Understanding immune-microbiota interactions in the intestine

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Summary

The past two decades have seen an explosion in research that aims to understand how the dynamic interplay with the gut microbiota impacts host health and disease, establishing a role for the gut microbiota in a plethora of pathologies. Understanding how health-promoting microbiota are established and how beneficial host–microbiota interactions are maintained is of immense biomedical importance. Despite the enormous progress that has been made, our knowledge of the specific microbiota members that mediate these effects and the mechanisms underlying these interactions is rudimentary. The dearth of information regarding the nature of advantageous host–microbiota interactions, and the factors that cause these relationships to go awry, has hampered our ability to realize the therapeutic potential of the microbiota. Here we discuss key issues that limit current knowledge and describe a path forwards to improving our understanding of the contributions of the microbiota to host health.

Abbreviations

ATP - adenosine triphosphate

CD - Crohn's disease

CRISPR - clustered regularly interspaced short palindromic repeats

IBD - inflammatory bowel disease

IL - interleukin

RAG - recombination activating gene

SFB - segmented filamentous bacteria

The gut microbiota as a regulator of host health

Born devoid of microbes, our intestines gradually become densely colonized by a complex collection of bacteria, eukaryotes, archaea and viruses that are collectively referred to as the gut microbiota. Rather than being mere passengers, the gut microbiota profoundly shapes many facets of host physiology, including aiding in the acquisition and provision of nutrients, educating the immune system, and providing resistance to invading pathogens.[1, 2] Although interest in this complex ecosystem is often touted as new, the microbiota has proved a source of fascination for scientists for well over a century. Indeed, immunological interest in the role of the gut microbiota can be traced to Elie Metchnikoff, who was obsessed with how the gut microbiota could be detrimental to health, and how this could be remedied through administration of health-promoting bacteria (probiotics), in the early twentieth century.[3] Although study of the microbiota long-remained a fringe area of research, a number of intrepid investigators continued to provide key insights into the contributions of our gut microbes to host health, laying the groundwork for modern microbiota research.[4-8] The field was reinvigorated with the advent of new and improved approaches for characterizing the composition of the microbiota and provocative findings that suggested the microbiota could modulate susceptibility to an ever-increasing variety of diseases.

The development of next-generation sequencing technologies, and analytic approaches accessible to non-experts, provided researchers with the opportunity to interrogate the structure of complex microbial communities at an unprecedented scale, advancing earlier efforts to understand the microbial and viral ecology of the intestinal tract.[6, 9-19] These approaches allowed researchers to define the microbiota composition from large numbers of individuals across time and geographical boundaries, at different developmental stages, and in response to a variety of perturbations.[20-22] These culture-independent approaches, which continue to grow in scale and resolution, revealed that the microbiota reaches a relatively stable and functionally mature state by 3 years of age[22] and exhibits large interpersonal variation,[14, 23] with each individual carrying a largely unique set of strains that persist for extended periods of time.[21, 24] Gut microbial community composition is modified by many environmental factors, such as geographical location,[21, 22] host diet[25] and administration of antibiotics[26] and other medicines.[27] Despite inter-individual differences in community composition, a core set of genes shared across individuals is encoded by the microbiota, suggesting that distinct community compositions can provide similar functional outputs.[13, 14] Strikingly, it was demonstrated that, relative to healthy controls, differences in microbial community structure (often termed dysbiosis) could be observed in individuals suffering from a variety of disorders including obesity,[28, 29] inflammatory bowel disease (IBD),[30-32] cardiovascular disease,[33] malnutrition,[34] and type 2 diabetes,[35] many of which had not previously been linked to the activity of microbes. Furthermore, transplantation of fecal microbiota from human donors with such diseases to recipient germ-free/gnotobiotic mice was shown to transmit features associated with the disease. This includes obesity,[29] malnutrition,[34, 36] autoimmunity,[37, 38] food allergy[39] and IBD,[40, 41] providing proof of principle of a causal role for the microbiota in elicitation or exacerbation of disease. Hence, a picture emerged where the unique constellation of microbial strains within an individual represented a critical factor that, in conjunction with host genetics and environmental influences, could influence disease susceptibility. The finding that alterations in microbiota composition were not merely correlated with disease, but could play a causal role in the development of a number of distinct host pathologies captured the imagination of scientists and the public alike, placing the microbiota at the forefront of biomedical research. Furthermore, experimental studies demonstrating that ‘microbiota repair', through administration of complete fecal communities or select microbial taxa, could alleviate disease in both humans and mice,[42, 43] highlighted the therapeutic potential of next-generation probiotics. This has led to a flurry of work from scientists in disparate fields to provide

insight into the microbes responsible for modulating host health, and has allowed microbiota researchers to adopt a framework akin to Koch's Postulates[44] for microbiota-driven phenotypes.

