 37, 38). In some studies, bacteria were introduced to the basolateral side of the culture to assess basolateral invasion (32, 34–38). Once infected, monolayers can be incubated for a minimum of 1 h (35) and up to 72 h (31) depending on the bacteria type and desired experimental settings. To eliminate unattached extracellular bacteria and

Table 1. *Summary of organoid-bacteria coculture studies*

Organoids	Bacteria	Coculture Method	Incubation Time	Key Findings	References
Murine ASC-derived intestinal organoids	*S. Typhimurium*	Cell mixture (intact organoids)	30 min	*S. Typhimurium* invaded organoids and caused morphologic changes, tight junction disruption, decrease in stem cell markers, and inflammation by activating NF-κB signaling pathway	Zhang et al. (23)
Murine ASC-derived intestinal organoids	*L. reuteri D8* *C. rodentium*	Cell mixture (intact organoids)	24–48 h	*L. reuteri* increased organoid size and epithelial cell proliferation through the activation of Wnt/β-catenin pathway *L. reuteri* reduced TNF-induced organoid damage and apoptosis and stem cell loss *L. reuteri* reduced *C. rodentium* colonization and protected against *C. rodentium*-induced damage	Wu et al. (24)
Murine ASC-derived intestinal organoids	*S. Typhimurium* *L. acidophilus*	Cell mixture (sheared organoids)	12 h (*L. acidophilus*) 24 h (*S. Typhimurium*)	*S. Typhimurium* induced epithelial villi damage and reduced organoid formation *L. acidophilus* alleviated intestinal damage by reducing TNF-α secretion, suppressing intestinal inflammation and enhancing mucosal barriers	Lu et al. (25)
Murine ASC-derived intestinal organoids	*L. monocytogenes*	Cell mixture (sheared organoids)	18 h	At early infection stage (1 h), L. monocytogenes invaded and reduced growth and proliferation of organoids, reduced ISCs number and expression of ISCs, goblet, and Paneth cells markers At late infection stage (18 h), severe organoid damage was observed but proliferation, ISCs and Paneth cell markers increased	Huang et al. (26)
Murine ASC-derived intestinal organoids	*L. monocytogenes*	Cell mixture (sheared organoids)	18 h	*L. monocytogenes* increased goblet and Paneth cells by inhibiting Notch signaling pathway	Zhou et al. (28)
Murine ASC-derived intestinal organoids	*L. monocytogenes*	Cell mixture (intact organoids)	18 h	*L. monocytogenes* increased the expression of cellular communication network factor 1 (CCN1) and the activation of TLR2/4 pathways inducing intestinal inflammation	Zhou et al. (29)
Murine ASC-derived intestinal organoids	*L. monocytogenes*	Cell mixture (intact organoids)	18 h	Quantitative proteomic analysis showed differentially expressed protein profiles in organoids infected with L. monocytogenes strains with different virulence	Zhou et al. (30)
Human ASC-derived colonoids	*Enterohemorrhagic E. coli*	ODM (apical surface)	4–18 h	*E. coli* colonized ODM apical surface and reduced expression of Mucin 2 and protocadherin 24 causing mucus layer and brush border damage	In et al. (32)
Human ASC-derived enteroids	*Enteroaggregative E. coli*	ODM (apical surface)	3 h	*E. coli* showed segment- and donor-specific adherence patterns with a sheet-like (duodenal and ileal), a mesh-like (colonic), and diffuse adherence in the jejunal ODM	Rajan et al. (33)
Human ASC-derived enteroid	*S. flexneri*	ODM (apical/basolateral surface)	3–24 h	*S. flexneri* infected ODM and replicated intracellularly	Ranganathan et al. (34)

Continued

Table 1.— *Continued*

Organoids	Bacteria	Coculture Method	Incubation Time	Key Findings	References
				ODM differentiation increased the apical invasion due to the presence of M cells and basolateral invasion was more efficient than apical infection *S. flexneri* increased IL-8 secretion and MUC2 expression in ODM	
Human ASC-derived enteroids	*S. flexneri*	ODM (apical/basolateral surface)	3 h	*S. flexneri* preferentially invaded ODM via basolateral surface and replicated intracellularly Infection increased proinflammatory cytokines and amino acid transporter SLC7A5 expression Disruption of tight junctions facilitated apical invasion	Koestler et al. (35)
Human ASC-derived organoids	*S. flexneri*	ODM (apical surface)	3 h	*S. flexneri*-specific bacteriophage killed *S. flexneri* strains and prevented bacterial adherence and invasion	Llanos-Chea et al. (36)
Human ASC-derived organoids	*S. Typhi*	ODM (apical surface)	4–5 h	Disruption of yrbE phospholipid transporter gene in *S. Typhi* reduced bacterial adhesion and induced production of a higher amount of IL-8	Verma et al. (37)
Human ASC-derived enteroids	Enterotoxigenic *E. coli*	ODM (apical surface)	4–8 h	ETEC increased intracellular cAMP which underwent apical export while cGMP mainly effluxed into the basolateral surfacec GMP production is modulated by heat-labile and heat-stable enterotoxins and other virulence factors (EatA, EtpA, and CfaE)	Foulke-Abel et al. (38)
Human ASC-derived colonoids	*B. adolescentis* *B. fragilis* *C. butyricum* *A. muciniphila*	ODM (apical surface) in Hemi-Anaerobic Coculture System (iHACS)	5 days	iHACS supported the propagation of *B. adolescentis* and colony formation of *B. fragilis* and *C. butyricum* *A. muciniphila* degraded the mucus layer and grew without exogenous mucin	Sasaki et al. (39)
Human/murine ASC-derived CRC organoids	*F. nucleatum* *E. coli* *H. pylori*	ODM (apical surface)	24 h	*F. nucleatum* downregulated expression of DNA repair protein (NEIL2) increasing DNA damage and production of pro-inflammatory cytokines	Sayed et al. (40)
Human ASC-derived colonoids	Enteroaggregative *E. coli*	ODM (apical surf)		EAEC Pic serine protease disrupted the mucus layer by inducing MUC2 degradation and increasing proinflammatory cytokine secretion	Liu et al. (41)
Human/murine ASC-derived enteroids	*S.Typhimurium*	ODM (apical surface)	7 h	*S.Typhimurium* replicated and induced IL-18 secretion in human and murine ODM Proinflammatory caspases restricted intracellular replication and IL-18 production in human (caspase 4) and murine (caspase 1) ODM	Holly et al. (42)
Human ASC-derived colonoids	Enteroaggregative *E. coli*	ODM (apical surface)	3–6 h	EAEC aggregative adherence fimbriae II is important for bacterial adherence and disruption of barrier integrity in ODM	Gonyar et al. (43)
Human ASC-derived enteroid and colonoid	*S. flexneri* *S. Typhi* *S. Typhimurium* *E. coli*	ODM (apical surface)	2–3 h	*S. flexneri* adhered to cecum and colon ODMs *S. Typhimurium* caused more damage to ileal monolayers while S. *Typhi* infected the cecum more efficiently	Nickerson et al. (44)

Continued

Table 1.— *Continued*

Organoids	Bacteria	Coculture Method	Incubation Time	Key Findings	References
				E. coli adhered to the epithelium with minimal invasion	
Porcine ASC-derived enteroids	Enterotoxigenic *E. coli*	ODM (apical surface)	2 h	Bacterial F4 fimbriae mediated adhesion of ETEC to epithelium in ODMs	Vermeire et al. (45)
Murine ASC-derived colonoids	*pks* + *E. coli*	Cell mixture (sheared organoids)/ODM (apical surface)	3 h	Organoids exposed to short-term *pks* + *E. coli* infection displayed CRC cell characteristics (increased proliferation, downregulated differentiation, Wnt-independence and genetic mutations)	Iftekhar et al. (27)
Human ASC-derived enteroids	Enteroaggregative *E. coli*	ODM (apical surface)	3 h	Hydrogels supported development and differentiation of ODM Soft hydrogels enhanced EAEC aggregate formation by impacting the mucus layer	Swaminathan et al. (46)
Human ASC-derived enteroids	Adherent-invasive *E. coli*	ODM (api)		SCFAs protected against AIEC invasion by inducing an anti-inflammatory response and enhancing epithelial integrity	Pace et al. (47)
Murine ASC-derived enteroids	*L. reuteri NPL-88* *S. Typhimurium*	ODM (apical surface)	3.5 h	*L. reuteri* prevented the decrease in the barrier integrity caused by *S. Typhimurium* infection	Anjum et al. (48)
Human PSC-derived enteroids	Probiotic *E. coli Nissle* Commensal *E. coli ECOR13* Pathogenic *E. coli O157:H7 PT29S*	ODM (apical surface)	24–36 h	Nonpathogenic *E. coli* rapidly replicated in ODM without causing epithelial damage or decreasing barrier integrity Pathogenic *E. coli* attached and replicated in ODM causing epithelial damage with a significant reduction in barrier integrity Biotin and L-fucose protected against pathogenic *E. coli* attachment and alleviated ODM damage	Small et al. (49)
Commercial human colonoids	Enterohemorrhagic *E. coli* *E. faecalis* *B. thetaiotaomicron*	ODM (apical surface)		EHEC attached to ODM causing AE lesions *B. thetaiotaomicron* and *E. faecalis* enhanced EHEC virulence and formation of AE lesions in ODM	Martins et al. (50)
Human PSC-derived organoids/ASC-derived enteroids	*S. Typhimurium*	ODM (apical surface)	2 h	*S. Typhimurium* infects ODM derived from PSC-derived organoids and ASC-derived enteroids Administration of IgA recombinant monoclonal antibody Sal4 IgA reduced bacterial invasion	Costello et al. (51)
Bovine ASC-derived enteroids	*M. avium paratuberculosis*	ODM (apical surface)	1–72 h	*MAP* can infect and replicate in ODM, and bacteria can be observed intracellularly	Blake et al. (31)
Human/murine ASC-derived enteroids	*S. Typhimurium* *L. monocytogenes*	Apical-out organoids	6 h	*S. Typhimurium* preferentially invades the apical surface while *L. monocytogenes* invade epith at cell extrusion sites and both exit within apically extruding epithelial cells	Co et al. (19, 20)
Human ASC-derived colonoids	*Enteroaggregative E. coli*	Apical-out organoids	3 h	EAEC clustering was observed on epithelial surface with/without MUC2 clusters EAEC epithelial binding mainly depends on functional mucus with some adherence linked to MUC2	Rajan et al. (52)

