Published in final edited form as:

Immunol Rev. 2017 September ; 279(1): 8–22. doi:10.1111/imr.12578.

Deciphering interactions between the gut microbiota and the immune system via microbial cultivation and minimal microbiomes

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Summary

The community of microorganisms in the mammalian gastrointestinal tract, referred to as the gut microbiota, influences host physiology and immunity. The last decade of microbiome research has provided significant advancements for the field and highlighted the importance of gut microbes to states of both health and disease. Novel molecular techniques have unraveled the tremendous diversity of intestinal symbionts that potentially influence the host, many proof-of-concept studies have demonstrated causative roles of gut microbial communities in various pathologies, and microbiome-based approaches are beginning to be implemented in the clinic for diagnostic purposes or for personalized treatments. However, several challenges for the field remain: purely descriptive reports outnumbering mechanistic studies and slow translation of experimental results obtained in animal models into the clinics. Moreover, there is a dearth of knowledge regarding how gut microbes, including novel species that have yet to be identified, impact host immune responses. The sheer complexity of the gut microbial ecosystem makes it difficult, in part, to fully understand the microbiota-host networks that regulate immunity. In the present manuscript, we review key findings on the interactions between gut microbiota members and the immune system. Because culturing microbes allows performing functional studies, we have emphasized the impact of specific taxa or communities thereof. We also highlight underlying molecular mechanisms and discuss opportunities to implement minimal microbiome-based strategies.

Keywords

gut microbiota; immune system; anaerobic cultivation; minimal microbiomes; Altered Schaedler Flora; lipids

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CONFLICTS OF INTEREST

The authors do not have any conflicts of interest

1 INTRODUCTION

The mammalian gastrointestinal tract is home to a dense microbial population, a community collectively referred to as the gut microbiota. These organisms live a mutualistic lifestyle with their host and provide it with numerous benefits—they supply micronutrients, increase food digestibility and nutrient absorption, influence immune maturation, offer colonization resistance against pathogens and secrete metabolites that aid in nutrition and whole body physiology (1, 2). Despite serving a valuable role in host biology, the gut microbiota has also been implicated in the pathogenesis of several immune-mediated human diseases, including allergy, asthma, multiple sclerosis, inflammatory bowel diseases (3–6). Consequently, much interest exists in understanding the mechanisms by which members of this complex community shape host physiology and immunity.

Studies in germfree mice highlight the importance of the gut microbiota to proper immunological development—these animals have reduced numbers of immune cells present in their intestinal lamina propria and smaller Peyer's patches compared to conventionally reared mice (7–10). Systemically, they exhibit a diminished capacity for antibody production, fewer plasma cells, smaller mesenteric lymph nodes, and reduced numbers of germinal centers versus their conventional counterparts (11). Immune maturation can be induced in germfree mice by colonization with a complex gut microbiota; however, some notable deficits and distinctions remain into adulthood depending upon the age of mice at the time of microbial exposure (12–16). Indeed, early life represents a critical window for proper immune development (17, 18). Infants are born sterile and establishment of the gut microbiota begins shortly after birth (19–21). During this time, the mammalian host is rapidly exposed to a myriad of different microorganisms (22, 23). The gut microbiota is quite unstable throughout the first months of life, is strongly influenced by breast-feeding, diversifies substantially with the introduction of solid food, and eventually stabilizes after the first two years of life (24, 25). Such temporal variability and succession dynamics add yet another level of complexity to dissecting the interplay between the immune system and gut microbes and are beyond the scope of this review.

Instead, we will focus on the dynamic interactions between cultured members of the microbiota and host immunity. Indeed, depletion of gut microbes in conventional mice via antibiotic treatment significantly impacts the immune system—it reduces spleen size, decreases immune cell numbers and alters expression of genes regulating inflammatory responses and production of antimicrobial factors such that these animals more closely resemble germfree mice than those with a conventional microbiota (26, 27). Cultivation of gut microbes represents an important technique for studying the relationship between this complex ecosystem and its host. Molecular-based methods represent another approach that has been reviewed many times elsewhere (28, 29) and will not be touched upon here. Because molecular techniques allow high-throughput analysis of entire systems, they are very useful for obtaining comprehensive and integrated views of complex interactions not permitted by more reductionist approaches. In contrast, the major advantage of cultivation approaches is the ability to characterize microorganisms in great detail, perform downstream functional studies and identify specific microbial bioactive compounds and their effects on

target cells (30). Contrary to frequent introductory statements in the literature that only minor fractions of the mammalian gut microbiota can be cultured, cultivation techniques can indeed capture a substantial amount of the bacterial diversity present in the mammalian gut: a comparison of thousands of high-throughput amplicon datasets with a catalogue of reference sequences from isolates revealed that 35% to 65% of microbial species in the human and mouse gut have representative strains in culture (31). Nonetheless, there are still numerous unknown taxa to be discovered. These taxa represent a considerable pool of novel functions, some of which may have a profound impact upon the immune system.

This review compiles knowledge about the regulation of immune responses by specific members of the gut microbiota, with primary focus on studies using cultured microbes as single strains or minimal consortia to investigate specific interactions at local (gut mucosal immune system) and distant body sites. Even though culture approaches are simplistic, they provide precise knowledge that is eventually useful for proper interpretation of results generated with more comprehensive systems-oriented approaches. We acknowledge that microorganisms other than bacteria can regulate immune responses. However, the bulk of information provided here concerns bacteria as they represent the dominant members of gut ecosystems and are the subject of most reports in the literature. Furthermore, there is exhaustive literature on probiotics and their impact on the immune system. However, because most probiotic studies are based on the use of generic strains and molecular mechanisms are rarely investigated (32, 33), we deliberately excluded probiotic studies from the present review. Finally, sophisticated *in vitro* systems and functional metagenomic approaches allow assessment of the impact of microbes on the immune system (34, 35). However, because these approaches are still in their infancy and findings eventually require validation in more complex models, this review focuses primarily on data obtained *in vivo*.

