ABSTRACT

The epithelial barrier in the gastrointestinal (GI) tract is a protective interface that endures constant exposure to the external environment while maintaining its close contact with the local immune system. Growing evidence has suggested that the intercellular crosstalk in the GI tract contributes to maintaining the homeostasis in coordination with the intestinal microbiome as well as the tissue-specific local immune elements. Thus, it is critical to map the complex crosstalks in the intestinal epithelial-microbiome-immune (EMI) axis to identify a pathological trigger in the development of intestinal inflammation, including inflammatory bowel disease. However, deciphering a specific contributor to the onset of pathophysiological cascades has been considerably hindered by the challenges in current in vivo and in vitro models. Here, we introduce various microphysiological engineering models of human immune responses in the EMI axis under the healthy conditions and gut inflammation. As a prospective model, we highlight how the human “gut inflammation-on-a-chip” can reconstitute the pathophysiological immune responses and contribute to understanding the independent role of inflammatory factors in the EMI axis on the initiation of immune responses under barrier dysfunction. We envision that the microengineered immune models can be useful to build a customizable patient’s chip for the advance in precision medicine.

Keywords: Immune response; Microphysiological system; Gut inflammation-on-a-chip; Microbiome; Co-culture; Organoid
Studying the human gut microbiome using in vitro models has been challenging because many commensal gut microbiome is oxygen-sensitive (8) and unculturable (9). The conventional static culture formats often cause the overgrowth of gut bacteria when co-cultured with epithelium, thus hampering the in vitro demonstration of host-microbiome crosstalk (10). Animal models harbor a vastly different composition of the microbiome population compared to that of humans, which compromises the translational value of experimental results (11). Moreover, although animal models have been used to induce disease-like symptoms and test for the validation of therapeutics, there have been notable limitations in the independent manipulation of the individual contributing factors (±immune element, ±gut microbiome), spatiotemporal modulation of the inflammatory triggers (e.g., before/after Ag presentation, directional introduction of the particular immune trigger), or in situ visualization at high resolution in real-time (e.g., time-lapse imaging of the fluorescently labeled cells under the controlled high-power magnification imaging). Thus, developing a translatable in vitro platform utilizing the gut-on-a-chip technology that allows the mechanistic investigation of the EMI axis is a promising technology in disease modeling (Fig. 1A and B). In this review, we aim to review the physiological role of the individual components in the EMI axis, unique techniques that have been developed to overcome the difficulty of mimicking such axis, and the future direction with the in vitro study of the EMI.

**Figure 1.** Microfabrication of a human gut-on-a-chip and microfluidic cultures of the EMI axis. (A) The gut-on-a-chip microfluidic device is fabricated using soft lithography method by placing each compartment in a layer-by-layer approach to build the central cell culture chamber with an upper (blue) and a lower (orange) microchannel (1 mm×1 cm×200 μm, width×length×height) and bi-lateral vacuum chambers (grey). (B) A schematic flowchart shows sequential steps to recreate intestinal EMI in a gut-on-a-chip by coating the central cell microchannels using ECM (“coating ECM”), followed by the attachment of epithelium (“seeding epithelium”), application of flow and mechanical stretching motions (“flow, stretching”), and maintenance of the steady-state physiological milieu including gut microbiome and immune cells (“EMI axis”). An inset photograph (top middle) shows a fully equipped gut-on-a-chip device linked to the silicone tubing that supplies culture medium (purple or yellow-green arrow heads) or cyclic vacuum suctions that induce peristalsis-like motions (green arrow heads). Schematics of the representative experimental steps (middle row) and corresponding micrographs of either the device or the cell morphology (bottom row). A zoomed-in snapshot in the left bottom shows the part (a light grey dashed box) of a PDMS porous membrane (25 μm in thickness). A phase contrast image in “seeding epithelium” shows the formation of an intestinal epithelial monolayer in the upper microchannel. An image in “flow, stretching” displays the villus growth under mechanically dynamic physiological conditions at 100 h since seeding. Finally, an overlaid image in the “EMI axis” shows the villus morphology (grey) and green fluorescent protein-labeled E. coli (green) after the co-culture for 24 h. Bars, 100 μm. Images were reprocessed from the references (65,66).