Continued

Table 1.— *Continued*

Organoids	Bacteria	Coculture Method	Incubation Time	Key Findings	References
Ovine ASC-derived organoids	*S. Typhimurium*	Apical-out organoids	6 h	*S. Typhimurium* attached to apical surface and invaded epithelial cells of apical-out organoids	Smith et al. (53)
Bovine ASC-derived enteroids	*M. avium paratuberculosis*	Apical-out organoids	24–72 h	Apical-out enteroids were permissive to *MAP* infection with intracellular bacteria observed 24 h postinfection	Blake et al. (31)
Human PSC-derived organoids	*L. casei* *B. longum*	Apical-out organoids	12 h	Both bacteria attached to and colonized apical-out organoids incubated in a hypoxic condition Both microbes upregulated expression of barrier integrity genes	Kakni et al. (54)
Murine ASC-derived enteroids	*B. thetaiotaomicron*	Microinjection	Overnight	*B. thetaiotaomicron* increased expression of *fut2* and surface fucosylation in enteroids indicating that *B. thetaiotaomicron* modulates intestinal epithelium to enhance bacterial growth	Engevik et al. (55)
Human PSC-derived organoids	*C. difficile*	Microinjection	Overnight	*C. difficile* reduced mRNA and protein expression of NHE3 ion transporter altering intestinal environment to support *C. difficile* growth	Engevik et al. (56)
Human PSC-derived organoids	*C. difficile*	Microinjection	2–12 h	*C. difficile* maintained viability within the organoids for up to 12 h resulting in disruption of intestinal epithelium and barrier functions via *C. difficile* TcdA toxin	Leslie et al. (57)
Human PSC-derived organoids	*S. Typhimurium*	Microinjection	3 h	*S. Typhimurium* invaded epithelial cells and altered gene expression Mutation in pathogenicity Island 1 (SPI-1) invasion apparatus reduced invasion capability	Forbester et al. (58)
Murine ASC-derived enteroids	*S. Typhimurium*	Microinjection	20 h	α-defensins in organoids restricted the growth of multiple *S. Typhimurium* strains	Wilson et al. (59)
Human PSC-derived organoids	*E. coli*	Microinjection	24–96 h	PSC-derived organoids maintained stable colonization of nonpathogenic *E. coli* resulting in mucus layer maturation, enhanced barrier functions and increased antimicrobial peptide secretion	Hill et al. (60)
Murine ASC-derived enteroids	*S. Typhimurium*	Microinjection	21 h	Exposure of apical surface to live *S. Typhimurium* induced immediate release of Paneth cell granules	Yokoi et al. (61)
Human PSC-derived organoids	*E. coli Nissle* *Enterohemorrhagic E. coli* *Uropathogenic E. coli*	Microinjection	72 h	EHEC and UPEC, but not *E. coli Nissle, caused* epithelial barrier damage *E. coli Nissle*, protected against barrier damage, apoptosis and oxidative stress via activation of host defenses	Pradhan et al. (62)
Human PSC-derived intestinal organoids	*L. rhamnosus* *L. reuteri* *L. plantarum*	Microinjection	12 h	*Lactobacilli spp.* survived and colonized mature organoids more efficiently due to enhanced intestinal epithelial integrity	Son et al. (63)
Human PSC-derived intestinal organoids	*Nonpathogenic E. coli*	Microinjection	24 h	Loss of stress response regulator gene RpoS significantly decreased *E. coli's* colonization	Barron et al. (64)
Human/murine ASC-derived enteroids	*S. Typhimurium*	Microinjection	16 h	Type III secretion system 1 enhanced *S. Typhimurium* invasion and colonization	Geiser et al. (65)

Continued

Table 1.— *Continued*

Organoids	Bacteria	Coculture Method	Incubation Time	Key Findings	References
Human PSC-derived intestinal organoids	*S. Typhimurium*	Microinjection	8–24 h	*S. Typhimurium* enhanced expression of proinflammatory genes and downregulated expression of cell cycle and DNA repair genes	Lawrence et al. (66)
Murine ASC-derived intestinal organoids	*L. monocytogenes*	Microinjection	1–16 h	*L. monocytogenes* translocated across goblet cells within membrane vacuoles via microtubule and internalins surface protein	Kim et al. (67)
Human ASC-derived intestinal organoids	*pks* + *E. coli*	Microinjection (repeated)	24–72 h	*pks* + *E. coli* caused DNA damage and induced a mutational signature in the organoids resembling the mutational signature detected in human CRC genomes	Pleguezuelos-Manzano et al. (68)
Human ASC-derived neonatal organoids	*Infant faecal microbiota*	Intestine-on-a-chip (apical surface)	72 h	Exposure to microbes from infants with necrotizing enterocolitis decreased expression of ZO-1 tight junction and increased barrier permeability	Lanik et al. (69)

A. muciniphila, *Akkermansia muciniphila*; *B. adolescentis*, *Bifidobacterium adolescentis*; *B. fragilis*, *Bacteroides fragilis*; *B. thetaiotaomicron*, *Bacteroides thetaiotaomicron*; *C. butyricum*, *Clostridium butyricum*; *C. rodentium*, *Citrobacter rodentium*; *C. difficile*, *Clostridioides difficile;* CCN1, cellular communication network factor 1; CRC, colorectal cancer; *E. coli*, *Escherichia coli*; *E. faecalis*, *Enterococcus faecalis*; EAEC, *Enteroaggregative Escherichia coli*; EHEC, *Enterohemorrhagic Escherichia coli;* ETEC, *Enterotoxigenic Escherichia coli; F. nucleatum*, *Fusobacterium nucleatum*; iHACS, cellular communication network factor 1; ISC, intestinal stem cell; *L. reuteri D8*, *Lactobacillus reuteri D8*; *L. acidophilus*, *Lactobacillus acidophilus*; *L. monocytogenes*, *Listeria monocytogenes*; *M. avium paratuberculosis*, *Mycobacterium avium paratuberculosis*; NHE3, Na^+ /H^+ exchanger 3; ODM, organoid-derived monolayer; PSC, pluripotent stem cell; *S. Typhimurium*, *Salmonella Typhimurium*; *S. flexneri*, *Shigella flexneri*; *S. Typhi*, *Salmonella Typhi*; SCFAs, Short-chain fatty acids; ZO-1, zonula occludens-1; UPEC, *Uropathogenic Escherichia coli*.

to assess bacterial adherence and invasion, monolayers are treated with media containing nonpermeable gentamicin for a short period postinfection (34, 35). Although the ODM method offers a simple approach for coculturing organoids with bacteria, it does not maintain the organoid 3-D structure. It is also an end point model that cannot be expanded or cultured post bacterial infection. Furthermore, in its basic form, transwell monolayers are not suitable for culturing strictly anaerobic bacteria. To overcome this, Sasaki et al. (39) developed a transwell-based hemi-anaerobic system with a hypoxic apical chamber and a normoxic basolateral chamber that supported the growth of several gut microbiota species (*B. adolescentis*, *B. fragilis*, *C. butyricum*, and *A. muciniphila*). The transwell method could also be improved by adopting the air-liquid interface (ALI) culture method where monolayers are established in transwell inserts until they reach confluency and the media is removed from the apical but not the basolateral chambers (70). The absence of culture media increases epithelial thickness and the number of differentiated cells such as mucus-producing goblet cells, enteroendocrine cells, and enterocytes and hence improves bacterial colonization (33).