2 SINGLE STRAINS, SPECIFIC EFFECTS

Germfree mice have been used extensively to study the impact of single microorganisms on immune responses. Born and raised in the absence of living body microorganisms, these mice allow targeted manipulations of the gut microbiota and determination of the impact of specific microbial strains, including characterization of underlying molecular mechanisms (36). Here, we highlight only a select set of experimental studies aimed at revealing the specific interactions between single bacterial species and the host (37), some of which utilized germfree mice while others employed mouse models with a complex microbiota (more examples are described in Table 1). For additional reading, we highly recommend Faith et al., 2014 (38), Tan et al., 2016 (39) and Geva-Zatorsky et al., 2017 (40), all of whom have reported extensively on strain specific effects in experimental model systems. The spectrum of host-microbe interactions is presented here by Phylum merely to facilitate discussion; we are mindful that phylogenetic clustering may not provide a consistent phenotype for all members that accurately predicts host responses. We highlight taxa belonging to the most abundant phyla in the murine microbiota (with Firmicutes and Bacteroidetes representing c.90% of commonly detected sequence diversity) as well as members of some other commonly detected but lowly abundant phyla such as Proteobacteria, Fusobacteria, Tenericutes, Deferribacteres, Verrucomicrobia and Actinobacteria (41). Finally, considering the width of strain level diversity (42) and rapid

exchange of genetic information (43) that exists within gut microbial ecosystems, the results discussed herein should not be considered as generalizable phenomenon applicable to all members of a particular species, but rather as examples of the complexity and uniqueness of their intricate relationships with the host.

2.1 FIRMICUTES

Clostridium spp—Given the phylogenetic diversity of the *Clostridium* genus across its many clusters (44), only a few major examples of species-specific interactions with the host are described here, the majority of which promote immune regulation. Conventional mice colonized with *C. butyricum* MIYAIRI 588 expanded the numbers of both IL-10 secreting macrophages (F4/80⁺ CD11b⁺ CD11c^{int}) and inducible CD4⁺ T regulatory (Treg) cells, the latter of which occurred via increased secretion of TGF-β1 by lamina propria dendritic cells (DC). Moreover, these enhanced regulatory responses were sufficient to prevent experimental colitis in mice via a TLR2-dependent manner (45, 46). When *C. ramosum* AO31, *C. sordelli* AO32 or *C. histolyticum* AO25 were individually mono-associated with mice, each similarly expanded both Foxp3⁺Roryt⁺CD4⁺ T cells and Foxp3⁺Roryt⁺Helios⁺CD4⁺ T cells in the colon and Foxp3⁺Roryt⁺Helios⁺CD4⁺ T in the small intestine compared to germfree mice (40). In contrast, *C. perfringens* ATCC 13124 did not promote expansion of either cell type, demonstrating that there are clear species-specific effects on immune modulation by members of the *Clostridium* genus (40).

Faecalibacterium prausnitzii—Another Firmicutes member often studied for its immunomodulatory properties is *Faecalibacterium prausnitzii*. The abundance of this taxa is routinely reduced in the gut microbiome of patients with inflammatory bowel diseases (IBD), particularly during flares (47–49). This decrease is presumably a consequence of an inflammation-driven increase in tissue oxygen tension that creates an environment unfavorable for *F. prausnitzii* (50, 51). However, the lack of *F. prausnitzii* may actively contribute to the disease process as this organism possesses anti-inflammatory properties and helps maintain epithelial cell integrity. *F. prausnitzii* A2-165 induces IL-10 production in human and murine dendritic cells and increases intestinal expression of mouse Claudin-4, which together may explain its protective role in colitis models (52–55). *F. prausnitzii* A2-165 may mediate these anti-inflammatory effects via secretion of effector proteins that inhibit NF-κB signaling in intestinal epithelial cells (56). Alternatively, *F. prausnitzii* may confer anti-inflammatory effects via the production of butyrate, which could alter the functions of macrophages and peripheral Treg cells via epigenetic modifications (57, 58). Additional studies are needed to define if these pathways are indeed regulated by *F. prausnitzii* *in vivo* to limit host inflammation.

Segmented Filamentous Bacteria (SFB – Candidatus savagella) (59)—SFB are Gram-positive clostridia-related bacteria best characterized as colonizers of the murine small intestine, with similar microorganisms found in humans, non-human primates, cats, dogs, and other animals (60–62). Through intimate contact with small intestinal epithelial cells (IEC), SFB induces the secretion of serum amyloid protein A (SAA) 1/2 (63, 64). In turn, SAA acts on CD11c⁺ dendritic cells to trigger production of IL-1β and IL-23—the latter of which stimulates group 3 innate lymphoid cells to release IL-22, which then synergizes with

IL-1 β to maximize IEC secretion of SAA (64). Additionally, IEC also release reactive oxygen species that, when combined with the actions of dendritic cells and CX3CR1⁺ macrophages, induce expansion of Th17 cells in the small intestine (64–68). Importantly, host-adaptation influences the capacity of SFB isolates to adhere to the epithelium and subsequently induce Th17 cells, as demonstrated by a comparison between mouse and rat isolates with reciprocal colonization of each strain in both hosts (64). Locally, SFB induction of the IL-17 axis is protective against *Citrobacter rodentium* colitis in mice (65). However, colonization of severe combined immune deficiency (SCID) mice with SFB precipitates the onset of severe colitis following adoptive transfer of CD45^{high}CD4⁺ T cells (69). SFB can also induce Th17 cells that migrate to distant body sites, such as the joints, to mediate arthritis (70). Collectively, this intricate cross-talk between SFB and the IL-17 axis clearly shapes immunity and may contextually precipitate disease in susceptible hosts. However, whether the same SFB-mediated immunological signaling described in mice is preserved in humans and other host species remains unknown.