Immune modulation by the gut microbiota

The gut microbiota profoundly impacts the phenotype and function of the host immune system, the details of which have been reviewed expertly elsewhere.[2, 45-47] Germ-free mice present with numerous immune defects, including impaired T-cell differentiation and accumulation in the intestine,[48-50] decreased production/secretion of immunoglobulin A (IgA),[8, 51] reduced anti-microbial peptide production,[52] and elevated levels of systemic IgE.[53] Importantly, many of these deficits can be reversed through administration of a fecal microbiota from murine and human donors, or their derived bacterial strains, as well as by fungi or viruses, reinforcing the potential contribution of non-bacterial members of the community.[48, 49, 54-58] Although not the first description of immunomodulation by the microbiota, the discovery that different frequencies of T helper type 17 (Th17) cells were present in the small intestine of C57BL/6 mouse substrains obtained from two different commercial vendors, and that this was attributable to the presence of a single microbe, segmented filamentous bacteria (SFB),[49, 55, 56] prompted intensive efforts to identify additional microbes that could control the development of various arms of the immune system. Understanding the molecular basis of such microbiota–host interactions could provide a basis to develop novel agents that shape immune function in a targeted fashion to benefit host health. However, the massive complexity of the gut microbiota poses a daunting challenge to researchers, and identifying effector strains (microbial taxa that shape phenotypes of interest) remains a major goal for the field. Several different strategies have been used to overcome these difficulties and provide detailed insight into immune–microbiota interactions.

Strategies to identify effector strains

An optimal strategy to identify effector strains should have the following key features; (i) require no prior knowledge of the effector strain; (ii) not be focused solely on highly abundant community members; (iii) be agnostic to currently known functions of different taxonomic classifications; (iv) provide strain-level resolution; and (v) be capable of identifying complex relationships where the effects of a particular microbe could be modified by other microbes.[59, 60]

The primary strategy for identifying effector microbes involves approaches that compare community composition (membership and relative abundance) in a community that modifies immune function with one that does not. This has been employed successfully in the identification of SFB as a potent stimulator of the intestinal immune system.[56] However, it has many limitations. First, strains whose abundance correlates with a phenotype of interest may not play causal roles in that phenotype.[61] Second, comparing the structure of distinct microbial communities that imprint different host phenotypes may lead to the generation of a large list of taxa that are differentially represented and therefore, potentially mediating the phenotypic difference, making the identification of target agents a difficult task. An elegant solution to this problem uses co-housing of mice that have been colonized with different microbiota that are associated with distinct phenotypes.[62, 63] As co-housing of different mice leads to incomplete exchange of gut microbiota, this approach generates ‘sublines' of mice harboring distinct mixtures of the two ‘parental' microbiota. By determining shared microbiota constituents that correlate with the phenotype of interest in the sublines and parental mice, the microbes responsible can be parsed in a more efficient fashion. Third, until recently, sequencing-based studies lacked strain-level resolution, and used ≥97% 16S rRNA gene sequence similarity as a proxy for identifying species. Yet, inferring function based on this level of taxonomic resolution is flawed given that strain-level variation, where strains of a given species vary in their function or the host phenotypes they imprint, is a well-

described feature of gut microbes (here we refer to strains as isolates of the same species that exhibit distinguishable genotypic or phenotypic characteristics[64]). For example, strains of *Bacteroides fragilis* that express the enterotoxin known as *B. fragilis* toxin can promote the development of colon cancer,[65] colitis,[66] and malnutrition-associated cachexia,[67] whereas *B. fragilis* toxin-negative strains lack this capacity. Conversely, a non-enterotoxigenic strain of *B. fragilis* is associated with the induction of regulatory T (Treg) cells and interleukin-10 (IL-10).[68] This is not a peculiarity of *B. fragilis*, and profound differences linked to strain-level variation among other gut bacteria have been documented.[69] Hence, efforts to validate findings based on sequencing, which typically involves the use of a commercially available strain of the species of interest, may lead to use of strains lacking the desired activity, deeming the effort a failure. Finally, even when key effector strains/strain consortia can be identified, they may not be themselves sufficient and instead require the presence of specific additional community members for the phenotype to manifest. For example, in the 'TRUC' model of transmissible colitis in T-bet$^{-/-}$RAG$^{-/-}$ mice, *Klebsiella pneumoniae* and *Proteus mirabilis* elicit the development of colitis, but only when in the presence of an endogenous microbial community.[70] Similarly, while *Helicobacter hepaticus* induces robust typhlocolitis in IL-10$^{-/-}$ mice, mono-colonization of germ-free IL-10$^{-/-}$ mice with *H. hepaticus* is insufficient to promote intestinal pathology.[71-73] Hence, microbes that do not vary between communities may be necessary to observe a phenotype and will be missed by sequencing-dependent efforts.

