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Inflammatory bowel disease as a model for translating the microbiome

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Abstract

The inflammatory bowel diseases (IBD) are among the most closely studied chronic inflammatory disorders that involve environmental, host genetic, and commensal microbial factors. This combination of features has made IBD both an appropriate and a high-priority platform for translatable research in host-microbiome interactions. Decades of epidemiology have identified environmental risk factors, although most mechanisms of action remain unexplained. The genetic architecture of IBD has been carefully dissected in multiple large populations, identifying several responsible host epithelial and immune pathways but without yet a complete systems-level explanation. Most recently, the commensal gut microbiota have been found to be both ecologically and functionally perturbed during the disease, but with as-yet-unexplained heterogeneity among IBD subtypes and individual patients. IBD thus represents perhaps the most comprehensive current model for understanding the human microbiome's role in complex inflammatory disease. Here, we review the influences of the microbiota on IBD and its potential for translational medicine.

Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are the two main forms of inflammatory bowel disease (IBD), both chronic immune-mediated diseases with typical onset during young adulthood and a lifelong course characterized by periods of remission and relapse. CD can involve any part of the gastrointestinal tract, but most commonly the ileum and proximal colon. UC is most often localized to the descending colon, but can occur

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pancolonically as well. Worldwide, there is a trend towards increasing incidence of both UC and CD, with a recent systematic review concluding that 75% of the studies of CD and 60% of those with UC confirm a secular trend in incidence of disease (Molodecky et al., 2012). The modest concordance even in monozygotic twins along with the relatively rapid temporal changes in IBD incidence over the past six decades and the changes in disease risk with migration suggest an important role for the environment in disease pathogenesis. While the disease is strongly linked to the microbiome (see below) and the environmental factors that can influence the microbiome, the details of this relationship are complex.

The Complex Interplay of Host and Microbe in IBD

In this era of the $1,000 genome, it is difficult to appreciate the degree to which our knowledge of the gut microbiota in IBD has built on more than 50 years of microbiology and immunology. Dawson and colleagues (Vince et al., 1972) cite a “resurgence of clinical interest in the role of the intestinal bacterial flora” in 1972, just as has occurred in the past few years. Three main developments prior to the advent of modern culture-independent (i.e., sequencing-based as opposed to culturing-based) studies sustained interest in the IBD microbiome: systematic culture-based profiles during early clinical management of IBD, similar profiles of the gut microbial response to treatment, and the advent of rodent genetic models recapitulating IBD symptoms. Investigation of IBD throughout the first half of the 20th century tested and ruled out any number of individual microbial pathogens as causative agents in the disease (Weinstein, 1961), but gut-resident microbes remained of interest due to their exposure and uptake during ulceration and barrier breach (Seneca and Henderson, 1950). Increasingly refined selective media and anaerobic culture conditions throughout the 1960s-70s produced conflicting results for changes in gut microbial load or profile during IBD (Cooke, 1967; Mallory et al., 1973; Vince et al., 1972; Wensinck et al., 1981). These results in many ways predicted those observed more recently using metagenomics: although slight changes in gut microbial residents were present in some subsets of IBD patients, they were heterogeneous both among disease subtypes and among individuals (Sartor, 1990).

In parallel, as treatments such as 5-aminosalicylic acid (5-ASA) was introduced for the disease (Gorbach et al., 1968), its effects on the microbiome were explored using similar techniques. It was rapidly determined that compounds such as salicylazosulphapyridine in particular were metabolized by gut microbes (Cooke, 1969), due to differential product metabolite profiles in germ-free and antibiotic-treated animals (Peppercorn and Goldman, 1972). Again, though, changes in microbial load or profile were modest (West et al., 1974), and the mechanism of action of 5-ASAs and their effects on the gut microbiome remain complex (Iacucci et al., 2010; Morgan et al., 2012). Treatments for IBD provided another route by which the role of the gut microbiome could be explored, however, since microbial changes induced by compounds alleviating disease might help elucidate the still-missing host-microbe links.

