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## Microbiome as an immune regulator in health, disease, and therapeutics

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### Abstract

New discoveries in drugs and drug delivery systems are focused on identifying and delivering a pharmacologically effective agent, potentially targeting a specific molecular component. However, current drug discovery and therapeutic delivery approaches do not necessarily exploit the complex regulatory network of an indispensable microbiota that has been engineered through evolutionary processes in humans or has been altered by environmental exposure or diseases. The human microbiome, in all its complexity, plays an integral role in the maintenance of host functions such as metabolism and immunity. However, dysregulation in this intricate ecosystem has been linked with a variety of diseases, ranging from inflammatory bowel disease to cancer. Therapeutics and bacteria have an undeniable effect on each other and understanding the interplay between microbes and drugs could lead to new therapies, or to changes in how existing drugs are delivered. In addition, targeting the human microbiome using engineered therapeutics has the potential to address global health challenges. Here, we present the challenges and cutting-edge developments in microbiome-immune cell interactions and outline novel targeting strategies to advance drug discovery and therapeutics, which are defining a new era of personalized and precision medicine.

### Graphical Abstract

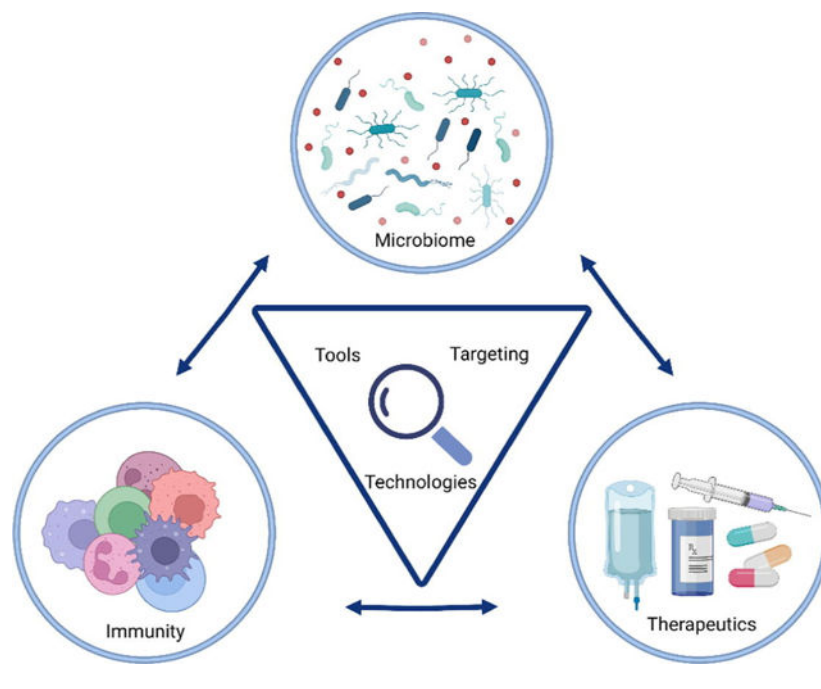
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#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



**Quote:** “Every Time We Have a Chance to Get Ahead, [Microbes] Move the Finish Line.” – Hidden Figures 2017.

## 1. Introduction

A diverse variety of microbes, colonizing by trillions, can be found throughout the human body in locations such as the gastrointestinal tract, respiratory tract, skin, and reproductive system. The communities of bacteria and other microorganisms create a localized microbiome that plays an essential role in health and disease [1]. The microbiome is not just a passive bystander, but it actively impacts multiple host functions, including circadian rhythm, nutritional responses, metabolism, and immunity [2]. Specifically, the microbiome plays multiple critical roles in the programming and development of major components of the host’s innate and adaptive immune system. Initially, the high-throughput DNA sequencing technology involved clustering reads based on bacterial and archaeal 16S ribosomal RNA amplicon sequences, followed by whole metagenomic and meta transcriptomic sequencing [3,4], and more recently multiplexed spatial imaging [5] endeavors are defining the function, spatial localization, and real-time activity of microbiomes and revealing interactions between microbial metabolism and host development.

The microbiome composition can differ markedly between individuals and can fluctuate within individuals based on changes in diet, exposure to new environments, and acute or chronic changes linked to disease. As demonstrated by the Human Microbiome Project, an individual’s early breastfeeding exposure, gender, and other demographic factors play a major role in defining communities of individuals with similar microbiomes [6]. Distinct body habitats have spatially diverse and niche-specific microbiomes present [7], which can lead to diseases and uniquely impact the performance of drugs and drug delivery systems.

A perturbation of the microbial community which overcomes the tolerance capabilities of the body, termed dysbiosis [8], and the expansion of specific microbial species over others, are implicated in the development of multiple diseases [2], including inflammatory bowel disease (IBDs) [9]; autoimmunity and allergic disorders [10]; Alzheimer's [11]; depression [12]; and cancers [13,14]. The gut microbiome is instrumental to the energy metabolism and immune response in the host and perturbations in beneficial host-microbe interactions cause aberrant activation of immune cells, consequently leading to diseases and metabolic disorders that involve immunity, including ulcerative colitis and Crohn's disease [15–18], obesity [19–21], diabetes [22,23], and non-alcoholic fatty liver disease [24], among others. Comparing the microbiomes of healthy individuals to diseased individuals shows that individuals facing acute or chronic illness have less diversity of microorganisms and are more susceptible to infection by foreign microbe populations, known as pathogens, due to the stunted capabilities of their immune systems [25–29].

Therapeutics and microbiome have a bi-directional impact on each other. In a recent drug screen [30], nearly one-fourth of more than 1,000 drugs, marketed for various conditions but not sold as antibiotics, had antibacterial effects when tested against 40 strains of human gut bacteria. In another study, 176 out of 271 drugs incubated with 76 human gut microbes from diverse clades were metabolized markedly to reduce the drug level by more than 20% [31]. By combining high-throughput genetic analyses with mass spectrometry, the study highlighted the causal links of the microbiota with interpersonal variability in microbiomes to interpersonal differences in drug metabolism [31]. These findings have implications for drug discovery, development, and deployment across a spectrum of disease conditions [31,32].

This review describes the human microbiome and its association with diseases and discusses strategies to target immune-microbiome interactions for drug discovery, screening, and development of novel therapeutics, including the modulation of microbial composition, as well as technologies for the improved study of drug-microbe-host interactions. Finally, we examine new directions for the field and discuss the implications these findings will have for medicine and global health moving forward.

## 2. The microbiome and disease

### 2.1. Human microbiome biology and pathophysiology

The human microbiota (or microbiome) consists of bacteria, archaea, viruses, and fungi which colonize and symbiotically reside at various interfaces throughout the body, and it is defined by the genomic expression of these co-residing species [33,34]. These epigenetic expressions and microorganism population densities are not only characteristic of specific organ systems but greatly vary between healthy and diseased states [35–37]. Two main organ systems whose microbiomes are heavily influenced by external factors are the respiratory tract (Fig. 1A) and the digestive tract (Fig. 1B), where the mucosal linings are constantly exposed to bacteria from the host's surrounding environment.

The complex compositions of respiratory and digestive tract microbiomes begin from birth, where initial microbial exposure and colonization stem from the rupture of amniotic

membranes and passage through the birth canal [47–49]. The childbirth delivery mode plays a critical role in the establishment of the early microbiome composition and the child's immune system, especially in the first year of development [50–52]. The delivery mode of birth, such as natural birth versus Cesarean section (C-section), and post-natal treatment affects the enrichment of certain species over others. Mainly, infants delivered by C-section do not receive their mother's commensal genera, such as *Bacteroides*, which are key microbes for immune development, are replaced by opportunistic strains characteristic of the hospital's environment [53,54]. Not only do C-sections lack positive microbes associated with neonatal brain, nervous system, and immune system development [55–57], but infants born through C-section have a higher likelihood of developing respiratory infections [52,58,59], allergies [60–64], asthma [65–67], and other metabolic, inflammatory, and immunological diseases [68–71]. This is followed by additional exposure of microbial species and bioregulation due to unique factors such as environment, breastfeeding, and nutritional choices [72–75].

The microbiome also plays a role in the link between human epigenetics and health. Epigenetics is defined as the study of phenotypic changes due to gene expression changes due to the mechanisms such as DNA methylation and histone acetylation that alter the packing of DNA and subsequently its ability to be transcribed and expressed [76]. These changes are regulated by other identified mechanisms including microbial metabolite crosstalk, diet, exposure to antibiotics, and environmental conditions (pH, oxygen, and temperature) that modulate expression [77]. One key attribute of many pathologies (including cancer, cardiac failure, chronic inflammation, and neurodegenerative diseases) is the overexpression of histone deacetylase (HDAC) which induces tighter packing of DNA on the histone protein and reduces the expression of the genetic code [78,79]. However, many gut bacteria produce HDAC inhibiting metabolites, like butyric and valeric acids, making microbiota vital in the body's ability to counter the initiation and progression of diseases [80]. This is especially important for understanding the link between the microbiota-gut-brain axis, where disturbances in the microbiomes composition or function can lead to both gastroenterological and neurological disorders [81].

Multitudes of factors, like sex differences, diet and nutrition, delivery mode, medication usage, genetics, and environment, play a role in the development of an individuals' microbiomes and can impact how an individual responds to drugs and therapies (Fig. 1C). There is also an observed heterogeneity between the genes of the same microbial species over time in a single individual. This is because bacteria can adapt to changing environmental conditions through a process called horizontal gene transfer (HGT), where genes encoded in the DNA or RNA of one microbe can be directly transferred to another microbe, leading to the addition or replacement of genes in the receiving microbe's genome [82]. This can alter the relationship a bacterium has with its host or allow it to obtain a competitive advantage against other microorganisms in the environment [83,84]. Mechanisms of HGT include transformation (DNA uptake from lysed cells), transduction (DNA introduction from a phage), conjugation (DNA transfer from conjugative pili), and through outer membrane vesicles [83]. Sequencing gut bacterial genomes from different populations worldwide has revealed that HGT frequently occurs in the gut microbiome of individual persons and even more so for those in industrialized populations [85]. From

a clinical perspective, HGT poses certain for drugs that interact with the microbiome extensively, such as antibiotics. The gut microbiome can adapt by acquiring mutations for antibiotic resistance genes, which can then be and then transferred to other local microbes through HGT. The collection of all intrinsic and mobile resistant genes is known as the “resistome” [86]. Studies have shown that individuals with a prevalent “resistome” are highly unresponsive to antibiotic treatment [87]. The emergence of these multidrug-resistant bacteria poses a threat for many in receiving life-saving treatments and must be key in considering treatment modalities moving forward.