Another commonly employed approach involves the use of anti-microbial agents to winnow the number of potential effector strains. These have included antibiotics that target specific classes of bacteria (i.e. Gram-positive, Gram-negative, anaerobes) and chloroform or ethanol treatment of intestinal material to enrich for spore-forming bacteria, which have also proved to be of major utility in identifying modifiers of the immune system.[48, 49, 54, 74, 75] However, such strategies are prone to some of the same issues listed above, and can preclude the identification of some complex relationships where distinct microbes must interact for a phenotype to manifest (for example if Gram-negative and Gram-positive bacteria cooperate). Moreover, some of these anti-microbial agents can also affect host physiology, which can confound interpretation of their effects.[76] Mono-colonization with large panels of microbes can also be used to determine their effects on host immune responses,[77, 78] but such efforts require that the microbial taxa used are sufficient to promote the phenotype of interest, require the ability of the specific taxa used to successfully mono-colonize mice, and will miss scenarios where microbe–microbe interactions are key to imprinting phenotypic responses.

More recently, researchers have developed and implemented newer, less biased approaches to address these limitations. The most fruitful of these methods involves assessing the coating of gut microbes with host-derived IgA, the dominant antibody class in the gut lumen, to identify immunogenic microbes within a complex community (i.e. microbes coated with IgA). In this strategy, microbes from intestinal contents that are coated by host IgA are detected through flow cytometry and IgA-positive and IgA-negative fractions purified by fluorescence-activated cell sorting are subjected to 16S rRNA gene sequencing for identification, or transferred to recipient gnotobiotic mice to determine their ability to transmit a phenotype[36, 41, 79, 80] (Fig.**1**). Using this approach to study microbiota from individuals with IBD, it has been demonstrated that bacteria enriched in IgA coating support development of more severe dextran sodium sulfate-induced colitis than non-targeted bacteria, identifying putative candidates that modulate the susceptibility to IBD.[41] A similar strategy identified a strain of *Escherichia coli* that was enriched for IgA targeting in microbiota from a subset of patients with Crohn's disease with spondyloarthritis and that could elicit potent activation of mucosal CD4^{+} T cells.[81] IgA targeting was also able to identify pathogenic *Enterobacteriacea*e from the microbiota of a child suffering from Kwashiorkor (a form of severe acute edematous

malnutrition) that could induce a high degree of mortality upon transplantation to gnotobiotic mice consuming a nutrient-deficient diet. In addition, IgA-targeted microbes from a healthy microbiota donor could in turn prevent this mortality phenotype, so pathogenic and protective microbes can be enriched for targeting by IgA and identified using this strategy.[36] This suggested that IgA targeting generally reflects microbes with immune modulatory potential, rather than pathogenic strains *per se*. However, a recent systematic assessment of the specificities of intestinal IgA has revealed a large degree of polyreactivity, suggesting that binding by IgA may not actually be due to B-cell stimulation by a given IgA-coated target,[82] but rather the expression of an epitope targeted by polyreactive IgA. Furthermore, this strategy captures only those microbes that have been bound by IgA, and microbes that evade antibody responses, or which shape immune responses without eliciting an immune response against themselves (for example, a microbe that enhances the fitness of a strongly immunogenic strain could alter immune responses in the gut without being recognized by the immune system) will not be detected using this approach.