2.2 BACTEROIDETES

Bacteroides fragilis—Although multiple members of the *Bacteroides* genus have been documented to impact host immune responses, our discussion here will focus specifically on the immunomodulatory properties of *B. fragilis*, which, depending on the strain, can induce either immunoregulation or inflammation. Colonization of germfree mice with *B. fragilis* NCTC 9343 induces a marked expansion of Tregs in the intestinal mucosa via TLR2 signaling—a response dependent on the presence of polysaccharide-A (PSA), a zwitterionic capsular polysaccharide. This response is sufficient to protect mice from both TNBS- and *Helicobacter hepaticus*-mediated colitis, in part, via an IL-10-dependent mechanism (40, 71–73). Dendritic cells, including plasmacytoid dendritic cells, have been shown to sense outer membrane vesicle (OMV)-associated PSA via Gadd45- α and TLR2 (74), which promotes CD4⁺ T cells to secrete IL-10 and suppresses Th17 cell responses (75); OMV signaling via *ATG16L1* and *NOD2* is also likely important (76) as *ATG16L1*-deficient dendritic cells are not able to induce Treg responses. *B. fragilis* NCTC 9343 can also mediate protection against oxazolone-induced colitis in a PSA-independent mechanism by providing a source of bacterial sphingolipids that limits expansion of colonic iNKT cells (77). In addition to regulating mucosal immunity, *B. fragilis* PSA can also impact systemic immune responses, including protection of experimental autoimmune encephalomyelitis (EAE) in mouse models through the action of TLR2 by inducing expansion of a specific subset of Treg cells (i.e., CD39⁺Foxp3⁺CD4⁺ T cells) (78–80).

In contrast to these examples of immune regulation, a subset of *B. fragilis* strains called enterotoxigenic *B. fragilis* (ETBF), including strain 86-5443-2-2, can induce pro-inflammatory responses, severe colitis and colonic tumorigenesis in mice; such responses are accompanied by Stat3 activation and a potent Th17 response that plays a causal role in disease (81). Interestingly, non-toxigenic strains of *B. fragilis* may mitigate the pathogenic effects of toxigenic strains by competitive exclusion mediated via the Type VI secretion system (82) and/or by reducing toxin expression (83). Together, these reports highlight the profoundly different interactions that can occur between the immune system and *B. fragilis* depending on which strain(s) are present in the host.

2.3 PROTEOBACTERIA

Escherichia coli—Diversity of the species *E. coli* represents a spectrum of host relationships that can range from mutualism to opportunistic and specialized pathogenesis (84). Here, we will discuss only the specific examples by which select strains of commensal *E. coli* are known to modulate host immunity. Studies in germfree guinea pigs, chickens and piglets were the first to describe that replicating *E. coli* could stimulate both mucosal and systemic immune responses (85–87). Since then, most studies seeking to define specific interactions between the host immune system and *E. coli* have focused on isolates such as the probiotic strain *E. coli* Nissle 1917, which provides protection against *Salmonella* (88) and pathogenic *E. coli* O157:H7 infections in mice via competition over nutritional resources (89). Nissle 1917 may further promote immune regulation by promoting the expansion of plasmacytoid DC, Foxp3⁺Roryt⁺CD4⁺ T cells and Foxp3⁺Roryt⁺Helios⁺CD4⁺ T cells in the colon and contraction of CD11b⁺CD11c⁺F4/80⁺ monocytes in the small intestine as compared to germfree mice (40).

In contrast to the immunoregulatory and protective effects of *E. coli* Nissle, other human commensal *E. coli* are hypothesized to contribute to the development of intestinal inflammation in a subset of Crohn's disease (CD) patients (90); these opportunists are known collectively as Adherent and Invasive *E. coli* (AIEC). AIEC strains are generally defined by their ability to adhere to and invade epithelial cells, replicate inside macrophages and induce TNF production from macrophages *in vitro* (91). Although detailed *in vivo* studies defining the immunological response induced by these strains are still needed, one recent study elegantly demonstrated that the IgA-coated AIEC strain A2 isolated from CD patients with peripheral spondyloarthritis (SpA), an extra-intestinal manifestation often found in patients with active IBD, induced both mucosal and systemic Th17 responses in germfree mice as compared to non-AIEC *E. coli* (92). Induction of such Th17 immunity required the *E. coli* to harbor the virulence-associated metabolic enzyme propanediol dehydratase, and colonization of genetically susceptible mouse models of colitis and arthritis led to more severe inflammation in both instances (92). Altogether, these findings highlight how complex and intricate the effect of various *E. coli* lineages can be in the host, ranging from responses that mediate protective immunity to immunopathology.

Klebsiella spp. and Proteus mirabilis—Other members of the family *Enterobacteriaceae*, including *K. pneumoniae* and *P. mirabilis* have been documented to also promote inflammatory responses and play a causative role in colitis. Using the TRUC (Tbet^{-/-} Rag2^{-/-}) mouse model, Garrett and colleagues showed that *K. pneumoniae* and *P. mirabilis* (strain name not provided) act in concert with the resident microbiota to elicit transmissible disease dependent on TNF signaling (93, 94). Disease was improved following the transfer of CD4⁺CD25⁺ regulatory T cells (93, 94), indicating that these bacteria negatively impact immunoregulation. More recent studies with two additional *Klebsiella* species describe the expansion of Th17 CD4⁺ T cells in both the small intestine and colon of mono-associated mice (39, 40), thereby suggesting a potential mechanism by which *Klebsiella* may contribute to inflammation. Colonization with *P. mirabilis* HI4320 can also elicit intestinal inflammation following intestinal epithelial injury; such disease is mediated by a bacterial hemolysin that activates NLRP3 signaling in Ly6C^{high} monocytes (95). These

monocytes require the chemokine receptor CCR2 to secrete IL-1 β and contribute to *P. mirabilis*-mediated disease progression (95).