The “germ theory of disease”, initially recognized in science and medicine through the work of the renowned “fathers of microbiology”, Robert Koch and Louis Pasteur, stipulates the potential of microorganisms to be disease-causing agents [88,89]. While their contributions elucidated the existence, correlation to specific diseases, and harmful health impacts of certain pathogenic microorganisms, there has been a long-standing tendency to generalize most external microorganisms as “germs”. However, in recent years there has been a paradigm shift in medicine recognizing the positive impacts of many microbial symbionts. For example, one study has shown that gardening is great for mental health not only as a recreational activity, but through an individual’s exposure to *Mycobacterium vaccae*, a bacterium found in the soil [90]. *Mycobacterium vaccae* has been shown to stimulate the host’s serotonin production, leading to a range of health benefits, especially for individuals who suffer from stress-related psychiatric diseases [91]. It has also been utilized by researchers in many therapeutic developments for stress-resilience [92], cognitive function [93], asthma [94], allergies [95], and tuberculosis [96].

In similar attempts to manipulate microbial populations through therapeutic means, supplementing prebiotics and probiotics into food have demonstrated positive impacts for gastrointestinal health. Prebiotics are defined as dietary components and chemicals that promote the growth of certain residing gut microbes over others; in contrast, the term probiotics refers to the direct delivery of bacteria cells to the host for integration into the microbiome [97]. Identification of probiotic bacteria has allowed for progress in the treatment of dysbiosis-linked diseases. For example, the *Lactobacillus* species have both health-promoting and protective properties against metabolic diseases [98].

Microorganisms are not only being increasingly appreciated for the dynamic roles they play in maintenance and homeostasis but are at the brink of therapeutic translation with more than 40 clinical trials seeking to obtain FDA (Food and Drug Administration) approval for targeted, therapeutic application [99]. There are various drug administration routes, often classified through the location at which the drug is administered and chosen carefully based on convenience, compliance, and the drug’s pharmacokinetics and pharmacodynamic profile [100]. With the convenience and cost-effective nature of oral administration, there is no surprise why this route is the most utilized in the field. For these drug formulations, the primary site of drug absorption is usually the small intestine, where several microbes reside, and the bioavailability of the medication is influenced by the amount of drug absorbed across the intestinal epithelium. [100] Yet, while manipulating microbiota for therapeutics may be promising in theory, the complex workings of the human body and microbiome are

not mutually exclusive studies have demonstrated several confounding variables, including nutrition may impact their efficacy and long-term benefits.

## 2.2. Challenges surrounding microbiome therapeutics

The therapeutic manipulation of gut microbiome-associated diseases remains challenging. A ten-year assessment indicates that ~30–50% of patients with IBD do not respond to current anti-inflammatory treatments, necessitating the need for new therapies [101]. For example, the delivery of bilirubin, an immunosuppressant, a potential therapeutic biomolecule for IBD has shown protective effects in experimental colitis [102]; however, its hydrophobic nature has limited its delivery efficiency and use in clinical translation. Another major challenge in microbiome research is a limited understanding of how to address the growing global phenomenon that is antimicrobial resistance. Although several global actions have been taken to address antimicrobial resistance (AMR), such as the World Health Organization's 2015 endorsement of the Global Action Plan (GAP) on AMR [103], it remains an area of concern for international coordinated action [104–107].

Critical work by Dr. Jeffrey Gordon has established the importance of understanding the microbiome in the lens of immunity, drug delivery, and nutritional sciences and the impact this knowledge can have on global health, particularly with malnutrition [108–110]. Severe acute malnutrition among children has been addressed in developing countries through standardized treatment protocols involving nutritional intervention [108–110]. However, there has long been a lack of understanding on how this intervention affects the gut microbiome composition, and how that correlates with therapeutic outcomes. One study conducted by Gordon and colleagues on a cohort of Bangladeshi children identified that children with a characterized immature or underdeveloped core microbiota were more likely to be malnourished, even amongst twins and triplets included for consideration of genetic and environmental factors [111]. This study established that early gut microbiome composition correlates to the development of malnourishment.

Continued work from Gordon and colleagues has applied this knowledge to the development of carefully designed nutritional formulations for modulation of the gut microbiome [108]. Their revised formulation, called microbiota-directed complimentary food 2 (MDCF-2), when compared to the standard supplementary food in a one-month trial on children between 12 and 18 months of age, was able to alter the microbiota composition of aged-matched healthy children and indicated improved health status of malnourished children as shown through plasma protein markers [108]. Similarly, to use the complimentary food approach to address and modulate microbiota composition in obesity, the group generated snack prototypes using plant fibers from different sustainable sources and were able to identify fiber specific alterations in the microbiome linked to changes plasma proteomes which indicate altered physiology [112]. A limitation to this study however is that it was performed on gnotobiotic mice, meaning it still lacks much work to translate to humans. These studies have contributed largely to the field, but additional investments into these efforts are direly needed, especially for improving the nutritional status of the global community [112].

Given that the gut microbiome is highly modulated by diet, another study aimed to determine how dietary intervention with plant-based fiber and fermented foods influence both the microbiome and immune system of healthy adults [113]. After a 17-week, randomized study using multi-omics such as proteomic and metagenomic sequencing alongside immune profiling, Wastyk et al identified that the two diets influenced the gut microbiota composition and host immune system distinctly. The consumption of fermented foods decreased levels of inflammation in participants, gathered through immune profiling of signaling proteins, cytokine levels, and immune cell composition in blood. In both diets, there was a high correlation between immune cell frequency and the short-chain fatty acids (SCFAs) found in the stool, specifically with an increase in B cell frequency observed with an increase in the SCFA, butyrate. The increase in microbiome diversity observed in the healthy participants from this study also correlated to the decreases in inflammation-linked signals and activity. Finding these links between diet and the immune status of individuals can potentially be used in therapeutic guidance and development.

Historically, it has been challenging to obtain enough subjects from different ethnic and geographic backgrounds in the study of microbiome compositional differences and responses. The inclusion of broader ranges of subjects in future microbiome research studies, such as those from underdeveloped countries and minority ethnicities/races, could help to gain a deeper understanding of the human microbiome in the lens of global health disparities and epidemiology [114]. Inclusion of individuals from non-Westernized countries is especially important for microbiome science given that recent literature points to there being extensive numbers of unexplored human microbiome species mainly harbored by individuals from these areas of the world [115]. With a more complete picture of the human microbiome comes the ability for us to use this information for improved drug design and delivery and more accurate tailoring of therapeutics.

The considerable variation in the microbiota between individuals, the limited understanding of the role played by microbiome across tissues and organs, and variation in drug metabolism impose a significant challenge to the next phase of research in this area. Technological advancements have enabled the discovery of the bidirectional effects of the microbiome on pharmaceuticals and therapeutics, however obtaining a deeper understanding of the interactions between microbes, immunity, and medicine is critical for the identification of novel therapeutic targets and improvements on current treatment modalities. The dysbiosis of the microbiome in the gut has been seen to have some level of cross talk on other organs, such as the lung through a proposed gut-lung axis [116]. Like the gut, the lining of the lungs also produces mucus that protects the body from the external environment that it is exposed to during breathing. The microbiota that resides within the lung plays a role in promoting immune tolerance [117]. Given the complexities of the microbiome-immune microenvironment in the gut and lungs, it is of vital importance for advancing personalized medicine to evaluate and effectively model how the microbiota affects relevant pathophysiological systems and employ methods to target immune-microbiome interactions.

### 3. The microbiome and immunity

The body's immune system orchestrates multiple regulatory functions and protective responses against pathogens in the external environment around us. These immune responses can be classified either as innate, being the first and fastest line of defense in the body, or adaptive, which constitutes the building of long-term, memory responses. The respiratory and digestive tracts are among the key mucosal immunity sites, which also have an active microbiome composition that shapes the local and systemic immune response. In fact, the microbiome can cause immunomodulation, such as an activation or suppression of immune function [118–120]. Hence, an in-depth understanding of the interplay between the immune system and microbiome is necessary for developing effective pharmaceuticals and other therapeutics.

#### 3.1 Innate immunity in the respiratory and digestive tracts

Innate immunity in the respiratory and digestive tracts is mediated through tissue-resident and circulating immune cells, such as macrophages, Natural Killer (NK) cells, dendritic cells (DCs), and innate lymphoid cells (ILCs) [121,122]. These cells are essential in recognizing and reacting to respired allergens, pathogens, and toxins in the respiratory and digestive tracts. The respiratory tract is generally categorized into the upper respiratory tract (nose and pharynx) and the lower respiratory tract (larynx, trachea, bronchi, lungs). The most common bacterial species of the respiratory tract include *Streptococcus pyogenes*, *Streptococcus viridians*, *Streptococcus pneumoniae*, *Corynebacterium diphtheriae*, *Staphylococcus aureus*, *Haemophilus influenza*, *Mycobacterium tuberculosis*, *Bordetella pertussis*, *Klebsiella* spp., *Neisseria meningitides*, *Mycoplasma pneumonia*, and *Pseudomonas aeruginosa*, among others [123]. In addition, the lungs may also harbor viruses (*Adenovirus*, *Rhinovirus*, *Influenza virus*, *Epstein Baar virus*, *Measles virus*, etc.) and fungal species (*Aspergillus* spp., *Candida albicans*, *Candida immitis*, *Candida neoformans*, etc.) [124]. The epithelial cells of the airway play a critical role as the first line of innate immune sensors.

In performing defense functions, the airway epithelium and submucosa regulate barrier tightness, secrete mucus and antimicrobials, and communicate with immune cell subsets through the production of cytokines, chemokines, and growth factors [125]. The airway epithelium can also sense pathogens through pattern recognition receptors (PRR) such as Toll-like receptors (TLR), Retinoic acid-inducible gene I (RIG)-I-like receptors (RLR), protease-activated receptors (PAR), and Nucleotide-binding and oligomerization domain (NOD)-like receptors [125].

Innate immunity in the gut includes mechanisms of immune regulation like that observed in the lungs. However, there are some identified differences between the lung and gut microbiome in terms of bacterial compositions and mechanisms of immunity. For example, the bacteria *Helicobacter pylori* shapes the population of local group 2 innate lymphoid cells (ILC2), the dominant ILCs in the stomach that are dependent on the microbiota and are highly responsive to IL-7-induced proliferation, whose production is instigated by the stomach microbiota [126]. ILC2s can also help in the production of IgA by secreting IL-5, which modulates stomach microbial composition [126]. Apart from the production of proinflammatory molecules and ILC2 signaling, the cellular compartments of the innate



immune system, including dendritic cells (DCs) and macrophages, also play an important role in combating pathogens during the innate immune response in the gut [127]. Here, the macrophages and DC cells can begin to process antigen and present the antigen to naïve T cells, initiating the adaptive immune response.