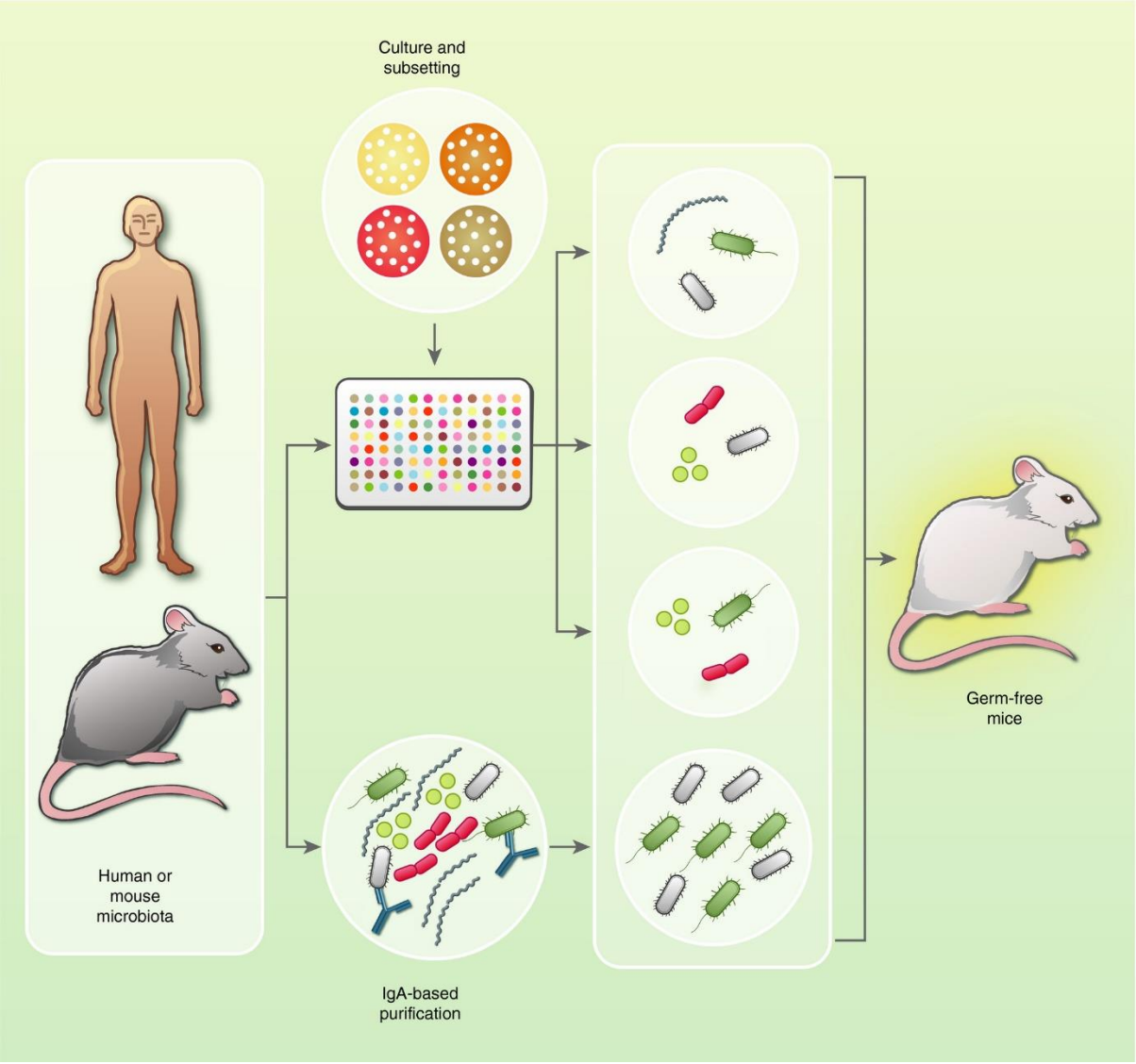

Figure 1- Identifying immunomodulatory microbes within the gut microbiota: As depicted above, newer strategies employ intensive culturing and microbiota subsetting, and/or IgA-based identification of immunomodulatory microbes, followed by selective reconstitution of germ-free mice, to map phenotypes to defined microbiota components. These efforts should provide key insights into the prominent modifiers of host immune phenotypes and help to develop next-generation probiotics that beneficially manipulate immune function.

One of us (PPA) recently developed a strategy, termed combinatorial gnotobiotics, that provides a more systematic assessment of the microbes that shape host phenotypes of interest.[83] This approach (described in ref.[59]) involves isolation of microbial strains from a community of interest in clonally arrayed culture libraries,[84] and administration of randomly selected microbial consortia of differing but known composition to gnotobiotic mice, to assess how individual microbes, either alone or together with other community members, modulate phenotypes of interest (Fig. **1**). This strategy has the advantage of providing strain-level resolution, requires no prior knowledge of the strain, is agnostic as to whether a host response targets the microbe, and by using subsets containing different numbers of microbes has the ability to identify higher-order interactions where two or more strains cooperate or compete to elicit a response. This strategy effectively identified complex interactions that regulated the cecal metabolite pool, and identified greater redundancy in colonic Treg cell accumulation than had been previously appreciated.[83] In addition to requiring a larger number of gnotobiotic mice than the other approaches discussed, a primary challenge of this strategy is the need to capture agents in pure culture, which would have missed the important contributions of SFB to mucosal immunity because of the difficulties associated with its cultivation.[85] Although much maligned, recent years have witnessed a rebirth of culture-based approaches to interrogate gut microbiota composition and function.[74, 84, 86, 87] Resistance to culture-based strategies can be explained in part by the notion that much of the microbiota was 'unculturable'.[87] A resurgence in efforts to culture these microbes, led by environmental microbiologists, has seen dramatic improvements in the proportion of the gut microbiota that can be isolated. In a landmark study, 212 different culturing conditions, involving perturbations to media composition, growth temperature and pH among other variables, were tested leading to the isolation of 340 bacterial species, many of which had not been previously found in the human gut.[86] Importantly, just 20 different conditions were sufficient to isolate a majority of the strains, and it has been demonstrated by other groups that a great deal of the diversity within a given community can even be captured using a single rich medium,[84, 88] enhancing the tractability of isolation efforts. As improvements continue to be made in culturability, aided by metagenomics-based identification of potential growth factors, isolation of strains of interest will likely be less problematic, thereby advancing studies that begin with a culture-based step.

