REVIEW ARTICLE

From germ-free to wild: modulating microbiome complexity to understand mucosal immunology

Carolyn A. Thomson[1], Sydney C. Morgan[1,2], Christina Ohland[2] and Kathy D. McCoy[1,2]✉

The gut microbiota influences host responses at practically every level, and as research into host-microbe interactions expands, it is not surprising that we are uncovering similar roles for the microbiota at other barrier sites, such as the lung and skin. Using standard laboratory mice to assess host-microbe interactions, or even host intrinsic responses, can be challenging, as slight variations in the microbiota can affect experimental outcomes. When it comes to designing and selecting an appropriate level of microbial diversity and community structure for colonization of our laboratory rodents, we have more choices available to us than ever before. Here we will discuss the different approaches used to modulate microbial complexity that are available to study host-microbe interactions. We will describe how different models have been used to answer distinct biological questions, covering the entire microbial spectrum, from germ-free to wild.

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INTRODUCTION

In recent decades, we have begun to uncover the extensive reach that host-microbe interactions have on our health, and a spotlight has been shone on our once "forgotten organ", the microbiota. Novel technologies, methodologies and in vivo microbiome manipulation now allow us to explore host-microbe interactions in detail, leading to the question of which method is best to unravel the impact of the complex interplay between host and microbes. Should one take a reductionist approach, using gnotobiotic animals to tease out the effects of specific microbes or microbial metabolites, or should we embrace more complex paradigms, such as the wild microbiome, with the aim of more accurately recapitulating the human condition? Of course, the answer is simple: it depends. Each layer of microbial complexity adds dimension and nuance to the field. In this review, we will discuss the context-dependent benefits of the different approaches available to study host-microbe interactions in vivo and examine how models with varying microbial complexity (from germ-free to wild) have been used to make key advances in biomedical research.

OUT WITH THE OLD? SPECIFIC PATHOGEN FREE MODELS

Since its advent in the 1960s, the specific pathogen free (SPF) model has become the gold standard for laboratory animal research. The history of this model and the advantages of using animals free of pathogens for advancing biomedical research have been reviewed extensively. In general, although ubiquitous in animal facilities across the globe, the SPF model has some major pitfalls that shouldn't be overlooked. These pitfalls are part of the reason for the diversification of methodologies used to study host-microbe interactions in vivo (Fig. 1).

Relatively speaking, the SPF microbiota is considered complex and diverse. In the main, colonization resistance renders the intestinal niche inaccessible to opportunistic pathogens, but also to the introduction of microbial species of interest. As such, whether they are used to induce dysbiosis or to create an empty niche prior to the introduction of novel microbes, microbial manipulation in SPF animals usually requires antibiotics. Unfortunately, these can have confounding, off-target effects on the host's immune system and effects on off-target microbes that are difficult to control,[1] thus limiting the effectiveness of using SPF animals to study host-microbe interactions. Even when experimental questions do not relate to the microbiome directly, the gut microbiota has a broad reach and variations in composition can both skew experimental outcomes and hamper experimental reproducibility. This might not be a problem if the SPF microbiota were standardized. However, there is enormous variability in the SPF gut microbiome composition from one facility to the next. Substantial differences in microbiome composition can even exist within barrier facilities: from one room to the next, or even from one cage to its neighbor. In fact, this specific pitfall has been utilized to identify novel host-microbe interactions. In 2009, Ivanov and colleagues discovered a large discrepancy between the abundance of mucosal T helper 17 (Th17) cells in two commercially available SPF C57BL/6 wild-type strains of mice.[2] Although genetically identical, only one group of mice were colonized with segmented filamentous bacteria (SFB) in their terminal ileums. This turned out to be a driving force behind Th17 cell induction in SPF mice from Taconic, not just locally but systemically.[2] Not only does this demonstrate that a single difference in microbiome community structure can drastically affect immune responses, but that vender-specific differences in the SPF microbiota can act as confounding experimental factors.

[1]Department of Physiology and Pharmacology, Snyder Institute for Chronic Diseases, Cumming School of Medicine, University of Calgary, Calgary, AB T2N 4N1, Canada. [2]International Microbiome Centre, Cumming School of Medicine, University of Calgary, Calgary, AB T2N 4N1, Canada. ✉email: kathy.mccoy@ucalgary.ca

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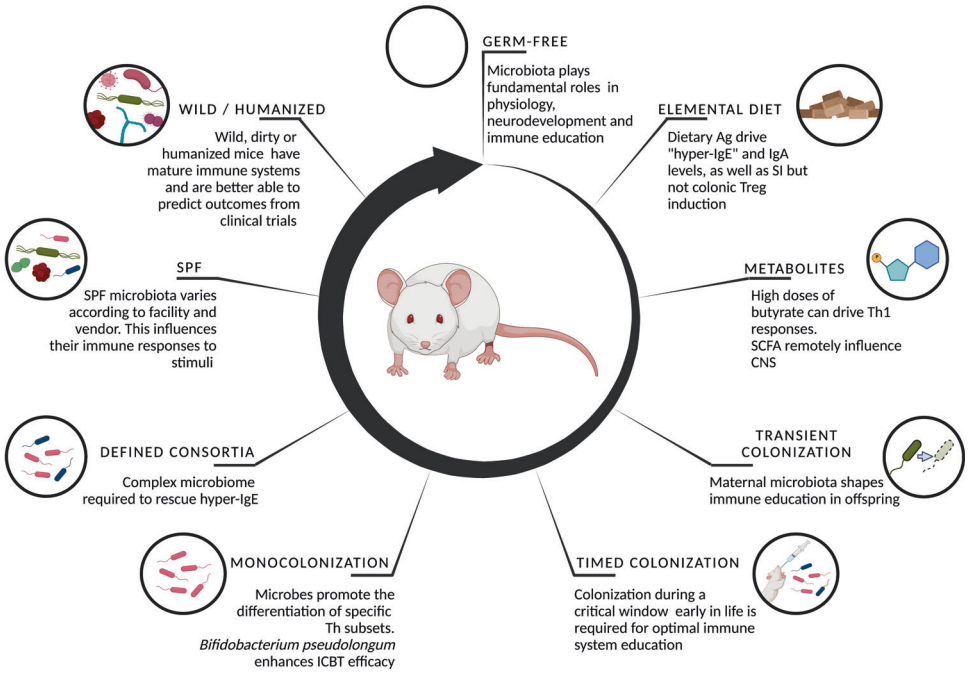