**Abbreviations**

3D, three-dimensional; AOI, anoxic-oxic interface; DC, dendritic cell; EMI, epithelial-microbiome-immune; GI, gastrointestinal; HuMiX, human-microbial crosstalk; MPS, microphysiological system; PDMS, polydimethylsiloxane; SCFA, short-chain fatty acid

**Author Contributions**

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EMULATING THE IMMUNE RESPONSE DURING THE INTESTINAL INTERCELLULAR CROSSTALK

The EMI axis
There has been substantial progress in developing a microfluidic system modeling the physiology and pathology of the GI tract (12-16). The human intestine is the most representative organ for the processes by which the tissue-specific immune system constitutively exerts homeostatic intercellular crosstalk with the host cells as well as the gut microbiome (17). Due to the spatial proximity (18) and biological interactions at the mucosal microenvironment (19), the major contributors involved in the multi-component interactions, including intestinal epithelium, gut microbiome, and the tissue-specific immune elements can be referred to as new terminology, “EMI axis.” These contributing cells should be involved in modeling the pathophysiology of inflammatory immune responses in a defined spatial structure. The prerequisites of each element to build inflammation models are summarized.

Epithelium
The intestinal epithelium is a primary cell type to form a physical tissue barrier that provides an interface between the lumen and the abluminal compartments. In this microenvironment, the EMI axis plays a key role in creating complex intercellular interactions, stimulations, and regulations (19). There are various intestinal epithelial cells, such as absorptive enterocyte, mucus-producing goblet cells, hormone-secreting enteroendocrine cell, anti-microbial peptide-releasing Paneth cells, Ag-permeable microfold (M) cells, and taste-sensing tuft cells (20-22). In general, absorptive and goblet cells are major contributors to recreate the epithelial barrier function by providing tight junction integrity and mucus layer, respectively. Hence, major cell lines such as Caco-2 (human colorectal adenocarcinoma, representing the absorptive enterocytes) (23) and HT-29 (human colorectal adenocarcinoma, representing the goblet cells) (24) have been predominantly used in biomedical researches or pharmaceutical tests. Anticipated physiological functions of intestinal epithelium include the expression of tight junction (e.g., zonula occludens 1, occludin, and claudin) (25) and adherence junction (e.g., E-cadherin) (26) and mucin (e.g., mucin 2) (27). However, immortalized cell lines are often derived from tumor cells (28) or been immortalized with a tumor Ag (29) with limited capability to recapitulate a coordinated function of multi-lineage cells (30). In recent days, biopsy-derived (31,32) or stem cell-derived (33) intestinal organoid culture method has been suggested to present diverse cell lineages compared to the immortalized single-lineage cell lines. The organoid cultures, however, have shown critical limitations in co-culturing living microbial cells due to the static culture nature and the enclosed lumen (34).

Immune components
Immune surveillance is critical to promote host defense as well as homeostasis (35-37), where tissue-specific immune elements control the primary immune responses in concert with systemic immunity. In the intestinal lamina propria, professional Ag-presenting cells (38,39) including dendritic cells (DCs), macrophages, and B lymphocytes as well as other local immune cells such as intraepithelial lymphocytes (40), innate lymphoid cells (41), and Th or Treg (42) closely communicate and interact to contribute to the intestinal homeostasis. In this unique spatial coordination, DCs (43) and macrophages (44) in the lamina propria collect exogenous Ags (e.g., infectious bacteria) (45) and share the epitopes of molecular components with B lymphocytes (46). In this event, the B cells that are homing from the mesenteric lymph node to the lamina propria transform into the IgA-producing plasma cells and produce secretory antimicrobial IgA (47,48). As given this example, immune cells
continuously interact with endogenous or exogenous Ags as well as with other adjacent cells, then contribute to induce inflammatory responses. Thus, involving the right type of tissue-specific immune cells in intestinal inflammation models is an essential design step to demonstrate immune-mediated interactions. Recently, incorporation of immune system components into these microphysiological systems (MPS) was accomplished, and a major advance in such technological breakthrough includes the spatiotemporal induction of immune cells as well as living microbial cells in the model. Currently, most of in vitro models that demonstrate human immune reactions have relied on the PBMCs derived from a drawn blood sample (13). Alternatively, the differentiated immune cells (e.g., DCs from monocytes) (14) can be enriched in vitro and introduced into the MPS model to induce physiological immune tolerance in the gut (14,15). However, compared to animal models, it has been still nascent to recreate a tissue-specific immunity in an in vitro model, which remains a critical unmet in disease modeling.