### 3.2. Adaptive immunity in the respiratory and digestive tracts

In the adaptive immune response, naïve T cells undergo a series of events including activation, proliferation, and differentiation until they obtain the effector T cell phenotype that allows them to mediate the immune response against the pathogen. T cells are directly involved in the killing of the infected cells and the release of regulatory and proinflammatory mediators necessary for clearance of infection. The humoral immune response mediated by B cells makes up the other part of the adaptive immune responses, where the end goal is the production of neutralizing antibodies that allow for the specific targeting and clearance of the pathogen. Several aspects of activation of humoral immune response using drug delivery systems are reviewed elsewhere by us [128].

Adaptive immune responses in the gut and lungs often occur across the multiple mucosal surfaces present in these anatomical locations, including the upper respiratory and gastrointestinal tract. In these sites, there is often both an innate and adaptive response underplay. Inductive mucosal sites are referred to as the mucosa-associated lymphoid tissues (MALT), which include the gut-associated lymphoid tissues (GALT), Bronchus-Associated Lymphoid Tissue (BALT), and the nasopharyngeal-associated lymphoid tissue (NALT) subcategories [129]. Since these areas are susceptible to infections, the body must be prepared with an appropriate immune cell lineup to fight off potential invaders. In each of these locations, there is a unique establishment of communication and crosstalk between microbes and immune cells, and disturbances in this microbial homeostasis can alter immune responses [130,131].

Mucus, a naturally produced biofluid, contains mucins, salts, lipids, proteins, and cellular signals that vary based on anatomical location and the function of the corresponding organ system [132]. Since mucus is constantly being created, degraded, and cleared along the mucosal linings, different areas vary in thickness, viscoelasticity, and performance of the mucus with maintaining and affecting microbiota [133]. In the gut, goblet and specialized mucous cells synthesize and secrete mucus to protect the secondary lymphoid organs (SLOs) within the MALT from exposure to the external environment [132]. SLOs are areas of immune lymphocyte cell activation and can be observed in the MALT as small clusters of B cells, T cells, and dendritic cells called Peyer's Patches. Stromal cells in Peyer's Patches, known as fibroblastic reticular cells (FRCs), play a key role in priming the intestinal microenvironment, especially during organogenesis [134]. During the development of Peyer's Patches, FRC lineages converge during organogenesis. These specific cell types govern intestinal immunity, including immune responses to SARS-CoV-2 antigen and other immunogens [134,135].

The MALT is covered and protected by a mixture of different specialized epithelial cells that form crypts and villi structures along the intestine [129]. Key signals and cells such as EMC3 and LGR5 + intestinal stem cells allow for maintenance and intestinal homeostasis

[136,137]. Within this epithelial lining are microfold (M) cells that allow for transport of luminal antigen toward the Peyer's Patches, allowing for immune activation, production of antibody-secreting cells and plasmablasts, and regulation of immunoglobulin A (IgA) in the gut [138,139]. Although studies have been conducted and have identified patterns among microbiota and immune dysfunction, the knowledge of the specific microbiota members and the mechanism in which they cause these effects is still limited [140]. The microbiome can imprint a class of T cells in the MALT called mucosal-associated invariant T cells (MAIT) which recognize microbial metabolites presented by MHC class I [141]. Studies by Biram et al have also identified key mechanisms which differ in the Peyer's Patches compared to the draining lymph node and spleen [142]. They not only identified the sites in the Peyer's Patch where B cell selection occurred and the cellular events that occurred before germinal center formation but also showed how the B cell receptor (BCR) regulated infiltration of B cells specific to the antigen in the germinal centers (B cell maturation process [128,143–145] and impeded clonal expansion in the subepithelial dome [142]. Another key finding with Peyer's Patches includes data that suggests that Gut-associated germinal centers undergo clonal selection and antigen-driven maturation in the absence of infection or immunization that is tunable via the presence and diversity of microbiota [146].

Other key environmental factors, like dietary fiber metabolites and exposure to microbiota over time since birth, also regulate lymphoid cell responses and affect resistance to immunopathology [147,148]. Morphologically, PPs have B cell niches, a subepithelial dome (SED) made up of a mixed cell cocktail of B and T cells, stromal cells, macrophages, and dendritic cells, as well as a follicle associated epithelium (FAE) [149]. The FAE consists of specialized intestinal epithelial cells and microfold (M) cells that allow for the transport of luminal antigen and bacteria [149]. When exposed to antigen and T cell-dependent activation, the PP's naive B cells can become activated, form a germinal center (GCs) and undergo proliferation, somatic hypermutation (SHM), and affinity-based selection before differentiation into memory B cells [150]. The process of somatic hypermutation is initiated by the presence of activation-induced cytidine deaminase (AID) [151]. Somatic hypermutation diversifies the V region of B cell genes through the introduction of point mutations that are selected for providing greater affinity for the antigen [151]. Class switch recombination, in contrast, does not affect the V region but increases the functional diversity of immunoglobulins by replacing the C<sub>μ</sub> region in the immunoglobulin gene, which is first expressed with another heavy-chain C region to produce IgG, IgA, or IgE antibodies [151]. T cell cytokines and receptors, like CD40L, play an important role in B cell stimulation, activation, and class switching to different antibody types [151]. These processes and responses have a crucial role in host defense against pathogens whose surface antigens cannot elicit peptide-specific T-cell responses. Under conditions where a pathogen is detected by lymphoid follicles, germinal centers form and respond.

The vast complexity of B cell and T cell activation and function in response is characteristic of the microbiome's homeostatic conditions and is dependent on the form of associated inflammation or disease state. Maintaining homeostasis is critical for an individual to remain healthy, and there are several ways in which adaptive immune cells contribute to stabilizing the microbiota or that are indicative of dysbiosis. In a positive case, the body's detection of the receptors TLR2-, MyD88, and PI3K-from bacterial lysates all resulted in the increased

proliferation of B cells producing IL-10, which both inhibits T cell activation and the release of proinflammatory cytokines by macrophages [152,153]. The resulting effect of this induction stabilized the mucosal homeostasis and relieved T cell-mediated colitis. In the case of Inflammatory Bowel Disease (IBS), the transfer of an IBS microbiota into a murine germ-free gut resulted in an increased number of T helper cells producing IL-2 (Th2) and IL-17 (Th17), which are critical for T cell growth factor and linking T cell activation to neutrophil innate immunity, respectively [154,155]. There was also a decrease in ROR $\gamma$ t + regulatory T cells (Treg), which help maintain tolerance to disturbances in the microbiota. Thus, from the increase in inflammatory response from the Th cells and loss of tolerance correlated to the loss of Treg cells, investigators were able to better understand the pathogenesis of IBS related to the microbiota.

Understanding how the introduction of microbes induces specific immune responses is especially important for predicting how a patient may contract a disorder through exposure to specific classes of microbes, or even how to develop preventative healthcare measures to entirely avoid these pathologies. Physical triggers are especially important to consider. The induction for Th17 cells, for example, is dependent on whether microbes can adhere to the intestinal wall within the gut. A study by Atarashi et al. demonstrated how Th17 cells were induced only when microbes had the ability to adhere to the epithelial cells lining the intestinal wall, whereas adhesion-defective microbes did not yield a Th17 induction [156]. There are also studies focused on how immune cell populations change over the lifetime of a tumor. Yu et al demonstrated with murine models that during tumorigenesis a high prevalence of CD8 + IFN + T cells within a tissue that develops a tumor and the other neighboring tissues, results in a greater likelihood of developing multiple tumors in subsequent stages of tumor metastasis [157]. Despite the important findings we can gain by studying the impacts of adaptive immunity on microbiota in healthy and diseased states, it is important to remember that microbiota composition is correlated to an individual's geographic population and can have a tremendous impact on an individual's responses to different bacterial infections, as demonstrated by Porras et al [158].

#### 4. Bidirectional impacts of microbiome and therapeutics

The human microbiome not only can affect host metabolic functions but also has powerful capabilities in the modulation of pharmacotherapies since microbes affect the pharmacokinetics and pharmacodynamics of drugs [159]. This unique balancing act between therapeutic drugs, the microbiome, and the targeted host has been complemented with the birth of Pharmacomicrobiomics [159]. There is a bidirectional impact of the microbiome on therapeutics and of therapeutics on the microbiome, although the mechanisms of these interactions are still not well characterized, especially when considering the role of immune cells and processes in the mix.

Several population studies have been conducted to evaluate the effect of common drugs on the microbiome and have found specific perturbations in those taking the identified drugs, either via a decrease or increase in certain taxa [160]. These bidirectional changes illustrate how commonly used drugs play a role in bacterial strains and population dynamics. Proton pump inhibitors are a class of drugs that are commonly used in the treatment of

Gastroesophageal reflux disease (GERD), peptic ulcers, and *Helicobacter pylori* (*H. pylori*) infection. These drugs alter specific taxa in the gut microbiome and can lead to decreased colonization resistance and the development of enteric infections, including *Clostridium Difficile* infections [160,161]. A population-based study found that those using Paracetamol and opioids had a higher abundance of *Streptococcaceae*, which plays a role in metabolic disease and function [162]. Selective serotonin reuptake inhibitors (SSRIs), which are commonly used for depression were negatively associated with *Turicibacteraceae* abundance [163]. Also, inhaled anticholinergic inhaled medications have been negatively associated with *Ruminococcaceae* and *Peptococcaceae* abundance and alpha composition which suggests that non-oral drug administration might also indirectly influence the gut microbiota [162]. The question of whether non-antibiotic drugs can also have some properties that affect microbial populations remains. One major study conducted to address this was able to assess over 1000 commonly used drugs that were identified as non-antibiotics. From this study, more than 24% of the drugs inhibited the growth of at least one bacterial strain in vitro [30].

Two different modes of action have been proposed to explain how drug usage influences gut microbial composition [160]. First, that drugs can facilitate the translocation of the microbiome from other sites in the body to the gut, thereby inducing dysbiosis. Second, that drugs directly affect the growth of bacteria due to interactions with the microenvironment. This second mode points toward the decrease of certain bacteria in a similar mechanism. Aside from the impacts that the microbiome has on drug metabolism and vice versa, there is significant potential for bioengineering approaches that use microorganisms as a therapeutic. For example, one study reported the successful use of a bacteriophage to decrease mortality by the alcoholic fatty liver through selective removal of *Enterococcus faecalis* bacteria, which is responsible for producing cytolytins that lead to deterioration of patients with alcoholic hepatitis [164]. However, the microbiome can potentially function through a third mechanism – modulation of the immune response.