Fig. 1 Murine models covering the whole spectrum of microbial diversity can drive forward biomedical research. We now have more choice than ever before when it comes to designing and selecting an appropriate level of microbial diversity for the colonization of our laboratory rodents, and different microbiotas can be used for different research questions. GF rodents are useful for investigating the far-reaching influence the microbiota has on host responses. By feeding GF mice an antigen-free, elemental diet, researchers can begin to unravel the specific influences that dietary, microbial, and environmental antigens (Ag) have on Ag-specific, adaptive immune responses. This has revealed a prominent role for dietary Ag in antibody class-switching, as well as IgA and Treg induction in the small intestine (SI). The administration of specific microbes or metabolites to GF mice can be used to gain mechanistic insights into host-microbe interactions. This has helped us to further understand the roles that SCFA play in mucosal immunity and CNS responses, and to clarify the specific microbes and microbial molecules that shape $CD4^+$ T cell differentiation. It has also helped us unravel a role for specific microbes, such as *B. pseudolongum*, in determining the efficacy of immune checkpoint blockade therapy (ICBT). Timing of colonization is important, and the introduction of a microbiota at different time points, coupled with the use of transient models of in vivo colonization, has helped uncover a critical window of opportunity during gestation and early life, when the establishment and maintenance of a healthy microbiota is crucial for long-term health. Defined consortia can be used to reduce the experimental variables associated with SPF and wild mice, while retaining a more physiological community structure. These have helped us to establish the degree of complexity required to rescue the hyper IgE phenotype associated with GF mice. Despite limitations, SPF mice are still used to define novel host-microbe interactions. For example, vendor-specific differences in gut microbiota community structure were utilized to identify SFB as a potent inducer of Th17 responses. However, SPF mice do not seem to have a diverse enough microbiota to drive sufficient immune maturation. Their immune responses resemble that of a neonate. As such, humanized mice, "dirty" mice, and wild mice are used to develop models that more closely recapitulate human responses. Mice with wild microbiomes have been shown to predict outcomes of human clinical more accurately.

In the case of SFB, this is likely to be particularly important for models where Th17 cells play prominent protective or pathological roles.[3] In addition to housing and commercial sources, genetic differences can also influence colonization. We may never know how many manuscripts have been published that erroneously link biological phenotypes to genotype, when instead they could have been microbially mediated.

Arguably, one of the greatest challenges with animal research is that findings often fail to be translated to the clinic.[4,5] Is this because humans and mice are fundamentally different, or does the SPF microbiota fall short of mimicking the human microbiota? Recent work shows a distinct "immature" phenotype of immunological responses in SPF mice compared to wild mice, laboratory mice with wild microbiomes or adult humans.[6] These findings suggest that greater microbial diversity might be required for optimal immune system education and, thus, the full translational potential of laboratory rodents. This will be discussed in more depth below.

Coupling the above pitfalls with the newfound importance of the gut microbiome in host health and wellbeing, should we continue using SPF models? Is it time we moved away from the 'old' and set our sights on new methodologies? The truth is that, despite limitations, the SPF model has been an affordable, accessible workhorse in biomedical research, and it isn't going anywhere anytime soon. Discoveries have been made using SPF rodents that have shed light on host-microbe interactions in barrier tissues including gut,[7–9] lung,[10–13] skin[14,15] and beyond. For example, a recent publication by Hosang and colleagues used intratracheal antibiotics to deplete the microbiome locally in the lung, but not in the gut. In doing so, they demonstrated a role for the lung microbiome in modulating susceptibility to experimental autoimmune encephalitis (EAE), a murine model of multiple sclerosis.[12] This seminal study provides evidence that the lung microbiome may have a more prominent role in immune function than was previously appreciated, and that targeting the lung microbiome might be a potential novel therapeutic approach.

Findings from studies using SPF mice have also paved the way for further research using more definitive approaches, such as gnotobiotics, to limit microbial diversity and richness. For example, in 2015 and 2016, the use of antibiotics in pregnant SPF dams was used to link the maternal microbiota to type I diabetes susceptibility in the offspring.[16,17] Although these studies don't rule out the confounding effect of the pups' own microbiota, they represented a breakthrough for the "developmental origins of health and disease" hypothesis and were amongst several publications that sparked a widespread interest into the maternal microbiome, as well as follow up studies using gnotobiotic models. Thus, although a useful and accessible tool, it is important

to remember and control for the confounding effects of antibiotic use and community variabilities, and to diversify as new methodologies become more and more accessible.

