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2 **Modeling Host-Pathogen Interactions in the Context of the Microenvironment:**

3 **3-D Cell Culture Comes of Age**

4

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39 **Abstract**

40 Tissues and organs provide the structural and biochemical landscapes upon which microbial
41 pathogens and commensals function to regulate health and disease. While flat two-dimensional
42 (2-D) monolayers composed of a single cell type have provided important insight into
43 understanding host-pathogen interactions and infectious disease mechanisms, these
44 reductionist models lack many essential features present in the native host microenvironment
45 that are known to regulate infection, including three-dimensional (3-D) architecture, multicellular
46 complexity, commensal microbiota, gas exchange and nutrient gradients, and physiologically
47 relevant biomechanical forces (e.g., fluid shear, stretch, compression). A major challenge in
48 tissue engineering for infectious disease research is recreating this dynamic 3-D
49 microenvironment (biological, chemical, physical/mechanical) to more accurately model the
50 initiation and progression of host-pathogen interactions in the laboratory. Here we review select
51 3-D models of human intestinal mucosa, which represent a major portal of entry for infectious
52 pathogens and an important niche for commensal microbiota. We highlight seminal studies that
53 have used these models to interrogate host-pathogen interactions and infectious disease
54 mechanisms, and we present this literature in the appropriate historical context. Models
55 discussed include 3-D organotypic cultures engineered in the Rotating Wall Vessel (RWV)
56 bioreactor, extracellular matrix (ECM)-embedded/organoid models and organ-on-a-chip (OAC)
57 models. Collectively, these technologies are providing a more physiologically relevant and
58 predictive framework for investigating infectious disease mechanisms and antimicrobial
59 therapies at the intersection of the host, microbe and their local microenvironments.

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60 **Introduction**

61 Mucosal surfaces lining the gastrointestinal, respiratory and urogenital tracts continuously
62 interface with the external environment and serve as a barrier against pathogens, commensals,
63 chemicals, drugs and toxins. These tissues possess a complex architecture with multiple cell
64 types organized into 3-D structures that facilitate tissue-specific functions. The biological,
65 chemical, and biomechanical characteristics that define microenvironmental niches along these
66 surfaces provide the structure and context upon which infection takes place. Pathogens have
67 adapted to detect specific host structures, polarity, and changes in local environmental stimuli
68 (pH, temperature, oxygen, nutrients, hormones, physical forces, etc.) to know where and when
69 to activate specific virulence programs during different infection stages (1-7). A major challenge
70 in tissue engineering for infectious disease research is recreating *in vivo* spatiotemporal
71 properties of dynamic 3-D microenvironments to more accurately model host-pathogen
72 interactions in the laboratory.
73
74 Historically, infectious disease has been commonly studied *in vitro* by assessing the interaction
75 of a single microbe with a single host cell type, with the latter grown as flat 2-D monolayers. This
76 reductionist approach has enabled important discoveries and advanced our understanding of
77 mechanisms that underlie infection and disease. However, the study of disease in isolation or
78 out of context can change the native behavior of both host and microbe, thus creating a barrier
79 for researchers to correlate *in vitro* and *in vivo* responses. In this data-rich period where multiple
80 -omics technologies are being synergistically applied for unparalleled insight into host-pathogen
81 interactions, it is critical to consider the context under which these investigations are performed.
82 Reconstructing host microenvironments is key, including 3-D tissue architecture, multicellular
83 complexity, microbiota composition/localization, oxygen tension, transport processes and
84 biomechanical forces (e.g., fluid shear, stretch, compression) (1, 8-11). Within this context, *in*
85 *vitro* models are positioned along a continuum between 2-D and 3-D, with flat monolayers of a

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86 single cell type representing the most basic system with more complex models located further
87 down the spectrum that recreate multiple aspects of the native tissue microenvironment (**Fig. 1**).
88 Since tissues and organs function in a 3-D context, consideration of proper structure is essential
89 for development of models that better mimic *in vivo* responses. Since no current *in vitro* model
90 fully accomplishes this task, multidisciplinary teams of biologists, engineers, physicists,
91 mathematicians and clinicians are creatively working together to develop next-generation 3-D
92 models with enhanced predictive capabilities to open new avenues for clinical translation.
93
94 Present-day 3-D culture techniques result from a series of progressive advances in tissue
95 engineering over the past century to better mimic the native structure and microenvironment of
96 normal and diseased tissues (reviewed in (12)). Indeed, long ago the cancer research
97 community recognized that appropriate modeling of the 3-D microenvironment is important for
98 mimicking disease, leading to development and application of 3-D organoid models developed
99 within or on top of extracellular matrix (ECM) (12-16). The bidirectional exchange of biological
100 and physical signals between cells and their microenvironment regulates cell structure/function
101 and is largely manifested by tensile connections between ECM, cell surface receptors (e.g.,
102 integrins), and the cytoskeleton to transduce signals to and from the nucleus (17-31). This same
103 structural network is also engaged by certain invasive pathogens (e.g., *Salmonella*, *Shigella*,
104 *Listeria*, rotavirus, influenza virus) that hijack and remodel host cell architecture to facilitate their
105 internalization, intracellular trafficking, and/or dissemination (9, 32-34). Similarly, we and others
106 have demonstrated that bacteria also respond to biomechanical forces like fluid shear, which
107 can regulate virulence, gene expression and/or stress responses (1-5, 35-47). Indeed, the
108 discovery of biomechanical forces as environmental regulators of microbial pathogenesis was
109 made by our team almost two decades ago with the discovery that fluid shear forces globally
110 reprogram *Salmonella* gene expression, stress responses and virulence (35). Fluid shear also
111 plays a central role in regulating a number of host responses, including differentiation (48-50).

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112

113 Although 3-D models have long been applied for cancer research (12-16), their utility remained
114 largely unincorporated by the infectious disease community until the late 1990s and early
115 2000s. As expected for many new ideas in an established field, the use of 3-D models to study
116 host-pathogen interactions was initially met with skepticism. The first reports of 3-D models to
117 study viral infections were by Long et al. in 1998 (rhinovirus) and bacterial infections by
118 Nickerson et al. in 2001 (*Salmonella enterica* serovar Typhimurium/*S*. Typhimurium) (11, 51).
119 Recently, infectious disease researchers have broadly embraced 3-D models for studying
120 pathogenesis mechanisms, biomarker discovery, and drug candidate screening. In this review,
121 we highlight key microenvironmental factors to consider when selecting *in vitro* 3-D intestinal
122 models to study host-pathogen interactions. We focus on three key technologies for model
123 development, 1) the RWV bioreactor, 2) ECM-embedded/organoid models, and 3) gut-on-a-chip
124 models, and propose a vision for future model advancements. We also provide proper historical
125 context for use of 3-D cell cultures in studying host-pathogen interactions, which is finally
126 gaining a critical mass of scientists who understand and appreciate the value of studying
127 disease in the proper context of tissue form and function.

128

129 **I. Microenvironmental cues in host-microbe interactions**

130 Mucosal tissue function and homeostasis are meticulously controlled by complex bidirectional
131 interactions between cells and their microenvironment (15, 20, 25, 27-29, 52-55). The
132 microenvironment includes 3-D tissue architecture, multiple cell types, ECM , innate immunity
133 mediators, indigenous microbiota, and physical forces. These factors are regulatory signals for
134 mucosal pathogens and may be beneficial or detrimental for infection (1-5, 8, 35-45, 47, 56-64).
135 Below we address key cellular, biochemical and biophysical cues that dictate infection outcome
136 and are important considerations when modeling host-enteric pathogen interactions.

