Salmonella enterica

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Salmonella enterica serovars are important pathogens of humans and animals that are responsible for enormous morbidity, mortality and economic loss worldwide. Models used to study the disease pathology so far have provided valuable advancements, however, the molecular complexity of its pathogenesis remains poorly understood, particularly in humans. Therefore there remains a disconnect between what works at the bench versus at the bedside, especially in case of vaccines. The development of organoids/enteroids offers a tremendous opportunity to bridge this gap by bringing human-specific factors into the research models as well as elevate our understanding of the interactions and crosstalk between multiple cell types and the microbiota with *Salmonella*. Thus the use of organoids in studying Salmonella biology has the potential for improving clinical outcomes and future prophylactic and therapeutic intervention strategies.

organoids enteroids Salmonella host-pathogen interactions model systems infectious diseases organotypic culture system

1. Salmonella enterica

Several members of the genus *Salmonella* are major foodborne pathogens [1]. It comprises two species: *S. bongori* and *S. enterica.* Almost all *Salmonella* organisms that cause disease in humans and domestic animals are serovars belonging to *S. enterica subspecies enterica*. Broadly, the diseases caused by *Salmonella* in humans are of two kinds: 1) systemic febrile illness termed typhoid/enteric fever, and 2) acute self-limiting gastroenteritis. The serovars that cause typhoid are referred to as typhoidal *Salmonella* and include *S. enterica subsp. enterica* serovar Typhi and *S. enterica subsp. enterica* serovar Paratyphi A, B, and C. *S.* Typhi causes approximately 76.3% of global enteric fever cases [2]. *S.* Typhi and *S.* Paratyphi are restricted to humans and higher primates, and clinical manifestations of the infection include sustained high fever, abdominal pain, headache, weakness, malaise, and transient diarrhea/constipation. Without appropriate and effective antibiotic therapy, the infection may lead to gastrointestinal bleeding, intestinal perforation, septic shock, and death [3][4]. The Non-Typhoidal *Salmonella* (NTS) serovars cause self-limiting gastroenteritis and include *S. enterica subsp. enterica* serovar Typhimurium and *S. enterica subsp. enterica* serovar Enteridis, which are the most prevalent clinical isolates, according to the World Health Organization (WHO). These pathogens are broader in host range and infect humans and animals such as poultry, cattle, reptiles, and amphibians. Infections with NTS typically involve self-limiting diarrhea, stomach cramps, headache, vomiting, and fever that resolve on their own; however, the infection can be severe in children and the elderly and can sometimes be fatal [5]. NTS serovars have been reported to cause an invasive infection similar to typhoid, particularly in sub-Saharan Africa, predominantly in children and HIV-positive adults, with several

co-morbidities such as ongoing or recent malaria infection and malnutrition contributing to higher mortality [6][7][8]. *Salmonella* infections represent a considerable economic burden and public health concern in both developing and developed countries. The Center for Disease Control and Prevention (CDC), estimates that 1.35 million infections by *Salmonella* occur in the United States. NTS causes more than 93 million global infections per year, with 155,000 deaths [9]. A study examining the global burden of diseases, injuries, and risk factors in 2017 reported 14.3 million cases of enteric fever globally, with a case fatality of 0.95% resulting in approximately 135,000 deaths [10]. Children, the elderly, and those residing in lower-income countries account for the greatest incidences [11][12].