IN WITH THE NEW? GNOTOBIOTIC MODELS

The term "gnotobiotic" is based on the Greek words "gnotos" (known) and "bios" (life); i.e., a defined microbiota. This covers everything from axenic or germ-free (GF) models, where animals are maintained in a sterile environment from birth, up to models using a more diverse, but controlled microbial consortia consisting of many species. Although not technically new, the technology required to maintain animals under GF conditions is now becoming more accessible to the average research lab. The power of having gnotobiotic models is that they allow us to: (i) unequivocally attribute host biological phenotypes to the microbiota, and (ii) investigate the effects of specific microbes or metabolites in vivo, either alone or in concert. The simplicity and specificity of these models render them a powerful tool for mechanistic studies into host-microbe interactions (Fig. 1).

Lessons from germ-free mice

As research into the effects of the microbiome on mucosal immunity has steadily increased over the past several years, so too has the use of GF mouse models. Devoid of all microorganisms, GF mice are the perfect tool for studying physiological, immunological, and neurological development and function in the absence of a microbiome. Building upon studies made using antibiotics to deplete the microbiota in SPF mice, GF research has revealed that the microbiome plays a role in shaping virtually every biological system. Since a comparison of GF mice and antibiotics treatment has recently been performed,[18] we will focus here on the use of GF mice to crystalize our understanding of microbial involvement in host responses.

A guiding hand in local physiological development. The gastrointestinal (GI) tract has the largest mucosal surface of the body as well as the largest bacterial community.[19] Therefore, it is not surprising to find major changes in the GI tract in the absence of microbes. GF mice exhibit architectural and functional abnormalities along the GI tract, such as increased small intestinal villi length, an enlarged cecum, altered mucus layers, and slower peristalsis.[20] The maturation signals for gut-associated lymphoid tissues (GALTs) are absent, leading to smaller Peyer's patches and mesenteric lymph nodes (MLN).[21] In contrast, no major morphological changes have been observed in other barrier sites, such as the skin or the lung, likely due to increased barrier thickness and/or lower microbial abundance.

Immune system education and maturation. Our immune system is heavily influenced by signals from the microbiome, which is apparent when one compares the immune system of GF mice to that of their SPF counterparts. One of the most prominent phenotypes associated with GF mice is their immature immune system. They are ill-equipped to handle microbial colonization, as evidenced by the reduced expression of antimicrobial peptides at barrier sites.[22–24] The innate immune cell landscape is also changed. This is best characterized in the intestine, which has fewer macrophages, mast cells, mucosal-associated invariant T (MAIT) cells, and in most reports, subsets of group-3 innate lymphocyte cells (ILC3).[25–29] Interestingly, the intrathymic development of MAIT cells requires presentation of a microbiota-derived metabolite.[30] The innate immune cells present in the gut also exhibit an immature phenotype, with macrophages lacking the hypo-responsiveness to lipopolysaccharide (LPS) necessary to tolerate the commensal microbiota and ILC3 failing to produce the necessary levels of IL-22 required for host defence, even after stimulation.[24,31,32] Collectively, these studies demonstrate that microbial cues are required to instruct and train innate immunity in the gut, however, the impact of the microbiome on innate immune cell function is likely to be global, particularly due to the dysregulated hematopoiesis observed in the GF bone marrow (for review, see McCoy and Thomson.[33]) Indeed, similar innate immune system deficits have been reported in other barrier sites, such as the skin[27,34] and the lung,[27] as well as the spleen,[35] liver[36] and brain[37] of GF mice.

The intestine hosts a richly populated and highly specialized adaptive immune system, which must strike a balance between protection from pathogens and tolerance of commensal microbes and food antigens. It is not surprising that microbes have a hand in maintaining this balance. In the absence of a microbiota, fewer effector T cells patrol the lamina propria[38] and fewer regulatory T cells protect the colon.[38–40] Antibody responses are disrupted, with fewer IgA-producing plasma cells populating the GALT, lamina propria, lung and bone marrow[41] and reduced circulating levels of all antibody classes with the exception of IgE, which is pathologically elevated.[42,43] To summarize, GF mice can be used to elegantly demonstrate the requirement of microbial cues in immune education and training.

Identifying a critical window in immune development. Maintaining gnotobiotic conditions has been a useful tool for facilitating the controlled introduction of a microbiota at different periods of development. While many of the immunological deficits associated with GF mice can be rescued by colonization anytime in life, some phenotypes can only be reversed if mice are colonized in early life. For example, the immunological maturation that coincides with a substantial shift in microbiome composition at weaning, termed the "weaning reaction", depends on early-life colonization.[44,45] Weaning appears to be a crucial time for microbe-mediated immune education, as colonization before but not directly after weaning had a lasting effect on mucosal immune responses, protecting mice from several experimental inflammatory models and tumor formation.[44,45] Exposing neonatal, but not adult, GF mice to an SPF microbiota has been shown to reverse the life-long overrepresentation of invariant natural killer T cells in the lungs and colon, thereby protecting them from the induction of experimental asthma or colitis.[46] Likewise, exposure to a 5-species consortium of commensal bacteria rescued the MAIT cell deficiency observed in the skin of GF mice, but only if the mice were recolonized as neonates.[27] Neonatal colonization with a diverse microbiota was required to reverse the characteristic "hyper-IgE" phenotype associated with GF mice.[42]

This critical period of microbe-mediated immune education may also be influenced by the lung microbiota. Despite having a lower biomass compared to other barrier sites, such as the GI tract,[47,48] compelling evidence is beginning to emerge that highlights the importance of the lung microbiome in immune function and pathology. By monitoring lung microbiome composition and immune phenotypes in the lung over time, Golwitzer et al. demonstrated that lung microbiome formation and maturation during this critical window is important for inhibiting allergic airway inflammation in mice.[49] It should be noted, however, that while numerous studies have attempted to attribute pulmonary phenotypes to the lung microbiome using GF mice,[50,51] these studies can be difficult to interpret as the absence of a gut microbiome has widespread effects on host immune function.