Clearly, no single approach encompasses all the discovery features required for a truly unbiased and fully systematic approach, nor are any of these strategies suited to all questions. Instead, leveraging the advantages of these different approaches may provide an optimal way towards increasing our understanding of gut microbiota regulation of immune function. As a proof-of-principle of the utility of combining these strategies, studies merging the power of sequencing of IgA-bound bacteria with isolation in culture and phenotypic assessment in gnotobiotic mice, have already been employed to identify potential harmful and beneficial bacterial taxa.[36, 41]

Of mice and men

An important facet of gut microbiota research using gnotobiotic mice involves the decision about whether to study microbes derived from humans or those obtained from mice (Fig.**2**). The rationale for studying human-derived microbes is self-evident, in that it appears more translationally relevant. Microbial communities from humans with various diseases of interest can be assessed for their capacity to transmit disease susceptibility and microbes implicated in the modulation of human health can be identified.[[29, 34, 36-42]] This approach has led to the successful identification of the gut microbiota as a critical regulator of a variety of diseases, and defined specific human-derived microbial taxa that can shape the host immune system.[54, 83] However, a number of caveats exist that should be considered. First, many human-associated microbial taxa poorly colonize mice, if at all,

and the relative abundance of those taxa that do successfully colonize may poorly reflect that observed in the donor community.[25, 89] The study of a complex human microbiota in gnotobiotic mice may therefore significantly overstate or underestimate the contributions of particular taxa to phenotypes of interest. Second, human-derived fecal microbiota communities may be less well adapted to the mouse intestine. When mice harboring a human-derived or mouse-derived fecal microbiota were co-housed to facilitate exchange of their respective microbiota, many strains from the murine-derived community were effectively able to invade the human-derived gut microbiota and stably establish themselves, while the members of the human-derived community were largely unable to establish themselves within the murine community.[90] These data suggest that although human-derived microbiota can form a stable community within a gnotobiotic mouse, they lack fitness in this environment relative to the murine community. Finally, host specificity in host–microbe interactions is a long-appreciated phenomenon (particularly in microbial pathogenesis)[91] and individual microbial taxa may have evolved to stimulate or evade the immune system of the host in which they normally reside. Isolates of SFB from mice and rats have shown exquisite specificity in the induction of intestinal Th17 responses, with rat-derived SFB priming Th17 responses in the rat but not the mouse, and mouse-derived SFB priming Th17 responses in the mouse but not the rat.[92] Moreover, some human-derived fecal microbiota were demonstrated to be poor stimulators of immune system maturation in selected lymphoid and non-lymphoid sites in gnotobiotic mice, providing reduced resistance to pathogenic intestinal infection when compared with the recipients of murine fecal microbiota.[55, 89] We posit that much may be learned about human immune interactions with their endogenous microbiota by comparatively studying other microbial communities in their natural habitat, i.e. the study of murine-derived microbiota in the mouse intestine may better delineate shared paradigms of host–microbiota interactions than the study of human-derived microbiota in gnotobiotic mice. This is not to suggest precluding the study of human-derived microbes in mice, from which we have already learned much,[25, 34, 36, 40-42, 50, 54, 83] but instead calls for judicious interpretation of data using such microbes. Indeed, study of the molecular basis by which human-derived microbiota adapt and evolve during colonization and long-term residence of the mouse intestine, in addition to delineating differences in the shaping of immune responses, represents a powerful strategy to uncover key facets of host–microbiota interplay. Complimentary approaches to the discovery process are also necessary given the mouse-intensive nature of most current strategies and the fact that important human–microbiota interactions may not be modeled in a murine system. For example, primary intestinal organoids represent a particularly useful tool to study human epithelium–microbe interactions for several reasons, including: they can be generated directly from humans (with or without disease-associated genetic polymorphisms); they can be genetically modified using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas technology; they recapitulate important epithelial cell lineages and physical features of the intestinal tract; and they can be seeded with microbes of interest.[93] Another promising approach is to identify factors like metabolites that can impact a response of interest and work to identify the microbes that are responsible for their production.[94, 95] As knowledge of the key mechanisms of host–microbiota interactions are revealed, our accuracy in predicting immunomodulatory activity based on the genomic content of a strain or community should markedly improve.