Salmonella is acquired via contaminated food and water. Luminal bacteria invade M cells and absorptive enterocytes via a specialized apparatus called the type three secretion system (T3SS) [13] encoded on the *Salmonella* Pathogenicity Islands (SPIs) [14]. The T3SS injects bacterial proteins into host cells allowing the bacteria to essentially commandeer host cellular processes to induce cytoskeletal rearrangements that engulf the bacteria into specialized vesicles called the *Salmonella* containing vacuoles (SCVs) [15]. This invasion process, with subsequent translocation across the epithelium, is followed by the uptake of the bacteria by macrophages and dendritic cells in the intestinal submucosa, where bacterial proteins interfere with phagolysosomal maturation and allow the bacteria to survive inside the cells [16][17][18]. NTS serovars undergo prolific growth in the intestine, while T3SS effectors induce fluid secretion and promote inflammation. Immune signaling via recognition of pathogen-associated molecular patterns such as lipopolysaccharide (LPS) and flagella also induce a robust inflammatory response, which actually provides *Salmonella* with a growth advantage over resident microflora [5][19][20][21]. The immune response eventually limits *Salmonella* growth; nevertheless, the short-term proliferation is sufficient to ensure propagation. Typhoidal *Salmonella* strains elicit a more attenuated inflammatory response in the intestine, especially in terms of limited neutrophil recruitment [3][22][23]. Bacteria migrate to the mesenteric lymph nodes (MLN) and systemically within the reticuloendothelial cells, and as free bacteria in the blood or lymph, to establish new foci of infection in the liver, spleen, bone marrow, and gallbladder [24]. At these new sites, the bacteria replicate and re-enter the intestinal lumen via secretion in bile, promoting the shedding of the bacteria to continue the cycle of new infections by contaminated food and water. In 3%–5% of cases, the bacteria can persist for long durations in the gallbladder, which serves as a reservoir of chronic infection [25]. Chronic infection with *Salmonella* has been found to be a risk factor for the development of malignant neoplasms, including gallbladder cancer [26][27] and colorectal cancer [27][28].

The emergence of multi-drug resistance to conventional antibiotics complicates the treatment of *Salmonella* infection [29][30][31][32]. Antibiotic treatment destroys the resident microflora, provides a niche for *Salmonella* to proliferate, and may lead to increased levels of bacterial shedding [33]. Additionally, bacterial populations that express an antibiotic-tolerant phenotype can evade treatment and persist, causing relapses of the infection as well as the evolution of bacterial virulence [34][35]. Asymptomatic carriers act as reservoirs, contribute to the continued propagation of the pathogen, and are particularly important for food safety considerations. Currently, there are no effective vaccines against gastrointestinal *Salmonella* infections. Several typhoid vaccines are available and licensed in many countries; however, robust protection is limited and has been associated with injection site reactions. Furthermore, the vaccines have not been widely adopted by public health programs [36]. With the significant aging populations in both developed and developing countries [37][38], more people are at risk for severe

consequences of *Salmonella* infections. Advances in the mechanistic understanding of *Salmonella* infections will facilitate the development of improved control strategies, particularly, safe and effective vaccines. The broad conservation of host responses as well as the molecular machinery used by *Salmonella* strains during infection of various hosts, namely T3SSs encoded by SPI1 and SPI2 that enable invasion of epithelial cells and subsequent intracellular survival, allows several model systems to be applied for *Salmonella* research [39]. Each non-human model has pros and cons and varies in the ability to recapitulate natural infection. Given the host-specific aspects of infection physiology, it is necessary to be cautious in applying the results from these model systems to human patients. Additionally, with *Salmonella* being an important tool to understand host physiology, metabolism, immune function, and interactions with microbes, human-specific investigations are valuable to the research community.

2. Model Systems to Study *Salmonella* Biology

Transformed cell lines such as Cos-1, MDCK, HeLa, HepG2, CaCo2, and T-84 have been used to carry out several fundamental studies on *Salmonella* pathogenicity, such as the identification of the T3SS and the SPIs [13][40]. However, these cells have drawbacks in that the cells do not adequately represent the physiological characteristics of normal human tissue [41][42]. Explant tissue cultures have organotypic properties that can be vital for studies on development and physiology but are limited by culturing difficulties and short life span [43][44]. Classically, animal models have offered solutions for several of these limitations, and have been used to corroborate data obtained from other model systems as well as to investigate the deeper molecular mechanism of infection. For example, *S.* Typhimurium is a natural pathogen of calves and causes gastroenteritis with clinical and pathological manifestations similar to humans, namely diarrhea, anorexia, fever, localized infection, and neutrophil infiltration [45][46]. Meanwhile, the bovine ligated loop was instrumental in characterizing fluid accumulation and host inflammatory responses following *S.* Typhimurium infection. For example, mutants lacking the invasion proteins SipA, SopA, and SopD were shown to have little to no effect on the ability of *S.* Typhimurium to invade epithelial cells, but were shown to reduce the fluid accumulation and neutrophil immigration in bovine loops [47][48].