Collectively, these observations have considerable clinical relevance considering the compelling epidemiological studies showing a link between childhood dysbiosis and the development of inflammatory disorders later in life. Collectively, these studies suggest that a critical window of opportunity exists early in life, when the establishment and maintenance of a healthy, diverse microbiota is crucial for long-term health outcomes.

Elucidating dietary effects on immune status independent from the microbiota. While eliminating the microbiome can help tease apart the contribution microbial antigens play in immune responses, it also provides an opportunity to determine how dietary antigens and/or environmental components can modulate immune responses independently of the microbiome. It is important to note that autoclaving or irradiating food and bedding does not eliminate microbial DNA, antigens, or endotoxins, all of which can induce expansion of B and T cells in the GALT, alter humeral responses and possibly impact the development of experimental inflammatory diseases.[52,53] The relative contribution of microbial, dietary, and environmental components to antigen-specific immune responses can be dissected by feeding GF mice diets that are devoid of dietary antigens and/or microbial products. For example, feeding GF mice a diet consisting of extensively hydrolyzed protein did not limit the hyper-IgE phenotype normally observed in GF mice.[42] However, feeding GF mice a chemically defined antigen free diet completely abrogated hyper-IgE, suggesting that the hyper-IgE phenotype in GF mice is driven by food antigens.[54] IgA levels also appear to be influenced by dietary antigens. Bunker et al. found that absence of dietary antigens partially reduced the number of IgA^+ plasma cells present in the small intestines of GF mice fed a chemically defined antigen-free diet, however, this had no bearing on levels of luminal IgA.[41] Moreover, Hara et al. found that dietary antigens induced T follicular helper cells and germinal centre B cells in the Peyer's patches leading to increased IgA production in the small intestine.[55] Food antigens are also responsible for the induction and maintenance of Tregs in the small intestine. Although the GF colon is depleted of extra-thymic Tregs, Treg numbers in the small intestine of GF mice remain intact, as demonstrated by Kim et al., who fed GF and SPF mice an elemental diet and noted a marked reduction of Tregs in the small intestine, regardless of colonization state.[39] These studies show the need for careful consideration of the various factors that influence intestinal immune responses, especially in the small intestine where dietary antigens are plentiful.

Microbial metabolites. GF mice can be a useful tool to assess the role of specific metabolites on host responses in the absence of a metabolically active microbiome. Short chain fatty acids (SCFA) are generally considered to be beneficial, immunoregulatory small molecules, produced by the microbiota following the fermentation of dietary fibres. However, this seems to be dose- and context-dependent. Although butyrate is a potent inducer of Tregs,[56] Kespohl et al. demonstrated that high doses of butyrate could drive acetylation at *Foxp3*, *Ifng* and *Tbx21* (T-bet) promotor sites, resulting in increased IFNγ expression by T cells. Feeding butyrate to GF mice resulted in increased Th1 responses and exacerbated dextran-sodium sulfate (DSS)-induced colitis.[57] Oral administration of succinate to GF mice has been shown to be sufficient to drive tuft cell hyperplasia, which may have downstream effects on Th2 responses.[58] Metabolites are small and can easily transverse epithelial and endothelial barriers. As such, their effects are not restricted to the intestinal lamina propria and several studies have demonstrated that oral metabolite administration can remotely influence central nervous system (CNS) responses, including blood-brain barrier permeability, microglial cell maturation and neuroinflammation.[37,59,60] Although these studies highlight a key component of the gut-brain axis, discussing them in full is outside the scope of this review. It is important to note that oral metabolite administration may not be a true reflection of metabolite production in the colon, as much will be taken up in the small intestine, which induces a distinct immunological response.[61,62]

Gnotobiotic colonizations

Restricting the microbiota to one or a few known species has some key advantages over SPF models. Experimental variables are markedly lessened, and specific research questions can be addressed that have helped researchers uncover novel scientific discoveries and mechanistic insights that would have been difficult in conventionally colonized animals.

Transient microbial exposure. Building on re-colonization studies, an elegant method to study the effects of timing is to use transient methods of colonization, for example using auxotrophic microbes that cannot survive in vivo. In 2010, Hapfelmeier and colleagues genetically engineered an auxotrophic strain of *Escherichia coli* and used it to demonstrate the dynamics of microbially-induced IgA responses in vivo.[63] More recently, we have used this model to transiently colonize GF dams during pregnancy to investigate the specific effects of the maternal microbiome on immune system development in the offspring. As dams were given sufficient time to return to GF status before giving birth to GF pups, exposure of the pups to microbial molecules could only occur in utero and via the milk. From this, we established that the mother's microbiota primes the intestine for colonization by inducing transcriptional reprogramming that included the upregulation of genes encoding antimicrobial peptides. Microbial signals from mom also increased numbers of intestinal macrophages and $NKp46^+$ ILC3. These changes, which were dependent on maternal antibodies, were associated with a reduction in inflammation and bacterial translocation when pups were colonized with commensal microbes later in life.[64] Since this work was published in 2016, manuscripts have emerged that demonstrate a key role for the maternal microbiota in modulating metabolism, susceptibility to metabolic syndrome and anti-viral immunity in the offspring.[65,66] Rather than using gnotobiotic conditions, these latter studies used surgical caesarian sections and cross-fostering experiments to attribute biological phenotypes to the maternal microbiome.