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138 **Cellular factors.** Intestinal mucosal epithelium contains an array of specialized epithelial and
139 immune cells that work in synergy to protect against infection by: (i) serving as a barrier against
140 luminal toxins, commensals and pathogens, (ii) sampling microbial antigens, and (iii) recruiting
141 innate and adaptive immune effectors (65). The intestine contains multiple epithelial cell types,
142 including enterocytes (absorptive functions), enteroendocrine cells (hormone secretion), Paneth
143 cells (antimicrobial production), goblet cells (mucin production), M cells (luminal antigen
144 sampling/induction of mucosal immunity), Tuft cells (Th2 immunity), and Cup cells (unknown
145 function) (66, 67). The intestine also contains immune cells for innate and adaptive responses to
146 pathogen attack, including macrophages, dendritic cells, T and B cells, including those
147 organized in lymphoid structures termed Peyer's patches, sites of induction of mucosal
148 immunity. As the body's largest immune organ, the composition, organization and function of
149 the intestine varies by region and consists of integrated cross-communication networks of
150 different cell types and effectors critical for protection against pathogens (described in (65, 68-
151 73)).
152
153 Epithelial cell polarity establishes barrier function, regulates uptake/transport of nutrients, and
154 maintains epithelial architecture (65, 74-76). In the intestine, apical surfaces face the lumen and
155 regions between villi/folds, lateral surfaces face adjacent cells, and basal surfaces face the
156 basement membrane and lamina propria. Along Peyer's patches and isolated lymphoid follicles,
157 the basal side of the follicle-associated epithelium overlies a subepithelial dome region
158 containing a mixture of immune cells (77). The distinct biochemical composition (e.g., protein,
159 lipid) of apical and basolateral surfaces facilitates their specific functions (76). Given that many
160 pathogens have evolved to recognize surface-specific molecules for attachment and/or to
161 disrupt barrier integrity to enable their uptake and dissemination (6, 75, 78-80), appropriately
162 modeling polarity *in vitro* is critical as pathogens infect host cells differently depending on
163 whether they are polarized or non-polarized (81-84). Maintaining barrier integrity requires proper

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164 expression and localization of tight and adherens junctions. Adherens junctions are mediated by
165 E-cadherin and catenin interactions, while tight junctions are composed of transmembrane
166 proteins (e.g., claudins, occludins) and cytoplasmic plaque proteins (e.g., zonula occludens).
167 While generally protective, junctional complexes are also exploited by pathogens to facilitate
168 invasion (75) and some enteric viruses utilize receptors localized to these junctions (78, 79).
169
170 Another major cellular component encountered by enteric pathogens is the diverse microbial
171 community – termed microbiota (referring to microorganisms) or microbiome (referring to
172 microbial genomes). The intestinal tract contains prokaryotes, viruses, archaea, and eukaryotes,
173 some of which protect the host against pathogen colonization by a variety of mechanisms,
174 including epithelial cell turnover, mucin synthesis, and triggering bacterial sensors on host cells
175 (85-87). Reciprocal interactions between host and microbiota contribute to tissue function and
176 homeostasis and determine microbiota composition, thereby playing an important role in
177 infection and disease (88). For example, intestinal microbiota regulate production of
178 antimicrobial peptides by Paneth cells (89) and shape immune responses by regulating
179 numbers, subsets, and/or functions of T, B and myeloid cells (65). Microbiota-induced changes
180 in immunity also determine intestinal microbiota composition (86, 90).
181
182 The intestinal microbiota is comprised of ~10^{14} bacteria (>1000 species), with Firmicutes and
183 Bacteroidetes most abundant (91-94). Interpersonal variation in intestinal microbiome occurs,
184 with each individual carrying a subset of the total known microbiome (95). Temporal and spatial
185 variation occurs throughout the intestinal tract (96, 97). Increasing data suggest a relationship
186 between an imbalanced intestinal microbiome and various diseases, including obesity,
187 inflammatory bowel disorders and cancer (98). The importance of gut microbiota to health is
188 highlighted by successful clinical application of fecal microbiota transplants from healthy

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189 individuals to patients with recurrent, antibiotic resistant *Clostridioides difficile* (*C. difficile*) (99-
190 101).
191
192 **Biochemical cues.** Mucosal tissues contain an array of small molecules, including innate
193 defense mediators that target pathogens and regulate downstream host defenses. Intestinal
194 mucus harbors compounds from the innate and adaptive systems that protect against microbial
195 insult, including digestive enzymes (e.g., lysozyme), lactoferrin, antimicrobial peptides,
196 complement, and antibodies (e.g., secretory immunoglobulin A/sIgA) (65). In addition, cells of
197 the innate defense system respond to pathogen-associated molecular patterns (PAMPs) using
198 pathogen recognition receptors (PRRs). Depending on the pathogen, PRR-mediated signal
199 transduction results in different cellular outcomes (e.g., cell proliferation, apoptosis, antimicrobial
200 peptide production, autophagy, cytokine secretion). Cytokine production leads to recruitment of
201 innate and adaptive immune effectors to the infection site, representing a bridge between these
202 two arms of immunity (65, 102).
203
204 Mucins are complex mixtures of high molecular weight, glycosylated macromolecules that bind
205 and remove pathogens and their products (7, 102). Enteric pathogens sense and respond to
206 cues within mucus and overcome this barrier to reach underlying epithelium (7). Normal
207 intestinal mucus consists of two layers: an outer layer colonized by microbes and a sterile inner
208 layer (103-105). The composition and thickness of these mucin layers varies throughout
209 intestinal regions to accommodate their different functions and microbial burdens. Within the
210 small intestine the inner and outer mucosal layers are thinner to facilitate nutrient absorption,
211 with thicker regions found towards the ileum where microbial burden is heavier (7). In the colon,
212 both layers are thicker to accommodate the burden of several trillion commensals (7). The
213 presence of sIgA and other mucin antimicrobials also serves to reduce bacterial colonization
214 (106).

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215

216 The ECM is another key contributor to tissue homeostasis. Historically neglected as a signaling
217 entity, seminal discoveries have revealed the central role of ECM in regulating tissue
218 architecture/function (20, 53). The ECM is a three-dimensional non-cellular scaffold comprised
219 of proteins (e.g., collagens, elastins, laminins, fibronectins), proteoglycans and water. Two main
220 types of ECM include: (i) interstitial connective tissue matrix, which serves as a cellular scaffold,
221 and (ii) basement membrane matrix, which separates epithelium from interstitium (107, 108).
222 ECM components also serve as ligands for cell receptors like integrins, which transduce
223 physical forces into biological responses (mechanotransduction). Additionally, immune
224 responses are mediated through interactions with the ECM (108, 109). Furthermore, the ECM
225 controls availability/release of growth factors and other signaling molecules (hormones,
226 cytokines) (108). The complexity, composition and structure of ECM are highly dynamic and
227 specific (as are the biochemical gradients it contains) and depend on tissue type, developmental
228 stage, and health/disease state (108).

229

230 **Biophysical forces.** The role of physical forces in cell and tissue development/function is as
231 important as those of genes and biochemical signals (28, 110). Physical forces regulate cell
232 proliferation, differentiation and homeostasis (111, 112). Forces experienced by intestinal cells
233 include fluid shear, pressure (113), and contractile peristalsis of muscles (114). Hydrodynamic
234 calculations suggest that fluid shear forces on the exposed epithelial brush border microvilli are
235 ~ 200 times greater than those between microvilli (< 0.01 dynes/cm^2) (115).

236

237 The cytoskeleton and its linkage with ECM play an essential role in enabling cells to sense and
238 respond to biophysical forces. While the governing role of ECM as a dynamic signaling entity
239 that regulates tissue form/function is now appreciated, it was initially considered a purely static
240 scaffold. However, tissue-specific architecture and function are regulated by the biophysical

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241 properties of ECM (20, 116, 117), which exerts physical influences transduced by cell surface
242 receptors through the cytoskeleton to the nucleus to ultimately alter cellular and molecular
243 properties. These structural networks are critical for regulating cell shape/architecture, and have
244 been modeled using the principle of tensegrity, which refers to structures that are stabilized
245 under continuous tension by balancing opposing tension and compression forces (27-29, 31).
246 The integration of biophysical forces across cells and tissues using this structural network
247 regulates a wide range of biological processes (e.g., cell proliferation, apoptosis, differentiation,
248 adhesion, migration, gene expression, architecture)(8, 20, 21, 23, 25, 27, 29-31, 55, 118).
249 Accordingly, ECM composition and stiffness are critical regulators of cellular responses (119,
250 120). These properties are continuously remodeled through the process of “dynamic reciprocity”
251 (17, 20, 53, 118), theorized by Bissell in 1982 to explain how signaling between the ECM and
252 nucleus regulate tissue function. This laid the foundation for modern 3-D cell culture approaches
253 used today (20, 108, 121). Not surprisingly, pathogen-ECM interactions play an important role in
254 mediating infection (122-127). In addition to impacting the host, physical forces also globally
255 alter bacterial gene expression, stress responses, and virulence in unexpected ways to
256 contribute to infection (5, 36-40, 47, 62-64).
257

258 **II. Modeling the microenvironment: 3-D models for infectious disease**

259 Several cell culture systems exist for the development and application of 3-D models of human
260 tissues for infectious disease research, including the RWV bioreactor, ECM-embedded scaffolds
261 (e.g., ECM extracts, purified ECM, or synthetic/semi-synthetic hydrogels), and organ-on-a-chip
262 (OAC) models. The choice of system to use depends on several factors, including the
263 experimental question being addressed, technical complexity, and cost and expertise for model
264 development. Different cell types in the native tissue (including immune cells) can be co-
265 cultured in these models to further enhance physiological relevance. Additionally, a single
266 epithelial cell type can spontaneously differentiate into multiple epithelial cell types normally