More importantly, the vast majority of studies on *Salmonella* pathogenesis have been conducted in the murine model, including studies for the development of new vaccines. This model has been useful in clarifying various aspects of in vivo *Salmonella* pathogenesis; however, it does have limitations with respect to its ability to faithfully reproduce all aspects of *Salmonella* infection in humans. *S.* Typhimurium causes two very different types of diseases in human and mice. In humans, the infection is localized, dominated by the infiltration of neutrophils and self-limiting. In mice, *S.* Typhimurium spreads systemically, with slow infiltration of mononuclear inflammatory cells and little or no localized intestinal tissue injury. The host responses involved are also dramatically different. Since the clinical manifestations and pathology of mice infected with *S.* Typhimurium resemble those observed in humans with *S.* Typhi infection, the model has been used as a surrogate to study typhoid pathogenesis [49]. A mouse model of *S.* Typhimurium-induced enterocolitis has been developed and involves pre-treatment of mice with a single dose of streptomycin. This procedure diminishes the colonization resistance by the commensal microbiota, allowing *Salmonella* (that carry the gene for resistance to streptomycin) to grow to high densities in the cecum and large intestine and trigger acute gastroenteritis [50]. Mouse models have also been developed to mimic chronic infections

of *Salmonella* observed in certain carrier individuals, which typically involve infecting susceptible mice with avirulent strains, or sub-lethal doses of *S.* Typhimurium in resistant mice. Experimental interpretations from the mouse model may not translate to human disease. *S.* Typhi and *S.* Typhimurium share about 89% of their genes, with approximately 500 genes unique to *S.* Typhimurium and 600 genes unique to *S.* Typhi, including the genes encoding typhoid toxin and the immunoprotective capsule [51][52]. *S.* Typhi and *S.* Paratyphi also have pseudogenes as well as small sequence differences in genes encoding the T3SS apparatuses and related effectors that may have important implications in pathogenesis. It is possible that virulence factors that may have no role in *S.* Typhi-mediated infection in humans may be important for mouse infection by *S.* Typhimurium and vice versa. Finally, *S.* Typhi genes required for causing typhoid but absent in *S.* Typhimurium cannot be studied easily using the latter. Typhoid fever can be induced experimentally by oral infection in higher primates or human volunteers, but these studies come with their own set of difficulties and ethical objections. Researchers have attempted to compensate for the discrepancies by developing humanized mice, i.e., immunodeficient mice engrafted with human hematopoietic cells [53][54], but these models are cumbersome, expensive, and still do not guarantee that mouse-specific factors will not add complexities or variations to the data generated. In addition, host genetic background has been found to play a role in susceptibility to invasive NTS infections, a concept that borders on the realm of personalized medicine [55]. Thus, organoids offer a promising model to mechanistically study host-specific aspects of infection.

3. Organoids/Enteroids in *Salmonella* Biology

Organoids consist of a three-dimensional (3D) organ-like structure that is made up of organ-specific differentiated cells of multiple lineages and that recapitulates the unique organizational and functional characteristics of the corresponding organ in vivo. The 3D structures are generated in vitro from cells with a whole range of origins, such as tissue segments [56][57] and their derived adult stem cells (ASCs) [58][59], transformed cell lines [60], and pluripotent stem cells (PSCs). These models bridge the gap between simple 2D cell culture and complex in vivo experiments. Organoids have the following characteristics: 1) self-organization: individual cells arrange in vitro into a 3D structure that mimics the in vivo organ or tissue, 2) multicellularity: organoids are composed of multiple cell types typically found in the organ or tissue in equivalent proportions, 3) functionality: the organoid structure should be able to execute at least some of the organ- or tissue-specific functions, and 4) sustainability: organoids can propagate indefinitely without requiring transformation by maintaining a pool of progenitor cells. In 2012, the International Stem Cell Consortium [61] set guidelines for the nomenclature to be used to define the 3D structures generated in vitro depending on their origin and cellular composition. When referring to intestinal organotypic models, 3D structures that are composed of just epithelial cell types are generally known as “enteroids” if derived from the small intestinal epithelium, or “colonoids” if derived from colonic origin. The term “organoid” is usually reserved for 3D structures containing more than one cell lineage. However, it should be noted that these guidelines have not been uniformly adopted by the field. Many researchers commonly use “organoids” as a blanket term for 3D structures derived from ASCs, PSCs, or comprising transformed cell lines that resemble in vivo 3D architecture and physiology.