Monocolonizations. Simplification of the mouse microbiome to a single strain of bacteria allows in depth, precise testing of how a single strain of bacteria can contribute to the immune response. This has been pivotal for determining the microbial factors driving $CD4^+$ T cell differentiation into T helper (Th) subsets. One keystone example of this was the discovery that SFB can single-handedly induce Th17 responses in vivo.[2] SFB is now used as a model Th17 cell inducer and mice monocolonized with SFB are used to further investigate Th17 responses, as well as other changes to the adaptive immune system, such as germinal centre expansion and IgA induction.[67–69] Tan et al. used gnotobiotic monocolonizations to screen the ability of numerous human commensals to induce a Th17 response in GF mice, and identified *Bifidobacterium adolescentis* as a potent inducer, on par with SFB.[70] Candidate microbes that induce other Th subsets have similarly been used to monocolonize GF mice, and this has shed light on microbial regulation of T cell differentiation, particularly that of inducible Tregs. For example, by activating TLR2, *Bifidobacterium bifidum* and *Bacteroides fragilis* share the capacity to induce Tregs in a polysaccharide-dependent manner.[71,72] The ability of individual microbes to shape $CD4^+$ T cell differentiation in vivo has been reviewed extensively recently.[73]

Monocolonizations are frequently used to study the immunomodulatory roles of specific metabolites. For example, feeding different SCFAs to mice monocolonized with either *E. coli* or *Bacteroides thetaiotaomicron* revealed that acetate selectively induces a T cell-dependent IgA response to *E. coli* but not to *B. theta*.[74] Our group also showed that monocolonization with *Bifidobacterium pseudolongum*, isolated from colorectal tumors following immune checkpoint blockade, increased the efficacy of anti-tumor immunotherapy.[75] *B. pseudolongum* produced the metabolite inosine, which translocated the intestinal barrier and had a direct effect on T cells via the adenosine 2A receptor (A2AR). A2AR activation increased IFN-γ production by $CD4^+$ and $CD8^+$ T cells, enhancing antitumour immunity. Such gnotobiotic

experiments demonstrate how a reductionist approach can be used to gain mechanistic insights that can be exploited for translational benefit.

Research using monocolonizations to study host-microbe interactions at mucosal sites is extensive and covering it all would be outside the scope of this review. Notably, however, Geva-Zatorsky et al. characterized the immunological impact of monocolonizing mice with 53 human commensals.[76] Despite potential limitations of colonizing mice with human microbes[77] (discussed below), these microbes induced immunological and transcriptional changes that were independent of phylogeny, demonstrating the functional redundancy present in our microbiomes. Interestingly, when comparing the effects of colonization with a mouse microbiota to colonization with a human microbiota, Gaboriau-Routhiau et al. found less functional redundancy with a species-specific microbiota.[78] Of note, the functional redundancy for metabolic functions appeared greater than for immunological changes.

Simple, defined microbial communities. Whether due to cross-feeding, competition or cooperation, bacteria respond differently alone than they do in communities.[79] For example, through molecular mimicry, *Lactobacillus reuteri* exacerbated the development of EAE, but only when mice were co-colonized with a novel strain of Th17-inducing *Erysipelotrichaceae*.[80] In contrast, disease progression in *L. reuteri* monocolonized mice was indistinguishable from that in GF mice, which are highly resistant to EAE,[81] and although *Erysipelotrichaceae* alone had a modest role in pathogenesis, disease progression in co-colonized mice was significantly more severe, suggesting that these microbes acted synergistically.

To study mechanisms of host-microbe mutualism in a more physiological setting, microbial consortia have been designed so that mice can be colonized with defined microbial communities. These have the advantage of being complex enough to facilitate a degree of immune maturation, yet simple enough to permit additional colonization by other single bacteria strains, thus allowing researchers to test responses to specific strains in a semi-mature immune environment. For example, the Schaedler Flora and then the Altered Schaedler Flora (ASF) were developed in 1965 and 1978, respectively, to move further along the complexity spectrum while still maintaining control of which bacteria are present. Now constituting eight bacterial members, this community remains a widely used gnotobiotic model to study host-microbiome interactions.[82]

The Oligo-Mouse Microbiota 12 (OMM12) consortia was created using 12 commensal isolates from the murine gut that span the 5 most dominant phyla.[83] OMM12 is highly reproducible between animal facilities, stable over multiple generations and provides increased colonization resistance compared to ASF.[83,84] Moreover, OMM12 induces a greater level of immune maturation compared to ASF, such as inhibition of hyper-IgE.[85] Yet, its limited diversity still allows the addition of bacterial species to build up microbial community complexity and probe microbial function. For example, the addition of *Extibacter muris* to the OMM12 revealed the capacity of this bacteria to produce secondary bile acids.[86] Tanoue et al. developed a defined microbial consortium consisting of 11 human commensal strains that stimulated increased levels of IFN-γ-producing $CD8^+$ T cells, providing pathogen resistance to *Listeria monocytogenes* and promoting the anti-tumor activity of immune checkpoint inhibitors.[87] Another microbial consortium comprised of 15 bacterial strains isolated from SPF mice was used to develop the GM15 gnotobiotic mouse model, which stimulated increased levels of serum antibodies and Peyer's patch development, phenotypically resembling SPF mice.[88]

Moving further along the spectrum of complexity, several groups have endeavored to assemble large, complex communities of microbes while maintaining gnotobiotic status. In 2011, Atarashi et al. discovered that a mixture of 46 *Clostridium* species from the murine microbiome potently induced colonic Treg in a TGF-β-specific manner. These provided protection against DSS- and oxazolone-mediated colitis and systemic IgE induction in BALB/c mice, following a prime and boost with ovalbumin and alum.[89] Treg induction was partially lost when mice were only colonized with 3 of the *Clostridium* strains. To facilitate clinical translation, this group then repeated this study using *Clostridium* species isolated from the human, rather than murine, microbiome. Colonization with 17 human *Clostridium* species was sufficient to phenocopy the Treg induction and regulatory phenotype induced by the 46 murine Clostridia.[90] Monocolonizing mice with individual strains from this humanized consortium only induced a partial phenotype, demonstrating the need for more complex microbiota for full immune activation.