Recently, a paradigm shift is seen in associating the interactions between the microbiome and cancer immune therapeutics in the development of anti-tumor efficacy. Various studies have shown that the gut microbiome may immunomodulate antitumor innate and adaptive immune responses. Paulos et al showed, using mouse models, that microbial translocation can augment the function of adoptively transferred self/tumor-specific CD8 + T cells through toll-like receptor (TLR4) signaling [165]. Sivan et al. showed that commensal *Bifidobacterium* can promote antitumor response to anti-PD-L1 checkpoint inhibitors [166]. They compared murine melanoma tumors in mice harboring distinct commensal microbiota and established differences in antitumor immunity. These differences were eliminated upon cohousing the mice or after fecal transfer. Lida et al further showed that gut microbiome dysbiosis using germ-free mice or antibiotics impaired the response of subcutaneous tumors to CpG-oligonucleotide immunotherapy and platinum chemotherapy [167]. These effects were through the modulation of tumor microenvironment where disruption of microbiome resulted in a poor response from tumor-infiltrating myeloid-derived cells, lower cytokine production and tumor necrosis after CpG-oligonucleotide treatment.

However, the relevance of the microbiome in anti-cancer therapy was only recently established in humans. Gopalakrishnan, V., et al. reported that the gut microbiome modulates response to anti-programmed cell death 1 protein (PD-1) immunotherapy in melanoma patients [14], thus, looking into manners in which maintenance of healthy gut flora could help in optimizing the efficacy of cancer immunotherapy treatments. Analysis of fecal microbiome samples of patients that responded to anti-PD1 therapy versus non-responders revealed significantly higher alpha composition, the relative abundance of *Ruminococcaceae* bacteria, and functional differences in gut bacteria in responding patients, including enrichment of anabolic pathways. In another study, Routy *et al.* showed that low levels of the bacterium *Akkermansia muciniphila* due to antibiotic consumption was associated with poor response to anti-PD-1 inhibitors in patients with epithelial lung and kidney cancers [168].

Gut microbes can also contribute to drug efficacy and safety by enzymatically transforming drug structure and altering drug bioavailability, bioactivity, or toxicity [169]. One example is the transformation of the cardiac drug digoxin into the cardioinactive metabolite, dihydrodigoxin, by *Eggerthella lenta* (*E. lenta*) residing in the gut microbiome [170]. There are a few proposed ways in which the microbiome interfaces and plays a role in cancer and vaccination, as highlighted in Fig. 2. The presence of bacteria allows for inherent innate sensing mechanisms and the metabolites secreted by the microbiota could also directly affect B cell activation and maturation in the gut. In addition, tumor microenvironments have been shown to have hypoxic conditions which could directly affect anaerobic bacteria populations to thrive in these localized tumor microenvironments (Fig. 2). Under medication treatment with antibiotics and immunotherapies, changes in microbial composition could directly affect or alter cancer cell-immune cell interactions (Fig. 2).

## 5. Microbiome and immune targeting strategies

Given the large role that the microbiome plays in immunity and vice versa, it is important to implement proper targeting strategies in the development of drug delivery systems. With more research being conducted on these key topics, including immunomodulation via microbial composition, pre and probiotic engineering, and targeting formulations, there could be a significant improvement in the treatment of disease.

### 5.1. Immunomodulation via microbial composition

Microbial composition and colonization play a role in pathology and disease state modulation of the immune system. Given the power that these microorganismal communities play, several strategies have been implemented to immunomodulate via control of the growth and presence of certain microbiota. Approaches such as fecal microbiota transplants (FMT) in which the microbiota from healthy donors are processed and transplanted into diseased patients have shown potential to treat *Clostridioides difficile* infection, IBD, cancer, colitis, and cirrhosis via immunomodulation [171]. Early-stage observations from clinical trials have shown promising results, including how this transplantation reduces gut microbial antibiotic resistance gene transfer and improves responsiveness to anti-PD1 immunotherapy in melanoma patients [172–174]. A Phase

1 clinical trial was conducted to assess the safety and feasibility of FMT followed by anti-PD-1 immunotherapy reinduction in 10 patients with anti-PD-1-refractory metastatic melanoma [172]. After the FMT and anti-PD-1 Therapy, PET-CT imaging followed outcomes on metastatic lesions visualized as black areas concentrated in the leg and groin [172]. For one patient, there was an observed increase in lesion sizes and number but after Day 67, complete resolution of all lesions was demonstrated via additional imaging [172]. Immunohistochemistry staining also succeeded in highlighting a clear increase in CD68 + cell infiltration in the gut lamina propria, an increase in the abundance of intra-tumoral CD8 + T cells, and visualization of tumor necrosis [172].

However, there are some limitations and challenges in the advancing of FMT as commonplace therapies. There is currently limited knowledge on the dose and composition of FMR necessary for effectiveness. This is likely attributed to the complexity and limitations on what is known to constitute a “healthy”, model microbiome. In addition, several studies must be undertaken to ensure reproducibility, safety, regulation, and scalability will be optimal for clinical translation and adaptation. Though FMT has shown promising and exciting results, FMT therapeutic treatment does not demonstrate itself to be the best or most sustainable option for clinical practice, especially until there are well established standard protocols and techniques.

## 5.2. Synthetic biology and nanotechnology approaches

Other exciting advancements include synthetic biology examples of engineered microbes and nanotechnology. Synthetic biology is used to tune a bacteria’s response cues, leading to targeted diagnostic readout [175–177]. In one case, murine-specific *Escherichia coli* was modified with a genetic toggle for the lacZ operon, where exposure to anhydrotetracycline (ATC) induces permanent operon expression [178]. The engineered bacteria were then placed within a murine *in vivo* model [178]. This study demonstrates, the utility of engineered bacteria for diagnostic activation in antibiotic resistance studies in addition to the need to validate *in vitro* models to *in vivo* responses which varied dramatically in this study and has been an ongoing concern for developing biomimetic *ex vivo* models. In another case, bacteria were engineered to protect commensal microbiota from dysbiosis induced by antibiotic treatment [179]. A strain of the *Lactococcus lactis* bacteria was engineered to degrade broad spectrum  $\beta$ -lactam antibiotics by programming them to produce the  $\beta$ -lactamase enzyme [179]. In this study, Cubillos-Ruiz et al ensured that their engineered live bacteria therapeutic was not capable of undergoing horizontal gene transfer via genetic engineering mechanisms, and when administered to mice treated with ampicillin, the group was able to show conservation of the gut microbiota while maintaining serum antibiotic levels required for treatment [179]. Bacteria have been recently engineered to therapeutically secrete protein tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) in response to highly controllable heat stimulation, which resulted in an inhibition of breast cancer tumor growth in mice *in vivo* [180]. The use of engineered bacteria for therapeutic modulation and protection of the microbiome provides new windows of opportunity for clinical translation in a broad variety of illnesses and could begin to address the spreading problem that is antibiotic resistance and dysbiosis.

In application, this is important for the development and translation of new vaccines, which face considerable challenges with the natural variation microbiota variation between patients and the poor understanding of how vaccines perform under these altered conditions [181]. Work from Pulendran et. al highlights that mice which fail to recognize flagella on gut bacteria resulted in poor germinal center formation [182]. This means that the TLR mediated sensing of flagellin plays a vital role in promoting plasma cell differentiation and the decimation of the gut microbiome in mice using antibiotics also leads to inadequate vaccine outcomes [182]. TLR5 in specific is a key player in the inflammatory response to pathogen expressing flagellum when they bypass the epithelium, the gut's the first layer of defense. Depletion of the microbiome in mice from antibiotic use can lead to unfavorable and ineffective outcomes in vaccination.

One clinical trial explored how antibiotic-induced perturbations in the gut microbiome affected the efficiency of an administered vaccine against H1N1 influenza [184]. Evidence points to the importance of microbiome composition with vaccination, high-lighting that lack of vaccination and exposure to flu strains critically affect neutralization and IgG/IgA binding response against H1N1 in antibiotic altered microbiome states [184]. Altered gut microbiomes also can enhance innate immunity and the expression of genes that produce key inflammatory response signals, such as transcription factor activating protein 1 (AP-1) and nuclear receptor 4A1 (NR4A1) [184]. Treatment with antibiotics may also alter seasonal influenza vaccine recipients' blood metabolome in part via the alteration of bile and lithocholic acids, which likely also affect inflammation and vaccine responses [128]. Antibiotic treatments have potential to be used for enhancement of inflammatory signatures, dendritic cell activation and manipulation toward desired metabolic trajectories. Hence, the gut microbiome is a critical factor to be considered as we move forward in the vaccine development landscape.

Fundamental investigations into poor immune responses to vaccines have driven investigation into how one can engineer vaccines to have a more favorable immune response. By engineering a nanogel vaccine that specifically target toll like receptor 2 (TLR2), a protein on mammalian cell membranes which begins signaling of proinflammatory cytokines in response to bacterial lipopeptides, traditionally unfavorable immune responses in mice were able to be improved as well as the vaccine trafficking and germinal center response [185]. TLR5<sup>-/-</sup> mice with altered microbiomes resulted in metabolic syndrome and diminished germinal center response to the nanovaccines [185]. The nanogel vaccines also changed gut microbiome composition which suggests a feedback loop with immune responses. Chronic treatment with antibiotics in this study showed poor vaccine responses as well, likely due to interleukin-6 expression levels in the mice. A promising highlight, however, is that low immune responses were rescued by the pyridine-poly (hydroxyethyl methacrylate) (*Pyr-pHEMA*) nanogel vaccine [185]. This study is the first to underscore the advantages of material-based nanovaccines, which offers immunomodulation for cases of gut inflammatory diseases as well as more robust vaccine response. Hence, there is a space for materials in the context of drug delivery as materials themselves could offer immunomodulatory properties and bias immune response toward more robust clinical outcomes in patients with underlying conditions linked to gut microbiome dysfunction and dysbiosis. Nanotechnology approaches have also been widely proposed in the case of

intestinal pathogens that contribute to cancer. One such microorganism is *H. pylori*, which has been associated with increased risk and prevalence of gut cancers. Knowing the role that these microbes play in cancer allows for smart microbe-inspired technologies and materials as well as the use of these bacteria as targets for future therapeutics [186].

### 5.3. Prebiotics and probiotic therapeutics

Identification of potential prebiotics and probiotics, as well as novel methods of delivery to patients, brings further insight into vaccine efficacy in relation to diet, microbiota stability, and healthy versus diseased microenvironments. Prebiotics, such as fructo-oligosaccharides and galacto-oligosaccharides, are non-digestible carbohydrates, commonly known as fiber, that are metabolized by the resident microbiota to support their health and growth [187]. Alternatively, probiotics refer to the microorganisms which may yield health benefits for the host in controlled amounts [188]. When prebiotics and probiotics are combined, these are known as Synbiotics [189]. Prebiotics may be delivered either through a patient's diet or formulated into an orally administered supplement. Since the polysaccharide inulin has been demonstrated to increase the efficacy of immune checkpoint blocker (ICB) therapy against anti-programmed cell death protein-1 ( $\alpha$ -PD-1), Han et al formulated a "colon retentive" oral gel which was able to support the host-resident microbiota and improve the T cell immunity of microbiota administered through ICB therapy [190]. In another recent study, Luccia et al worked to improve the immune response of malnourished patients to an oral cholera toxin vaccine through a synbiotic approach [191]. This involved transplanting the microbiota of Bangladeshi children with severe acute malnutrition (SAM) into a murine gut, then administering a specially formulated prebiotic-enhanced diet and exposure to supportive probiotics through a shared cage strategy [191]. With this strategy they demonstrated the success of combining prebiotic and probiotic therapies and their success as adjuvants for modulating the immune response, which may be extended to a variety of other vaccine targets. However, it is important to note that the current understanding of what foods can be considered prebiotics and whether they are "beneficial" is generally relative and variably defined across the field. More so, recent arguments could extend the definition of prebiotics beyond fiber-containing foods to peptides and lipids which have benefits for colon-resident microbiota [192].