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267 found in the parental tissue and undergo self-assembly into tissue-like structures using all of
268 these 3-D technologies. To date, most *in vitro* infection studies have been performed using cell
269 lines; however, there is a push to develop models using either primary and/or stem cells to
270 better mimic the native tissue. To explore the integration of different environmental signals in
271 regulating infection, a hierarchical series of increasingly complex 3-D model systems comprised
272 of different cells types can be developed and applied in parallel under differing experimental
273 conditions (e.g., different oxygen tensions, physical forces).
274
275 **RWV-derived 3-D models.** The RWV bioreactor is an optimized form of suspension culture that
276 facilitates formation of self-organizing 3-D tissue-like aggregates by allowing cells the spatial
277 freedom to co-localize and self-assemble based on natural affinities within a low fluid shear
278 environment (**Fig. 1B, panel a**) (8, 128). Fluid shear influences cell proliferation, differentiation,
279 morphology and function (30, 115, 129-141). Models developed within the dynamic RWV
280 environment experience excellent mass transfer of nutrients/wastes and exhibit enhanced
281 structure, differentiation, function, and multicellular complexity relative to 2-D monolayers (11,
282 81, 142-155). Along these lines, observations from the 1970s showed that flotation of collagen
283 gels led to a more permissive environment for cellular differentiation (12, 156, 157). Moreover,
284 the low fluid shear environment in the RWV is also physiologically relevant to that encountered
285 by pathogens in low shear regions of the infected host, including intestine (38, 115, 130-132).
286 Accordingly, the RWV is also used to culture pathogens to study the role of fluid shear and
287 mechanotransduction in regulating microbial pathogenesis and host-pathogen/commensal
288 interactions (1, 35-41, 45-47, 62, 64, 146, 158-168).
289
290 The RWV is among the most extensively used approaches to develop 3-D models to study host-
291 pathogen interactions. It was the first technology used to develop 3-D models for infection
292 studies with bacterial (*Salmonella*) and viral (rhinovirus) pathogens (11, 51). A range of RWV-

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293 derived 3-D models have been developed using cell lines, stem cells, and/or primary cells,
294 including small and large intestine (11, 81, 142, 144, 146, 147, 153, 169-178), lung (145, 148,
295 179-183), liver (149, 154, 175, 184, 185), bladder (8, 186-188), reproductive tissues (150-152,
296 189-191), heart (192-194), prostate (143, 187, 195), pancreas (196, 197), nervous tissue (183,
297 198-200), blood-brain barrier (201), skin (202), eye (203), bone, joint or disc (204-208) and
298 tonsil (209), among others. These studies demonstrated that RWV-derived models exhibit
299 enhanced *in vivo*-like characteristics, including: spontaneous differentiation into multiple cell
300 types that self-organize into 3-D structures (**Fig. 1B, panel d**), polarization, appropriate
301 expression/localization of adherens/tight junctional complexes, metabolic product secretion,
302 gene expression, cytokine production, responses to antimicrobials and microbial products,
303 support of commensals, and/or susceptibility to infection (8, 11, 81, 142-154, 169-195, 198-
304 209). In addition, RWV models have been advanced to incorporate immune cells to study their
305 role in host-microbe interactions (172, 176, 178, 181).
306
307 Models are typically initiated by harvesting monolayers, combining cells with porous ECM-
308 coated microcarrier scaffolds and loaded into the RWV. Scaffold and ECM porosity allow the
309 basal side of cells to experience autocrine/paracrine communications, aiding cellular
310 differentiation/responses in a manner reflecting *in vivo* tissues. This differs from monolayers
311 where cells proliferate on impermeable surfaces, thus hindering proper communications across
312 apical and basolateral surfaces. Additionally, models may be developed scaffold-free or using
313 non-microcarrier scaffolds (e.g., decellularized tissues) for transplantation (180, 182, 210). Once
314 developed, distribution of 3-D models into multi-well plates lends to their experimental
315 tractability for infection assays, as their structural/functional integrity remains intact following
316 seeding. Alternatively, pathogens or compounds can be directly added to the RWV to study
317 interactions under physiological fluid shear. One key advantage of RWV culture is the

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318 production of large numbers of cells (~10^7-10^8 per culture). Below we discuss RWV-derived 3-D
319 models of human intestinal mucosa.
320
321 *RWV-derived intestinal models.* We began using the RWV to engineer 3-D models of human
322 intestine for infection studies in the late 1990s after realizing that available models for studying
323 bacterial pathogenesis lacked multiple aspects of the *in vivo* microenvironment (11). RWV-
324 derived 3-D models have enabled the study of host-microbe interactions relevant to different
325 regions of the intestinal tract, including small intestine (11, 170) and colon (81, 144, 146, 147,
326 153, 172, 173, 176, 178). Imaging of these models revealed enhanced 3-D architecture relative
327 to monolayers, including the presence of extensive 3-D folds and microvilli, that more closely
328 resembled what is observed *in vivo* (**Fig. 1B, panel d**). These 3-D models are essentially
329 "inside-out" such that the apical/luminal side faces the media and the basal side faces the
330 scaffold, allowing for straightforward introduction of pathogens, toxins, and antimicrobials at the
331 apical surface, as commonly occurs *in vivo*. Collectively, these models have shown
332 physiologically relevant expression and localization of key cellular components, including
333 junctional proteins (e.g., ZO-1, occludin, symplekin, E-cadherin, β-catenin, desmosomes),
334 secretion of basal lamina components (e.g., collagen types II, III, IV, laminin, vimentin,
335 fibronectin), brush border formation with villin, and/or mucus secretion (11, 81, 144, 146, 147,
336 153, 170, 172, 173, 176, 178). Spontaneous cellular differentiation into multiple lineages found
337 in the intestinal epithelium is also observed, including enterocytes, M cells, goblet cells, and/or
338 Paneth cells (enteroendocrine cells were not evaluated) (11, 81, 147, 153, 172, 176). The
339 presence of multiple cell types within a model (e.g., epithelial and immune cells) enables study
340 of their combined effects on infection, and in particular, pathogen co-localization patterns with
341 different cell types. An example is described below using an advanced 3-D RWV co-culture
342 model that combined human colonic epithelium with phagocytic macrophages to study infection
343 by different *Salmonella* pathovars (172). Primary human lymphocytes have also been

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344 incorporated in a 3-D co-culture model of intestinal epithelium to study *Salmonella* infection
345 (176).
346
347 RWV-derived intestinal models have contributed to the study of a variety of pathogens such as
348 *S.* Typhimurium (including multidrug resistant ST313), *S.* Typhi, Enteropathogenic *Escherichia*
349 *coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), *Cryptosporidium parvum*, and human
350 enteroviruses including coxsackievirus B (CVB) and poliovirus (11, 81, 144, 146, 147, 153, 172,
351 173, 176). Studies with *S.* Typhimurium using 3-D models of small and large intestine displayed
352 marked differences from monolayers in colonization, tissue morphology, apoptosis,
353 prostaglandin and cytokine expression (11, 81, 153). The responses of these 3-D intestinal
354 models to *S.* Typhimurium challenge were highly predictive of *in vivo* responses in
355 humans/animals (11, 81, 153), including rapid repair of the small intestine (initial site of
356 *Salmonella* pathogenesis) and significant damage to the colon (primary site of pathogenesis)
357 (211). These models were also the first *in vitro* systems to challenge the widely accepted
358 paradigm established using monolayers that *Salmonella* Pathogenicity Island-1 (SPI-1) Type
359 Three Secretion System (T3SS) is required for invasion of intestinal epithelium (81, 153).
360 Historically, studies with monolayers contradicted *in vivo* observations wherein successful
361 animal infections were possible with T3SS SPI-1 mutants (212, 213), and clinical isolates of
362 *Salmonella* lacking SPI-1 function were isolated from foodborne disease outbreaks in patients
363 experiencing gastroenteritis (214). Using a 3-D intestinal model comprised solely of epithelial
364 cells, Radtke et al. demonstrated that SPI-1 mutants and a *Salmonella* mutant lacking all known
365 T3SS (SPI-1, SPI-2, and the flagellar system) still exhibited high levels of invasion relative to
366 wild type (although approximately 0.5-1 log lower) (153). As expected, in monolayers these
367 mutants exhibited little-to-no invasion (<10 CFU); a finding which does not reflect *in vivo*
368 observations (153). Thus for the first time, an *in vitro* intestinal epithelial model was able to
369 parallel *in vivo* results by supporting *Salmonella* invasion independently of SPI-1. These findings