One of the earliest studies that utilized organotypic 3D structures to investigate *Salmonella* pathology was carried out by Nickerson, CA et al., in 2001 [62]. The authors generated 3D organotypic cultures by growing human embryonic intestinal cell line Int-407 in rotating wall vessel (RWV) bioreactors and subsequently infected the cells with *S.* Typhimurium. The resulting infection was quite different from what had previously been observed in monolayer cultures. There was minimal loss of structural integrity, lower ability of the bacteria to adhere to and invade epithelia, and lowered expression of cytokine in 3D Int-407 aggregates as compared to infected Int-407 monolayers. Since the authors observed that the 3D Int-407 aggregates more closely resembled in vivo characteristics (tissue organization, tight junctions, apical-to-basal polarity, microvilli development, expression of extracellular and basement membrane proteins, and greater M cell glycosylation pattern), the authors concluded that the infection phenotypes observed in the 3D aggregates were likely representative of an in vivo infection. This study laid the groundwork for the use of 3D organotypic cultures to study *Salmonella* biology. The following section will highlight research performed in both mouse and human organotypic models that have improved our understanding of *Salmonella* pathogenesis (Figure 1).

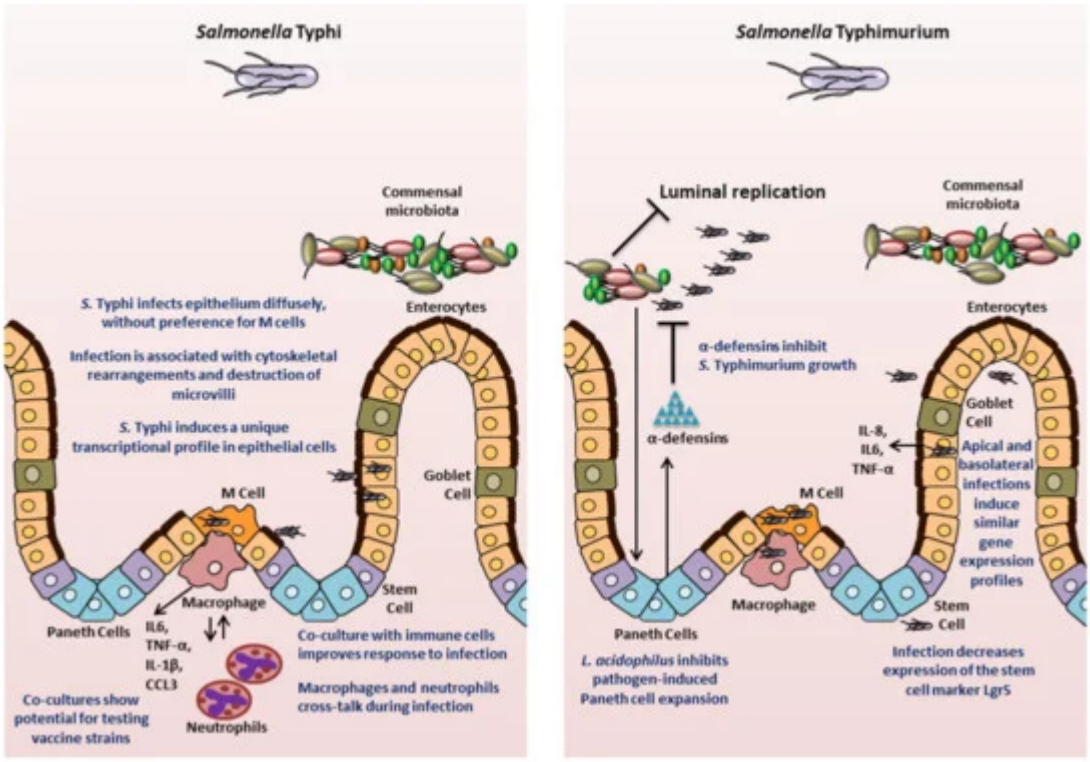

Figure 1. Insights into *Salmonella* pathogenesis from intestinal organoids/enteroids. The key findings for *S.* Typhi and *S.* Typhimurium are highlighted.

3.1. Mouse-Derived Models

Following the establishment of protocols to generate crypt-derived mouse intestinal enteroids (referred to as organoids by the authors) by Sato et al., Zhang and colleagues [63] in 2014 utilized the system to analyze the interaction of *S.* Typhimurium with epithelial cells. The authors visualized bacterial infection, while also observing bacterial-induced disruption of tight junctions, activation of the nuclear factor kappa-light-chain-enhancer of

activated B cells (NF-kB)-mediated inflammatory response, and a decrease in the stem cell marker Lgr5. The authors noted that these observations were similar to findings in animal models. The caveat to this study is that the *Salmonella* were not delivered into the lumen of the enteroids, the location of the initial contact of bacteria with epithelial cells in vivo; instead, the bacteria were added to the medium and came in contact with the enteroids basolaterally. Nevertheless, this study established mouse-derived enteroids as a model system for studying *Salmonella* infection biology.