Beyond simply integrating naturally beneficial probiotics into a patient's microbiota as a means of treatment, there are a variety of cases where engineered bacteria have been successfully tailored for applications such as anti-cancer therapeutics. Engineering controllable probiotics can be promising in other applications like preventing drug-resistant bacterial infection [193] as well as in addressing colon cancer [194]. Bacteria over millennia have evolved and adapted to be excellent carriers and survivors in the body. Hence, it is of no surprise that biologists have wondered whether these bacteria can become "living machines," hacked and reprogrammed for the benefit of human health and medical therapeutics. Chowdhury et al. have further developed this idea by developing bacteria with programmed circuitry that can hone into tumor microenvironments, produce nanobodies, and release them in a localized manner after lysis. Here, the programmed bacteria produced anti-CD47 nanobodies which blocked the anti-phagocytic CD47 receptor that is expressed across various cancer cell lines [195]. This prevents the inhibition of phagocytic cells in



the tumor microenvironment while also boosting immune response and honing due to the presence of lysed bacteria, causing an overall increase in the activation and efficacy of this treatment in cancer. Further work has applied this technology to poorly immunogenic or “cold” cancers [196]. This same strategy, if well designed, could also be applied in other health applications either as a standalone therapeutic option or in combination with other pre-established treatment modalities.

Just as microbial composition can be modulated with multiple engineering strategies, the mucus along the gut and lungs also can be engineered to produce more favorable immune responses and outcomes [197]. Similarly, certain beneficial bacteria can be used as bioagents for therapeutic and diagnostic purposes. Advances in nanotechnology and materials science have allowed for the fabrication of protective coatings and new delivery mechanisms for bacteria-based therapies. This provides more control over therapeutics. For example, bacteria can be engineered to be reactivated on demand using a triggerable, pH-responsive nanocoating [198]. This allows for the bacteria to be engineered to remain inactive until after gastric emptying, which allows for improved oral availability and treatment efficacy. Applications of bacteria clinically in the past have shown low treatment efficiency, which is associated with a rapid clearance and inflammatory side effects caused by the immunogenicity of bacteria. To combat the challenge of unfavorable immunogenicity, therapeutic bacteria can be coated with biological membranes that mimic the host mammalian cells. Studies conducted by Cao et al. have shown that by membrane coating probiotic bacteria there was low inflammatory response and accumulation, slow elimination by macrophages, and unaltered bioactivity which is promising for improving treatment efficacies while avoiding side effects [199].

Nanoparticles can also be engineered to target, activate and deliver siRNA to macrophages that can as a result significantly improve their ability to combat bacterial infections [200,201]. As shown by Kim et al, a fusogenic nanoparticle construct with the macrophage targeting peptide, CRV [202], efficiently delivers siRNA payload against the Irf5 gene [200]. This porous silicon nanoparticle formulation inhibited proinflammatory signaling pathways and allowed for targeted and high payload delivery [200]. In mice, a 480% gene silencing in activated macrophages was observed *in vivo* as well as improved response to therapeutics in both Gram-positive and Gram-negative models of bacterial infection in the muscle and lungs [200]. Lung infections, like pneumonia and cystic fibrosis (CF), present major challenges clinically with appropriate treatment modalities. Though antibiotic drugs have been the traditional treatment for these infections, many have investigated another promising therapeutic agent: phages. They especially show potential in addressing difficult to treat bacterial infections because the mechanism of action for phage induced lysis of bacteria is separate from that of antibiotics. This feature of phage therapy indicates that the complex treatment of multidrug resistant bacteria can be addressed. Engineered polymeric microparticles have been proposed as a drug delivery vehicle for getting active phages into the deep lung via dry powder inhalation for the lysis of bacteria [203]. These microparticles, composed of PLGA, when tested showed a reduction of *Pseudomonas aeruginosa* bacterial infection and mortality in mice, indicating a promising and potentially clinically translatable option for addressing intractable lung infections [203]. In the case of CF, it is not well known how fungi affects bacteria in the lungs. *Scedosporium* and *Lomentospora* species of

filamentous fungi that are typically found in CF lungs and present peptidorhamnomannans (PRMs) in their cell wall that affect adhesion and cell-host interactions [204]. One group tested isolated PRMs from these species of fungi and found that they possess antibacterial and anti-biofilm effects, highlighting them as a potential target and future therapeutic for treatment of CF [204]. In specific, PRM isolated from *L. prolificans*, *S. aurantiacum*, *S. boydii*, and *S. apiospermum* were found to be especially effective against *B. cepacia* and MRSA [204]. However, *P. aeruginosa* and *E. coli* showed resistance to all PRM, meaning that this potential targeting and treatment option could be limited in its range of application [204].

#### 5.4. Microbiome targeted medicines

Formulation-based engineering approaches are important for the development of locally targeted treatments that promote microbial replenishment and repair. In cases like inflammatory bowel disease (IBD), there has historically been a limitation on anti-inflammatory treatment efficacy, meaning that there is a need for novel therapeutic options. One such option of interest is the immunosuppressive catabolite and antioxidant, bilirubin, which was once considered by the medical community as a futile by-product of the body [205]. Over time, this paradigm shift on bilirubin has attracted several researchers in considering this molecule in therapeutic development. A limitation in the delivery of bilirubin is its high hydrophobicity, which causes increased binding to plasma albumin and limits its clinical translatability [206]. However, researchers have taken approaches to modify and improve the delivery of bilirubin. Specifically, Lee et. al has showed that hyaluronic acid-bilirubin nanoparticles (HABNs) were able to encapsulate bilirubin aqueously despite being water-insoluble, these HABNs are successful in replenishing the gut commensal microbiome, regulating immune response and restoring the gut barrier in murine inflammatory colitis (Fig. 3) [207]. This advancement in the delivery of a previously troublesome chemical target highlights the importance of using advanced chemistries and materials engineering to improve drug delivery. Moving forward, techniques and approaches such as this can not only open doors for delivering other insoluble molecules of interest but also will likely lead toward a future where nanomedicines are personalized to specific microbial signatures in disease and personalized immune-modulating nanomedicine treatments.

We have previously discussed in Section 3 the variety of ways in which innate and adaptive immunity plays, as well as physical mechanisms within the microbiota, lead to healthy and diseased states. By formulating targeted drugs to promote or inhibit surface receptor interactions is a key approach to not only reducing inflammatory responses, but also in resolving the underlying disorders [209]. One example of this is Enterome Biosciences' development of a fimbrial adhesion inhibitor treatment known as Sibofimloc, which blocks the adhesion of invasive *Escherichia coli*, and has demonstrated inflammation reduction in patients suffering from active Crohn's Disease [210].

Outside of targeting microbiota, there are many companies working on drugs that manipulate the microbiota's metabolizing capabilities to treat a variety of other disorders, especially concerning the gut-brain and gut-lung axes. Dr. Sarkis Mazmanian and

his colleagues have previously demonstrated that patients with neuro-divergent and neurodegenerative conditions, such as autism spectrum disorder (ASD), schizophrenia, and Parkinson's Disease, have different microbiota compositions compared to neurotypical controls [211,212]. Most importantly, they have highlighted how microbial metabolite products directly affect nervous system communications, the potential for drug delivery via the gut to bypass the blood–brain barrier, and have even initiated Phase 1b/2a clinical trials for an oral therapeutic designed to regulate anxiety in ASD patients [213].

Critical work is also underway in developing therapeutics to modulate the gut-lung axis, with high clinical importance following the outbreak of Sars-CoV-2 (COVID-19). With the outbreak of the Sars-CoV-2 pandemic, numerous research groups have led investigations to understand how the gut-lung axis facilitates the course of Sars-CoV-2 infection and subsequent healing, as detailed in a review by de Oliveira et al [214]. These investigations range from studies analyzing the changes in pulmonary and gut microbiota compositions, impacts on the immune system's ability to clear Sars-CoV-2, related symptoms of the disease in both the pulmonary and digestive tracts, and the beneficial effects of probiotic administration as a means for treating this disease.

Probiotic therapies for pulmonary microbiota have also been extended beyond infectious disease applications to better treat chronic illnesses and metastatic lung cancer. Noci et al compared the responses of probiotic and antibiotic aerosolized therapies in countering immunosuppression in the lungs, which is often linked to the lungs' high susceptibility as a secondary organ site for tumor metastasis from other organ systems [215]. Here, they demonstrated antibiotic treatment led to a decrease regulatory T cell prevalence while probiotic treatment led to maturation of antigen presenting cells (APCs) responsible for immunosurveillance, ultimately leading to the body's ability to effectively launch an immune response against metastatic cancers infiltration to the lung. Similarly, others have used nebulized combinations of antioxidants and antibiotics for delivery in treating idiopathic pulmonary fibrosis [216].

While there is promise for therapeutic treatments that function through microbiota targeting and manipulation, there are still many challenges to overcome. These challenges include a low correlation in microbial composition changes between patients in response to these drugs and the need for more advanced biosensors and 3D culture platforms, as well as regulations and best practice policies set forth by the FDA [217]. In the next section, we will explore how these tools are being developed to address this wide array of challenges.

## 6. Advancements and challenges in therapeutic screening tools

The development of quality tools for the study of microbiome has enabled a better understanding of the clinical importance of this microbiome in health. However, there are still limitations with the tools currently in use for studying the microbiome, especially in that there is not sufficient use of technological advancements for analyzing how it plays a role in therapeutic efficacy. Though there are still many challenges in tools for sampling and analyzing, we propose that certain pushes and applications of technologies could aid in addressing these issues moving forward. Certain technologies in specific have already shown

much promise in addressing these limitations, such as the use of spatial mapping, machine learning, and organoid-on-chip technologies (Fig. 4).