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370 demonstrate the enhanced capability of RWV models to predict *in vivo*-like pathogenic
371 mechanisms.
372
373 Host-pathogen-commensal and host-commensal interactions have also been investigated using
374 RWV 3-D intestinal models (173, 178). Commensal microbes naturally enhance intestinal
375 mucosal barrier function against pathogen colonization through complex mechanisms not yet
376 fully characterized (215). Naturally occurring probiotic strains of bacteria are being exploited as
377 a strategy against pathogens to combat ongoing problems of antibiotic resistance. Treatment of
378 a 3-D intestinal model with *Lactobacillus reuteri* or its antimicrobial metabolite, reuterin, before
379 or after challenge with *S*. Typhimurium reduced adhesion, invasion and intracellular survival of
380 this pathogen as compared to untreated cells (173). This was the first study to report the effect
381 of reuterin on the enteric infection process for any mammalian cell type. A 3-D intestinal co-
382 culture model containing immune cells was used to profile responses to both free secretory IgA
383 (SIgA) and SIgA complexed with a commensal strain of *E. coli* (178). Application of free SIgA to
384 the model induced upregulation of MUC2, IL-8, and polymeric immunoglobulin receptor (pIgR),
385 secretion. When SIgA was complexed with *E. coli* and applied to the model, these responses
386 were down-regulated relative to models treated with free SIgA.
387
388 Barrila and Yang et al. reported advancement of a 3-D HT-29 colon model to include phagocytic
389 macrophages, thereby improving its physiological relevance to study aspects of the innate
390 immune response to infection (172). Characterization of this co-culture model revealed
391 macrophages integrated between and underneath epithelial cells, while preserving epithelial
392 tight junctions and presence of multiple epithelial cell types, including enterocytes, M cells and
393 goblet cells (172). Macrophage phagocytosis was confirmed by evaluating their ability to engulf
394 inert, bacteria-sized beads. Contribution of macrophages to *Salmonella* infection was assessed
395 using *S. enterica* pathovars with differing host tropisms and disease phenotypes, including the

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396 well-studied sequence type ST19 Typhimurium strain SL1344, which causes disease in a wide
397 range of hosts, the multi-drug resistant ST313 Typhimurium strain D23580, and the human-
398 specific *S.* Typhi strain Ty2. Although classified as Typhimurium, ST313 strains display genome
399 degradation similar to human-adapted Typhi, and are associated with devastating epidemics of
400 blood-borne infections in sub-Saharan Africa (216). Bacteria were cultured aerobically or
401 microaerobically prior to infection to simulate oxygen environments encountered before and
402 during intestinal infection. Colonization of all strains was reduced in the co-culture model
403 containing macrophages relative to the epithelial model, indicating antimicrobial function of
404 macrophages. Although ST313 are considered 'highly invasive' due to the systemic infection
405 they cause, D23580 was not highly invasive in the 3-D models, but instead exhibited enhanced
406 survival/replication, thus providing clues as to what drives their pathogenicity. Pathovar- and
407 oxygen-specific differences in host cell co-localization patterns were also observed (**Fig. 1B,**
408 **panel g**), indicating the ability of these advanced models to distinguish between closely related
409 *Salmonella* serovars, thus providing a unique advantage over models composed of a single cell
410 type (172).
411
412 RWV-derived intestinal models are also valuable for investigating host-pathogen interactions for
413 which conventional cultivation strategies are unable to adequately model *in vivo* complexity.
414 Recently, a 3-D colonic model was applied to study human CVB (147); a pathogen for which *in*
415 *vitro* and *in vivo* models may not fully model the enteral infection route in humans (147, 217-
416 220). Comparisons between polarized 2-D and 3-D cells revealed that the 3-D model displayed
417 an enhanced number of viral particles secreted into the media at early stages of the viral life
418 cycle, which did not coincide with increased host cell destruction relative to monolayers (147).
419 These data suggest that 3-D models exhibit an enhancement in non-lytic release of viral
420 particles, which might result from morphological changes (e.g., enhanced brush border
421 formation) in 3-D cells. Similarly, another 3-D colonic model was used to study *Cryptosporidium*

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422 *parvum*, a parasite for which there is a lack of physiologically relevant *in vitro* and *in vivo* models
423 (144). Following *C. parvum* infection, morphological changes were observed that were
424 consistent with those from colonic biopsies of infected patients (144). These studies further
425 emphasize the critical importance of model complexity and physiological relevance as
426 determinants in enabling host-pathogen interactions.
427
428 In summary, 3-D RWV intestinal models are powerful tractable research tools that advance the
429 study of host-pathogen interactions. These models can be modularly altered to incorporate
430 different cell types (including patient-derived cells), ECM, commensal microbiota, physical
431 forces, etc, akin to *in vivo* scenarios, increasing their relevance. Their tissue-like architecture,
432 differentiation and polarization, enhanced expression/localization of junctional proteins, and
433 mucin production are necessary components of an effective barrier to invading pathogens.
434
435 *Limitations and future directions of RWV-derived 3-D models*. Although many key
436 structural/functional characteristics of parental tissues have been successfully recapitulated
437 using RWV models, several limitations remain. The full extent of 3-D architecture, multicellular
438 complexity and array of physical forces of *in vivo* tissues has not yet been attained. Ongoing
439 studies are further enhancing these features, plus incorporating patient-specific immune cells
440 and fecal microbiota, and achieving vascularization and innervation. Models can be costly due
441 to high media consumption required for culturing large numbers of cells, however researchers
442 can scale down. Although bead porosity facilitates apical/basal cytokine secretion and there is
443 excellent access to the apical side of the models, there is currently an inability to sample the
444 basal side. This also prevents measurement of transepithelial electrical resistance (TEER),
445 which measures electrical resistance across a monolayer as a proxy for assessing barrier
446 integrity (221). The technique involves using two electrodes, one in contact with cells on a
447 semipermeable membrane (e.g., apical side) and the other in a different chamber containing

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448 culture medium (e.g., basal side). With most RWV models grown on tiny (~175 μm) microcarrier
449 beads, these measurements are not currently possible with off-the-shelf technology. This
450 challenge will likely be surmounted with custom electrode design to accommodate current RWV
451 models or the use of alternative scaffolds. Currently, immunofluorescence imaging of
452 cytoskeletal and tight junctional markers represents an alternative method to evaluate model
453 integrity. As these models grow in size and complexity, introduction of vasculature and nerve
454 cells will be important. Finally, current models are not easily amenable to chronic infection due
455 to lack of perfusion once removed from the RWV, however inclusion of automated waste
456 removal and nutrient delivery during infection will facilitate this approach.
457
458 **3-D organoid models.** The term organoid ('organ-like') has been used to describe a variety of
459 3-D models that resemble *in vivo* tissues. Historically, this included models engineered with
460 different technologies using cell lines, stem cells, primary cells, or tissue explants either
461 embedded in, or cultured on top of, ECM scaffolds that allow cells to self-assemble into 3-D
462 structures (8, 12, 144, 146, 147, 170, 172, 222-230). Advances in stem cell biology led to a
463 recent terminology shift to more specifically define organoids as 3-D models derived from stem
464 cells, progenitor cells or primary explants (223, 231-239). Herein, we focus on 3-D models
465 cultured within a 3-D ECM matrix that fit this definition. It is important to emphasize that current
466 models are based on decades of work by pioneering cell biologists that laid the foundation for
467 the current organoid field (reviewed in (12)), representing an advancement and merging of old
468 and new technologies to enable novel discoveries (12, 229, 240). Models cultivated using thick
469 ECM matrices have deep roots in tissue engineering and cancer biology, where they were
470 applied to develop advanced models enabling the study of a variety of biological mechanisms,
471 particularly with regards to understanding the interrelationship between tissue structure and
472 function (12). This effort resulted in a critical mass of scientists who now recognize the

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473 importance of 3-D models for infection and are bringing elegant advances to the field, but may
474 not be fully aware of their historical context.
475
476 A range of different organoid models have been established, including small and large intestine
477 (230, 231, 233, 235, 241-269), lung (270-275), stomach (276-283), breast (55, 284, 285), brain
478 (286-288), liver (223, 289, 290), pancreas (223, 291, 292), gall bladder (293), eye (294), kidney
479 (295), prostate (223, 296, 297) and reproductive tract (298, 299), among others. Relative to
480 monolayers, these models more closely mimic endogenous tissues, including organization and
481 spontaneous differentiation of multiple cell types into physiologically relevant 3-D structures
482 (**Fig. 1B, panel e**), expression and localization of tight junctions, mucus production, polarity,
483 gene expression, cell viability and proliferation, cytokine production, responses to antimicrobials,
484 support of commensals and susceptibility to infection (12, 55, 223, 227, 229-236, 238, 239, 241-
485 267, 270-320).
486
487 To develop 3-D organoid models, stem cells or tissue explants containing stem cells are used.
488 Biopsies may be treated with a dissociation agent and/or mechanically disrupted prior to
489 embedding into ECM. Stem cells isolated from biopsies can be pre-differentiated into progenitor
490 cells and further differentiated into ECM-embedded organoids. Differentiation into committed
491 cell types is enabled by stepwise supplementation and/or removal of signaling factors during
492 culture (250, 252, 253, 255, 265, 276, 279, 304, 321-323). Purified ECM components and
493 mixtures can be used, including Matrigel, a laminin-rich ECM isolated from chondrosarcomas
494 (324, 325). Synthetic hydrogels help circumvent challenges associated with Matrigel, including
495 batch-to-batch variation and potential carcinogenic issues connected with tumor-derived
496 matrices (230).
497