Helicobacter hepaticus—Enterohepatic *Helicobacter* species such as *H. hepaticus* and *H. bilis* are well-known for playing a causative role in the development of liver tumors as well as intestinal inflammation and carcinogenesis in immunodeficient mice (96–98). The immunological mechanisms by which these organisms contribute to disease are best described for *H. hepaticus* and will therefore be highlighted here. Although *H. hepaticus* (NCI-Frederick isolate 1A) induces Th1 responses specific to its flagellar hook protein (FlgE) during colitis in *IL-10*^{-/-} mice (99), additional studies using 129/SvEv *Rag2*^{-/-} mice demonstrated that IL-23, rather than IL-12, secreted by CD11c⁺ monocytes was essential for developing intestinal inflammation (100–102). IL-23 induced by *H. hepaticus* 1A can further exacerbate disease by activating ILC-3 cells, a sub-population of innate lymphoid cells (103). IL-1 β and MyD88 signaling also play critical roles in disease (104, 105). In immunocompetent mice, however, the relationship between *H. hepaticus* and the host immune system appears very different. Colonization of wild-type mice with *H. hepaticus* 1A promotes the development of Treg cells (106). Chow and Mazmanian proposed that *H. hepaticus* actively induces a tolerogenic response from the host through the action of a Type VI secretion system and perhaps via transcriptional suppression of genes such as TLR4, NF- κ B and IL-17R in IECs. This directed effort to modulate the function of IECs may prevent the development of antigen-specific CD4⁺ T cell responses and intestinal inflammation (107, 108). Although further investigation is still required, it seems that in this instance the pathobiont alters host responses such that the host-microbiota balance tips from tolerance to inflammatory.

2.4 FUSOBACTERIA

Fusobacterium spp—Following characterization of the microbiota associated with colorectal cancer, a potential pathogenic role for *Fusobacterium* species emerged (109). Using the Apc^{Min/+} mouse model for tumorigenesis, *F. nucleatum* (strain EAVG_002; 7/1) was shown to not only potentiates tumor growth but also elicit a pro-inflammatory response similar to that of fusobacteria-associated human carcinomas, including infiltration of myeloid cells (109). Additional studies revealed that *F. nucleatum* uses its Fap2 protein to bind to a carbohydrate moiety present on tumor cells (110) and render them resistant to NK and T cells cytotoxic activities via direct interaction with the cell surface receptor TIGIT (111). Interestingly, mono-association of wild-type mice with *F. nucleatum* F0419 induced either little or no expansion of Th17 cells in the colon and small intestine, respectively, (39, 40), indicating that this organism may only significantly modulate host immune responses during disease. In contrast, *F. varium* robustly stimulates the mucosal immune system when it is mono-associated with wild-type germfree mice. *F. varium* AO16 expanded the numbers of CD103⁺CD11b⁺ dendritic cells, Foxp3⁻Roryt⁺CD4⁺ T cells, Foxp3⁺CD4⁺ T cells and Foxp3⁺Roryt⁺Helios⁻CD4⁺ T cells in the colon; however, it did not induce any changes in the populations of immune cells present in the small intestine (40). *F. varium* also influenced host gene expression patterns in a site-specific manner, including up-regulation of genes involved in triglyceride and arachidonic acid metabolism in the colon and down-regulation of genes in the small intestine involving bile acid and retinol metabolism and those

belonging to the cytochrome p450 and Reg3 antimicrobial families (40). Kasper and colleagues hypothesized that such a distinct transcriptional signature could be acting to permit *F. varium* colonization of the mouse intestine (40). Importantly, these findings highlight the distinct influence of *F. varium* and *F. nucleatum* on host phenotypes, including immune responses.

2.5 ACTINOBACTERIA

Bifidobacterium adolescentis—Similar to SFB, mono-colonization of germfree mice with *B. adolescentis* L2-32 markedly promoted the expansion of Th17 cells in the small intestine without provoking intestinal inflammation (39). However, *B. adolescentis* L2-32 induced host gene expression patterns distinct from those of SFB, including genes in MHC II-dependent antigen presentation and muscle-related pathways, the latter of which suggests that non-hematopoietic cells may play a role in communicating with the host immune system during *B. adolescentis* colonization. Of particular interest was the finding that *B. adolescentis* L2-32, like SFB, exacerbated disease in a murine model of arthritis (39). These results demonstrate how the behavior of a gut symbiont can vary from commensal/mutualistic to pathobiont depending on the strain and/or its relationship with the host.

3 A COMMUNITY IS MORE THAN THE SUM OF ITS PARTS

Many studies described above as well as other reports in the literature used germfree animals to test the effects of colonization by single bacterial species on host responses. Although these systems are very powerful for dissecting individual microbe-host interactions and for demonstrating causality, they are somewhat artificial because the complete absence of endogenous microorganisms most likely influences the nature and amplitude of these interactions. Hence, there is a growing interest in developing defined microbial communities of reduced complexity, referred to as minimal microbiomes (10). Figure 1 illustrates concepts for establishing these model communities based on two main approaches detailed in the paragraphs below. It is of course important to eventually test the health-modulating properties of strains under native conditions in animal colonized with endogenous complex communities (112). However, minimal microbiomes allow detailed mechanistic investigation under controlled conditions.

3.1 HYPOTHESIS-DRIVEN ASSEMBLAGE VIA STRAIN SYNTHESIS

Hypothesis-driven assemblage consists of combining known microorganisms previously cultured and characterized. The hallmark of this approach is to work with single microorganisms that must be isolated from a complex starting environment.

Cultivation of individual species can be a substantial challenge for certain microorganisms that are highly sensitive to changes in physicochemical properties (e.g. redox potential or nutrient availability). Hence, strain selection hypotheses as generated by high-throughput sequencing are often hampered by the uncultured nature of candidate taxa (113). So far, the assemblage of minimal bacterial consortia has been primarily driven by expert knowledge of the phylogenetic and functional diversity of the native complex ecosystem under investigation and of phylogenetically-related single microorganisms that can be cultured.