### 6.1. Microbiome sampling products and biosensors

Moving forward with microbiome research and dysbiosis, it is important to be able to non-invasively sample and test the microbiome for studies. Advancements by multiple groups have proposed the use of smart ingestible materials to accomplish *in vivo* sampling of the gut microbiome [218,219]. Having a non-invasive, site differential platform such as this to obtain samples more easily would be beneficial for the microbiome research community and would foster more identified microbial species in pathogenesis and disease progression. Increased attempts to do just this have occurred throughout the past decade. For example, studies ranging from microelectromechanical systems (MEMS) technology approaches to develop a remote-controlled drug delivery to a microbiome sampling capsule system for the goal of diagnosing and treating gastrointestinal diseases have been explored [220–222]. Subsequent studies in the field have continued to make strides in the engineering of products to non-invasively sample the microbiome for further analysis and diagnosis, including some hydrogel materials-based approaches [223,224]. However, despite these considerable efforts there are still roadblocks in the use of microbiome sampling products, mainly related to having accurate representation of the microbial community. These roadblocks are even more apparent when it comes to sampling the microbiome of the respiratory tract, especially since there is a difference in biomass and accessibility to the lower respiratory tract for sampling [225–227]. Hence, there is an urgent need to continue refining these devices for optimal, non-invasive sampling of the microbiome across various regions of the digestive and respiratory tract.

A platform for sampling microbiota could also provide insight on a specific patient's intestinal health and can allow for physicians to make more informed decisions on the best treatment options for each patient based on their unique microbiome signature and compare to the patterns seen data patients with similar signatures and diseases. With the recent advances and pushes toward personalized medicine, it is clear how a highly thorough, predictive technology could help the next generations of physicians and drug delivery specialists. There is also much promise in the development of infection and microbiome activity-based diagnostics moving forward. The microbiome often produces enzymes and metabolites which are essential for survival. These enzymes and markers can be used as biomarkers for clinically and pharmacologically relevant applications such as monitoring and evaluating therapeutic treatment regimens and global surveying of the microbiome [228]. One enzyme of interest is Bile salt hydrolase (BSH), which is essential for metabolite processing in the intestinal microbiome. A promising biotechnology being developed is a bioluminescent probe which monitors the activity of bile salt hydrolase, which could be beneficial in understanding how BSH modulating drugs play a role in therapeutic intervention [229]. In this study, Khodakivskyi et al demonstrate how these metabolic probes can be used to identify prebiotics that increase levels of this beneficial BSH and linked BSH activity and inflammatory bowel disease [229].

For respiratory tract biosensing, pivotal work by Chan et al has developed protease-sensitive breath biomarkers for the lung [230]. These nano sensors are activity based, meaning that exposure to extracellular proteases such as those present in respiratory disease states, cleave the conjugated peptide substrates and release volatile organic compound reporters which can be exhaled and detected via mass spectrometry [230]. This technology could potentially be used for microbiome specific detection in the lung across a variety of disease states and conditions. These and other biosensor approaches are promising for microbiome monitoring and sensing; however, they are still limitations of these biosensor approaches, including various parameters (sensitivity, ease of use, etc.) that must be refined before translation into clinical practice [231,232].

## 6.2. Spatial-Omics and multiplexed imaging

Mass data obtained from novel and advanced technologies in gene sequencing and high throughput analysis of the microbiome have highlighted that the communities and species involved are highly diverse. However, much remains to be done within this area. For one, there are limited tools that give insight on the way that these complex communities are organized in across the space and over time, which is clearly important for understanding the crosstalk and interactions which are occurring between microbes and the host. Strides have been taken to address this, as seen by seminal work from Shi et. al. Using high phylogenetic resolution microbiome mapping by fluorescence in situ hybridization (HiPR-FISH) technology, they were able to use binary encoding, spectral imaging and machine learning algorithms to create complex maps of microbial species [5]. This approach allowed for the unique labelling of more than a thousand different isolates and as well as insight into the spatial organization of microbes at the single-cell resolution level.

Recent work by Feng et al has also applied multiplexed imaging techniques to perform in situ imaging of both bacteria and tumor tissue using their designed prokaryotic and eukaryotic hybrid probes for in situ imaging (PEHPSI) system [233]. This study observed that there is an enrichment of gram-negative bacteria in the breast cancer tissue microenvironment and a significant correlation between these bacteria and CD8 + T cell presence in HER2 + breast cancer subtype, providing insightful information on microbiome-immune cell interactions in cancer [233]. Given that it is crucial to understand tumor microenvironment - microbiome interactions, being able to co-detect cancer subtypes alongside bacterial communities provides spatiotemporal information which can help further facilitate our understanding of the mechanisms and how microbial populations contribute to disease pathogenesis and treatment.

Moving forward, this technology can be broadly applied to not only visualize interplay between microbiota and host cells but also to evaluate drugs in context of spatiotemporal distribution and therapeutic response [234]. Antibiotic resistance continues to be a grand challenge in the field and often this innate ability for microbes to evolve and adapt against drugs can be related to horizontal transfer, the carrying over of genes across bacterial species. In specific with antibiotic resistance, these genes which are conserved and shared contribute to a “resistome” and allow for better survival when put up against antibiotic drugs [83]. Hence, it is important to build technologies and robust techniques which can

follow this mechanism of horizontal transfer moving forward and perhaps design the next generation of drugs to outsmart microbes in this battle seen with antibiotic resistance. These analysis techniques can also be used to inform drug design and development and potentially even lower the costs of therapeutics currently seen in the market. Collection of microbiome data across various individuals can help identify how certain microbial species could be used in combination with already existing drug formulations. In addition, by having longitudinal studies of individual patients one can observe over time the impact of microbial composition with clinical outcomes.

### 6.3. Machine learning and Sequencing/Bioinformatics tools

Advanced artificial intelligence techniques such as machine learning algorithms have been applied to the development of microbiome targeting and therapeutics [235]. Machine learning is broadly defined as the process of fitting computational models to data, often ones which are highly complex and multi-faceted [236]. This can be used for predicting outcomes based off training to a specific dataset and holds much promise when it comes to the mass information one obtains from biological –omics sequencing. Approaches to integrate these learning algorithms to the microbiome include those which aim to predict biomolecular protein–protein interactions between microbiota and the host [237]. Over the years, there has been a steady development of both reference-based prediction tools, like Mangosteen and MIMOSA, as well as machine learning prediction approaches, like Melonpan [238,239]. These approaches have been used for predicting metabolite profiles given microbiome sequencing data. When comparing the reference based computational approach to machine learning approaches, it has been highlighted that machine learning was able to be trained to predict metabolites most accurately [238,239]. Within the broad machine learning umbrella lie more specific learning methods such as deep learning, artificial neural, and random forest non-linear models, all which have been applied to the microbiome space [236,240–246]. Although machine learning has had a significant impact on biological research and is expected to continue doing so, it also does have its limitations such as natural variance in biological data and potentially not having sufficient data for prediction or understanding complex and undefined areas of biology. However, with growing amounts of detailed data from patients with known outcomes and profiles, machine learning can potentially be used in the future to inform industry and healthcare professionals on decision making on therapeutic interventions.

The future breakthroughs in microbial macromolecules and metabolite sequencing, advanced bioinformatics software tools and development of higher resolution imaging modalities that highlight the microenvironment conditions and microbe-cell interactions will likely be of high impact and interest for microbiome research [247–249]. Microbiome related –omics data processing and bioinformatics tools provide essential examples of how these approaches can further inform and answer key scientific questions about the microbiome, therapeutics and clinical outcomes. These technological advancements will allow for faster discovery of “-omic” differences between species, environmental factors, and anatomic locations, such as immune follicles. Some advances in this understanding are currently underway. For example, complete genome sequencing of murine Peyer’s patches-derived *Lactobacillus taiwanensis* CLG01 strain points toward this being a potential

probiotic with antibacterial and immunomodulatory systemic characteristics [250]. In addition, deeper understanding of the infamous p53 gene, which is an important coding signal in cancer, has been seen to change from tumor suppressive expression to oncogenic depending on the signals and interactions with the gut microbiome based partially on ChIP-seq data analysis and 16S rRNA sequencing [251]. Proteomics and other data analysis techniques can also help in characterization of human tissue and specifically, the use of this type of data analysis in organoid models to study different diseases [252,253].

#### 6.4. Development of organoid modelling Technologies:

The modelling and exploration of the functionality of the human microbiome has been aided by the development and establishment of animal models [254]. A humanized animal model of the gut microbiome can be instrumental for mechanistic understanding of microbial activities as well as preclinical development of therapies. In fact, studies have clearly demonstrated that humanized gnotobiotic mouse models have contributed to the advancements in biomedicine, bridging human and animal physiology in health and disease [255]. However, inadequate understanding of the full intricacies of the gut microbiota and lack of standardized protocols in these models place major constraints on translatability of the system. Other modelling techniques, hence, can provide a better understanding and mimicry of the biological processes occurring with the microbiome and immunity.

The rise of organoids, self-organized organ-like cell aggregates that originate from multipotent stem cells, has allowed for advanced levels of biomimicry to be achieved [256,257]. This unique technology has become a promising area of biomedical research which holds potential in both research and medicine. Across the scientific community, organoids look to be the next top model for studying human biology and are a significant step forward for personalized medicine. Significant strides have been taken in understanding and developing robust intestinal and lung organoids [258–265]. Polyethylene glycol (PEG) hydrogels have been used for generating human intestinal organoids for *in vivo* delivery as a therapeutic [266–268]. Other gut and intestinal organoids have also been used as research tools and models for digestive tract diseases such as inflammatory bowel disease (IBD) [269–273]. Others have sought to bridge the gap of understanding intestinal microbiota function regarding luminal physiology through organoid culture and microbiota supplementation through microneedle injection and high-throughput culture [274].

Recapitulating certain biological and mechanical cues in gut and lung organoids, such as the contractile nature of the tissue, has been an area of interest in the field which has shed light on the importance of signaling pathways and stem cell differentiation [275–277]. When it comes to lung organoids, similar advancements have been seen that allowed for improved modelling of the physiological environment *in vitro*. For example, lung organoids and models have been used to model lung branch *ex vivo* morphogenesis and study pharmacokinetics of inhaled drugs [278–280]. However, lung organoids can be limited in their use for personalized medicine in that for certain cases such as in modeling lung cancer, it can become quite difficult to distinguish between tumor and healthy organoid tissues and keep track of the cell states [281].

The development of immune competent organoids has been an advancing scientific pursuit as of late but to a much more limited extent. Intestinal organoids have begun to interface with immune biology to recapitulate drug effects and overall responses in cases like with coronavirus and enterovirus infections [282–285]. Initiatives such as the Organoid Cell Atlas project focuses on characterizing organoids and in vitro cellular systems at the single-cell level to help facilitate the use and manufacturing of obtained single cell and genomics banked data for application in human organoids [286].