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498 *3-D intestinal organoids.* Sato et. al. (250) and Ootani et al. (254) independently reported
499 conditions enabling long-term *in vitro* culture of mouse intestinal crypts containing Lgr5+ stem
500 cells (as well as purified Lgr5+ stem cells that generate villus-crypt-like structures (250)). These
501 approaches used either Matrigel (250) or collagen (254) in combination with supplementation of
502 Wnt agonist R-spondin1. Sato et al. also included epidermal growth factor to enable crypt
503 growth, and noggin to facilitate passaging (250). These models displayed a polarized,
504 multicellular epithelium (enterocytes, goblet cells, Paneth cells, enteroendocrine cells) organized
505 into a central lumen lined by villus-crypt-like structures (250, 254). Murine intestinal organoids
506 developed from single Lgr5+ stem cells also developed into these multicellular structures (250).
507 Subsequently, additional factors were included to enable human colonoid culture (265).
508
509 The NIH Intestinal Stem Cell Consortium defined a standardized nomenclature to reflect model
510 sources, approaches and *in vitro* structures (326). Structures directly isolated include epithelial
511 sheets, crypts and organoids (crypts and surrounding mesenchymal elements) (326). Various
512 structures produced *in vitro* from small intestine include enterospheres (rounded epithelial cyst-
513 like structures); enteroids (formation of budding crypts from enterospheres); and induced
514 intestinal organoids (multicellular clusters from induced embryonal or pluripotent stem cells;
515 e.g., induced human intestinal organoids) (326). Analogous colonic structures include
516 colonospheres, colonoids and colonic organoids (326). It is common to see terms used
517 interchangeably and the nomenclature will likely evolve as the field expands.
518
519 Model infection can be accomplished by: 1) addition of pathogen directly to the media (basal
520 side), 2) microinjection into the lumen (**Fig. 1B, panel b**), 3) shearing of models followed by
521 pathogen addition, and 4) disruption of 3-D models into flat monolayers followed by pathogen
522 addition (231, 238). Consideration of the normal infection route is critical. Direct addition to the
523 media is easiest; however, for pathogens that infect via the apical/luminal side, this represents a

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524 non-physiological route of infection. Microinjection is technically challenging, but preferable for
525 pathogens that normally infect from the lumen. Due to challenges associated with
526 microinjection, there is a growing tendency to mechanically dissociate organoids into smaller
527 pieces or completely dissociate into monolayers on Transwell inserts or plastic (238, 262, 282,
528 314, 315). This approach has been successful for a number of studies, including cultivation of
529 norovirus (315), a major advance in the field. Use of Transwell inserts also facilitates TEER
530 analysis and easier cytokine sampling from the apical/luminal side of the model.
531
532 When dissociating 3-D models prior to infection, it is important to note that this disconnects their
533 form and function similar to disrupting primary tissue into monolayers and may render them less
534 predictive for some (not all) phenotypes. In this approach, use of Transwell inserts are
535 preferable over plastic as the former display improved physiological relevance over conventional
536 monolayers (327). Additional profiling should confirm the extent to which the dissociated model
537 may have de-differentiated and additional culture time may be required to re-establish
538 polarity/barrier function. Key findings should be validated using intact organoids and
539 microinjection to avoid artifacts. Additionally, since ECM-pathogen interactions are important for
540 infection dynamics (61), infection surfaces should not contain ECM components not typically
541 found in that location *in vivo* (e.g., lumen) if the pathogen would not normally encounter it.
542
543 A variety of pathogens have been studied using 3-D enteroid/colonoid/organoid models
544 including *Salmonella*, *C. difficile*, EHEC, EPEC, Enterotoxigenic *E. coli* (ETEC), Norovirus,
545 rotavirus, enteroviruses, *Toxoplasma gondii* and Coronaviruses (231, 232, 234-236, 239, 241-
546 246, 258-264, 267, 269, 308-320, 328). The first infection using induced human intestinal
547 organoids (iHIOs) was performed using human rotavirus, which lacks robust *in vitro* culture
548 systems (316). Both laboratory and clinical rotaviruses replicated in iHIOs and were detected in
549 epithelial and mesenchymal cells (316). Crypt-derived enteroids also supported rotavirus

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550 replication and were used to assess antiviral efficacy against patient isolates (245, 267).
551 Ettayebi et al. made a significant advance by the successful *in vitro* culture of human norovirus
552 (HuNoV), known for its lack of a reproducible culture system (315). The authors initially cultured
553 3-D intestinal organoids and then dissociated them into monolayers on plastic or Transwell
554 inserts (315). Successful viral replication was observed and only enterocytes were infected with
555 HuNoVs regardless of strain or intestinal region from which the model was derived. Additional
556 viral models including those using enteroviruses (e.g., CVB, Echovirus 11, and Enterovirus 71)
557 have identified the cell type-specific nature of these infections and the virus-specific nature of
558 innate immune signaling in response to infection (328).
559
560 Enteroid models were also used to study *S.* Typhimurium and *E. coli*. Zhang et al. (241) and
561 Wilson et al. (244) used crypt-derived enteroids to study *Salmonella* infection. *S*. Typhimurium
562 successfully colonized the model (241, 244) and infection responses aligned well with *in vivo*
563 observations, including disruption of tight junctions, inflammatory responses, and decreased
564 stem cell numbers (241). Forbester et al. infected iHIOs with *S*. Typhimurium and observed
565 physiological transcriptomic and cytokine profiles (258). Injection of *E. coli* O157:H7 into iHIOs
566 containing neutrophils led to loss of actin, epithelial integrity disruption, induction of
567 inflammatory cytokines, and neutrophil recruitment (**Fig. 1B, panel h**) (261). In contrast,
568 commensal *E. coli* was retained within the lumen with no loss of model integrity. Infection of
569 colonoid-derived Transwell models identified MUC2 and protocadherin-24 as early EHEC
570 infection targets (262). Colonoids were initially cultured in 3-D followed by dissociation onto
571 Transwells. Model differentiation correlated with expression of differentiation markers, increased
572 TEER, and microvilli (262). EHEC preferentially colonized the differentiated model relative to an
573 undifferentiated control, reducing colonic mucus and inducing microvilli damage. A similar
574 approach was applied to study EPEC and ETEC infections in co-culture models containing
575 macrophages (314). Inclusion of macrophages in the bottom chamber of the enteroid-derived

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576 Transwell model enhanced barrier function, increased epithelial height, and altered cytokine
577 responses relative to the control. EPEC increased total macrophage numbers and induced
578 projections that extended into the epithelium, while ETEC induced macrophage extensions
579 across the epithelium to the apical surface. Presence of macrophages in the co-culture model
580 enhanced barrier function and correlated with decreased numbers of ETEC relative to the model
581 lacking immune cells.
582
583 iHIOs were also used to study *C. difficile* infection (CDI) (259, 260, 263, 264). CDI patients
584 secrete acidic mucus consisting primarily of MUC1, with decreased MUC2 and altered
585 oligosaccharide composition relative to healthy patients (260). Injection of the pathogen alone
586 into iHIOs decreased MUC2, while whole CDI stool supernatant was required to induce patient-
587 like oligosaccharide composition changes (260). iHIOs were also used to investigate non-
588 toxigenic and toxigenic strains of *C. difficile* and purified toxins, TcdA and TcdB (263). Injection
589 of the toxigenic isolate or purified TcdA led to loss of barrier function, while iHIOs injected with
590 the non-toxigenic strain remained intact. Separately, colonoids helped identify Frizzled proteins
591 as receptors for the TcdB toxin (264).
592
593 In summary, 3-D organoid models are advancing mechanistic understanding of host-microbe
594 interactions due to their enhanced 3-D architecture, presence of Lgr5^{+} stem cells together with
595 multiple cell types and other functional properties. In addition, patient organoid 'biobanks' have
596 been established and are facilitating fundamental research and clinical applications (231, 232,
597 329, 330). One exciting example of the applicability of these models is the use of patient-derived
598 organoids to predict drug responses for cystic fibrosis treatment (223, 232, 251, 308, 330, 331).
599
600 *Limitations and future directions of 3-D organoids*. As for other models, organoids have
601 limitations that researchers are working to overcome. Variability and quality control challenges