Bacteria can also be introduced into the organoid lumen via microinjection. This allows direct contact between the bacteria and the apical surface of the intestinal epithelium. Before microinjection, intestinal organoids are grown into relatively large cystic organoids (>200 μm diameter) (55, 57, 71) and to allow easier access to the lumen, organoids are ideally seeded into BME in a microinjection dish (58) or regular petri dish instead of multi-well plates. To perform microinjections, specific micropipettes and microinjection systems are required. Preparing good micropipettes with an appropriate tip size is a critical step for successful microinjection. Previous studies have mainly used thin-wall glass capillaries pulled using a micropipette puller. The micropipettes can be manually broken to obtain the desired tip size (55–57, 59, 61) or beveled (65) to facilitate the injection process and minimize damage to organoids. Other factors to consider when performing microinjection are the injection volume, time, and pressure to avoid organoid rupture or leaking of the injection content. To date, multiple studies have used this approach to inject different types bacteria including *B. thetaiotaomicron* (55), *Clostridioides difficile* (*C. difficile*) (56, 57), *S. Typhimurium* (58, 59, 61, 65, 66, 72, 73), *E. coli* (60, 62, 64, 68), *L. monocytogenes* (67), and *Lactobacillus spp (L. rhamnosus, L. reuteri, and L. plantarum)* (63) (Table 1). Overall, one of the key advantages of microinjection is that it allows the delivery of microbes into the lumen that closely mimics the physiological environment of the microbes' habitat such as oxygen concentration and contact with the apical surface of the epithelium. It has been shown that the organoid lumen has a low oxygen concentration and can support the growth of both aerobic and obligate anaerobic bacteria for extended periods (74). However, the manual microinjection process is time-consuming and labor-intensive and hence inappropriate for high-throughput applications. To overcome this, Williamson et al. (74) developed a high-throughput semiautomated microinjection system that was able to inject several organoids in a short period (~90 organoids/h). Using this system, individual aerobic (e.g., *E. coli* and *Yersinia pseudotuberculosis*) and anaerobic (e.g., *B. adolescentis*) microbes, and microbial communities derived from stool filtrates, were successfully injected and sustained within the organoid lumen for up to 72 h and 96 h, respectively. This provides a promising solution for microinjection automation; however, further

research is needed to establish fully automated microinjection systems.

To allow easier access to the organoid apical surface without microinjection, researchers have successfully developed 3-D organoids with reversed epithelial polarity referred to as "apical-out" organoids. These organoids can be established by treating established 3-D basal-out organoids with EDTA (19, 20, 52, 53) or cell recovery solution (75) to dissolve the BME. The released organoids are then cultured in suspension in low attachment cell culture plates for 72 h (19, 20). Alternative to using established basal-out organoids, apical-out organoids have also been generated directly by seeding isolated crypts (31) or human ESC-derived hindgut spheroids (54) in suspension culture in the absence of BME. In the suspension culture, epithelial polarity spontaneously reverses so that the apical surface is facing outward in direct contact with the culture media. To create organoid-microbe coculture, organoids are incubated in bacteria-containing media for a specific period (19, 53). This approach has been used to culture several microbes with intestinal organoids including *S. Typhimurium* (19), *L. monocytogenes* (19), *E. coli* (52), *MAP* (31), and *Lactobacillus Casei* (*L. casei*) and *Bifidobacterium longum* (*B. longum*) (19) (Table 1). Although this method is scalable and maintains the 3-D structure of the organoids, it is an end point culture and generally has a short life span (up to 7 days) (54). It is also not an ideal method for creating a coculture with strictly anaerobic microbes. To overcome this, Kakni et al. established hypoxia-tolerant apical-out organoids with organoid-microbe coculture incubated in hypoxic conditions (5% O_2) for 12 h. This study showed that hypoxia did not impact the organoids' barrier integrity and supported the colonization of the gut anaerobes *L. casei* and *B. longum* (54).

As mentioned earlier, each method has its own strengths and limitations and is chosen according to the type of bacteria used and research questions to be addressed. For instance, aerobes and facultative anaerobes can be studied using cell mixture, basic ODMs or apical-out organoids, whereas microinjection and ODMs within oxygen-controlled systems are more appropriate for strict anaerobes. Nevertheless, despite the current limitations, present organoid-bacteria cocultures have been widely used to enhance our understanding of the pathogenesis of enteric pathogens, the protective effects of commensal microbes and the role of microbes in the development of intestinal diseases as highlighted in Table 1. For example, coculture models have been extensively used to characterize the adherence, invasion, and virulence factors for important human enteric pathogens (*S. Typhimurium*/*Typhi, L. monocytogenes*, *E. coli*, and *S. flexneri*). Furthermore, using these systems, it has been demonstrated that gut commensals e.g., *L. reuteri* and *L. acidophilus* protect against pathogen-induced intestinal damage (24, 25, 48). It has also been shown that microbes such as *F. nucleatum* (40) and *pks+ E. coli* (27, 68) potentially contribute to colorectal cancer (CRC) development by causing DNA damage, inflammation, and genetic mutations.

Organoid-Viruses Coculture

In addition to bacteria, the intestine is home to a complex community of viruses, referred to as the gut virome. These viruses interact with the intestinal epithelium and gut bacteria and contribute to intestinal health and disease (76). The intestine can also be exposed to infectious viruses that directly infect and replicate within the intestinal epithelium (77). Intestinal organoids offer a useful tool for studying epithelial-viral interactions and modeling enteric viral infections. To date, several studies have cultured viruses with intestinal organoids, mainly focusing on infectious viruses including rotaviruses (78–82), noroviruses (82, 83), enteroviruses (84), adenoviruses (85, 86), coronavirus 2 (SARS-CoV-2) (87), astroviruses (88), and reovirus (89) (Table 2). Generally, the two main methods for culturing viruses with intestinal organoids are infection of intact or sheared organoids or using 2-D ODMs, whereas the microinjection method has been used in one study only (Fig. 1). Using the disrupted organoid approach, Finkbeiner et al. used human PSC-derived intestinal organoids for modeling rotavirus infections. Mature organoids were cut open using tungsten needles to expose the luminal epithelium, infected with viral inoculum, and then resuspended in Matrigel. Histological and molecular analyses confirmed viral replication in the organoids (78). In another study, differentiated human ASC-derived intestinal enteroids were disrupted by vigorous pipetting to expose the apical surface and then inoculated with rotavirus (79, 80). Rotavirus inoculum has also been added directly to intact organoids and then re-embedded in Matrigel (81). Similarly, Drummond et al. used human enteroids derived from fetal intestinal tissue to model different types of enterovirus infections. In this study, the viral suspension was added directly to the ASC-derived enteroids and incubated for 24 h. Viral RNA was detected within the enteroids that was associated with antiviral and inflammatory responses (84). To model adenovirus infection, human ASC-derived enteroids with/without shearing were mixed with clinical isolates and prototype strains of adenoviruses. Sheared enteroids were incubated with viruses then infected enteroids were reseeded in Matrigel whereas unsheared enteroids were directly reseeded in Matrigel with viral lysates (85). In another study, SARS-CoV-2 was cultured in horseshoe bat and human ASC-derived enteroids and colonoids. Differentiated 3-D cultures were mechanically sheared and incubated with viral clinical samples and infected organoids were reseeded in Matrigel (87). Furthermore, in a study by Zou et al. (82), 3-D differentiated enteroids were released from Matrigel and incubated with rotavirus inoculum to study rotavirus infection. Using an alternative method, the same study used the ODM approach for norovirus culture. Differentiated ODMs were established in transwell inserts or multi-well plates and the viral inoculum was added to the monolayer and incubated for up to 72 h (82). Several other studies have also used the ODM method to model norovirus, adenovirus, astrovirus, and reovirus infections (83, 85, 88, 89) whereas microinjection has been used in one study only to deliver mouse adenovirus into the lumen of mouse enteroids (86).

Using intact organoids allows for viral infection of epithelial cells; however, it does not mimic in vivo infection via the apical surface whereas shearing organoids will expose both the apical and basolateral surfaces to viral particles. Both methods are relatively easy to perform and could be used for isolating and cultivating viruses for clinical diagnosis and modeling viral infections. Alternatively, microinjections provide a more precise representation of apical infection. ODM