Biomaterials are used in the generation of organoids because of their ability to provide a carefully engineered scaffold with cellular cues for the clusters of stem-like cells to grow and begin developing on. Because of this, it is not a surprise that many advancements in materials science and manufacturing have become the foundation for smarter approaches in organoid growth and design. For example, different types of hydrogels have been engineered to support the growth of intestinal organoids and to promote favorable immune responses [287–291]. Different manufacturing advancements in biomaterials have also allowed for more control of tissue shape and spatiotemporal architecture. Technologies such as bioprinting have also allowed for complex organoids to be developed either through using cells and materials or predeveloped organoids as building blocks to creating macroscale tissues *ex vivo* [292].

There are several limitations of organoids in the context of microbiomes and therapeutics. One such limitation is that few organoid models are capable of directly integrating of the human gut microbiome since cells are still susceptible to bacterial infection in the dish and eventual cellular death. Headway in the gut organoid space has started to address this co-culture issue but still requires an added level of complexity and advancement to truly mimic *in vivo* conditions. In addition, different classifications of bacteria, such as anaerobes and aerobes, require different parameters for survival in culture in a way like microbiota *in vivo*. Most established organoid systems have several limitations including reproducing the level of physiological function, heterogeneity, and the ability to monitor readouts such as metabolite production to name a few [257]. However, many of these limitations can now be combatted using rising engineering technologies and approaches, which will hopefully allow for further exploration and acquisition of knowledge in this field.

### 6.5. Development of Organoid-on-Chip modeling technologies

Biomaterials have also affected the way in which organoids can be put onto other platforms, such as microfluidic chips, to better recapitulate environmental cues. Integrating microfluidics to develop organoid-on-a-chip platforms is advantageous in that they facilitate better nutrient disbursement, gas exchange, and recapitulate 3D tissue architecture and physiology across the body [256,293,294]. Specifically, advances with gut-on-chip microfluidic models have seen great development over the past years, with different platforms aiming to recapitulate important gastrointestinal features such as peristalsis, tissue structure and flow and its induced shear stresses, and even more recently the co-culture with bacterial species [295–298]. However, limitations continue with the ability to culture bacteria alongside these organoid platforms, especially since most are not compatible with the 0.5% luminal oxygen levels required of anaerobic bacteria [299]. Additional roadblocks



with using microfluidic organ-on-chip platform continues to be that not all physiological phenomena like vascularization and presence of lymphatics are not easily integrated into the system [300]. Although there are certain limitations of organoid modelling, microfluidic integrated models have shown great potential as a strategy for both understanding disease mechanisms and screening therapeutics [298,301].

Recent breakthroughs in intestinal organoid on-chip technologies now include more control over morphogenesis and tissue architecture. Work from Nikolaev et al has shown how epithelial organoids can be engineered to self-organize into crypts and villi-like structures to develop more physiologically relevant mini-intestines [302]. The work of Dr. Nancy Albritton's group has also achieved these architectures [303], in addition to devices analyzing factors that impact the microbiome including the mucus deposition, self-sustaining oxygen gradients, and chemical gradients [296,304]. This addition of mechanical and structural cues at the cellular level might seem minor but new findings in transcriptomics point to microfluidic conditions being more favorable for certain cell types, like epithelium, compared to static conditions [305–307].

Similarly, lung-on-chip platforms have advanced recently to model diseases and assess immune responses [308]. With nanomedicine growing in clinical translation, an area of application for these modeling platforms includes using them to monitor the transport, adsorption, and efficacy of various nanoparticles in vitro [309,310]. Most lung-on-chip platforms lack integration of microvasculature networks and the presence of interstitial fibroblasts. To address this, Mejías et al designed a microfabricated device that behaves and mimics the human lung interstitial-airway interface, which after characterization point toward the fabrication being a more pathophysiological relevant model for complex pulmonary disease such as CF [311]. Other lung-on-a-chip have also used stretchable materials like elastin and collagen to create stretchable alveoli to mimic breathing movements with more tunability with thickness, composition, and stiffness [312]. Biomimetic validation of these platforms has only recently been achieved, where these models present a noteworthy potential to model the complex biology of immune cells and microbiota cross-talk. However, when integrating the microbiome into these modeling platforms, it is important to consider how experimental conditions such as the choice of cell culture media, oxygen levels, and microbial composition might influence antibiotic, activity, and immune studies [313].

## 7. Future Directions:

The key impacts of microbiome research are the development of novel technologies and techniques for identifying immune-microbiome interactions and targets, especially in how these engineered constructs may translate to the formulation and delivery of clinical therapeutics. The potential impacts that this type of research can provide span across multiple areas of medicine in a personalized fashion. In addition to advancements currently being done in synthetic biology, pre/probiotic engineering, nanotechnology, organoids, microfluidics, and 3D printed technologies, a new area which may further pave the way in microbiome research includes work within modelling the human microbiome-gutbrain axis [314–318]. Findings in this field have shown that interactions between gut microbiota and

cells such as enteric neurons and local lymphoid cells can regulate blood glucose and shape IgA responses, which opens more potential paths and targets for future therapies [319,320].

The importance of understanding the human microbiome echoes through society to this date, especially as we monitor and reflect upon the COVID-19 pandemic that has induced serious global health concerns. The COVID-19 causative virus, Severe Acute Respiratory Coronavirus 2 (SARS-CoV-2), has been shown to infect human gut enterocytes that express ACE2 [321–323] and alter the respiratory and gut microbiota of infected individuals [324–327]. The severity of COVID-19 is also linked with the microbiome and could be tied to disease prognosis [41,325,328]. Though some of these studies remain limited in their patient size and disease time-line, the findings highlight an interesting and under-recognized perspective on COVID-19 immunity and the microbiome. These findings hold powerful clinical implications and potential solutions, such as using short-chain fatty acids like butyrate for clinical diagnosis and precision intervention techniques and prebiotics to modulate and inhibit certain identified bacterial culprits related to worse clinical outcomes [329]. With this disease and beyond, the microbiome can be the missing piece needed to unleash a revolution in personalized medicine.

The medicine of tomorrow will be shaped by the microbiome findings of today. With these key pieces of information and the exemplary engineering and scientific advancements in immune-microbiome interactions, a new era of personalized and precision medicine will be born. One in which both medical treatment and drug design and development will be guided not just by a patient's medical history but by their distinct microbiomes as well.