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602 between experimental preparations includes: 1) heterogeneity in size, shape and viability of
603 organoids within a culture and across different samples; 2) batch-to-batch variability in Matrigel
604 or other ECM, and 3) batch-to-batch variability in growth factor sources. Organoid infection
605 presents challenges as described above. Media cost is high if scaling up due to reliance on
606 specific growth factors. Incorporation of the full array of cell types found *in vivo*, including the
607 diverse collection of immune cells and microbiota has not been attained. Organoid models also
608 lack spontaneous M cell formation (252, 332). Pre-treatment of *in vitro* models with RANKL,
609 exposure to lymphocytes or infection with pathogens like *S*. Typhimurium can induce M cell
610 formation (332-334). Although the mechanism by which M cells spontaneously differentiate in
611 RWV models (11, 153, 172, 176) is unknown, it is possible that the low fluid shear suspension
612 culture environment is important, since flotation of ECM scaffolds was more permissive for
613 differentiation as compared to surface-attached ECM (12, 156, 157). Since organoid models are
614 typically ECM-embedded, another limitation is that the application of the range of biomechanical
615 forces found *in vivo* is limited; however, an iHIO model containing functional neurons that
616 enabled peristalsis-like contractions was reported (257). Combinations of technologies,
617 including organoid-derived 3-D models developed using the RWV bioreactor (203) and organ-
618 on-a-chip (335) are further expanding these capabilities. TEER measurements are also not
619 currently possible with intact organoid models due to their size, structure and because they are
620 ECM-embedded. Some studies have dissociated organoids into 2-D on Transwells to facilitate
621 these measurements, although there can be disadvantages to using this approach, as
622 discussed.
623
624 **Organ-on-a-chip models**. Advanced microfluidic and microfabrication technologies are being
625 broadly applied to develop "organ-on-a-chip" (OAC) models that mimic key aspects of *in vivo*
626 microenvironments. Rather than focusing on recreating the 3-D structure of the entire tissue,
627 this technology aims to recreate a microscale model of the local 3-D architecture and spatial

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628 distribution of dynamic tissue interfaces to mimic tissue- and organ-level functions (336). These
629 devices are designed with micrometer-sized fluidic channels separated by thin, flexible porous
630 membranes that enable development of different tissues in adjacent chambers, while retaining
631 their ability to interact (**Fig. 1B, panel c**) (336-340). These features allow flexibility to model
632 active processes within a tissue, such as vascular-like perfusion. One exciting functional feature
633 engineered into the design of many of these devices is the capability to apply dynamic forces
634 across the tissue to model fluid shear and peristalsis (335, 341-344).
635
636 OAC models vary in complexity, ranging from simple systems containing a single perfused
637 chamber and cell type, to more advanced chips that contain several microchannels,
638 membranes, and assorted cell types, thereby allowing the reconstruction of multiple tissue
639 interfaces (336). Microengineering techniques for these devices have been extensively
640 reviewed (336, 339, 345-350). Chips are commonly made of a silicone polymer called
641 polydimethylsiloxane (PDMS), which is compatible with many cell types and has several
642 advantages, including optical transparency for easy imaging, low cost, flexibility, and high gas
643 permeability (336, 340, 345, 351). PDMS does carry some disadvantages (discussed below), so
644 other options are being explored (351, 352). Depending on experimental requirements, chip
645 design and approaches for tissue development can be altered. Porous membranes can be
646 coated with a variety of matrices/scaffolds (336, 340, 345, 346, 353). Moreover, 3-D bioprinting
647 techniques are facilitating complex spatial patterning of cells and scaffolds (353). Although
648 traditional electrodes used for TEER measurements do not accommodate the small culture area
649 of most OAC models (221), recent studies have integrated custom electrodes (354).
650
651 A variety of OAC platforms have been derived from cell lines, stem cells, and/or primary cells,
652 including small and large intestine (335, 341-343, 354-357), lung (358-362), liver (363-370),
653 kidney (371-373), heart (374-378), cornea (379), skin (380), nervous tissue (381-384), bone

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654 (385, 386), reproductive tract (387), blood/endothelium and blood-brain barrier (388-394),
655 among others. Once developed, these models typically retain their structural and functional
656 integrity for several weeks (model-specific), further lending to their experimental tractability.
657 Similar to the other 3-D models discussed, OAC models exhibit *in vivo*-like characteristics,
658 including spontaneous differentiation into multiple cell types, polarity/barrier function, formation
659 of local 3-D structures (**Fig. 1B, panel f**), responses to biophysical forces, cytokine production,
660 gene expression, mucus production, responses to nanoparticles and drugs, support of
661 commensals, responses to microbial components (e.g., LPS), and/or susceptibility to microbial
662 infections (335, 336, 340-343, 355-378, 380-395). The application of physical forces across
663 several of these models alters physiological responses, including changes in:
664 expression/localization of tight junctions, barrier integrity/function, polarity and differentiation,
665 cell viability, size, morphology, ECM production, integrin expression, enzyme activity, cytokine
666 responses, chemical/gas exchange gradients, molecular transport, drug responses, bacterial
667 colonization, virion-related cytopathic effects, and/or formation of 3-D structures (e.g., villi) (335,
668 341-344, 346, 359, 360, 362, 372, 373, 377, 378, 385, 388, 389, 396). Importantly, several
669 models have been advanced to incorporate immune cells (343, 360, 397). Below we discuss
670 examples of gut-on-a-chip models that have been applied to study pathogens or commensals.
671
672 *Gut-on-a-chip models.* The Ingber laboratory developed a series of 'mechanically active' gut-on-
673 a-chip models and applied them to study host-microbe interactions (341, 343, 344). They initially
674 constructed a PDMS chip containing two microfluidic channels separated by a flexible, porous
675 ECM-coated membrane (341). Colonic cells were seeded in the upper channel under low fluid
676 shear stress (0.006 - 0.06 dyne/cm^2) and medium also flowed in the bottom chamber. The chip
677 was engineered with dual vacuum chambers on either side of the main microchamber to enable
678 application of a physiological cyclic strain across the membrane to mimic intestinal peristalsis.
679 This led to a highly polarized columnar epithelium and spontaneous formation of 3-D villi-like

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680 folds with basal proliferative cells in the crypt region. Model characterization revealed well-
681 formed tight junctions, mucus production, and multiple intestinal epithelial cell types (absorptive,
682 goblet, enteroendocrine and Paneth cells) (341, 342). The ability of this model to support
683 commensal colonization was assessed using *Lactobacillus rhamnosus* (LGG). Colonization of
684 LGG improved barrier function and was supported for greater than a week without impacting
685 model integrity, consistent with previous *in vivo* observations for probiotics. The model was also
686 applied to study host-virus interactions using CVB (**Fig. 1B, panel i**) (344). Exposure of CVB to
687 the apical surface led to successful viral replication, induction of cytopathic effects (CPE) and
688 polarized (apical) release of pro-inflammatory cytokines. Infection of the basal side led to
689 decreased viral titers and lower CPE, with apical secretion of pro-inflammatory cytokines.
690
691 The above gut-on-a-chip model was further advanced to include immune cells (peripheral blood
692 mononuclear cells/PBMCs) and/or endothelial cells (vascular or lymphatic) (343). This
693 combination of models enabled exploration of the interplay between these factors (and others)
694 in bacterial overgrowth and inflammation in the onset of intestinal injury. Synergistic effects
695 between PBMCs and either non-pathogenic *E. coli*, pathogenic enteroinvasive *E. coli* (EIEC), or
696 purified LPS led to altered barrier function and changes in villus architecture. Similarly, the
697 presence of both PBMCs and LPS led to polarized secretion of basal pro-inflammatory
698 cytokines, which stimulates recruitment of additional immune cells in an *in vivo* scenario.
699 Exposure of the PBMC-containing model to a therapeutic formulation of probiotic bacteria
700 increased barrier function. The formulation reduced EIEC-induced intestinal damage in the
701 model lacking PBMCs, but in the presence of immune cells only delayed injury onset. Cessation
702 of cyclic stretching led to enhanced bacterial overgrowth, even under constant media flow.
703
704 *Limitations and future directions of OAC models*. While there are many advantages to OAC
705 models, there are limitations. Many of these models have multiple cell types which exhibit