Since this initial study, enteroids have been used to interrogate various aspects of *Salmonella* pathology, including investigating cell types that were previously not accessible to study in vitro. Farin and colleagues [64], in a 2014 study, used mouse intestinal enteroids to study the control of Paneth cell (PC) degranulation in response to bacteria or bacterial molecules such as LPS. The authors found that PC degranulation did not occur upon stimulation with microbial molecules or *Salmonella,* but was induced by a novel mechanism requiring only the presence of recombinant interferon gamma (IFN-γ) [64]. In another study, Wilson and colleagues [65] interrogated the antimicrobial role of Paneth cell α-defensin peptides. The authors developed small intestinal epithelial enteroids from both wild-type mice or mice mutated for α-defensin production (*Mmp7*$^{-/-}$ mice, MMP7 is a matrix metalloprotease that is required to generate bactericidally active α-defensins in mice [66]), and infected the enteroids with *S.* Typhimurium by microinjecting the bacteria directly into the lumen. The absence of mature α-defensins reduced the intra-luminal bacterial killing, which could be partially restored by the expression of the human defensin HD5 [65]. This study demonstrated the contribution of α-defensins to the innate immune response to *Salmonella,* which previously had been a challenge to examine since most of the earlier experimental systems inadequately recapitulated in vivo cellular processes [67].

Salmonella has been suggested to contribute to the development of cancer by epidemiological studies [27]. Scanu and colleague [68] probed this phenomenon in a 2015 study using the case of gallbladder cancer (GBC). The authors derived gallbladder enteroids from mice carrying mutations that inactivate p53 and are known to be found in GBC patients in India, where the disease is prevalent. When exposed to wild-type *S.* Typhimurium, single cells derived from the gallbladder enteroids carrying these predisposing mutations generated new enteroids that exhibited growth factor independence, which is one of the hallmarks of transformation, and had histopathological features consistent with neoplastic transformation, thus establishing a direct association between *Salmonella* and cancer. To delve into the mechanism of this transformation, the authors looked at the *Salmonella* T3SS-mediated activation of AKT or mitogen-activated protein (MAP) kinase pathways, which have been shown to be elevated in human cancers. The signals activated by AKT and MAPK were found to be key in driving the cellular transformation and were sustained even after the eradication of the *Salmonella* infection. Studies have shown that AKT and MAPK pathways are activated by other bacteria and viruses that have been associated with various cancers [69][70][71][72]. Although the authors employed a murine gallbladder enteroid model and *S.* Typhimurium to study GBC, the authors proposed that the AKT and MAPK pathways are activated by both *S.* Typhimurium and *S.* Typhi serovars, and contribute to development of cancer that is associated with chronic *S.* Typhi infection in humans. Chronic infection by *Salmonella* has also been found to be a risk factor for developing cancers in the ascending and transverse colon [28]. However, detailed mechanistic studies of *Salmonella*-associated colon

carcinogenesis need to be carried out and organoid model systems may prove to be extremely useful for this purpose.

Finally, another important aspect of *Salmonella* is the interaction of the pathogen with the host microbiome. Lu and colleagues [73] recently demonstrated that *Lactobacillus acidophilus*, a well-established probiotic bacterium, can alleviate damage caused by *S.* Typhimurium. Earlier studies had shown that *L. acidophilus* can inhibit adhesion of *Salmonella* to CaCo2 cells [74]. In this study, the authors extended the mechanism of protection to include the effects on the host. *L. acidophilus* altered the differentiation of epithelial cells in crypt-derived enteroids by impeding the *Salmonella*-mediated expansion of Paneth cells [64], thus maintaining homeostasis and appropriate epithelial composition during the infection. This study not only improved our understanding of the role of *L. acidophilus* in protecting the epithelial lining, but also demonstrated the ability to include microbiota-specific analyses to study *Salmonella* infection with enteroids/organoids.