The Altered Schaedler Flora (ASF) is such a consortium that consists of 8 bacterial strains originally isolated from the intestine of mice (114). Since its establishment in the 1970s and 1980s, it has been used often as a reference defined microbiota and has therefore generated the highest number of publications describing the effects of a minimal bacterial consortium on the immune system. The ASF community has been reviewed recently in detail (115) and we have listed key studies pertaining to ASF-induced immune responses in Table 2.

Although major immune parameters are normalized in ASF mice such that they closely resemble their conventionally-raised counterparts, the limited number of strains in the ASF community and the primary rationale for their selection (to standardize mouse models prior to introduction into barrier production and limit colonization by enteric pathogens) explain why not all microbial and host-derived functions are present in ASF-bearing mice, including Th17 cell maturation, numbers of IgA+ plasmablasts, and the expression of RegIII- γ (115). The SIHUMI microbiota represents another consortium of 8 strains (originating this time from the human gut), which harbors metabolic functions not described in ASF mice, such as mucin or bilirubin conversion (116, 117). SIHUMI has been utilized in mouse models of colitis, where inflammatory scores were mirrored by the production of IFN- γ , IL12-p40 and IL17 by colonic tissue explants and by mesenteric lymph node cells stimulated with lysates from members of the SIHUMI community (118); however, additional SIHUMI-induced effects on the immune system have otherwise not been described.

The low complexity of ASF and SIHUMI makes them versatile systems appropriate for testing the impact of colonization by additional strains. However, there are very few corresponding reports in the literature (119–122), and none on the role of specific bacteria in the regulation of immune responses. This may be due in part to the limited public availability of minimal consortia and gut bacterial strains in general, which prevents experimental implementation. The latter shortage has been at least ameliorated by recent projects focused on the cultivation of mammalian gut bacteria and the discovery of novel taxa (123–125), including our own work that cataloged bacterial strains from the mouse intestine (126). This resource has already been used successfully for the generation of a modular minimal consortium, referred to as Oligo-MM (oligo-mouse microbiota), which conferred colonization resistance against the enteric pathogen *Salmonella enterica* serovar Typhimurium (127). The Oligo-MM model opens new avenues for detailed mechanistic studies in gnotobiotics under standardized and close-to-native conditions, including effects on immune responses.

Another recent study pushed the current limits of minimal microbiome-based approaches by colonizing mice with a mixture of 92 cultured bacterial strains (128). The paper focused on describing metabolic functions of the microbiota—their impact on the immune system was not described. Nevertheless, the approach is elegant and the work offers new perspectives for the field. Although the authors reported a core of colonizing strains that formed stable, dominant communities across each animal, not all 92 strains established in the mice. Variations were detected for a few taxa and may increase when population densities are lower than those detectable by sequencing. Moreover, bacterial spatial distribution in the gut was not investigated, nor was colonization of different mouse groups with the defined complex mixture. The latter experimental aspect has a high chance to result in varying microbiota composition; one could take advantage of this inter-individual variability by

combining it with metabolome characterization, antigen profiling, and detailed immunophenotyping to unravel microbe-host interactions in a highly controlled manner.

Finally, one additional line of promising future research is the use of models other than mice that are better suited for translational medical research, especially pigs (129, 130). Decades ago, studies in germfree pigs/piglets highlighted the impact of gut microbiota on the immune system (131, 132). To the best of our knowledge, there have been very few attempts to implement minimal microbiomes in pig models (133). Logistics associated with the maintenance of gnotobiotic pigs and limited availability of microbial strains from the pig intestine are sound explanations for this paucity of data. However, the impact of demonstrating the causal effects of specific gut microbiota members on immune regulation in animal models with better clinical relevance would match the effort required for such work.

3.2 FUNCTIONAL ENRICHMENT VIA COMMUNITY REDUCTION

Instead of following a culture-based, reductionist approach as described in the previous section, the functional enrichment workflow relies on serial reduction of starting community's complexity to obtain the most simplified consortium associated with the given phenotype of interest. This approach can include a combination of *in vitro* and *in vivo* enrichment strategies, as pictured in Figure 1.

By focusing on target functions of the native ecosystem (e.g. the ability to induce a certain disease phenotype), the major advantage of this second approach to generate minimal microbiomes is that it bypasses the major risk of not recapitulating the desired phenotype when working with single isolates or a combination thereof. However, the workload associated with multiple germfree experiments necessary for functional enrichment can rapidly become a bottleneck, and the final consortium of reduced diversity may still contain unknown taxa that complicate interpretation of results.

To the best of our knowledge, there are very few examples of functional enrichment approaches that generated defined communities. Although not related to immune responses, a recent seminal study illustrates well the principle of *in vivo* community reduction (134). The authors showed that providing a diet low in fermentable polysaccharides to mice previously colonized with a human fecal microbiota reduced the relative abundance of many taxa (as detected by sequencing), illustrating a loss of dominant microbiota members. Although this effect was reversed when a high polysaccharide diet was re-introduced to the same mice, constant feeding of the low polysaccharide diet across four generations resulted in irreversible, long-term loss of taxa.

Another example of effector strain enrichment, this time in the context of inflammation, is the *in vitro* enrichment of lactobacilli from the murine gut for treatment of *Citrobacter rodentium* colitis. These lactobacilli reduced disease severity, improved gut barrier function and decreased mRNA expression of IFN- γ and TNF in the distal colon (135). Of note, treatment of *C. rodentium* infected mice with MRS alone (the medium used for *in vitro* enrichment) also tended to provide similar benefits and was associated with increased abundance of endogenous lactobacilli.