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706 enhanced 3-D architecture; however, the vast array of native heterogeneous cell types found *in*
707 *vivo* still need to be incorporated and different laboratories are optimizing ECM composition and
708 structure. Along these lines, to our knowledge, no one has yet reported the presence of M cells
709 in gut-on-a-chip models. There is also a strong push for physically linked multi-organ models, or
710 "humans-on-chips" (339, 398). Another limitation is the PDMS material commonly used for chip
711 construction which can absorb small hydrophobic molecules, and interfere with drug screening
712 and cell signaling analysis (339, 351, 352). There are also risks of uncrosslinked PDMS
713 leaching into the culture if the curing process is incomplete, causing cell damage (351, 352).
714 While the small number of cells required can be considered advantageous, in some cases,
715 larger numbers of cells (10^7-10^8) may be required depending on the experiment. Infection
716 studies typically involve many permutations, and it is not uncommon to use several multi-well
717 plates within a single experiment. For example, during colonization assays, samples are
718 harvested at different times and plated for viable bacteria, while others are processed for
719 downstream analyses. Thus, it will be beneficial to incorporate multiple 3-D model systems into
720 infectious disease research depending on the experimental question being addressed, as no
721 single model system is sufficient to address all infectious disease experimental scenarios.
722
723 **Conclusions**
724 Over the past two decades, a multidisciplinary consortia of researchers have been creative in
725 developing 3-D intestinal models of increasing complexity that better mimic the biological,
726 chemical and physical microenvironments of the endogenous tissue for studying host-microbe
727 interactions. These models have been developed using a variety of approaches and are being
728 applied to understand the dynamic relationship between the host, pathogens and commensals
729 that dictate infection outcome and for development of new treatment/prevention strategies.
730 Collectively, these models have ushered in a new era for infectious disease research by offering
731 predictive *in vitro* translational platforms. Indeed, the establishment of 3-D intestinal models and

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732 their application as human surrogates for infectious disease research have provided specific
733 examples of how the study of microbial pathogenesis can be advanced by using appropriate,
734 biologically meaningful models.
735
736 We are still in the infancy of learning how to build more realistic 3-D tissue models and there
737 remain an endless number of questions and hypotheses to test about how infection actually
738 happens in the body. Continued model advancement to better recapitulate the *in vivo* tissue
739 microenvironment coupled with the application of multiple 3-D model systems will lead to
740 increased translation of research discoveries to practical and significant outcomes. Such
741 advances will be pivotal for the success of personalized medicine approaches using patient-
742 specific normal and diseased cells, and incorporation of the full repertoire of immune cells to
743 predict clinical correlates of protection for vaccine development.
744
745 Toward this goal, we must deeply comprehend 3-D tissue/organ structure and function, the
746 associated microenvironment, and the microorganisms to be studied. It is likewise important that
747 we are aware of and acknowledge the rich history and work of researchers who have long
748 applied 3-D tissue modeling to study host-pathogen interactions. Accordingly, we should revisit
749 past research in the field to help us understand and guide our direction. While it remains a
750 daunting task to gain a complete understanding of infectious disease, the alignment of
751 multidisciplinary research teams dedicated to the establishment of 3-D models that reconstruct
752 the architecture and function of the *in vivo* organ and their application for host-pathogen
753 interaction studies make this an exciting time to be a scientist!
754
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759 **Figure legend.**

760

761 **Figure 1. Recreating the complex intestinal microenvironment to study host-pathogen**
762 **interactions. (A)** *In vitro* model advancement from 2-D to 3-D by incorporation of physiological
763 factors to better mimic the *in vivo* environment. **(B)** Three-dimensional approaches routinely
764 used to develop advanced intestinal models: (a) RWV bioreactor, (b) organoids, and (c) OAC.
765 (d) Scanning electron micrograph/SEM showing an RWV colon model. (e) Light micrograph of
766 an enteroid model. (f) SEM of a gut-on-a-chip model. (g) Oxygen-dependent host cell co-
767 localization of *S*. Typhimurium in RWV 3-D co-culture model of intestinal epithelium and
768 macrophages. Following aerobic culture of bacteria, no macrophages were found, but following
769 microaerobic culture macrophages were present and either empty (left inset) or contained
770 internalized bacteria (right inset). Macrophages (CD45, yellow), *Salmonella* (green; white when
771 overlaid with CD45), nuclei (DAPI, blue). Scale bar = 10 µm. (h) iHIOs injected with *E. coli*
772 O157:H7. Nuclei (blue), neutrophils (CD11b, red) and *E. coli* (green). Scale bar = 100 µm. (i)
773 CVB-infected gut-on-a-chip. CVB (green), F-actin (red), nuclei (blue). Lumen shown in (A)
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1921 **Author Bios**

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1924 **Jennifer Barrila** is an Assistant Research Professor at the Biodesign Institute, Arizona State
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1926 received her Ph.D. in Biology from Johns Hopkins University, where she structurally
1927 characterized the SARS coronavirus main protease to facilitate structure-based drug design.
1928 Her postdoctoral work at the Biodesign Institute used innovative culture systems to investigate
1929 how biomechanical forces regulate host and pathogen responses during infection. In 2010 she
1930 was promoted to Assistant Research Scientist and in 2013 to Assistant Research Professor.
1931 Her current research involves the development and application of multicellular 3-D models of
1932 human intestine to investigate the role of cellular biomechanics in host-pathogen-microbiota
1933 interactions. Her biomedical research has flown on several NASA spaceflight missions to the
1934 International Space Station to study the influence of biophysical forces on infection. In 2014 she
1935 received the Thora W. Halstead Young Investigator's Award.
1936
1937
1938 **Aurélie Crabbé** obtained her PhD in Bioscience Engineering at the Vrije Universiteit Brussel,
1939 Belgium. During her PhD she received a fellowship of the Belgian American Educational
1940 Foundation to perform research in the laboratory of Prof. Cheryl Nickerson at the Biodesign
1941 Institute at Arizona State University. She then received a postdoctoral position in the Nickerson
1942 lab, where she developed and used physiologically relevant models of the lung mucosa to
1943 explore host-pathogen interactions. She is currently a team leader in the Laboratory of
1944 Pharmaceutical Microbiology at Ghent University under the tutelage of Prof. Tom Coenye,
1945 through an Odysseus fellowship of the Research Foundation Flanders. Her research focuses on
1946 understanding how the host, microbiome and their interactions influence antimicrobial agent
1947 efficacy and inflammation in chronic lung infections. To this end, *in vivo*-like models of lung
1948 epithelium, the microbiome or both are used, to facilitate translation of *in vitro* results to novel
1949 therapeutic approaches.
1950
1951
1952 **Jiseon Yang** is an Alfred P. Sloan postdoctoral fellow (NASA joint program) at the Biodesign
1953 Institute, Arizona State University/ASU. She received her M.S. in Microbiology studying
1954 *Salmonella* pathogenesis from Pusan National University(South Korea). She subsequently
1955 worked with Dr. Roy Curtiss III and Dr. Josephine Clark-Curtiss(ASU) on developing a
1956 2nd generation lysis system in recombinant attenuated *Salmonella* vaccines to deliver
1957 *Mycobacterium tuberculosis* antigens. She received her Ph.D. in Microbiology under Dr. Cheryl
1958 Nickerson(ASU), where she characterized virulence, stress and molecular genetic responses of
1959 invasive, multidrug-resistant nontyphoidal *Salmonella* to physiological fluid shear. She was co-
1960 first author on the first report of an RWV-derived 3-D intestinal co-culture model (epithelial cells,
1961 macrophages) to study *Salmonella* pathogenesis. She currently studies how microbes inhabiting
1962 built environments influence human health and systems integrity using the International Space
1963 Station as a microbial observatory to reveal new insight into how interspecies interactions within
1964 microbial populations can adapt/evolve over time.
1965
1966
1967 **Karla Franco** received her Bachelor's degree in General Science from Pontificia Universidad
1968 Catolica de Puerto Rico. She became interested in microbial pathogenesis during a year-long
1969 NIH training fellowship (ASU PREP). She enrolled in the Microbiology Ph.D. Program at Arizona
1970 State University, where she received a two year fellowship from the NIH IMSD program. As a