These gnotobiotic models allow the finely tuned dissection of how specific microbes within a community can mediate distinct immunological phenotypes. The same precision is not possible with more complex communities, such as SPF, or wild microbiome studies. New microbial consortia are now being developed and analyzed in in vitro cultures. For example, ten strains of human bacteria were selected for their combined capacity to efficiently convert dietary fibers into SCFA required for intestinal health.[91] While this synthetic Diet-based Minimal Microbiome has been analyzed in vitro, the functional impact of such consortia could also be studied in vivo in gnotobiotic mice, although the metabolic activity of these microbes in vivo will be heavily influenced by their fitness to the rodent diet.

The mycobiome and the virome

An emerging field that has been long overlooked is the mouse and human mycobiome. Thus far, most microbiome studies and gnotobiotic models have focussed on the impact of bacteria, but we are also colonized with a diverse community of viruses, protozoa, archaea, and fungi that interact with the mucosal immune system.[92–94] In fact, when introduced to SPF mice following antibiotics treatment, or to steady-state ASF mice, a consortium of mucosa-associated fungi was shown to alter epithelial transcription and permeability, induce intestinal Th17 responses and protect mice from colitis via IL-22- and $CD4^+$ T cell-dependent mechanisms.[95] These data suggest a role for the mucosal mycobiome in galvanizing barrier function. Moreover, when five specific human-associated yeast species were introduced into GF and OMM12 mice, they robustly impacted local, systemic, and distal immune responses.[96] Most notably, fungal exposure modulated inflammation following induction of DSS colitis or ovalbumin-induced airway inflammation.[96] Thus, adding fungal species into gnotobiotic models may be necessary to fully understand how the microbiome and mycobiome collaborate to modulate the mucosal immune system.

Using gnotobiotic mice to study host effects of the virome is in its infancy but has the potential to provide important information. Infection of GF mice with murine norovirus, an enteric RNA virus, was sufficient to restore intestinal morphology and stimulate immune development comparable to bacterial colonization.[97] Gogokhia et al. treated GF mice with a cocktail of bacteriophage isolated from *E. coli* and found that was sufficient to increase the number of IFN-γ-producing T cells in the Peyer's patches to levels found in SPF mice, indicating that bacteriophage can directly affect mucosal immunity.[98] Additional studies using gnotobiotic mice to investigate bacteriophage effects on gut bacteria and host function are warranted.

DRIVING TRANSLATION THROUGH INCREASING MICROBIAL DIVERSITY

A fundamental problem with using laboratory rodents for preclinical research is that a large proportion of findings fail to be translatable, leading to the 'prediction problem' currently plaguing translational research. Most mouse models cannot