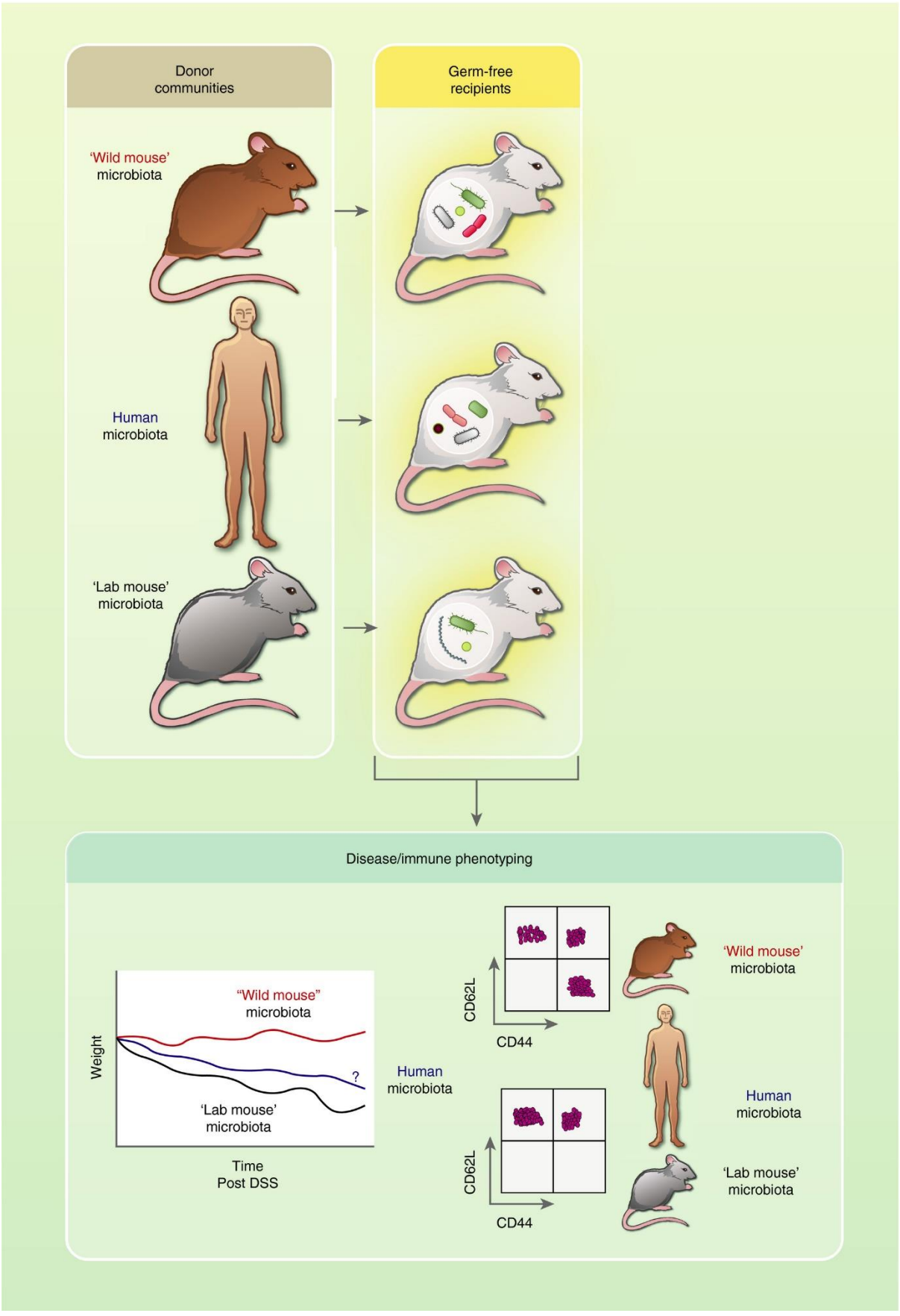

Figure 2 - Optimizing microbial communities to model microbiota–host interactions: The best source of gut microbiota to allow faithful modeling in mice of human host–microbiota interactions remains an important question. Recent work