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1971 member of the Nickerson laboratory, she has spent the last three years investigating the role of
1972 mechanotransduction in regulating the phenotypic and molecular genetic responses of
1973 *Salmonella* Typhimurium.
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1976 **Seth D. Nydam** received his doctorate in veterinary medicine from Cornell University and his
1977 Ph.D. from Washington State University. His graduate studies in Dr. Douglas Call's laboratory
1978 centered on the type III secretion system of *Vibrio parahaemolyticus*, after which he joined Dr.
1979 Cheryl Nickerson at Arizona State University for postdoctoral training to explore 3-D cell culture
1980 and its interactions with resident microflora and *Salmonella* pathogenesis. He is currently the
1981 clinical veterinarian at Arizona State University's Department of Animal Care and Technologies,
1982 where he leads the clinical team and provides research support and oversight. His continuing
1983 interests include microbial pathogenesis, animal models in infectious disease research, and
1984 teaching.
1985
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1987 **Rebecca J. Forsyth** received her B.S. in Microbiology from Arizona State University in 2008.
1988 She first joined the Nickerson laboratory as an undergraduate student, was subsequently hired
1989 as an Assistant Research Technologist and was later promoted to the positions of Associate
1990 Research Specialist and Senior Research Specialist. She used a variety of 3-D models,
1991 microbes and model host organisms in her infectious disease research. She was passionate
1992 about using "outside-of-the-box" approaches to solve important biomedical health issues,
1993 including the use of the spaceflight platform and the RWV bioreactor to study microbial
1994 physiology and host-pathogen interactions.
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1997 **Richard R. Davis** is a Senior Research Specialist in the Biodesign Center for Immunotherapy,
1998 Vaccines, and Virotherapy at Arizona State University. He earned his B.A. in Anthropology in
1999 2001 and a B.S. in Microbiology in 2007 from Arizona State University. He joined the Nickerson
2000 laboratory as an undergraduate student, was subsequently hired as an Assistant Research
2001 Technician and was later promoted to the positions of Research Specialist and Senior Research
2002 Specialist. His research over the past eleven years has focused on using both the microgravity
2003 platform (including six spaceflight experiments) and the RWV bioreactor to study the effect of
2004 physical forces on microbial pathogenesis and host-pathogen interactions.
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2007 **Sandhya Gangaraju** received her Master's degree in Biochemistry in 2003 from the University
2008 of Ottawa, Canada. In 2003, she joined the National Research Council, Canada as a research
2009 technical officer and viral facility manager in the department of neurogenesis and brain repair.
2010 She implemented lentiviral technology to deliver neurotropic factors to neural cells and
2011 developed transwell assays to study neutrophil transmigration through endothelial cells. In 2014,
2012 she joined the Biodesign Institute, Arizona State University as a principal research specialist
2013 and compliance officer for the Center for Biosignatures Discovery Automation, where she
2014 managed the cell culture facility, lead experimental design for students and junior staff
2015 members, and optimized working protocols for microfluidic devices for studying three-
2016 dimensional tissue environments. Most recently, she joined the Nickerson laboratory as a
2017 principal research specialist where she uses the RWV and the spaceflight platform to study
2018 microbial pathogenesis and for 3-D tissue engineering.
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2021 **C. Mark Ott** received his B.S. in Chemical Engineering from the University of Texas at Austin in
2022 1982, his M.B.A. from Louisiana State University in 1989, and his Ph.D. in Microbiology from
2023 Louisiana State University in 1998. He has published extensively in the areas of microbial
2024 ecology in spacecraft, human and microbial responses to spaceflight, and the development of
2025 advanced tissue culture models to investigate infectious disease. For the past 20 years, Dr. Ott
2026 has served as a technical lead in the Johnson Space Center Microbiology Laboratory, which is
2027 responsible for mitigating infectious disease risk during human spaceflight. His responsibilities
2028 include the assessment of microbial risk and development of spaceflight requirements based on
2029 vehicle and mission architecture as well as crewmember, food, and environmental monitoring.
2030
2031

2032 **Carolyn Coyne** completed her Ph.D. at the University of North Carolina at Chapel Hill, USA,
2033 where she studied the human respiratory epithelium. She then carried out her postdoctoral
2034 fellowship at the Children's Hospital of Philadelphia (CHOP), Pennsylvania, USA, and the
2035 University of Pennsylvania, Philadelphia, USA, where her research focused on identifying the
2036 mechanisms by which enteroviruses invade the gastrointestinal epithelium and blood–brain
2037 barrier endothelium. She joined the University of Pittsburgh, Pennsylvania, USA, as a faculty
2038 member in 2007, where her work continued to focus on defining the mechanisms by which
2039 viruses breach cellular barriers. Dr. Coyne's laboratory also studies how the human placenta
2040 restricts viral infections. Her research interests also include the development of primary—and
2041 cell line-based models of cellular barriers, focusing on both the GI tract and placenta. Her
2042 research interests include enteroviruses and flaviviruses, with a particular emphasis on the
2043 strategies by which these viruses bypass cellular barriers.
2044
2045

2046 **Mina J. Bissell** is Distinguished Scientist, the highest rank bestowed at Lawrence Berkeley
2047 National Laboratory and serves as Senior Advisor to the Laboratory Director on Biology. She is
2048 also Faculty of four Graduate Groups in UC Berkeley: Comparative Biochemistry,
2049 Endocrinology, Molecular Toxicology, and Bioengineering (UCSF/UCB joint). Having challenged
2050 several established paradigms, Bissell is a pioneer in breast cancer research and her body of
2051 work has provided much impetus for the current recognition of the significant role that
2052 extracellular matrix signaling and microenvironment play in gene expression regulation in both
2053 normal and malignant cells. Her laboratory developed novel 3D assays and techniques that
2054 demonstrate her signature phrase: after conception, "phenotype is dominant over genotype."
2055 Bissell has received numerous honors and awards and is an elected Fellow of most U.S.
2056 honorary scientific academies. She has published over 400 publications and continues to
2057 engage in full-time research, among other scientific activities.
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2059

2060 **Dr. Cheryl A. Nickerson** is a Professor in the School of Life Sciences at the Biodesign Institute,
2061 Arizona State University. She received her Ph.D. in Microbiology from Louisiana State
2062 University. Her postdoctoral training in *Salmonella* pathogenesis was done with Dr. Roy Curtiss
2063 III at Washington University in St. Louis. She initiated her ongoing studies into the connection
2064 between cellular biomechanics/mechanotransduction and host-pathogen systems biology after
2065 joining the faculty at the Tulane University School of Medicine in 1998. Her development of
2066 innovative model pathogenesis systems includes 3-D organotypic tissue culture models to study
2067 host-pathogen interactions, and approaches that characterize pathogen responses to
2068 physiological fluid shear forces encountered in the infected host, and in the microgravity
2069 environment of spaceflight. She received the Presidential Early Career Award for Scientists and
2070 Engineers, NASA's Exceptional Scientific Achievement Medal, is an American Society for
2071 Microbiology Distinguished Lecturer, and was selected as a NASA Astronaut candidate finalist.

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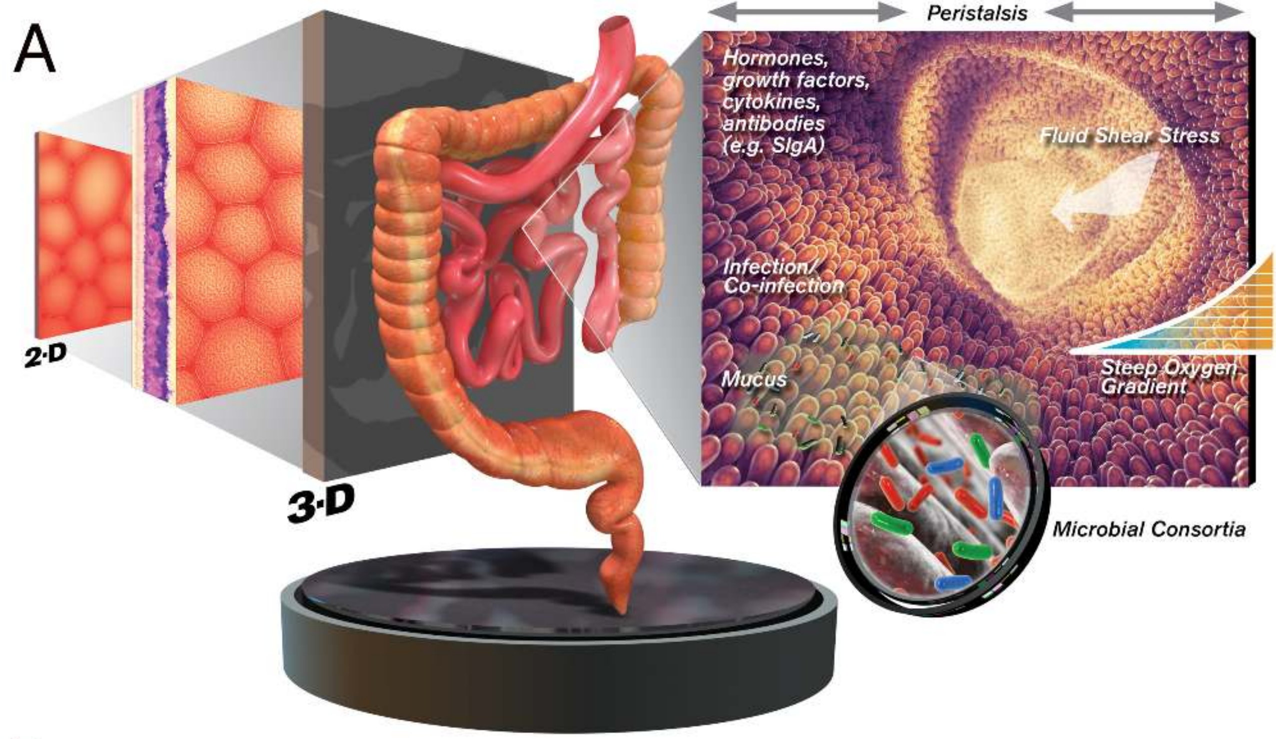

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