3.2. Human-Derived Models

The potential to gain significant insight into *Salmonella* pathogenesis is particularly relevant in relation to the use of human-specific organoids, especially since these models possess human genetic specificities absent in mice. Studies have interrogated the usefulness of intestinal enteroids/organoids derived from human ASCs or PSCs to understand complex interactions between the epithelium and *Salmonella*. In 2015 Forbester and colleagues [75] used RNA sequencing to examine the epithelial transcriptional signature following injection of *S.* Typhimurium into the lumen of organoids derived from human induced PSCs (hiPSCs). The analysis showed significant up-regulation of genes for cytokine-mediated signaling, NF-κB activation, angiogenesis, and chemotaxis. Enhanced release of pro-inflammatory cytokines IL-8, IL-6, and TNF-α was also confirmed. The findings were consistent with prior studies in animal and mouse organoid models, thus establishing the human organoids as a viable infection model for *Salmonella*. The study also demonstrated that a noninvasive mutant strain (deficient in *invA* gene) could be used in the model to examine *Salmonella* pathogenicity and the functionality of defined mutants [75]. Furthermore, the authors generated an RNA sequencing data set following basolateral administration of *Salmonella* to the organoids. Interestingly, 49 of the 100 most highly upregulated genes were also significantly induced in the data set obtained by microinjecting the bacteria for apical infection. The data provide credence to the results of the Zhang and colleagues study [63], which documented similar patterns of gene expression upon basolateral administration of *Salmonella* to mouse organoids as had been observed earlier in literature with other model systems. Thus, the hiPSC organoids maintain a conserved response to *Salmonella* infection and provide a human-specific model for pathogenesis studies.

A subsequent study further demonstrated the validity of hiPSCs as a model to study human-specific responses to *Salmonella* infection. Using the same model system, the role of the cytokine IL-22 in priming intestinal epithelial cells towards a more effective response to *S.* Typhimurium was also explored [76]. The study showed that IL-22 pre-treated hiPSC-derived organoids increased phagolysosomal fusion leading to enhanced antimicrobial activity. Thus, this study confirmed earlier observations made in mouse organoids [77].

The fidelity of organoid-derived data in representing human disease was further demonstrated in 2018 by Nickerson, KP and colleagues [78], who compared infection of human tissue biopsies and human intestinal enteroid-derived monolayers seeded on a 2D Transwell system, and observed that the enteroid-derived epithelial monolayers recapitulated *S.* Typhi infection observations made in the tissue biopsy model. The authors also carried out transcriptional profiling of both the host tissue and the bacteria in order to determine early critical interactions. Infection with *S.* Typhi significantly down-regulated several host genes, including those involved in activation of the mucosal immune response, bacterial clearance, and cytoskeletal rearrangement. Interestingly in this model, a down-regulation of SPI1 genes in *S.* Typhi was observed. This work demonstrated that *S.* Typhi reduces intestinal inflammation by limiting the induction of pathogen-induced processes through the regulation of virulence gene expression, which is a characteristic feature of human infection with *S.* Typhi. Transmission electron microscopic comparisons of the tissues and human organoid-derived epithelial monolayers showed that the monolayers reproduced the cytoskeletal arrangements, microvilli destruction, and vesicle-bound bacteria observed in tissues. There were no changes observed in paracellular permeability, increased death of host cells, or bacterial association with M cells, suggesting divergence from *S.* Typhimurium infection in mice. This study highlights the ability of organoids to compare human-specific responses to each *Salmonella* serovar, which is important in the context of translational capacity for developing prophylactic or therapeutic intervention strategies against *S.* Typhimurium versus *S.* Typhi infections.

Despite the multiple advantages of the organoids as an experimental system, the technology is still in its infancy and has certain limitations. The complex structure of the organoids poses a practical limitation in accessing the internal luminal compartment. Researchers have used microinjections to access the apical epithelium. This approach may preserve the internal microenvironment, but is resource-intensive, may not allow synchronous exposure and suffers from variability in the volume that can be injected due to heterogeneity of the organoid/enteroid sizes. In addition, the lumen of 3D organoid/enteroid accumulates cellular debris, which may bind bacteria or hamper interactions with the apical membranes. Researchers have turned to organoid/enteroid-derived 2D monolayers to better access the apical side of the model and enable more efficient, user-friendly analyses in a multiple-well plate format. However, this modification can limit the number of processes that can be interrogated, especially when considering the lack of 3D structure. Interestingly, Co and colleagues [79] demonstrated in a recent study that the polarity of human enteroids could be reversed such that the apical surface faced the medium and was readily accessible. The enteroids released mucus and extruded cells outwards into the culture medium rather than having the cells embedded in the basement membrane. Using enteroids with reversed polarity, the authors showed that *S.* Typhimurium invades and induces actin ruffles more efficiently at the apical surface compared to the basolateral surface. The authors observed a more diffuse process of epithelial invasion rather than invasion only or predominantly at the M cells [79], which confirmed the *S.* Typhi observations by Nickerson, KP et al. [78].