suggests that human-derived microbiota do not fully recapitulate the immune phenotype seen using murine-derived communities. In addition, the gut microbiota harbored by 'wild' (feral) mice may imprint a mature immune phenotype more comparable to that of adult humans than to the immature immune system found in common laboratory mice, priming development of more activated CD8⁺ T cells and providing increased resistance to weight loss induced by treatment with azoxymethane plus dextran sodium sulfate. Identification of the mechanisms that underlie these phenomena will facilitate the development of microbial consortia that allow more accurate representation of human physiology in laboratory mice.

Using the elegant approaches discussed above, the identity of specific microbes with potent immunomodulatory activity has been elucidated, especially those that shape the intestinal T-cell compartment. A variety of bacterial strains from distinct phyla, operating individually or as part of multi-strain consortia, prime the accumulation of peripherally derived colonic Treg cells and the production of the immune suppressive cytokine IL-10. These include *Clostridia* from clusters IV, XIVa and XVIII (Firmicutes),[48, 54] various *Bacteroides* species (Bacteroidetes)[68, 78, 83, 96] or *Helicobacter* species (Proteobacteria),[97-99] *Bifidobacterium bifidum* (Actinobacteria),[100] and the Altered Schaedler Flora (multiple phyla).[101] An isolate of *Bifidobacterium adolescentis* (an Actinobacteria) obtained from humans that could induce Th17 cells was also identified, confirming that this was not unique to SFB.[102] A cocktail of human-derived strains from multiple phyla promote the development of interferon-*γ*-secreting CD8⁺ T cells[50] and intraepithelial lymphocytes are also potently regulated by gut microbes, with a murine-derived strain of *Lactobacillus reuteri* shown to elicit intraepithelial $CD4^+CD8\alpha\alpha^+$ T-cell generation,[75] while SFB is known to increase T-cell receptor αβ-positive intraepithelial lymphocytes.[51] Mechanistically, microbial metabolites have been shown to mediate many of these effects. Short-chain fatty acids, products of microbial fermentation in the gut, promote Treg cell accumulation,[103-105] in addition to regulating many other immune effector functions.[106-108] Microbiota-derived ATP can stimulate Th17 development,[109] and tryptophan breakdown products like indole-3-lactic acid promote the development of intraepithelial $CD4^+CD8\alpha\alpha^+$ T cells through stimulation of the aryl hydrocarbon receptor.[75] Bacterially derived polysaccharides such as polysaccharide A from *B. fragilis,*[68] *β*-glucan/galactan polysaccharides from *Bifidobacterium bifidum*[100] or crude carbohydrate preparations from *H. hepaticus*[110] can prime Treg or IL-10 production. Intriguingly, atypical antigen acquisition pathways[111] have also been shown to be important for SFB-induced Th17 development. Collectively, these studies demonstrate that the intestinal immune system can be activated by a diverse array of microbial taxa through a variety of distinct mechanisms, highlighting a large degree of redundancy in the processes which help to maintain these mutualistic relationships. Interestingly, some of the microbial taxa that impart beneficial effects on the immune system have also been implicated in the pathogenesis of inflammatory disease models, and so rather than being inherently beneficial, their positive effects are dependent on host factors and the context of the interaction.[61, 112-115] Additionally, an important time-window where colonization must take place has also been established for particular phenotypes, emphasizing the importance not just of exposure to select microbes, but of encounter at the appropriate developmental stage.[116-118] Given the shorter lifespan of mice relative to humans, it will be critical to determine how such developmental windows translate between species, and the consequences for interventions that aim to repair poorly developed or damaged microbiota for therapy.

Developing a consistent approach to assessing microbiota effects

The identification of select microbial taxa that modulate various arms of the intestinal immune system that have variable presence in mouse facilities[49, 75, 99, 119] points to an unwanted source of variability in mouse models, highlighting the need for more defined microbiota to facilitate comparison of data across different laboratories. Although co-housing of different groups of mice has been reported to be able to transfer microbiota-dependent phenotypes in many instances, as