Table 2. *Summary of organoid-viruses coculture studies*

Organoid	Virus	Coculture Method	Incubation Time	Key Findings	References
Human PSC-derived intestinal organoids	Human rotavirus	Cell mixture (sheared organoids)	6–24 h	Organoids support replication of laboratory and clinical rotavirus strains (viral proteins, viral RNA and infectious progeny viruses were detected in organoid cells)	Finkbeiner et al. (78)
Human ASC-derived intestinal enteroid	Human rotavirus	Cell mixture (sheared organoids)	1–24 h	Rotaviruses infected differentiated enterocytes and enteroendocrine cells and viral replication varied in enteroids from different patients Infection caused luminal expansion resembling virus-induced diarrhea	Saxena et al. (79)
Human ASC-derived intestinal enteroids	Human rotavirus	Cell mixture (intact organoids)	1–1.5	Viral infection upregulated type III IFN response. Exogenous, but not endogenous, IFN restricted viral replication	Saxena et al. (80)
Murine and human ASC-derived organoids	Human rotavirus	Cell mixture (intact organoids)	1–24 h	Human organoids are more permissive to viral infection (viral RNA and infectious viral particles detected) Antiviral IFN-α or ribavirin treatment suppressed viral replication	Yin et al. (81)
Human ASC-derived intestinal enteroids	Human rotavirus/ norovirus	Cell mixture (intact organoids)/ODM (apical surface)	1–72 h	Rotavirus and norovirus replicate in enteroid epithelium Enteroid differentiation is crucial for the success of viral infection	Zou et al. (82)
Human ASC-derived intestinal enteroid	Human norovirus	ODM (apical surface)	1–96 h	Multiple HuNoV strains can replicate in the enterocytes of ODM Bile is crucial for strain-dependent viral replication Viral neutralization and inactivation can be assessed in ODM	Ettayebi et al. (83)
Human ASC-derived fetal enteroids	Human enterovirus	Cell mixture (intact organoids)	24 h	Enteroids are permissive to enterovirus infection with virus-specific cell tropism Enteroids produce virus-specific antiviral and inflammatory responses postinfection	Drummond et al. (84)
Human ASC-derived enteroids	Human Adenovirus	Cell mixture (sheared organoids)/ODM (apical surface)	45 min to 2 h	Prototype and clinical strains of HAdV can replicate in enteroids HAdVs showed sensitivity to type I and III IFNs in ODMs Different strains preferentially infect different types of epithelial cells	Holly et al. (85)
Murine ASC-derived enteroids	Mouse adenovirus	Cell mixture (sheared and intact organoids)/microinjection	30 min to 24 h 3–8 days	MAdV-2, but not MAdV-1, can replicate in mouse enteroids α-defensins secreted by enteroids enhanced viral infection	Wilson et al. (86)
Bat/human ASC-derived intestinal organoids	SARS-CoV-2	Cell mixture (sheared organoids)	2 h	Bat and human enteroids supported infection and replication of SARS-CoV-2	Zhou et al. (87)
Human ASC-derived intestinal enteroids	Human Astrovirus	ODM (apical surface)	1–24 h	HAstV strains can infect intestinal enteroids HAstV VA1 can infect and replicate in different cell types in enteroids derived from different intestinal regions Enteroids showed enhanced antiviral immune response compared to Caco-2 cells	Kolawole et al. (88)
Murine ASC-derived intestinal enteroids	Reovirus	ODM (apical surface)	1–24 h	Reoviruses (T1L and T3D) can infect enteroids but vary in the level of apoptosis and cell death in infected enteroids	Brown et al. (89)

ASC, adult stem cell; ODM, organoid-derived monolayer; PSC, pluripotent stem cell.

is a more standardized and reproducible method and as such can be used for testing antiviral therapeutics. As shown in Table 2, organoid-virus cocultures have been primarily used as a tool for modeling viral infections to uncover new aspects of viral replication, pathophysiology, and cell-specific tropism in the intestine. These coculture models are particularly useful for cultivating viruses that are difficult to culture in vitro such as noroviruses (83). Furthermore, the cellular diversity of intestinal organoids has allowed the identification of cells that are permissive versus those that are resistant to a specific viral infection (84, 85). They also have been used for testing the efficacy of antiviral agents such as IFN-α or ribavirin treatment against rotavirus infection (81).

Organoid-Immune Cell Coculture

To add further complexity to the intestinal organoid model, immune cells have been added to replicate the in vivo microenvironment and to study epithelial-immune interactions. Typically, 3-D organoids and monolayer culture methods have been used in coculture models with immune cells (Fig. 1), such as peripheral blood mononuclear cells (PBMCs) (90) and specific immune cell types such as T lymphocytes (91–100), neutrophils (101), monocytes (102, 103), macrophages (99, 101), and dendritic cells (104, 105) (Table 3). When coculturing immune cells with organoids, the end measures need to be considered. For example, immune cells can be added to Matrigel along with intact organoids or dissociated organoid cells to measure cellular interactions (90–95, 97–99, 102, 104–107). When embedded in Matrigel, immune cells can move freely within the Matrigel dome to interact with the basal surface of the epithelium (94, 107). Alternatively, immune cells can be added to the media surrounding the Matrigel-embedded organoids to assess the effect of immune cell secretions (100). Microinjection has been discussed previously as a method for transplanting microbes into organoids, however, macrophages have also been successfully transplanted into the organoid lumen to study the gut immune response to lipopolysaccharide (103). Organoids grown as a monolayer culture with immune cells added to the apical compartment of transwell inserts allow for visualization or measurement of macrophage and $CD8^+$ T cell chemotaxis, along with permeability measurements (99). Alternatively, immune cells can be added to the basolateral side of the transwell inserts to measure the effect of the immune-secreted cytokines on monolayer development and functionality (96, 100, 101, 107). To improve the coculture, an inflammatory cytokine cocktail or IL-2 can be added to an immune-organoid coculture to study disease pathologies such as Crohn's disease (CD) (109), or intestinal maturation (96).

Within the intestinal microenvironment, immune cells interact with epithelial cells via direct contact or by secreting soluble factors such as cytokines. Mixing immune cells with intact or sheared organoids facilitates direct contact and interaction with epithelial cells and this is useful for studying immune-mediated epithelial injury. Using ODM in transwell systems is more appropriate to assess the impact of immune cell-secreted factors and immune cell intraepithelial migration behavior in response to tissue injury or infection. Overall, immune-organoid coculture provides a more complex model to replicate the in vivo intestinal microenvironment and presents a promising model that can be used to study immune-epithelial interactions and advance precision medicine. Table 4 demonstrates the key findings from organoid-immune cell coculture-based studies. Generally, the cocultures have been used to recreate the intestinal epithelial niche and to study epithelial-immune interactions, including its impact on epithelial development (91, 96, 100, 104) and pathophysiology of intestinal immune-related disorders such as graft-versus-host disease (93) and inflammatory bowel diseases (97, 106). In addition, studies have used cocultures to assess drug and immunotherapy responses (90). For instance, autologous T lymphocytes can be enriched for tumor-specific cytotoxic lymphocytes in organoid-killing assays. Three-dimensional tumor organoids are stimulated to enhance antigen presentation and then cocultured with activated lymphocytes to assess the sensitivity of the tumor organoids to a T cell-mediated attack (90).

Organoid-Stromal Cell Coculture

Stromal cells (fibroblasts and myofibroblasts) are important elements of the intestinal microenvironment that provide support for intestinal epithelial cell growth and differentiation. To establish organoid-stromal cell coculture, stromal cells can be cultured in close contact with organoids by mixing them with intact or sheared organoids, culturing them as monolayers underneath the epithelial layer or they can be cultured separately using ODMs in transwell systems (Fig. 1) (Table 4).

First, stromal cells can be mixed directly with isolated crypts, organoid fragments, or intact organoids and cultured in Matrigel (110–112). For example, using this method, murine CRC organoids were dissociated and mixed with murine colonic fibroblasts and seeded in Matrigel for 48 h. The coculture systems revealed that colonic fibroblasts regulate signaling pathways in epithelial cells and hyperactivate the PI3K pathway in CRC organoids with *Kras* and *Trp53* mutations (110). Fibroblasts can also be added to the media of organoid cultures embedded in BME (113). In one study, human ASC-derived colonoids were cultured in transwell inserts within a glass bottom plate for 4 days. Dissociated inflammation-associated fibroblasts were then added to the media of the colonoid culture. The coculture resulted in increased organoid swelling, disruption of barrier integrity, DNA damage, and proliferation dysregulation (113).

Alternatively, fibroblast monolayer cultures can be established and then crypts or organoid cells overlaid on the top of the fibroblast layer (114–118). In this method, Matrigel containing the epithelial cells is allowed to polymerize to form a thick layer on the top of the fibroblast monolayer. Sufficient amount of Matrigel depending on the well size is required to support the growth of 3-D organoids (119). This approach has been used to coculture murine/human intestinal subepithelial fibroblasts (ISEMFs) with murine/human intestinal crypts or enteroids to assess their impact on enteroid growth. For example, monolayers were generated by culturing the ISEMFs for 24 h and then crypt or enteroid fragments suspended in Matrigel were added to the ISEMF culture. The results showed that ISEMFs promote enteroid growth and budding formation for an extended period of time (114, 115). In another study, patient-derived normal and short bowel syndrome enteroids were plated over a preconfluent human ISEMFs monolayer culture. When coculturing short bowel syndrome enteroids with ISEMFs, the enteroid surface area significantly increased compared with enteroids cocultured with normal ISEMFs (117). A similar method has been used to create the coculture but without adding Matrigel to organoid suspension. Wallisch et al. (120) developed a protocol of organoid-fibroblast coculture by culturing fibroblasts for 3 days and then organoids suspended in Matrigel-free fibroblast media were seeded on top of the established fibroblast culture.

Another method is to coculture stromal cells with 2-D ODMs in a transwell system where organoids are seeded in the apical chamber whereas confluent fibroblast culture is grown in the bottom well (121–123). Hirokawa et al.