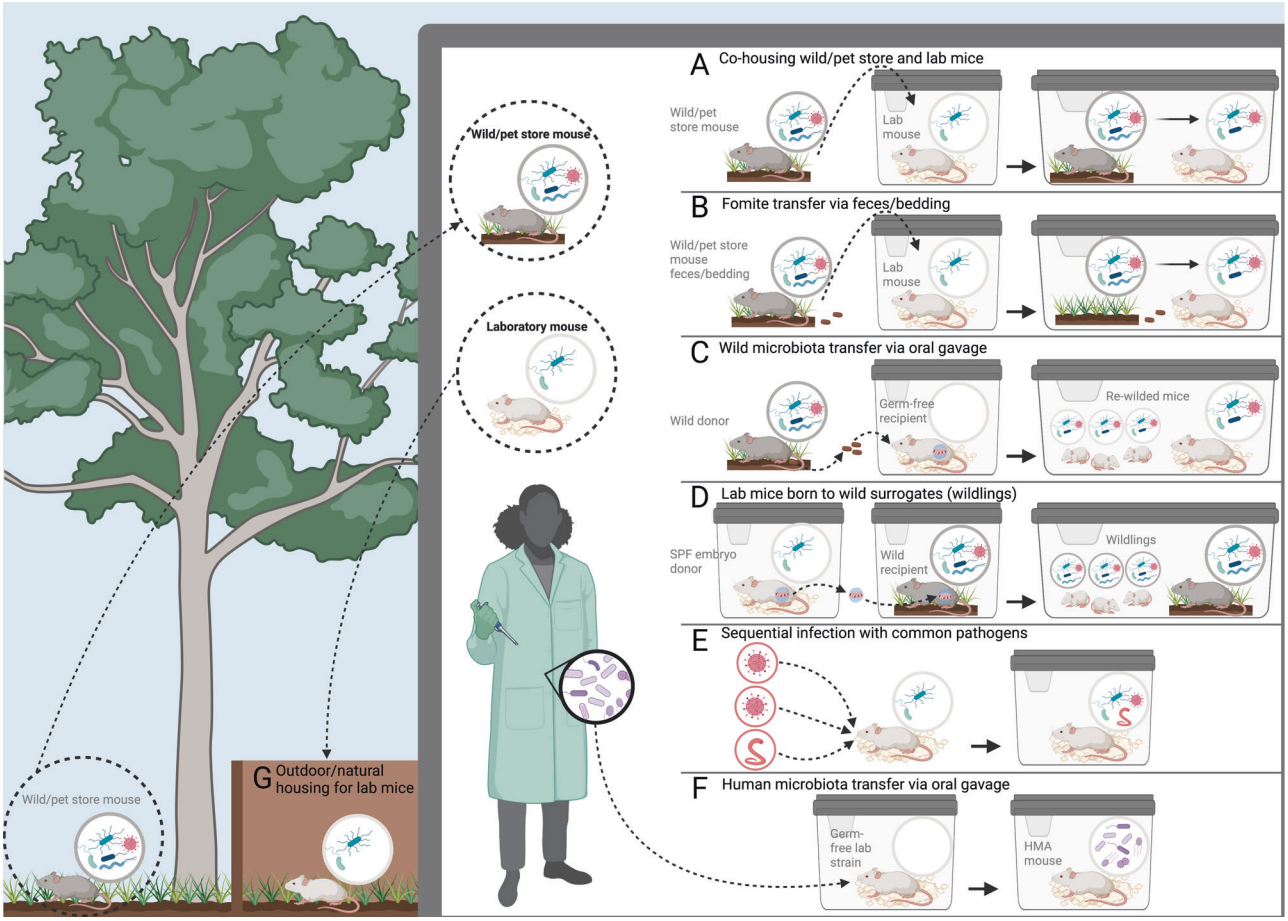

Fig. 2 Methods of generating mouse models for translational research. A Co-housing SPF lab mice with wild/pet store mice to create 'dirty' mice. **B** Microbial/fomite transfer of feces/bedding from wild/pet store mice into cages containing SPF lab mice. **C** Oral gavage of ileocecal material from wild mice to laboratory mice (pregnant status not required during microbiota transfer). **D** Generating 'wildling' mice, i.e., laboratory mouse strains born to wild surrogates, resulting in vertical wild microbiota transfer to laboratory strains. **E** Sequential infection with common pathogens such as viruses and helminths to create lab strains with primed immune systems. **F** Human microbiota associated (HMA) mouse models created via human microbiota transfer into germ-free mice. **G** Re-wilding laboratory mouse strains via outdoor housing.

accurately predict human responses,[5] and clinical trials in general have very low success rates.[4,99] With an immune system more closely resembling that of a neonatal human,[6] SPF mice often respond to immunological challenges differently than humans. To counter this, new mouse models have been developed to improve the predictive ability of translational research (Fig. 2).

Human microbiota-associated mouse models

Human microbiota-associated (HMA) mice are created via microbiota transplant of human feces into GF mice, which can be stably maintained in a laboratory setting.[100,101] Although often referred to as 'humanized' it is important to note that HMA mice have not been humanized through human tissue or hematopoietic cell engraftment. Although not all microbes will engraft in the recipient mice,[102] the HMA microbiota is still more complex and diverse than SPF mice and HMA mice are metabolically distinct from SPF mice and more closely correspond to the donor sample.[103] As such, they have helped further our understanding of host-microbe interactions in the context of human immunity. For example, Planer et al. showed that HMA mice were able to recapitulate the variable IgA responses of human donors,[104] demonstrating that microbiota composition plays a role in determining IgA responses.

HMA mice have been very useful for studying whether the human microbiome can transfer a disease phenotype to recipient mice and for elucidating causal relationships.[105] The use of HMA mice has been used to study the role of the microbiome in many different diseases (reviewed in Walter et al.[106]) and has provided great insights. However, as in all microbiome studies, utilizing HMA mice requires careful consideration of experimental design, discussed in detail by Walter et al.[106]. For example, there is a high risk of pseudoreplication: fecal samples are collected from few human donors (sometimes as little as one) and transferred to higher numbers of mouse recipients, thereby artificially inflating the number of biological replicates.[106] Moreover, mice are not humans and have not evolved with a human microbiota. This may sound trivial, particularly because it is the role of the human microbiome in health and disease that has captivated microbiologists and immunologists alike. However, these differences in evolutionary compatibility between host and microbiota could have important implications for the applicability of research involving HMA models. For example, Chung et al. observed that colonization of GF mice with human microbiota did not lead to the same degree of immune maturation as colonization with a mouse microbiota,[77] suggesting that a host-specific microbiota is required. Therefore, researchers have begun looking for other ways to improve the immune responses of laboratory mice using microbes (and pathogens) that co-evolved with mice, rather than humans.

'Dirty' mouse models: wild mouse models and their derivatives

Other methods used to expand and diversify the microbiota of laboratory mice, while retaining genetic uniformity, include co-

housing them with pet store mice, transferring bedding and feces between the cages of pet store mice and SPF mice, or intentionally infecting SPF with common pathogens.[6,107–110] Using these methods to generate 'dirty' mice has been shown to reverse some of the immunological deficits associated with SPF mice[6] and dampen humeral responses to vaccine challenge; an important factor when assessing vaccine efficacy.[107,109]

In the last decade, a major advancement toward generating translationally relevant mouse models is the characterization and utilization of the wild mouse microbiome. This has provided some hints as to why many pre-clinical trials involving laboratory mice have failed to translate to humans. The gut microbiota of laboratory mice is significantly different from that of wild mice (*Mus musculus domesticus*), likely due to differences in environment, diet, stressors, host genetics, and pathogen exposure. Two studies by the same group found that laboratory mice had a significantly higher relative abundance of Firmicutes bacteria, but lower Proteobacteria compared to wild mice.[111,112] Other groups found that Firmicutes was the most frequently identified phylum in the gut microbiota of wild mice,[113,114] but with marked seasonal changes.[115] Wild mice have an enriched mycobiome, as well as an increased pathogen load.[111,113,116,117] Collectively, these differences are associated with substantial changes in leukocyte maturation, education, activation and turnover.[118–121] However, it should be noted that some clinically relevant bacterial families, such as *Akkermansiaceae*, *Streptococcacea*e, and *Enterobacteriaceae*, are generally undetectable in wild rodents but have been acquired by SPF mice within the laboratory environment over time.