**Gut microbiome**

The gut microbiome can contribute to maintaining, and perturbing, the EMI axis by producing short-chain fatty acids (SCFAs), including acetate, propionate, and butyrate (49), and by releasing bacterial endotoxins (e.g., LPS) (50) or secreting polysaccharide A (51). SCFAs are the primary bacterial metabolites that are used as a major energy source for intestinal epithelium (52). The human commensal gut microbiome plays a pivotal role in degrading non-digestible fibers (i.e., prebiotics), including inulin (53), fructo-oligosaccharide (54), and galacto-oligosaccharides (55), and producing the SCFAs via symbiotic cross-feeding interactions in the colon (56). In addition, the intestinal microbiome can shape host immunity, where another major role of SCFAs is the orchestration of the balance of Th vs. Treg cells (49). Thus, stable maintenance of this syntrophic intercellular interaction of the commensal gut microbiome is important to sustain the physiological intestinal homeostasis in the EMI axis in a model (57). It is noted that the majority of intestinal bacteria are obligate anaerobic bacteria (58), and these anaerobic gut bacteria support intestinal homeostasis in the EMI in a mucosal anoxic-oxic interface (AOI) (59). The recent development of an AOI in a microfluidic gut-on-a-chip (60) enabled us to investigate the key physiological interactions of obligate anaerobic gut bacteria with the intestinal epithelium. We will further discuss how these technical challenges have been overcome in the in vitro recreation of AOI in the following section.

**Prerequisites for modeling the EMI axis**

**Reverse engineering**

Based on the reverse engineering approach (61), experimental demonstration of the EMI axis in vitro requires the “breakup” of individual components and their functionality from the complex microenvironment of the gut into the level of single components. Next, each component needs to be integrated independently, or collectively, to reform the sequence of interactions that induce intercellular crosstalk with a spatiotemporal resolution. As a reductionist’s approach, simplified but physiologically relevant inter-connectivity between different cell types in the EMI axis can be established by adding the uncoupled elements (i.e., microbiome, epithelium, and immune cells) one-at-a-time or by removing a particular cell type in a defined culture format. More importantly, once a system is accessible to reconstitute a functional simulacrum in vitro, different types of cells from various sources (e.g., different patient donors, a defined pathobiont in the GI diseases, a distinct consortium of microbial communities, or differentiated immune cells after the clonal expansion) can be adapted to better mimic individual target tissues and organs.
Recreating the tissue interface

Since these multiple cell types within the EMI axis are localized in the three-dimensional (3D) microenvironmental structure, recreating the lumen-capillary or lumen-lymphatic tissue interface is of great importance when working to demonstrate a polarized tissue organization and directional stimulation of microbial (i.e., luminal) or immune cells (i.e., capillary or lymphatic) in the model. To recreate an organ-level, tissue-specific microenvironment of the human living gut, the establishment of an intact intestinal epithelial barrier is a prerequisite to building the lumen-capillary tissue interface (25,26). The intestinal epithelial layer needs to possess similar compositions of differentiated lineages, including absorptive, mucus-secretory, enteroendocrine, and paneth cells (27,62-64). As previously mentioned, mucus production, expression of tight junction proteins, and production of antimicrobial peptides are major functional prerequisites in an epithelial layer. Also, the histogenesis of the 3D villus microarchitecture that displays the crypt-villus characteristics is also important for illustrating the effect of 3D topology on microbial niche formation, stem cell regeneration, and immune-microbiome interactions. Another key requirement in the intestinal modeling is the demonstration of physical deformations that mimics the biomechanics of bowel movement, so-called peristalsis (12,65). The macroscopic mechanical deformations and the microscopic villus motility are necessary, not only for showing the mixing and propulsion of bolus (66) but also for reflecting the villous morphogenesis (12), regenerative cellular signaling (67), and epithelial differentiation (68).