Table 3. *Summary of organoid-immune cell coculture studies*

Organoid	Immune Cell	Coculture Method	Incubation Time	Key Findings	References
Murine ASC-derived enteroids	Murine $CD4^+$ and $CD8^+$ T cells	Cell mixture (intact organoids)	14 days	T cells grew and developed intraepithelial lymphocyte features (membrane projections and movement in organoid epithelium)	Rogoz et al. (94)
Murine ASC-derived intestinal organoids	Murine IELs	Cell mixture (intact organoids)	7 days	IELs were maintained and expanded inside and outside organoids and showed migration and movement capacity across epithelium	Nozaki et al. (95)
Human ASC-derived CRC organoids	Human PBMCs	Cell mixture (sheared organoids)	14 days	PBMCs coculture with tumor organoids upregulated $CD8^+$ T cells and increased IFN-γ secretion with response variable response observed in organoids from different patients	Dijkstra et al. (90)
Human PSC-derived intestinal organoid	Human Jurkat T cells	Intact organoids in transwell (T cells on basal side)	2 days	Stimulated T cells increased organoid size and buddings by producing paracrine factors (IL-2) and upregulating IL-2R Coculture increased the expression of intestinal maturation genes and functionality of organoids	Jung et al. (96)
Murine ASC-derived intestinal organoids	Murine lamina propria leukocytes/ BMDCs	Cell mixture (intact organoids)	4 days	Immune cells from dendritic cell-specific TGF-β receptor 2 knockout mice resulted in morphological changes, depletion of goblet cells and activation of Notch signaling in organoids via E-cadherin-mediated adhesion	Ihara et al. (104)
Human ASC-derived fetal enteroids	Human lamina propria fetal $CD4^+$ Tem cells	Cell mixture (sheared organoids)	10 days	Low fetal intestinal $CD4^+$ T cells supported epithelial growth while high numbers suppressed ISC proliferation via TNF-α production	Schreurs et al. (91)
Human/murine ASC-derived intestinal organoids	Human/murine $CD4^+$ and $CD8^+$ T cells	Cell mixture (sheared organoids)	7 days	Activated T cells induced ISC damage and suppressed organoid formation and survival via IFN-γ production	Takashima et al. (92)
Murine ASC-derived enteroids	Murine BMDCs	Cell mixture (media of intact organoids)	48 h	LPS-activated BMDCs caused rounding and circularity in wild-type and Nfκb1-deficient but not Nfκb2-deficient enteroids by increasing cytokine production Dendritic cells modulate NF-κB2 signaling in epithelial cells	Jones et al. (105)
Human ASC-derived intestinal organoids	Human macrophage/ Neutrophils	ODM (immune cells on the basolateral side)	48 h (monocytes) 4 h (neutrophils)	Coculture system allows the assessment of phenotypic and functional changes in epithelial and immune cells	Staab et al. (101)
Human/ murine ASC-derived intestinal organoids	Human T cells	Cell mixture (intact organoids)	8–48 h	T cells reduced viability and size of murine organoids with mutation in intestinal *Atg16L1* (autophagy gene) by increasing inflammatory cytokines and necroptosis Inhibiting necroptosis or interferon signaling protects against T-cell killing in human organoids from individuals with *ATG16L1* variant	Matsuzawa-Ishimoto et al. (93)
Human PSC-derived organoids	Human monocytes	Cell mixture (intact organoids)	72 h	Monocytes migrated and adhered to organoid basal layer resulting in differentiation to an intermediate phenotype with increased expression of CD14 and CD16 surface markers	Jose et al. (102)
Murine tissue-derived/ human PSC-derived intestine organoids	Murine/human lamina propria ILC1	Cell mixture (sheared/ intact organoids)	4–7 days	Murine ILC1 increased expression of crypt stem cell marker Cd44 and enhanced growth of CD44v6 + crypt buds by increasing TGF-β1 levels and β-catenin accumulation Human ILC1 from patients with IBD increased CD44v6 expression and regulated extracellular matrix remodeling	Jowett et al. (106)

Continued

Table 3.— *Continued*

Organoid	Immune Cell	Coculture Method	Incubation Time	Key Findings	References
Human ASC-derived intestinal organoids	Human MLs	Cell mixture (intact organoids)	48 h	Autologous mucosal T cells infiltrated and induced epithelial cell death in CD organoids by increasing pro-inflammatory cytokines/chemokines Blocking lymphoepithelial interactions via CD103 and NKG2D antibodies inhibited T cell detrimental effects	Hammoudi et al. (97)
Murine ASC-derived intestinal organoids	Murine IELs	Cell mixture (intact organoids)	48 h	IELs coculture with intestinal Atg16L1-deficient organoids protected against Paneth cell death by secreting API5 Recombinant API5 prevented Paneth cell death in CD organoids with *ATG16L1* allele which is genetically susceptible to Paneth cell death	Matsuzawa-Ishimoto et al. (98)
Murine tissue-derived/human PSC-derived intestine organoids	Murine/human ILCP	Cell mixture (intact organoids/ ODM (ILCP on the basolateral side)	7–14 days	Organoid epithelium supports the proliferation and maturation of murine and human ILC with tissue-specific imprint	Jowett et al. (107)
Human PSC-derived intestinal organoids	Human monocytes/macrophages	Microinjection	14 days	Monocytes differentiated within organoids into macrophage-like cells with short-elongated projections, phagocytic vacuoles, and short pseudopodia Macrophages produced soluble cytokines and expressed distinct polarization markers	Tsuruta et al. (103)
Human ASC-derived CRC organoids	Human M2 macrophages/$CD8^+$ T cells	Cell mixture (intact organoids)	4 days	M2 macrophages enhanced survival and numbers of CRC organoids Treatment with C19 (an inhibitor of colony-stimulating factor 1 receptor) decreased organoid numbers, induced organoid fragmentation and enhanced $CD8^+$ T cell chemotaxis	Lv et al. 2024 (99)
Human ASC-derived fetal intestinal organoids	Human lamina propria T cells	Cell mixture (media of intact organoids)/ ODM (T cells on basolateral side)	72 h	T cell promoted maturation of fetal intestinal epithelium by increasing brush border gene expression and TEER measurements via IFN-γ production	Giugliano et al. (100)
Human ASC-derived intestinal organoids	Human macrophages	Intestine-on-a-chip	6 days	Stimulation of coculture with LPS/IFN-γ increased production of pro-inflammatory cytokines from macrophages and epithelial cells	Beaurivage et al. (108)

API5, apoptosis inhibitor 5; ASC, adult stem cell; BMDC, bone marrow-derived dendritic cell; CRC, colorectal cancer; IEL, intraepithelial lymphocyte; ILC1, type-1 innate lymphoid cell; ILCP, innate lymphoid; TEER, transepithelial electrical resistance; IBD, inflammatory bowel disease; cell precursor; ISC, intestinal stem cell; ML, mucosal lymphocyte; ODM, organoid-derived monolayer; PBMC, peripheral blood mononuclear cells; PSC, pluripotent stem cell; TGF-β, transforming growth factor-β.

cocultured subepithelial myofibroblasts isolated from mouse colon and L cells (noncolonic murine fibroblast) with intestinal crypts. Crypts plated in transwell inserts were placed in a multi-well plate containing the support cells (L cells and subepithelial myofibroblasts). Coculturing with subepithelial myofibroblasts enhanced the formation of 3-D colonoids whereas L cells showed an inhibitory effect on colonoid growth (121). In a study by Salari et al., confluent differentiated and undifferentiated ODMs cultured in transwell upper chambers were cocultured with human colonic myofibroblasts in the lower chambers. The coculture resulted in increased transepithelial electrical resistance (TEER) measurement and higher activity of Na^+/H^+ exchange on the apical membrane (122). Using the same method, human ODMs in transwell inserts were established from 3-D organoids derived from healthy individuals or patients with cystic fibrosis. The inserts were then transferred to culture plates containing a confluent myofibroblast culture in the bottom wells and cocultured for 4 days. The coculture increased the expression of TMEM16a, a Ca^{2+}-activated anion channel that modulates the activity of cystic fibrosis transmembrane conductance regulator membrane protein (123). To assess the importance of physical contact in organoid-stromal coculture, Lei et al. (124) used mixed cell culture and transwell method and reported that fibroblasts cultured in a close contact culture had a more significant impact on supporting enteroid growth compared with those in the transwell coculture.

As mentioned earlier, stromal cells can be cocultured with or without spatial separation with organoids. Using the cell mixture approach allows for contact-based interactions and is more appropriate for studies focusing on investigating the role of stromal cells in intestinal tissue development and repair. The contact-less coculture approach is suitable for