Perhaps the best known example of a successful community reduction approach in the context of immune modulation is the induction of Treg cells by a mixture of butyrate-producing clostridia (136, 137). The authors followed an elegant procedure with several steps of culture enrichments (e.g. using chloroform) prior to inoculation into germfree recipient mice; eventually they cultivated single strains and characterized a 17-member clostridium cocktail (138). Unfortunately, the authors did not provide full taxonomic classification of the strains that, although published, still do not have standing in the nomenclature and are not publically available. Moreover, effects of metabolic functions other than butyrate production (e.g. bile acid dehydroxylation by the *Clostridium scindens* strain) may be worth investigating.

The minimal microbial consortia described here clearly impact host physiology—some are even documented to influence immune responses. Although the exact mechanisms underlying these host-microbe interactions have yet to be described, they likely involve microbial metabolites capable of modulating cellular microenvironments. In the next section, we give examples of microbiota-derived metabolites that can influence immune responses, with a special focus on lipids.

4 LIPIDS AND THEIR IMPACT ON IMMUNE RESPONSES

Lipids represent a very complex family of dietary, host, and microbial compounds. Their direct roles in regulating immunity has been reviewed elsewhere (139, 140); however, the impact of gut microorganisms on lipid-based modulation of the immune system is less known. The immunoregulatory activities of short-chain fatty acids (SCFA) produced by gut bacteria via the fermentation of carbohydrates and proteins has been already studied and reviewed extensively (141, 142) and will be described only briefly in the next section. The gut microbiota can also metabolize dietary lipids, cholesterol, and host-derived cholesterol metabolites such as steroids and bile acids (143–145). Hence, gut bacteria have the potential to alter the bioavailability and bioactivities of these compounds and thereby alter corresponding signaling networks. When investigating the effects of gut microbial metabolites, it is important to keep in mind that the primary function of the digestive system is efficient absorption of dietary components and that many absorptive processes, including those for lipids, take place in proximal parts of the small intestine, whereas the densest microbial communities are found in the colon. That being said, the amount of metabolites that reach distal parts of the intestine may still be substantial depending on environmental (e.g. diet) and host genetic factors (e.g. transporter efficacy, *de novo* synthesis capacities). Combined with the fact that lipid-dependent signaling pathways are sensitive, it is sound to assume that the gut microbiota influences lipid-driven regulation of immune responses. The implementation of minimal microbiome-based approaches for studying these interactions will open new avenues of research and applications and facilitate testing the causative role of gut microbes in health regulation and identifying direct versus indirect effects.

4.1 SHORT-CHAIN FATTY ACIDS (SCFA)

SCFA are metabolic by-products of gut microbiota fermentation of dietary substrates, including fibers. The most abundant of these well-studied bacterial metabolites include

acetate, propionate, and butyrate, the latter of which serves as an important fuel source for IECs (146). Additionally, SCFA regulate the functions of a wide variety of both innate and adaptive immune cells primarily via the G protein-coupled receptors (GPCRs) GPR43 and GPR41. SCFA can also directly impact the actions of immune cells via GPR-independent mechanisms, including inhibition of histone deacetylase (HDAC) activity, which regulates gene transcription (142). Both mechanisms can be observed in SCFA regulation of innate immune cells, including macrophages. Butyrate in particular limits intestinal macrophage production of the pro-inflammatory mediators nitric oxide, IL-6, and IL-12 (57) and regulates intestinal macrophage polarization via histone acetylation (147). Butyrate also signals via Gpr109a on colonic macrophages to limit inflammation and maintain intestinal homeostasis (148). SCFAs can also induce tolerogenic responses in murine mucosal dendritic cells (149) and impart anti-inflammatory effects onto human monocyte-derived dendritic cells (150). SCFAs also impact the functions of polymorphonuclear cells (PMN, e.g., neutrophils), another type of innate immune cell. SCFA promote neutrophil activation and migration via upregulation of adhesion molecules and increased chemokine production (151, 152). However, they can also dampen neutrophil effector functions such as phagocytosis, bacterial killing and cytokine production (153).

Many reports in the literature also describe the profound impact of SCFA on the adaptive immune system, particularly T cell phenotypes. Butyrate induces differentiation of colonic and peripheral Treg cells in mice, potentially via histone modification of the Foxp3 locus (58, 137). Additionally, acetate and propionate regulate colonic Treg cell numbers and functions via GPR43 signaling in mice (154). More recently, optimal development of memory CD8⁺ T cells has been shown to require acetate (155). Humoral immune responses are also influenced by SCFA. Recently, Kim and colleagues showed that SCFA regulate metabolism and gene expression in B cells, which enhances antibody production (156). Additional studies revealed that acetate influenced vitamin A metabolism via GPR43 signaling in dendritic cells, which ultimately promoted intestinal B cell production of IgA (157). Additional studies are needed to determine if SCFA also impact humoral immunity via HDAC activity.

Considering that SCFA are absorbed by the gut and reach systemic circulation via the portal vein, these immunomodulatory metabolites can impact both local and systemic responses and may be beneficial for treating immune-mediated diseases. The anti-inflammatory properties of SCFA treatments have been observed in mouse models of colitis (137, 158) as well as in human patients, who experienced some improvements in disease following colonic irrigation with SCFA (159). Probiotic treatment with butyrate-producing bacterial strains such as *Clostridium butyricum* represents another potential therapy and showed promising results when administered to ulcerative colitis patients (160). Studies in mice suggest that *C. butyricum* limits colitis by triggering IL-10 production from intestinal macrophages (45). More recently, *C. butyricum* was shown to attenuate steatohepatitis in mice partially through butyrate-induced immunomodulation in the liver that included promoting Treg and limiting Th1 and Th17 cell development (161). Additional efforts to identify strains of gut bacteria that can provide a source of SCFA for treatment of inflammatory diseases are clearly warranted.

Such therapeutic approaches may also one day benefit patients with systemic immune-mediated diseases. Mice fed a high-fiber diet experienced increased circulating levels of SCFA and were protected against allergic airway disease (162). Treatment with propionate seeded the lungs with dendritic cells impaired in their ability to induce T-helper type-2 cell effector functions. In another pre-clinical study, acetate and propionate protected mice against development of experimental autoimmune encephalomyelitis, which was associated with an increase in Treg cells and a decrease in Th1 cells; however, providing SCFA in the drinking water or feeding a high-fiber diet exacerbated antibody-induced arthritis (163). Such dichotomous findings emphasize caution as these novel treatments are explored further.