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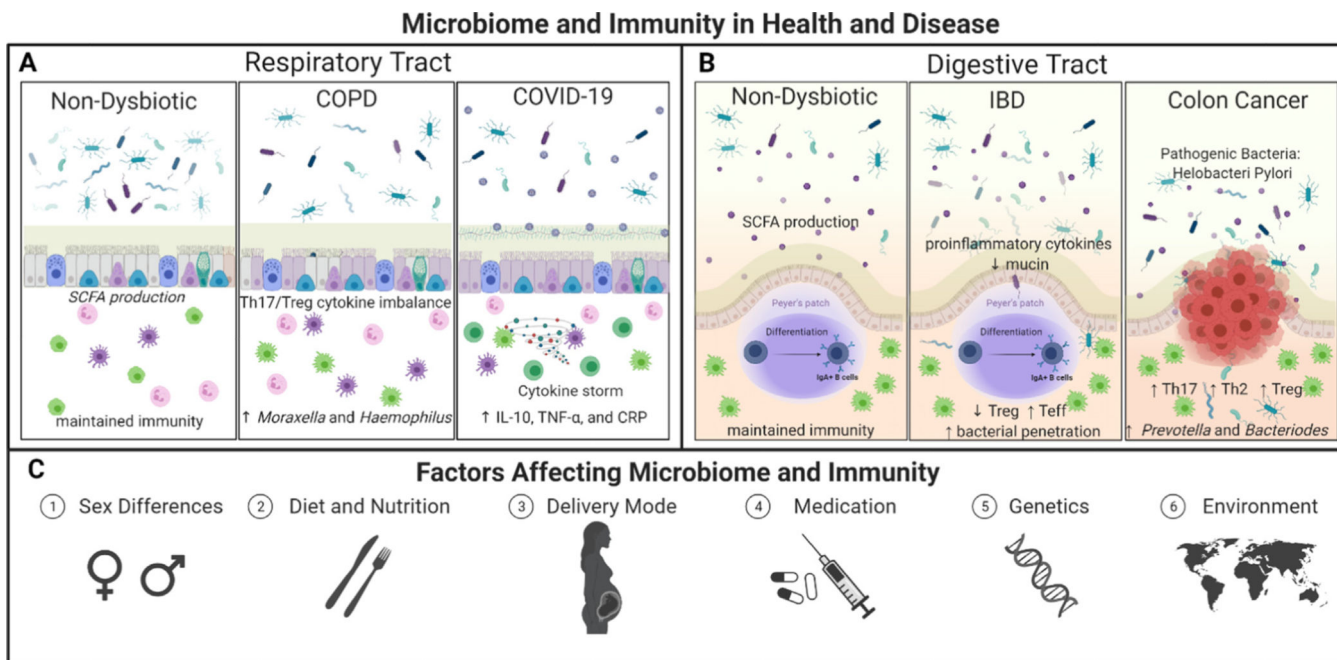
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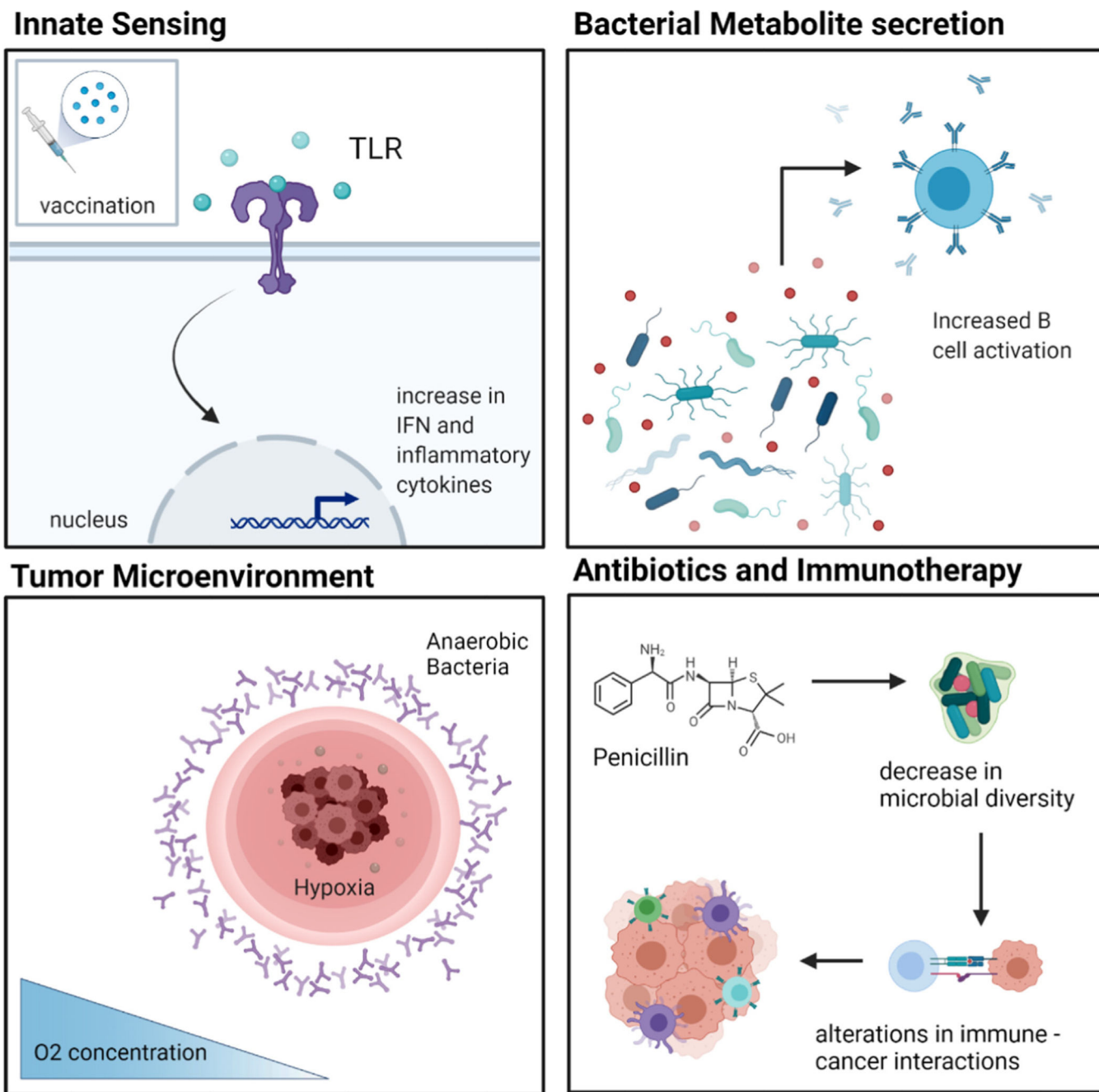
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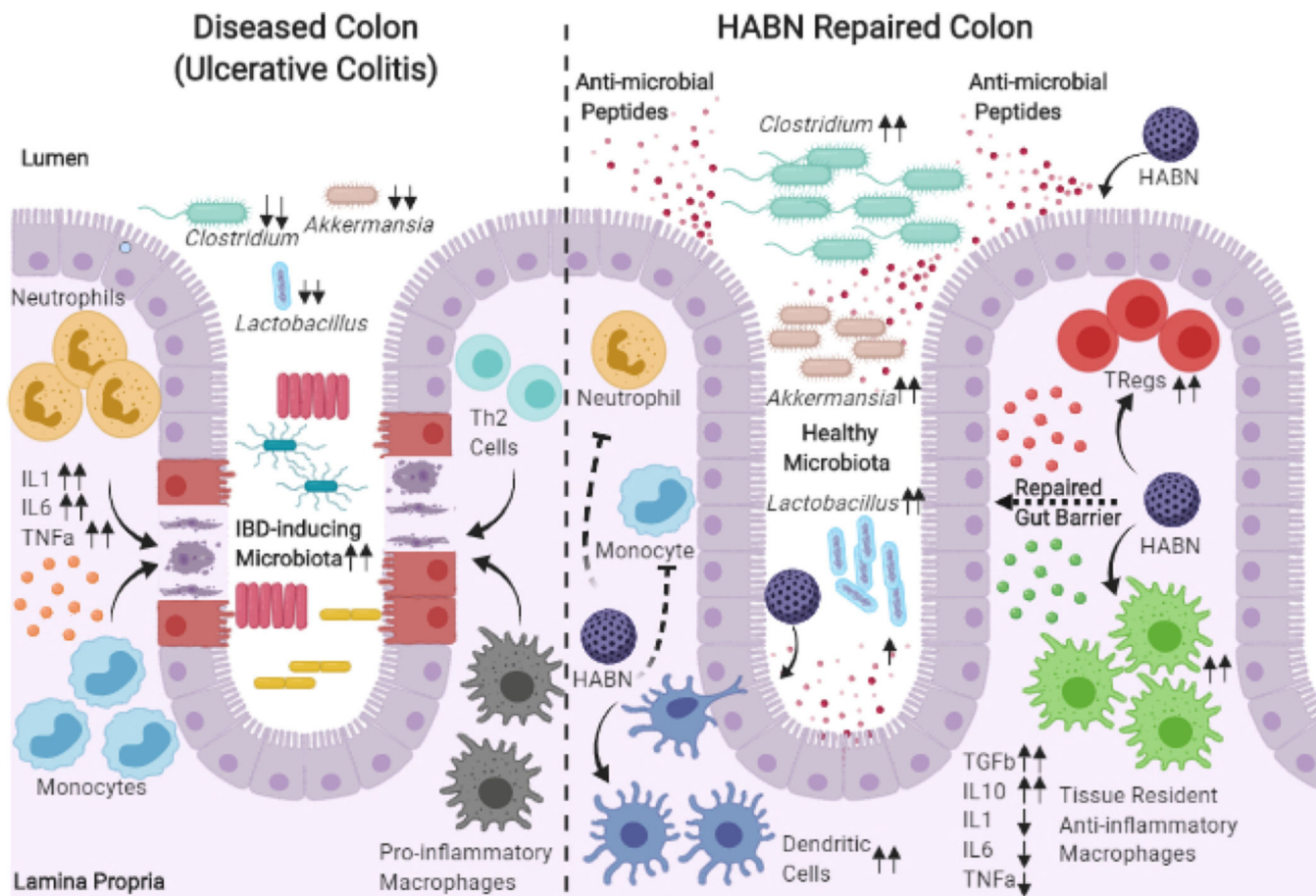
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**Fig. 1.** Landscape of Microbiome and Immunity. There is a dysregulation of the microbiome populations under health and diseased state conditions, especially in the A) respiratory tract [38–43] and B) the digestive tract [44–46]. C) Various factors influence the microbiome composition and the immune responses in humans, including sex, diet and nutrition, delivery mode, medications such as antibiotics, genetics, and the environment.

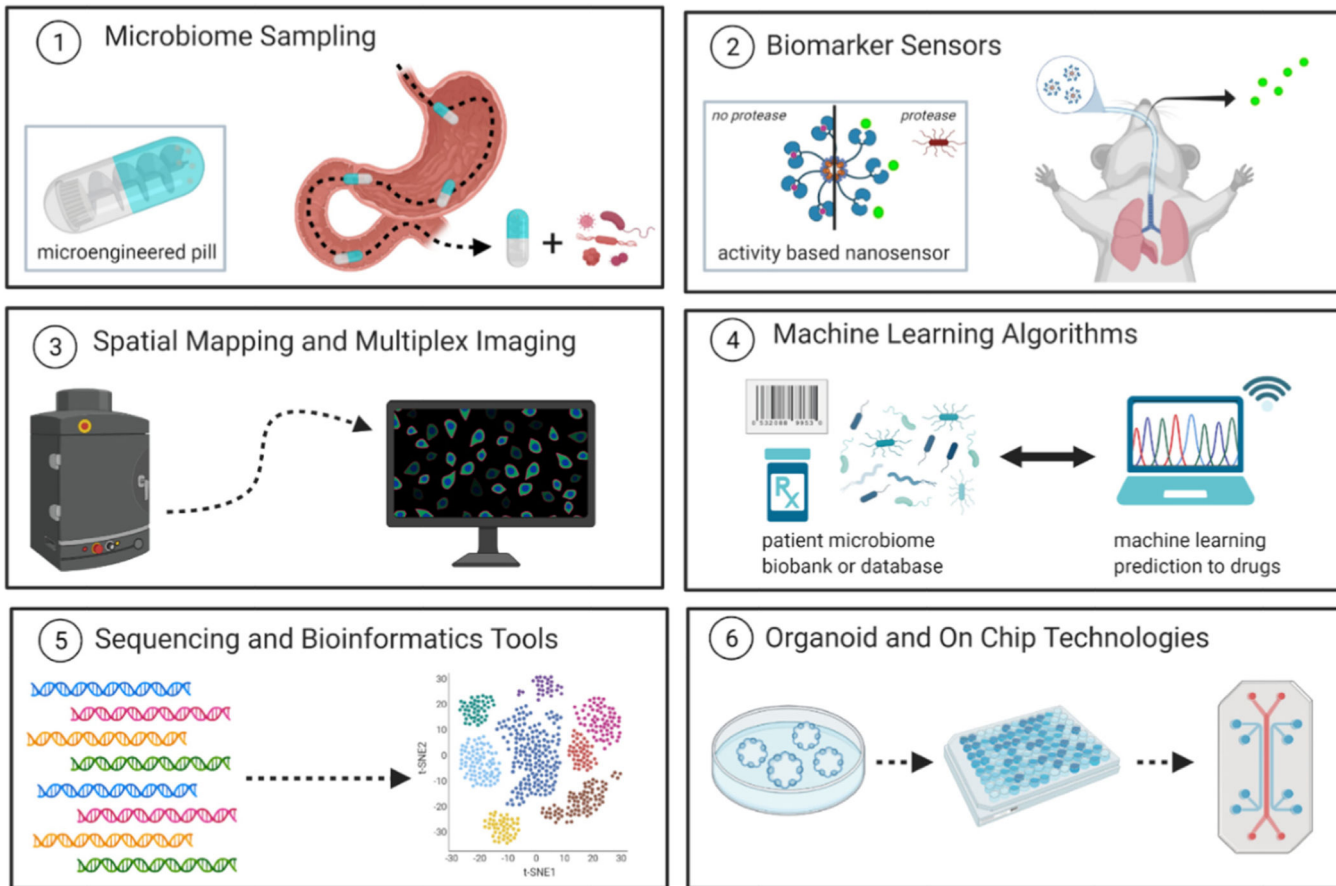


**Fig. 2.** Microbiome immune interactions in cancer, infectious disease, and vaccination. Mechanisms by which innate sensing, metabolite secretion, tumor microenvironments and the use of antibiotics combined with immunotherapies can affect the gut microbiome, immune cell response and native tissue.



**Fig. 3.** HABN repair of the Colon. Visual representation of HABN approach by Lee et. al allows for delivery of insoluble bilirubin and repair of the colon and gut microbiome [207]. Adapted with permission from Singh [208].

### Microbiome Tools, Technologies and Techniques



**Fig. 4.** Microbiome Tools and Technologies of the Future. Several advances in microbiome analysis tools and technologies have allowed for new potential ways of looking into the ways in which pharmaceutical drugs interact with the immune system the microbiome and overall human health.