Table 4. *Summary of organoid-stromal cell coculture studies*

Organoid	Stromal Cell	Coculture Method	Incubation Time	Key Findings	References
Murine ASC-derived intestinal organoids/CRC organoids	Murine colonicfibroblasts	Cell mixture (sheared organoids	48 h	Colonic fibroblasts regulate acute intercellular signaling and hyperactivate PI3K pathway in colonic organoids with *Kras* and *Trp53* mutations	Qin et al. (110)
Murine ASC-derived intestinal organoid	MEFs/ primary stromal cells	Cell mixture (epithelial crypts)	5 days	*Wnt*-deficient crypts coculture with Wnt3A-expressing MEFs partially restored organoid formation Stromal cells expressing *Wnts* and *Rspo3* supported organoid formation from *Wnt*-deficient crypts without RSPO1 supplementation	Kabiri et al. (111)
Murine ASC-derived intestinal organoid	Fibroblasts	Cell mixture (intact organoids)	4 days	$CD90^+$ fibroblasts express growth factors (Grem1, Wnt2b, and R-spondin3) and Sema3 protein family and support organoid growth	Karpus et al. (112)
Human ASC-derived colonoids	Human normal/IAFs	Cell mixture (intact organoids)	24 h	IAFs caused colonoid expansion and swelling, barrier integrity disruption, DNA damage and proliferation inhibition via prostaglandin E2/prostaglandin receptor EP4 signaling pathway	Dong et al. (113)
Murine ASC-derived enteroids	Murine ISEMFs	Culture layers (bottom layer: ISEMFs)	6 days	ISEMFs increased growth and crypt-like budding by supporting stem cell niche ISEMFs deficient in cell-secretion regulating protein (*epimorphin*) enhanced the budding and surface area of enteroids	Vishy et al. (114)
Human ASC-derived intestinal enteroids	Human/murine ISEMFs	Culture layers (bottom layer: myofibroblasts)	2–56 days	Human infant ISEMFs support long-term enteroid growth by supporting ISC growth and proliferation	Lahar et al. (115)
Murine ASC-derived enteroids	Murine ISEMFs	Culture layers (bottom layer: ISEMFs)	7 days	ISEMF coculture resulted in larger enteroids and enhanced expansion with no changes in morphology	Jabaji et al. (116)
Human ASC-derived enteroids	Human ISEMFs	Culture layers (bottom layer: ISEMFs)	7 days	ISEMFs isolated from patients with short bowel syndrome (SBS) increased SBS enteroid surface due to the inhibition of bone morphogenetic protein signaling	Gazit et al. (117)
Murine ASC-derived intestinal organoid	Murine intestinal myofibroblasts	Culture layers in transwell system (bottom layer: myofibroblasts)	7 days	Myofibroblasts enhanced growth and viability of crypts and survival of organoid culture	Pastuła et al. (118)
Murine ASC-derived intestinal organoids	Murine intestinal fibroblasts	Culture layers (bottom layer: fibroblasts)	4 days	Intestinal fibroblasts supported growth of intestinal organoids without growth factors supplement	Lee et al. (119)
Human CRC-derived organoids	Human fibroblasts	Culture layers (bottom layer: fibroblasts)	24 h	CRC organoids can be cocultured with fibroblasts without adding BME to create a system for drug testing assays	Wallisch et al. (120)
Murine ASC-derived colonoids	Murine ISEMFs/L cells	Culture layers in transwell inserts (bottom layer: fibroblasts)	10 days	Myofibroblast coculture improved colonoid formation efficiency L cells inhibited the colonosphere/colonoid formation capacity	Hirokawa et al. (121)
Human ASC-derived colonoids	Human colonic myofibroblasts	ODM (basolateral side)	4 days	Myofibroblasts cocultures increased transepithelial electrical resistance with claudin-2 downregulation Coculture showed high sodium-hydrogen exchange activity in apical compartment mediated by Na^+/H^+ exchanger 2	Salari et al. (122)
Human ASC-derived colonoids	Human colonic/rectal myofibroblasts	ODM (basolateral side)	4 days	Myofibroblasts coculture increased expression of calcium-activated anion channel (TMEM16a) in ODMs of healthy individuals and cystic fibrosis patients TMEM16a does not function as an apical anion channel in colonic epithelium	Salari et al. (123)
Murine ASC-derived enteroids	Murine ISEMFs	Culture layers (bottom layer: ISEMFs)	7 days	ISEMFs resulted in larger enteroids by producing soluble factors that support ISC growth and differentiation ISEMF coculture is critical for the success of enteroid engraftment and proliferation in *vivo*	Lei et al. (124)
Human ASC-derived colonoids	Human colonic fibroblasts	Intestine-on-a- chip (basal channel)	8 days	Coculture with growth hormone-expressing fibroblasts increased proliferation and DNA damage and decreased p53/p21 levels in epithelial cells	Chesnokova et al. (125)

ASC, adult stem cell; BME, basement membrane extract; CRC, colorectal cancer; IAF, inflammation-associated fibroblast; ISC, intestinal stem cell; ISEMF, intestinal subepithelial fibroblast; MEF, murine embryo fibroblast; ODM, organoid-derived monolayer.

studies focused on understanding the impact of stromal cell secreted factors and paracrine signaling on epithelial cell development and barrier functions. As shown in Table 4, current organoid-stromal cell cocultures have been mainly used to study fibroblasts as a niche component to assess their role in supporting epithelial cell growth, proliferation, and differentiation (111, 114, 115, 118, 124) or the pathophysiology of intestinal diseases such as CRC (110), inflammation (113), short bowel syndrome (117), and cystic fibrosis (123).

Organoid-Neural Cell Coculture

Enteric neurons and glia are also key components of the intestinal microenvironment. Like stromal cells, neural cells can be mixed with organoid/organoid fragments, cultured as monolayers beneath organoid culture, or added to the bottom chamber of transwell systems (Fig. 1) (Table 5). In the first method, neural cells are mixed with intestinal crypts or spheroids/organoids and cultured in BME (126–130). Workman et al. incorporated neural cells into organoids by mixing neural crest cells with mid/hindgut spheroids stage of iPSC-derived organoids. Neuroglial and neural crest cells were detected in the organoid mesenchyme following 28 days of coculture (127). Other methods involve mixing organoids with established neural cultures or using the transwell system (131–133). In one study, neural crest stem cells were established and seeded in a Matrigel-coated plate and then 3-D organoids were released from Matrigel and added to the wells containing the neural crest stem cells culture (131). In the same study, neural crest stem cells were also cocultured without direct contact with organoid cells that were cultured in the lower chamber whereas neural crest stem cells were cultured in the upper chamber of a transwell system. The study showed that coculture with physical contact had a more profound impact on neuronal cell maturation and colon enterochromaffin cell differentiation compared with transwell-based coculture (131). Using a similar method, human-induced neural stem cells were added to the lower chamber of transwell inserts with ODM cultured in the upper chamber (133). Alternatively, Levin et al. cultured intestinal crypts suspended in Matrigel on the top of one segment of longitudinal muscle myenteric plexus isolated from a mouse small intestine. In this coculture, longitudinal muscle myenteric plexus with peristalsis was maintained for up to 13 days in the presence of enteroids. This model also supported the proliferation of the key longitudinal muscle myenteric plexus cells (neurons, glia, fibroblasts, and smooth muscles) in addition to enteroid epithelial cells (134).

Like immune and stromal cells, neural cells can be cultured with/without physical contact with organoids depending on the type of study being conducted. The basic cell mixture-based coculture allows for creating neural-epithelial structures to study contact-dependent cellular interactions. Conversely, multi-layer and transwell cocultures are more suitable for studying interactions mediated by released soluble factors such as neurotransmitters and neuropeptides. Compared with other intestinal organoid-cocultures, fewer studies have been conducted using organoid-neural cell cocultures and these have mainly focused on developing coculture models (127, 130, 133, 134) with few investigating the impact of bidirectional interaction between neural and epithelial cells on cell growth, differentiation, and maturation (Table 5). For example, it has been shown that neural cells are critical for the differentiation of specific epithelial cells including enterochromaffin cells (131).

Multicomponent Coculture

To further enhance the complexity of the organoid models, researchers have attempted to coculture organoids with more than one component e.g., organoids-microbe-immune cells coculture. To date, several studies have developed tricultures such as cultures that include organoids-*E. coli*-polymorphonuclear neutrophils (PMNs) (136), organoid-*E. coli*-macrophages (137), organoids-*L. reuteri D8/L. acidophilus*-Lamina propria lymphocytes (138), organoids-*F. nucleatum*-tumor infiltrating lymphocytes (139), and organoid-*S. flexneri*-PMNs (140) (Table 6). For instance, Karve et al. used the microinjection technique to deliver *E. coli* into the lumen of the organoids. Four days later, PMNs were added directly to the media of the injected organoids and incubated for 22.5 h. The results showed that PMNs can migrate through the organoid tissues and colocalize with bacteria within the lumen of infected organoids (136). In another study, an organoid-lamina propria lymphocyte coculture was created by mixing lamina propria lymphocytes and organoids in Matrigel. *L. reuteri D8/L. acidophilus* was then added to the coculture media (138). A similar approach was used by Gao et al. (139) where organoid fragments were mixed with *F. nucleatum* and tumor infiltrating lymphocytes in Matrigel and incubated for 48 h. Alternative to 3-D organoids, ODMs cultured in the apical chamber of transwell inserts have been used to create multicomponent cocultures. In this approach, transwell inserts are inverted and the immune cells seeded and allowed to attach to the bottom side of the inserts. The inserts are then turned back to their original position and bacterial inoculum is added to the apical compartment and cultured for up to 24 h. This method has been used to create organoid-*E. coli*-macrophages (137) and organoid-*S. flexneri*-PMNs (140) cocultures.

Furthermore, organoid-fibroblast-macrophage cocultures have been established. In one study, dissociated murine CRC organoids were mixed with murine colonic fibroblasts and murine primary macrophages and cultured in Matrigel for 48 h. This allowed the study of epithelial oncogenic mutations in the presence and absence of stromal and immune cells (110). In addition, to create a tri-culture, intestinal organoids, vagal neural crest cells, and macrophages were mixed and resuspended in Matrigel. This study showed that adding vagal neural crest cells did not affect the establishment of macrophages in the developing intestine (141). In another study, an organoid-myofibroblasts-neurons coculture was established. Murine ODMs were established in the apical chamber whereas murine myofibroblasts and enteric neurons and glia were cultured in the lower chamber of the system. Using this system, the authors showed that subepithelial cells modulate the differentiation of ISCs, the release of cytokines, and barrier integrity (142).