4.2 OTHER LIPIDS

Lipid-binding receptors are known to influence inflammatory responses (140). Because the nature of these responses depends on lipid type and amount, the metabolic functions of gut bacteria such as fatty acid isomerase (164), lipid storage (165, 166), breakdown of triglycerides by lipase activity (145), or conversion of cholesterol (143, 167) are likely to influence immune reactions. For instance, cholesterol has the potential to induce intestinal inflammation and chemical inhibition of cholesterol absorption was shown to diminish systemic inflammatory markers and the severity of atherosclerosis (168, 169).

Bile acids represent one family of cholesterol-derived metabolites produced by the host that are found in high concentrations in the intestine, are metabolized by gut bacteria, and influence multiple inflammatory and metabolic signaling pathways. Genetic variants in one of the main bile acid-responsive transcription factor FXR (farnesoid X receptor) have been associated with the occurrence of inflammatory bowel diseases (IBD) (170), and alterations of bile acid pools have been reported in IBD patients (171). Mechanistic studies in mice demonstrated that interfering with bile acid signaling via FXR and TGR5 (also known as GPBAR1, G protein-coupled bile acid receptor 1) influence TLR-dependent pathways, chemically induced colitis, and NLRP3-dependent inflammasome activation (172–174). *In vitro*, bile acids regulate cytokine expression and cellular stress pathways (175–177). In contrast to these clinically or mechanistically relevant data, there is no experimental evidence for the impact of microbial conversion of bile acids on the diversity and activity of immune cell populations. In this respect, the use of minimal microbial consortia varying in their ability to produce specific secondary bile acids will be of help. Pioneering studies based on the colonization of germfree rodents with bile acid-converting strains have been reported (178, 179), but pathophysiological effects were not studied and the strains were not well characterized and are no longer available. More recent work successfully established a gnotobiotic mouse model colonized with a minimal bacterial consortium producing secondary bile acids (180, 181); however, the strains are also not readily available and the impact on immune responses was not investigated.

5 CONCLUSIONS AND OUTLOOK

Work in the 1960s first demonstrated the important influence of gut microorganisms on mouse phenotypes (182). Today there is a renewed interest in understanding the impact of

changes in microbiota composition (e.g. due to animal housing) on immune responses (65, 183). It is therefore of utmost importance to dissect the effects of specific gut bacteria on the host so we can identify the microbe-host interaction networks that shape the immune system, with the ultimate goal of manipulating these interactions for positive health outcomes. The isolation and culture of microorganisms is essential to this process as it enables working with specific taxa (as single strains or simplified communities) in downstream experiments.

Catalogues of human and mouse gut bacteria have been expanded substantially thanks to recent culture-based work (123, 124, 126). Such strain collections represent an important foundation for controlled, mechanistic studies. Efforts to isolate and identify additional species should be continued or even intensified in the near future, especially with respect to efficient taxonomic description of novel taxa (184, 185) and to functional characterization of strains and their impact on immune functions (40). For the latter purpose, public accessibility of strains is paramount for performing mechanistic analyses (186). In future work, it will also be important to assess in greater detail the role of gut microorganisms other than bacteria in modulating the immune system.

Research based on the use of minimal microbiomes is now entering an accelerated phase of development. We have new opportunities to implement microbial consortia that better resemble native gut communities; such advances will also provide standardized models that are comparable between labs. Most importantly, minimal microbiomes can be used in a modular fashion—when combined with an ever-increasing availability of strains, they will facilitate determining the effects of specific taxa in the presence of endogenous microorganisms and thereby provide an important proof-of-concept in pre-clinical research. To support the modular design of minimal microbiomes, future work should implement novel bioinformatic approaches that infer the best possible mixtures of cultured strains (e.g. based on sequencing data from dysbiotic native communities). Model communities can also be used to study host specificity of colonization and immune system maturation. Without going into detail because already discussed in detail elsewhere (10, 126, 187–190), the study of underlying molecular mechanisms using strains from various host species will also be a fascinating avenue of future research.

Although microbiome and host-gut microbe interactions research has garnered much attention in the last decade, descriptive and pre-clinical studies still dominate the literature and translation into human settings has been rather slow. *Clostridium difficile* enteric infections represent a showcase for the efficient use of minimal microbiomes in the clinic (191); however, this advancement likely relates to exclusion of an undesirable strain by commensals via microbe-microbe interactions and does not involve modulation of host responses as is likely needed for treatment of immune-mediated diseases. Manipulation of immune functions with microbiome-based therapeutics will likely require a more tailored approach that involves targeting specific host-microbe relationships. We may also need to intervene during the host's early life when gut communities are established and the immune system educated (18). Finally, we should also consider that targeted microbiome modulation could also improve the efficacy of conventional medications intended to augment immune

responses (192). These and other microbiome-based interventions that modulate immunity represent an exciting new horizon for biomedical research.

Acknowledgments

This work was supported in part by the German Research Foundation (grant no. CL481/2-1). A.E.R.T. acknowledges funding from the National Institute of General Medical Sciences of the National Institutes of Health (P20GM104320), the Crohn's and Colitis Foundation of America (grant # 3578), and the University of Nebraska–Lincoln.

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FIGURE 1.