An issue with working with captive wild mice is that it is hard to delineate between the effects of genes and the effects of the microbiota. Genetic variation can be controlled for using wild microbiota exposure experiments, where laboratory mice are given a wild microbiota via different methods (Fig. 2). These include: (i) introducing fecal matter from wild mice to GF mice by oral gavage;[112] (ii) transferring embryos from lab mice to wild surrogate dams to generate "wildlings";[111] (iii) "re-wilding" laboratory mice by housing them in an outside enclosure that recapitulates the habitat of wild mice;[122] or (iv) housing them with bedding from their natural environment.[123] Each approach results in genetically identical laboratory mice with wild microbiomes that can be stably transferred and maintained over generations, thus facilitating microbiome diversification while preventing confounding results from genetic variability. Although similar, their resulting microbiomes may not fully converge with those of wild mice, as has been described for wildlings,[111] possibly due to differences in host genomes and diets. Importantly, when compared to SPF mice, 're-wilding' results in immune system reprogramming, particularly in the spleen, MLN and peripheral blood[111,124] and alters susceptibility to infection in a context-dependent manner, with re-wilded mice being more susceptible to *Trichuris muris* infection but protected from lung pathology and death following a lethal dose of influenza A.[112,122] Interestingly, the leukocyte profile in the gut of wildlings was bimodal, with 50% of wildlings clustering with SPF mice and the rest clustering with wild mice, suggesting a dual influence of the host and microbial genome on mucosal immunity.

In contrast to the intestine, the lung microbiota was relatively similar between SPF, wild-derived and wild-caught mice, with Betaproteobacteria being the most abundant phylum across multiple hygiene status, followed by Firmicutes.[125] *Ralstonia* was the most abundant genus in SPF, wild-derived and wild-caught mice, followed by *Lactobacillus* in SPF mice and *Helicobacter* in the wild-caught mice. Wild-derived mice showed an intermediate phenotype, with similar levels of *Lactobacillus* and *Helicobacter*. Despite genus-level similarities, the overall community composition was significantly different among lung microbiota from these different groups of mice. It is possible that these differences are sufficient to shape the local innate immune cell landscape, as mast cells are enriched in the lung parenchyma of wild but not SPF mice.[123] Yeh et al. found that housing SPF mice in a semi-natural environment consisting of farmhouse-sourced bedding was sufficient to increase mast cell recruitment to the lung.[123] Although environmental components played a role in driving this phenotype, microbial communities present in the mice, or the bedding were not characterized in this study. Mast cells play a key role in IgE-mediated allergic responses and are present in abundance in the human lung parenchyma.[126] Thus, murine colonization status and/or environmental antigen exposure likely has important implications for the translational potential of mouse models in asthma and allergy research.

Comparisons have also been made between the skin microbiomes of SPF, wild mice, and wildlings.[111,127] However, despite wildlings possessing a similar skin microbiota to wild mice that differed from their SPF counterparts, the immune landscape of their skin more closely resembled that of laboratory mice, despite the differences in bacterial communities of these two mouse populations.[111] Thus, the host genome may play a larger role in determining the immune landscape in the skin than the local microbiome.

The potential for incorporating wild mice in pre-clinical trials

Due to their differentially educated immune systems, mouse strains with 'wild' microbiomes may better predict human outcomes in clinical trials. Recently, Rosshart et al. elegantly demonstrated the superiority of wildlings, compared to SPF mice, when it came to accurately predicting the outcomes of two clinical trials, which had previously failed to be translated from SPF mice to humans.[111] When assessing the ability of CD28-superantigen to induce Tregs therapeutically, pre-clinical trials showed a robust Treg expansion in rats that protected them from various autoimmune disorders, including EAE (previously reviewed extensively.[128]) However, the CD28-superantigen human clinical trial resulted in overt over-activation of inflammatory T cells followed by cytokine storm and a lack of Treg expansion.[129] These catastrophic effects were recapitulated wildlings but not SPF mice.[111] Similarly, when TNF-α blockade was used at high doses to treat septic shock, increased mortality resulted in the trial being halted early.[130] Following LPS-induced endotoxemia, TNF-α blockade was sufficient to protect SPF mice but not wildlings, which exhibited a similar level of mortality to control animals.[111] Dirty mouse models may also be useful for translational research as SPF mice had more "human-like" immune responses to vaccines after they were co-housed with pet store mice, housed with bedding from pet store cages or exposed to common pathogens.[107,109] Collectively, these studies demonstrate the powerful potential of microbial diversification as a prerequisite for pre-clinical research, and highlights a potential role for wild mice, or mice with a wild or diverse microbiome, in pre-clinical trials.

CONCLUSIONS

Advancements in biomedical research require high-quality basic research coupled with translationally powerful models that can successfully predict clinical trials. There is no "one-size fits all" approach. Due to its far-reaching influence, microbiome diversity and community structure can majorly impact the suitability and effectiveness of different rodent models to address specific scientific questions, with microbial diversity and pathogen exposure emerging as a missing link in translating findings from rodent models to the clinic. In contrast, when it comes to mechanistic studies and reverse-translational research, arguably, less is more. Gnotobiotic models in genetically identical mice vastly limits experimental variables and allows for specific research questions to be addressed with precision.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Correspondence and requests for materials should be addressed to Kathy D. McCoy.