Overall, creating multicomponent cocultures allows for a more accurate representation of the in vivo structure and cellular interactions within the intestine. As indicated in Table 6, these models have been used to characterize multicellular interactions during intestinal development and regeneration (138, 141, 142), epithelial and immune responses

Table 5. *Summary of organoid-neural cell coculture studies*

Organoid	Neural Cell	Coculture Method	Incubation Time	Key Findings	References
Murine ASC-derived intestinal organoids	Murine primary neurons	Cell mixture (crypts)	7 days	Neuronal cell coculture prevented loss of and sustained survival of DCLK1^{+} tuft cells via cholinergic signaling Adding neurons increased the number and size of organoids in the presence of DCLK1^{+} tuft cells	Westphalen et al. (126)
Human PSC-derived intestinal organoids	Human PSC-derived NCCs	Cell mixture (mid/hindgut spheroids)	28 days	NCCs migrated and differentiated into neurons and glia with neuronal activity in organoid mesenchyme Organoids in coculture developed functional neuroglial structures with Cajal interstitial cells	Workman et al. (127)
Murine ASC-derived intestinal organoids	Murine primary enteric neurons	Cell mixture (crypts)	3–5 days	Enteric neurons increased fucosylation levels and Fucosyltransferase 2 expression in organoids via VIP- VIPR1 signaling	Lei et al. (128)
Murine ASC-derived intestinal organoids expressing human α-synuclein	Murine Nodose ganglia neurons (lacking α-synuclein)	Cell mixture (intact organoids)	5–8 days	Organoids attracted and connected with nerve fibers which grew in close contact with EECs basal surface α-synuclein protein transferred from EEC onto nerve fibers processes around basal surface of organoid	Chandra et al. (129)
Human PSC-derived intestinal organoids	Human PSC-derived vNCCs	Cell mixture (hindgut spheroids)	28 days	Coculture with vNCCs resulted in innervated intestinal organoids allowing for studying enteric neuropathies	Loffet et al. (130)
Human PSC-derived intestinal organoids	Human ESC-derived NCSC	Culture layers (bottom layer: NCSCs)/transwell system (NCSCs in upper chamber)	7 days	NCSCs coculture enhanced morphological maturation of epithelial cells and differentiation of enterochromaffin cells by producing substance P	Che et al. (131)
Human ASC-derived colonoids	Human neuronal cells (LUHMES)	Cell mixture (intact organoids)	2–4 days	Coculture with COMT-knockdown neurons (lacking an enzyme that degrades catecholamines) increased expression of TNF-α and NF-kB p65 (proinflammatory signals) and decreased IκB-α (anti-inflammatory signal) compared to coculture with naïve neuronal cells	Zhou et al. (132)
Human ASC-derived intestinal organoids	hiNSCs	ODM (basolateral side)	–	Coculture enables studying neuro-epithelial interactions in response to xenobiotic exposure	Wang et al. (133)
Murine ASC-derived enteroids	Murine enteric neural cells from LMMP	Culture layers (crypts on top of LMMP culture)	6 days	Coculture supported proliferation and differentiation of mesenchymal (neurons, glia, fibroblasts and smooth muscle cells) and enteroid epithelial cells	Levin et al. (134)
Human ASC-derived colonoids	Murine myenteric neurons	Intestine-on-a- chip	6 days	Neurons developed extended projections and created connections with epithelial cells	De Hoyos et al. (135)

ASC, adult stem cell; EEC, enteroendocrine cell; ESC, embryonic stem cell; hiNSC, human-induced neural stem cell; LMMP, ongitudinal muscle myenteric plexus; NCC, neural crest cell; NCSC, neural crest stem cell; PSC, pluripotent stem cell; VIP, vasoactive intestinal polypeptide.

to enteric pathogens (136, 137, 140), and cellular signaling involved in the development and treatment response in CRC (110, 139). For instance, in one study, organoid-microbe-immune cells coculture revealed that the enteric pathogen *E. coli* induced neutrophil intraepithelial chemotaxis by increasing IL-8 expression (136). Similarly, in another study, *E. coli* infection enhanced macrophage epithelial adherence and phagocytic activity and hence reduced the number of viable bacteria in the infected organoids (137).

Microfluidic Intestine-On-Chip: an Emerging Approach for Developing Organoid Coculture

Microfluidic intestine-on-chips are cell culture systems used to recreate the intestinal structure, cellular complexity, and luminal flow. They allow for easier access to both apical and basolateral surfaces of the intestinal epithelium and precise control of chemical, biological, and mechanical parameters and hence have wider applications for studying intestinal physiology and pathology (144). Intestine chips are usually made of polydimethylsiloxane silicone polymer and consist of multiple channels. In these systems, cells are usually seeded on a BME-coated porous membrane that separates the chip's channels into apical and basolateral sides. These devices can use traditional intestinal epithelial cell lines such as Caco-2. However, recently there has been a shift toward using intestinal organoids given the cellular diversity of this model that offers a better representation of the in vivo transcriptome and functionality of the human intestine compared with Caco-2 cells (145). The intestine-on-chips method has been used to create two-way and three-way coculture systems in multiple studies.

The basic chip usually consists of two channels representing intestinal lumen and blood capillaries and as such they have been used to coculture endothelial cells with intestinal

Table 6. *Summary of multi-component coculture studies*

Organoids	Component 1	Component 2	Coculture Method	Incubation Time	Key Findings	References
Murine ASC-derived intestinal organoids/CRC organoids	Murine colonic fibroblasts	Murine Macrophages	Cell mixture (sheared organoids)	48 h	Fibroblasts hyperactivated PI3K pathway in colonic organoids with *Kras* and *Trp53* mutations and upregulated macrophage signaling pathways (NF-κB, MAPK, and PI3K) Epithelial cells upregulated MAPK and P38 signaling in fibroblasts	Qin et al. (110)
Human PSC-derived organoids	Commensal/pathogenic *E. coli*	Human PMNs	Microinjection of *E. coli*/ adding PMNs to organoids media	23 h	PMNs did not affect bacterial growth/recovery Pathogenic bacteria upregulated IL-8 inducing neutrophil chemotaxis and translocation of PMNs into organoids	Karve et al. (136)
Human ASC-derived enteroids	*E. coli*	Human macrophages	ODM in transwell (bacteria on apical side and macrophages on basolateral side)	24 h	Macrophages induced morphological changes, modulated cytokine release and increased epithelial thickness and barrier integrity of ODM *E. coli* increased macrophage adherence, intraepithelial projections and phagocytic capacity Macrophages decreased number of viable bacteria and partially prevented barrier integrity disruption without causing an inflammatory response	Noel et al. (137)
Murine ASC-derived intestinal organoids	*L. reuteri D8* *L. acidophilus*	Murine LPLs	Cell mixture (LPLs mixed with intact organoids in Matrigel & bacteria added to media)	24 h	LPLs increased surface area, number of proliferative cells and expression of Paneth and stem cell markers in the organoids *L. reuteri D8* increased organoid surface area and budding, alleviated TNF-α- induced damage and increased expression of Paneth and stem cell markers and IL-22 secretion in the presence of LPLs	Hou et al. (138)
Human ASC-derived CRC organoids	*F. nucleatum*	Human TILs	Cell mixture (sheared organoids mixed with bacteria and TILs)	7 days	*F. nucleatum* enhanced response to PD-L1 blockades by reducing tumor cell proliferation and increasing apoptosis, increasing $CD8^+$ and IFN-γ^+ $CD8^+$ TILs, activating STING signaling and upregulating PD-L1 expression in CRC organoids	Gao et al. (139)
Human ASC-derived enteroids	*S. flexneri*	Human PMNs	ODM (bacteria on apical side and PMNs on basolateral side)	2 h	*S. flexneri* induced migration of PMNs toward ODM apical side and enhanced bacterial phagocytosis and killing and modulated cytokine release	Lemme-Dumit et al. (140)

Continued

Table 6.— *Continued*

Organoids	Component 1	Component 2	Coculture Method	Incubation Time	Key Findings	References
Human PSC-derived intestinal organoids	Human PSC-derived VNCCs	Human PSC-derived macrophages	Cell mixture (VNCCs, macrophages and organoids mixed in Matrigel)	7–14 days	Macrophages migrated and proliferated within organoids and modulated growth and metabolism in the developing intestine Enteric neurons had no impact on number of macrophages in organoids	Song et al. (141)
Murine ASC-derived organoids	Murine Myofibroblasts	Murine enteric neural cells	ODM (neural cells and myofibroblasts on basal side)	3 days	Enteric neurons modulated ISC fate and promoted differentiation into enteroendocrine cells Myofibroblasts and enteric neurons increased expression of actin and ZO-1, reduced monolayer permeability and enhanced release of certain cytokines in coculture	Puzan et al. (142)
Human ASC-derived intestinal organoids	Human intestinal endothelial cells	Human PBMCs	Intestine-on-a-chip	3 days	PBMCs migrated to epithelial channel in response to nutritional deficiency-induced inflammation	Kim et al. (143)