From complex gut microbial community to defined experimental systems. Prerequisite for the workflows presented here is an animal model (a mouse in the present example) with a specific phenotype (e.g. chronic disease with a clear measurable endpoint) or a physiological parameter of interest (e.g. maturation of specific immune cell populations). The phenotype/parameter of interest can be associated with defined gut microbiota signatures and must be absent in germfree animals. Starting from a gut sample collected from an animal with the specific feature of interest, two routes of action can be followed: (A) The classical route is based on cultivation and isolation of strains (e.g. microbial colonies on nutrient agar) with the aim of identifying single components of the complex ecosystem of origin that can drive the phenotype. Each isolate is characterized taxonomically and functionally and deposited in public culture collections for downstream access. Based on expert knowledge or assisted by computational methods, single strains are combined together and used to colonize germfree animals with the goal of mimicking the disease phenotype or physiological parameter of interest. (B) A second, less explored route is the selection of microbial communities with the aim of obtaining the most reduced complexity still associated with the phenotype of interest. This can be achieved via iterative association of germfree animals (circled black arrow) in the presence of selective forces (e.g. special diet or antibiotics), and/or *in vitro* enrichment of specific microbes (e.g. via selective media or simply by serial dilution) prior to colonization of germfree animals. The advantage of performing an *in vitro* step prior to association with mice is bulk selection of microorganisms that can indeed grow under laboratory conditions, which can substantially facilitate downstream identification of single members of the final minimal microbiome. The progress of community reduction can be monitored throughout

the workflow using targeted (16S rRNA amplicon) or shotgun metagenomic sequencing. Of note, workflow B can be followed by A (dashed arrow) for detailed characterization of single community members. The new gnotobiotic models created by either approach can ultimately be used for detailed experimental testing of microbe-microbe and microbe-host interactions.

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Table 1

Impact of select microbial strains on the immune system in gnotobiotic mouse models

Strain	Reference	Impact on phenotype
<i>Acinetobacter lwoffii</i> F78	Geva-Zatorsky et al. 2017 (40)	Repressed colonic IL-22 production
<i>Akkermansia muciniphila</i> ATCC BAA-835	Derrien et al. 2011 (193)	Modulated colonic expression of host genes involved in antigen presentation, B and T cell receptor and leukocyte trafficking, IL-4 signalling and complement and coagulation cascades
<i>Bacteroides caccae</i> ATCC 43185	Faith et al. 2014 (38)	Increased number of CD4+ Foxp3+ Treg cells in the colonic lamina propria
<i>Bacteroides intestinalis</i> (no strain designation)	Faith et al. 2014 (38)	Increased number of CD4+ Foxp3+ Treg cells in the colonic lamina propria
<i>Bacteroides thetaiotaomicron</i> ATCC 29148	Faith et al. 2014 (38); Geva-Zatorsky et al. 2017 (40); Hoffmann et al. 2016 (38, 40, 194)	Increased number of CD4+ Foxp3+ Treg cells in the colonic lamina propria
<i>Bacteroides thetaiotaomicron</i> ATCC 29741	Geva-Zatorsky et al. 2017 (40)	Increased number of CD4+ Foxp3+ Treg cells in the colonic lamina propria
<i>Bacteroides vulgatus</i> mpk	Muller et al. 2008 (38, 40, 195)	Induced DC production of IL-6 in the absence of other pro-inflammatory cytokines
<i>Bacteroides vulgatus</i> (no strain designation)	Faith et al. 2014 (38)	Induced expansion of colonic Treg cells
<i>Bacteroides vulgatus</i> ATCC 8482	Geva-Zatorsky et al. 2017 (40)	Induced expansion of plasmacytoid dendritic cells
<i>Candida albicans</i> B311 (type A)	Jones-Carson et al. 1997 (196)	Induced development of memory T cells in a B cell-deficient mouse
<i>Candida albicans</i> SC5314	Faith et al. 2014 (38)	Induced production of IFN- γ , IL-17 and IL-22
<i>Clostridium sordellii</i> AO32	Geva-Zatorsky et al. 2017 (40)	Repressed colonic IL22 production
<i>Parabacteroides distasonis</i> (no strain designation)	Faith et al. 2014 (38)	Increased number of CD4+ Foxp3+ Treg cells in the colonic lamina propria
<i>Ruminococcus gnavus</i> ATCC29149	Geva-Zatorsky et al. 2017 (40) Hoffmann et al. 2016 (40, 194)	Up-regulated expression of host genes related to antimicrobial responses as well as amide, lipid and tryptophan metabolism in the colon

Table 2Impact of defined minimal bacterial consortia on immune parameters *in vivo*

Minimal consortium (host species)	Reference	Impact on host phenotype
ASF (mouse)	Jergens et al. 2007 (120)	Induction of systemic IgG
ASF (mouse)	Ivanov et al. 2008 (197)	Lack of Th17 cells and increased proportions of Foxp3+ cells in the small intestinal lamina propria; reduced proportion of IgA+ cells compared with SPF mice
ASF (mouse)	Hapfelmeier et al. 2010 (198)	Induction of IgA in duodenum
ASF (mouse)	Feng et al. 2010 (199)	Homeostatic and spontaneous proliferation of CBir1 TCR transgenic T cells in RAG-knockout mice
ASF (mouse)	Geuking et al. 2011 (200)	Activation and de novo generation of colonic Treg cells
Bristol microbiota (pig)	Laycock et al. 2012 (133)	Increase in serum levels of immunoglobulins
ASF (mouse)	Mosconi et al. 2013 (201)	Induction of Thymic stromal lymphopoietin (TSLP) mRNA expression in colonic lamina propria; Induction of IL-17 and IFN- γ in TSLP-knockout mice but not C57BL/6 wildtypes
ASF (mouse)	Natividad et al. 2013 (202)	RegIII- γ expression in ileum and colon lower than that induced by a complex microbiota
17-member clostridia cocktail (mouse)	Atarashi et al. 2013 (136)	Treg cell maturation
SIHUMI (mouse)	Eun et al. 2014 (118)	Increased IL-17, IL12-p40, and IFN- γ protein secretion by colonic tissue explants and MLN from IL10-knockout mice

ASF, Altered Schaedler Flora; MLN, mesenteric lymph node; SIHUMI, simplified human intestinal microbiota; SPF, specific pathogen-free