Microbial co-culture

Typically, microbial co-culture can be performed by adding an inoculum of microbial cells on the apical compartment of the epithelial monolayer that has reasonable barrier integrity for a limited period within a day (69). As previously discussed, this limited culture period is mainly due to the overgrowth of microbial cells, where an accumulation of bacterial organic wastes rapidly diminishes the pH of culture medium, followed by the damage of epithelial barrier. Thus, the proposed model should maintain a controlled microbial population without undesirable bacterial overgrowth (66). To study on a longitudinal host-microbiome interaction and monitor how the immune cells respond to the microbiome and their products in the EMI axis, it is necessary to recreate an intact epithelial layer that provides a physical barrier and prevents aberrant transmigration of microbial cells from the lumen to the capillary side. This physical compartmentalization also contributes to control the infiltration of immune cells from the vascular side to the lumen side. In this experimental setup, it is critical to flow culture medium and exert mechanical deformations to suppress bacterial overgrowth (60,66,68). Moreover, the majority of the gut microbiome comprises obligate anaerobic bacteria. Thus, it is also critical to create a local oxygen gradient and establish an in vitro AOI in the model. An AOI can be accomplished by several approaches. The easiest method is to perfuse anoxic and oxic culture media into the upper and lower microchannels, respectively, of a two-channel microfluidic device (e.g., gut-on-a-chip) (60). This method has the advantage to circumvent using complex equipment, including an anaerobic glove box or an oxygen controller (70). Alternatively, the “human-microbial crosstalk (HuMiX)” model employs the perfusion of anoxic and oxic culture media by continuously infusing the nitrogen gas into the adjacent microchannel. The oxygen level can be detected by an oxygen sensor (71) and controlled, in which a facultative and an obligate anaerobic bacteria were co-cultured in the HuMiX model to assess the effect on the epithelial cells. Another method to grow anaerobic gut bacteria is to fabricate the anaerobic chamber while using a calibrated optical probe system to verify the hypoxic conditions within the microfluidic culture system (70). An AOI can lead to better stability in the maintenance
of diverse composition of the fecal gut microbiome, which may allow us to investigate the host-microbiome crosstalk to the homeostasis of the GI tract of the intestinal disease (60). Such an AOI together with physiological flow and mechanical motions in an organ-on-a-chip may overcome the limited longitudinal host-microbiome crosstalk observation in the conventional static in vitro cultures (60,70,71).

**Modeling the pathophysiology of inflammatory immune responses**

**MPS to model the physiology of an EMI axis**

The MPS refers to a microfluidics-based experimental microcircuit that provides an in vivo-relevant and accessible microenvironment driven by a reverse engineering approach with high modularity to engineer each comprising element on-demand (61). The human gut-on-a-chip is a representative MPS that models the intestinal physiology (66,68) and the pathophysiology that occurs in the EMI axis of various GI diseases (12,13). In the following sections, we will focus on the introduction and application of the gut-on-a-chip microsystem in host-microbe co-cultures and inflammatory immune responses. The gut-on-a-chip is made of polydimethylsiloxane (PDMS), a transparent, elastic, and gas-permeable silicone material, and has 2 superposed microchannels separated by a porous, flexible, extracellular matrix-coated PDMS membrane (Fig. 1A). The human intestinal epithelium can be cultivated on the porous membrane in the upper microchannel, forming an intact intestinal epithelial barrier and thereby recreating the luminal microenvironment of the gut. On the other side of the membrane, either lymphatic or capillary endothelium can be grown, thus creating a counter microenvironment in the lower microchannel that represents the lymphatic or capillary vasculature. Epithelial cells in the gut-on-a-chip can form a 3D microstructure (72) reminiscent of in vivo intestinal villi, where gut microbiome and immune cells can be co-cultured in the upper and lower channels, respectively, to recreate the EMI axis on-chip (12,13) (Fig. 1B). Optionally, it is possible to grow anaerobic gut bacteria once after the AOI condition is established and stabilized (60). Finally, PBMCs as immune elements can be introduced into the lower microchannel to emulate the recruitment of immune cells from the nearby capillary vasculature (12,13). Recent studies demonstrated the introduction of tissue-specific differentiated immune cells such as DCs or macrophages (14), which may lead to more physiological biomimicry. However, as previously mentioned, the preparation of the various immune elements for the inflammatory modeling of the human intestine has been nascent, where aggressive collaborations between biomedical engineers and immunologists have been appreciated.