Current organoid/enteroid models are devoid of muscles, innervation, vascularization, and immune cells. There are a couple of approaches being carried out to increase the complexity of organoid models, including co-culturing techniques. In 2011, Salerno-Goncalves and colleagues [60] generated an organotypic model using the human ileocecal adenocarcinoma cell line HCT-8 and adding primary endothelial cells, fibroblasts, and peripheral blood

mononuclear cells (PBMCs), which they used in a 2019 study to probe the crosstalk between these cell types during infection with *S.* Typhi, *S.* Paratyphi A, or *S.* Paratyphi B [80]. An ECM composed of collagen-I enriched with other gastrointestinal basement proteins was embedded with the fibroblasts and epithelial cells, and transferred to an RWV bioreactor containing epithelial cells. Under low microgravity and low shear conditions, the HCT-8 cells behaved as multipotent progenitor cells and gave rise to multiple cell types, including absorptive enterocytes, goblet cells, and M cells. After one to two weeks, PBMCs were added to the system. The co-culture model was then infected with the various *Salmonella* serovars to compare responses to the three strains. The authors found that the presence of the immune cells in the model resulted in secretion of the cytokines IL-1β and CCL3, while secretion of cytokines IL-6 and TNF-α was enhanced. Using depletion experiments, the authors showed that macrophages were the PBMC cell type responsible for the enhanced secretion of IL-6 and TNF-α. The authors further used the Transwell system to show that supernatants from organotypic models built with whole or macrophage-depleted PBMCs infected with the three *Salmonella* strains varied in their ability to elicit transmigration of macrophages and neutrophils [80]. Interestingly, the two immune cells displayed crosstalk during infections with *S*. Paratyphi A and *S*. Paratyphi B, such that the presence of macrophages in the co-culture reduced neutrophil migration as compared to the system built without macrophages [80]. This study illustrates that co-cultures can aid in probing the contribution of immune cells to *Salmonella* infection at the mucosal surface. Finally, this model has also been used to assess the inflammatory response to several candidate *S.* Typhi vaccine strains in comparison to the response elicited by the oral vaccine strain Ty21a strain and its parent wild-type Ty2 strain [81]. Salerno-Goncalves and colleagues [81] found that specific changes to the genetic makeup of the candidate vaccine strains (in the form of deletions of specific metabolic genes) elicited host changes in intestinal permeability, inflammatory cytokine secretion, as well as activation of innate immunity pathways. Higgins and colleagues [82] also used the model to test the inflammatory response of an *S.* Typhimurium vaccine strain that they generated. These studies highlight the usefulness of co-cultured organoid/enteroid models in assessing important factors to be considered while designing vaccines.

Schulte and colleagues [83] generated a co-culture system of human intestinal epithelial cell line (Caco-2), primary human microvascular endothelial cells, primary intestinal collagen scaffold, and PBMCs in a Transwell set up. Using GFP-labeled *S.* Typhimurium, microscopy, and flow cytometry, the authors demonstrated that the bacteria can be found in epithelial but not endothelial cells, thus modeling the epithelium-restricted infection of humans with *S.* Typhimurium. These findings are in contrast to those of Spadoni and colleagues [24] in the mouse model of *S.* Typhimurium infections where a breach of the gut-vascular barrier by the bacteria was observed. The endothelial cells respond to the infection process by bringing about changes in the transcription of various genes and releasing the phagocyte chemoattractant IL-8. Such models, ideally with enteroid/organoid-derived cells replacing cell lines where used, should prove to be extremely useful and versatile in interrogating the role of different immune cells, vasculature, and the related crosstalk with epithelial cells during infection with *Salmonella,* especially for *S.* Typhi, where the bacteria spread systemically both as free bacteria and within reticuloendothelial cells [84].

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