L. reuteri D8, *Lactobacillus reuteri D8*; *L. acidophilus*, *Lactobacillus acidophilus*; *E. coli*, *Escherichia coli*; *S. flexneri*, *Shigella flexneri*; *F. nucleatum*, *Fusobacterium nucleatum*. ASC, adult stem cell; CRC, colorectal cancer; ISC, intestinal stem cell; ODM, organoid-derived monolayer; PBMC, peripheral blood mononuclear cell; PMN, polymorphonuclear neutrophil; PSC, pluripotent stem cell; LPLs, lamina propria lymphocytes; TILs, tumour infiltrating lymphocytes; VNCC, vagal neural crest cells; ZO-1, zonula occludens-1.

organoids to study epithelial-endothelial cross talk and to assess drug pharmacokinetics (145–148). For example, Yin et al. (147) used a two-channel intestine-on-chip to coculture jejunal enteroids with human umbilical vein endothelial cells. The endothelial cells were cultured in the lower channel and allowed to attach to the basal side of the chip membrane. Dissociated enteroid cells were seeded on the apical side of the membrane in the upper channel. Cells were subjected to media flow with/without cyclic mechanical stretch. Confluent and polarized epithelial and endothelial monolayers were developed on both sides of the membrane. Epithelial monolayers showed progressive differentiation over time mimicking normal intestine structure and functions in vivo (147).

In the context of host-microbe interaction, some studies have used intestine-on-chips to coculture aerobic and anaerobic bacteria with intestinal epithelium. In addition to using Caco-2 cell-based chips to create a coculture with *B. fragilis* (149), microbes from fecal suspension (150), strains of gut commensals (VSL#3 probiotics), and the enteric pathogen *E. coli* (151), studies are using organoid-based chips for microbial modeling (69, 149). For example, to study the role of microbes in the pathophysiology of neonatal necrotizing enterocolitis, an intestine-on-chip device was used to coculture epithelial cells with endothelial cells and gut microbiota. Three-dimensional human neonatal enteroids from healthy and neonatal necrotizing enterocolitis subjects were seeded in the BME-coated membrane in the top channel of the chip. Cells were supplied with media and exposed to cyclic peristaltic-like mechanical strain for 6 days. The intestinal endothelial cells were then seeded in the bottom channel and supplied with the appropriate media. Intestinal bacteria enriched from neonatal necrotizing enterocolitis fecal samples were then introduced to the top channel and cultured for up to 27 h (69). In addition, researchers have developed a transwell-based microfluidic platform "GuMI physiome" that recreates the apical and basolateral sides of transwell inserts with controlled flow rates and oxygen concentrations on each side. In this system, epithelial cells were seeded in six culture inserts integrated into the platform. To maintain the coculture, microbes were introduced via an injection port, anoxic media was pumped into the apical unit, and oxygenated media was pumped into the basolateral unit. This platform was used for the coculture of oxygen-sensitive *Faecalibacterium prausnitzii* (*F. prausnitzii*), *Eubacterim rectale* (*E. rectale*), and *B. thetaiotaomicron* for up to 96 h. Increased bacterial density after 48 h of coculture showed that the platform supports the growth of these microbes (152).

Intestine-on-chip systems can also be used to coculture organoids with stromal, neural, and immune cells (108, 125, 135, 143, 153, 154). Using an intestine-on-chip composed of top, bottom, and two lateral channels, colonic fibroblasts were cultured in the bottom channel whereas colonoid cells were seeded on the top channel for 8 days with mechanical stretch applied on days 3 and 4 of coculture. Coculture with growth hormone-expressing fibroblasts led to increased epithelial cell proliferation and DNA damage accumulation in epithelial cells (125). Furthermore, using a microfluidic device, de Hoyos-Vega et al. cocultured intestinal epithelial cells with enteric neurons. The device is composed of two adjacent channels connected with microgrooves. Epithelial cells from human intestinal organoids were seeded in one compartment whereas murine myenteric neurons were cultured in the other compartment 24 h after epithelial seeding. On *days 4–6* of coculture, neuronal cells developed neural

projections through the microgroove making connections with epithelial cells (135). Furthermore, epithelial-immune cell (108) and epithelial-endothelial-immune cell cocultures have been created (143). Kim et al. used two-channel chips and cultured human intestinal enteroid cells in the upper channel and endothelial cells in the lower channel. Isolated PBMCs were introduced via the basal channel and allowed to adhere to the endothelial layer. PBMCs were able to migrate to the epithelial channel in response to nutritional deficiency-induced inflammation (143). In addition, Nikolaev et al. developed a platform to coculture intestinal organoids with various cell types. This system supported the complete life cycle and growth of the intestinal parasite *Cryptosporidium parvum* and facilitated the coculture of epithelial cells with nonepithelial cell types such as endothelial cells, macrophages, and myofibroblasts (153).

CONCLUSIONS AND FUTURE DIRECTIONS

This review highlights recent advancements in the application of intestinal organoid cocultures to address the many questions related to intestinal physiology and disease. Despite significant improvements over the past decade, current organoid coculture approaches have several limitations in representing the complex and multifaceted interactions that occur in vivo. In terms of organoid-microbe coculture, access to the lumen and creating the optimal conditions for the growth of aerobic and anaerobic bacteria remain key challenges. Furthermore, most of the research has been focused on modeling individual facultative anaerobes associated with enteric infection with a limited number of studies focused on the complex gut microbial community. The gut microbial community consists of obligate anaerobes that survive in the presence of minimal oxygen levels as the apical surface is exposed to less than 1% oxygen in vivo (155). Developing high-throughput automated microinjection technologies or creating bioengineering-based approaches to control oxygen exposure to both the apical and basolateral epithelium will enable the coculture of gut microbiota communities with organoids.

Other nonepithelial cells have been cocultured in two-way or three-way cultures, however, complex multicellular cocultures are still limited. To achieve this, each cell type needs to be provided with optimal growth conditions and cultured in arranged structures that resemble the intestinal architecture in vivo. Finding a balance of media that contains appropriate growth factors/nutrients for each of the cultured components is essential. For example, Levin et al. used a media that supported stromal, neural, and epithelial cells in coculture (134). Other cell-specific considerations also need to be considered, such as the effect of cytokine supplements required for culturing immune cells on the organoids. In the case of immature PSC-derived organoids, coculture with T lymphocytes can induce in vitro maturation via IL-2 (96), highlighting the need to characterize the effect of mixing different media on each of the cell types.

Microfluidic intestine-on-chip offers a promising solution for improving intestinal organoid coculture systems to address the current limitations. The key advantage of these devices is that they can be controlled to recapitulate the intestinal microenvironment. This includes culturing different cells in different channels, supplying each cell type with appropriate media, exposing cells to regular luminal flow and peristaltic-like mechanical stretch, and therefore closely recreating intestinal structure and physiology. Luminal flow allows the continuous removal of debris and dead cells from the intestinal lumen that improves the lifespan of the culture and enhances cellular differentiation and the efficacy of microbial colonization. Furthermore, mechanical stretch replicates the shear stress that promotes the polarization, differentiation, and functionality of intestinal epithelium (153). Currently, this technology is still in its infancy, however, in the future, it could enhance scalability and allow for better modeling of the interactions between different components of the intestinal microenvironment under various conditions.

Finally, it is also worth mentioning that in addition to the coculture approaches discussed in this review, other methods such as ALI organoid cultures, fine-needle aspiration organoids, and tissue-engineered organoids can also be used to create complex and multicellular organoid systems comprising both epithelial and nonepithelial cells. As briefly mentioned earlier, ALI organoid cultures involve culturing the basal surface of the organoids submerged in culture media while the apical surface is exposed to air resulting in a more differentiated and mature epithelial layer with enhanced functionality (156). Furthermore, it has been shown that culturing ASC-derived organoids using the ALI technique allows for culturing epithelial cells along with mesenchymal and immune cells without the need to add them exogenously (157, 158). For instance, Santos et al. used ALI culture to generate patient-derived organoid models of celiac disease. These ALI organoid cultures, which incorporated diverse types of immune cells and stromal cells alongside the epithelium, provided a valuable platform for studying the autoimmune response to gliadin treatment (157). Similarly, Neal et al. established ALI patient-derived organoid cultures from mouse tumors or human biopsies and demonstrated that these cultures preserved the tumor epithelium, tumor-associated fibroblasts, and tumor-infiltrating immune cells that facilitated modeling of the tumor immune microenvironment (158). Fine-needle aspiration organoids are established from biopsy samples collected via the minimally invasive fine-needle aspiration technique. This method can be used for culturing tumor-derived organoids that closely resemble the histology of native tumors and allow the co-isolation of tumor-infiltrating immune cells that can be cocultured with organoids (159). In addition, tissue-engineered organoids involve the use of tools to enhance the complexity and structure of the current 3-D organoid models. This includes using hydrogels, scaffolds, and 3-D bioprinting and microfluidic devices to closely mimic the in vivo cell-cell and cell-extracellular matrix interactions in the organoid culture. This approach facilitates the construction of complex intestinal organoid structures that can be used in personalized and regenerative medicine in a scalable manner (160).

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