**Demonstration of immune-mediated inflammatory responses**

Notably, the microengineered models of the human intestinal EMI axis enable us to recapitulate the pathophysiology of immune-associated inflammatory responses by leveraging the modularity and accessibility (12,13,60,66). For example, a different strain of bacteria or a bacterial LPS was introduced to the luminal upper microchannel lined by an intact epithelium (50) to interrogate how the specific luminal perturbation provokes the epithelial damage and inflammatory reactions. LPS produced by gram-negative bacteria is a potent ligand of the TLR4 (50). An excessive amount of LPS (15 µg/mL) introduced to the gut-on-a-chip that emulates an outgrowth or infection of Gram-negative bacteria resulted in the disruption of the villous epithelial morphology as well as the barrier function (12). Interestingly, in vitro immune responses occurred when the microbial and immune cells co-stimulated the epithelial layer, by which the recruitment of PBMCs, activation of intercellular adhesion molecule 1 molecules on the endothelial surface (Fig. 2A), disruption of the villous microarchitecture, expression of TLR4 on the villous epithelium, and the directional
secretion of pro-inflammatory cytokines (Fig. 2B). On the contrary, an independent stimulation of each cell type in the EMI axis did not show any inflammatory responses (12).

The pathomimetic gut inflammation-on-a-chip
Since gut inflammation involves multifactorial crosstalk followed by simultaneous pathological outcomes, it is challenging to unravel the onset trigger of the complex inflammation process. Indeed, it is noted that experimental animal models are impossible to independently uncouple the inflammatory factors in the EMI axis and identify a specific inflammatory trigger that develops the disease cascade. To surmount this caveat, MPS models may be a compelling alternative to dissect the mechanistic contribution of individual inflammatory factors in various combinations. The “gut-inflammation-on-a-chip” for instance allowed us to match the cells in the EMI axis one-at-a-time and assess inflammatory readouts to identify which factor is a critical trigger that initiates the entire inflammatory cascade (13). Briefly, an intact intestinal epithelial barrier is necessary and sufficient to sustain the physiological homeostatic tolerance in the co-presence of both luminal (e.g., LPS or gut bacteria) and immune elements (e.g., PBMCs) (13). Under the complex intercellular crosstalk in the EMI axis, spatiotemporal inflammatory responses such as oxidative stress, inflammatory epithelial injury, secretion of inflammatory cytokine, immune cell recruitment or infiltration, and microbial vascular invasion were observed during the dysregulated epithelial barrier, suggesting that the maintenance of a good barrier function is pertinent to the intestinal homeostasis (Fig. 3).