noted above, the exchange of microbiota between adult mice is incomplete. In the absence of gnotobiotic facilities, current best practice for minimizing microbiota variables recommends using heterozygous or wild-type littermate controls in studies of genetically modified mice, as well as standardizing housing conditions and diets across experimental groups.[120] In addition, pedigrees can be tracked, to enable retrospective analysis of 'cage effects' that may have arisen as the result of distinct parental microbiota. However, these processes will not homogenize microbiota across breeding cohorts in different specific-pathogen-free (SPF) facilities, meaning that phenotypes that can be influenced by microbiota may not appear readily reproducible. Indeed, for the purposes of knowledge discovery, it could be argued that global homogeneity is undesirable, as such discrepancies provide a basis for identifying microbiota constituents that regulate phenotypes.[56, 75] As an example, one can consider the conflicting reports on the incidence of spontaneous intestinal inflammation in *Tlr5*$^{-/-}$ mice[121, 122] or dextran sodium sulfate-induced colitis susceptibility in *Nlrp6*$^{-/-}$ mice,[123-125] highlighting the contextual expression of these phenotypes depending on environmental factors.

In addition to variability in microbiota, another caveat of using laboratory mice to model the human immune system was highlighted by the finding that the immune system of adult laboratory mice housed under SPF conditions is phenotypically representative of a newborn's immature immune system, in contrast to the mature immune system found in adult mice living in the wild or in pet stores.[126] The adult-like immune phenotype of wild or pet-store mice has been linked to infectious agents,[127] but a gut microbiota from wild mice, devoid of known mouse pathogens, also beneficially modified the response of gnotobiotic mice to infectious or chemical insult, relative to a microbiota derived from SPF laboratory mice.[128] Hence, although the SPF mouse has been of enormous value in removing confounding variables like infections, a reassessment of the optimal microbiota composition for laboratory mice appears to be in order (Fig. **2**).

Crucially, SPF conditions are currently defined by the absence of specific pathogenic microbes rather than being defined by the microbes that are present. Furthermore, the precise criteria used to classify colonies as SPF varies among vendors and facilities, with many potent immunomodulatory microbial taxa, such as SFB and *Helicobacter* spp., not routinely included in the list of proscribed microbes. New microbial cocktails containing known immunomodulatory microbes as noted above, in addition to non-pathogens found in wild mice that shape host physiology (yet to be described), could be developed and used similarly to the Altered Schaedler Flora[129] to provide the consistency desired by researchers, while improving the translatability of mouse systems. However, this approach poses many challenges. For example, developing a consensus as to which microbes should be used in such a cocktail and implementing strategies that allow for maintenance of these microbes without the invasion of true pathogens or other immunomodulatory microbes, is far from simple. Generation and characterization of purified stable consortia, potentially composed of bacteria, fungi or viruses that provide a more homogeneous microbiota, driving a consistent level of immune maturation that more accurately reflects the immune-activation levels in adult humans, is an essential first step. It is very likely that there will not be a single microbiota type that is relevant for all questions, and the most appropriate community will critically depend on the human population to be modeled, as well as the environmental challenges under investigation. However, the community should be fully characterized, should have minimal variance over time and should reflect the human population to be studied as closely as possible. These consortia could then be used to regularly seed breeder colonies to help ensure their sustained presence within a colony, but the large-scale feasibility of such an approach and the precise protocols required to ensure consistent microbiota composition remain to be fully determined. Furthermore, this approach would require careful monitoring, maintenance of high levels of hygiene and husbandry and strict control of diet.

The expansion of gnotobiotic facilities will likely expedite the generation of new ‘minimal essential' stable consortia, with parallel characterization of the level of immune maturation in the murine host. In the meantime, in addition to the breeding and husbandry strategies outlined above, investigators should consider reporting the composition of the microbiota of mice used in their studies wherever possible. Much in the same way that sentinel mice are used to declare a colony as SPF, assessment of the microbiota of sentinels using 16S rRNA gene sequencing or shotgun metagenomic sequencing could be reported in published manuscripts, addressing an important variable that could lead to issues associated with reproducibility of data. Of course, because of constraints in resources and infrastructure, this reporting is unlikely to be feasible in all cases, but as sequencing costs reduce and bioinformatic training becomes more routine, reporting of microbiota composition should become a standard practice in many institutes. In addition, making such data available should also facilitate *in silico* approaches by other investigators to identify potential members, or interacting cohorts of microbes, that regulate immune maturation or experimental phenotypes. Together, such efforts will iteratively improve our understanding of how microbiota shape host immunity and disease phenotypes.

Conclusion

We are at an exciting time in microbiota research, with an increasing number of diseases being connected to the dynamic functions of the microbiota. The next steps, including the development of novel probiotic strains to treat disease, will require detailed understanding of the most beneficial microbes for host health, the context in which they operate, and molecular-level details underpinning these effects. Indeed, with the strategies described here, allied to efforts to characterize the mechanisms of action of the microbiota and microbiota-derived products in health and disease, we are well on our way.

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