Other models for mimicking inflammatory responses in vitro
In a similar approach, there have been a number of MPS models that mimicked the intestinal EMI axis to build intercellular inflammatory interactions in vitro. Using a sequentially connected porous inserts (15) or a microfluidic device (14), differentiated subsets of immune cells, including tissue-specific macrophages or DCs, were added to the MPS device into either luminal or vascular compartment to induce immune responses in the intestine (14). For example, 2 microbial cells, *Lactobacillus rhamnosus* and *Candida albicans*, were co-cultured in
Under the formed EMI, probiotic effects of *L. rhamnosus* against *C. albicans*-induced tissue injury and inflammatory responses was tested. However, no mechanical deformations to emulate the bowel movement existed, which can affect bacterial growth rate and less physiological. Another MPS model combined an *ex vivo* tissue explant that secures the intestinal EMI axis to an engineered circuit (16). This study employed resected large intestine segments of mice and directly connected this resected "tube" to the device to maintain the original intestinal architecture and the homeostasis created by flowing the culture medium in the luminal side. In this integrative *in vitro-* *ex vivo* configuration, the differentiated intestinal epithelium, immune cells, and enteric neuronal cells were successfully co-cultured to investigate the role of the gut bacteria in activation of Th17 or Treg cells as well as the enteric nervous system (16). However, a limited longevity of the system due to the use of *ex vivo* tissue hampers a long-term study of the EMI crosstalk, and human intestinal EMI cannot be applied to the devised platform.

**FUTURE PERSPECTIVES**

The impact of a human MPS platform has been outlined in various contexts in biomedical and clinical applications. By leveraging the modular accessibility, a simple microfluidic device can grow different types of cells for mimicking the pathophysiological immune responses of diverse organs and tissues, by which the tissue-specific immunological crosstalk can be uncoupled and recoupled in a defined space and time. By the phenomenal advances in stem cell- or tissue-derived organoid cultures, a modular MPS system allows for the collection of patient-specific organoids, immune cells, and other surrounding cells (e.g., fecal microbiome, tissue-derived mesenchymal stromal cells) (70,73) to build a better model that recapitulates a disease-specific milieu in a patient’s "avatar model" (Fig. 4). There has been substantial progress in developing microfluidic chip systems modeling the physiology of various organ systems, including the heart (74), liver (75), lung (76), kidney (77), and GI tract (12-16).
There is a notable limitation of the 3D intestinal organoid system, which is that it prevents access to the apical side of the epithelium for studying the interactions with dietary constituents, microorganisms, and pharmaceutical compounds transported through epithelial cells (34,78). However, organoid-derived epithelial layers have been shown to grow while maintaining the accessible apical surface on MPS systems (31,32,72). This approach may allow for a more physiologic reflection of genetic variants and inter/intra-heterogeneity of human chronic diseases such as cancers (79), lead to the discovery of the independent effect of disease triggers at various immune microenvironments (13,80,81), and result in validation of the pharmacological responses of immunotherapeutic drugs in different race/ethnicity backgrounds (82,83). It may also be possible to precisely evaluate the independent contribution of immune elements on the intestinal tissue homeostasis and regeneration using an organ chip model. For instance, germ-free animals often show abnormal or non-physiological immune activations regardless of a lack of exogenous perturbations, where the hyper-reactivity in immune responses resulted in false-positive or negative outcomes (84).

We envision that the application of the human organ-on-a-chip can be a discerning strategy to preclude the possible limitation of germ-free models and to concordantly understand the role of immune components in diseases.

Regardless of technological advances and progress, there are several challenges to be further delineated and discussed. A notable limitation includes the restricted resource of human immune cells, suggesting that substantial collaborations between biomedical engineers and immunologists to explore a robust protocol for the enrichment of patient-specific immune cell subtypes (e.g., induced pluripotent stem cell-derived immune cells) (85). A considerable collaboration with experts in the biobanking communities (86) and scientists who have explored liquid biopsy technologies (87) is a crucial component for securing precious and valuable bioresources. There may be an alternative avenue for immunologists to contemplate using the MPS as a comparative model to build a rare immunological disease that has been poorly established using mouse models (88). The transdisciplinary approach may contribute to bridge the gap between in vivo, ex vivo, in vitro, and in silico models and reinforce to discover the breakthrough to explore disease mechanism and test new therapeutics.
As a concluding remark, advances in human organs-on-chips and their integrations can potentially contribute to unraveling the immunological contributions in health and disease. We envision that a pertinent gut inflammation-on-a-chip model will be a cornerstone in the development of new disease models that include tissue-specific or systemic immune cells as well as the disease-specific immune milieu.

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