Similarly, the third major development in IBD as a model for complex microbial disease was the proliferation of genetically modified animal systems in the 1980s–90s (Sartor, 1995). Once genetic ablation and, eventually, genetic replacement systems joined chemical perturbations as rodent models for intestinal inflammation (Kontoyiannis et al., 2002;

Rivera-Nieves et al., 2003; Wirtz et al., 2007), it became relatively easy to test whether changes in the microbiome could shift disease outcome in these IBD models. It was demonstrated that most IBD-like symptoms were attenuated or abrogated by antibiotics and germ-free conditions and restored or exacerbated by subsequent colonization (Rakoff-Nahoum et al., 2004; Rath et al., 2001; Sadlack et al., 1993; Taurog et al., 1994). This behavior in germ-free and subsequently recolonized inflammation models provided, and continues to provide, one of the strongest arguments for the close involvement of the gut microbiome in IBD. The diversity of rodent genetic models that induced IBD-like histopathology helped the field rapidly converge on hypotheses that remain central to investigations today: maintenance of effector, regulatory, and T helper cell subsets; gut microbial involvement; and complex, non-Mendelian genetics encompassing a range of microbial sensing, antimicrobial, inflammatory, and innate and adaptive immune regulatory pathways (Bouma and Strober, 2003; Rakoff-Nahoum et al., 2004). It is, of course, important to remember that animal models rarely perfectly recapitulate human disease, and these developments were paralleled by further direct, translational evidence of microbiome involvement such as improved clinical outcome in the case of fecal stream diversion (Rutgeerts et al., 1991) and the modulation of outcome and recurrence by antibiotics (Bernstein et al., 1980; Rutgeerts et al., 1995). By the time of the first studies in IBD using culture-independent techniques comparable to those available today, the potential molecular players were thus well-established, but as yet without molecular data on gut microbial structure or function (Xavier and Podolsky, 2007).

Human Genetic Mechanisms of Microbial Interaction in IBD

The past decade has seen substantial advances in our understanding of the pathogenesis of IBD, and in particular the complex interaction with functional alterations in the immune system. Genome-wide association studies have identified 163 distinct loci that confer risk of or protection from the development of CD and UC with a substantial portion of these loci (110 of 163) common to both diseases (Jostins et al., 2012). The role of genetics in the development of these diseases appears greater for CD than UC, with roughly twofold greater variance explained by associated loci. However, given that IBD-associated genetic variants are present in many individuals who do not develop disease, as well as the suggestion that classic loss-of-function variants play only a disease initiation role in pathogenesis, a full explanation of disease complexity will require substantially more knowledge (Knights et al., 2013).

Many of the pathways in IBD are specifically known to have heterogeneous effects when activated in different cell types, and these cellular outcomes may be compounded to affect disease. For example, in epithelial cells, autophagy pathways play a key role in bacterial clearance; however, in macrophages, the same autophagy genes affect the ability of cells to secrete interleukin-1β (IL-1β), a key inflammatory mediator involved in host defense (Lassen et al., 2014). Furthermore, IL-1β can act through both innate lymphoid cells and $CD4^+$ T cells to stimulate IL-17 and IL-22 secretion and induce chronic intestinal inflammation (Coccia et al., 2012), demonstrating that the same cytokine can act on multiple arms of the immune system to promote inflammation.

IBD-associated loci as a whole can be grouped into several important pathways including the innate immune response, maintenance of intestinal barrier function, autophagy, endoplasmic reticulum stress, microbial defense and antimicrobial activity, goblet cell function, epithelial restitution, generation of reactive oxygen species, pathways that determine tolerance and training of innate immune cells, and maintenance of balance between T helper 17 (Th17) cells and Treg cells. For example, several risk loci including *HNF4A, CDH1, MUC19, ITLN1, PTGER4, PTPRS,* and *GNA12* are all involved in processes related to the maintenance of the epithelial barrier (Khor et al., 2011), while *ATG16L1, IRGM,* and *DAP* influence autophagy. The risk loci also highlight the importance of various cytokine signaling pathways, in particular those mediated by IL-10, IL-23, and IL-27. IL-23 in particular is influenced by several of the risk loci – at least *JAK2, STAT3, ICOSLG, TYK2,* and *TNFSF15* – and represents one of the major influences on the disease.

The pathways affected by IBD risk (or protective) alleles sometimes appear to act in synergy with each other such that two processes (for example, autophagy and endoplasmic reticulum stress) operating within the same cell type result in an environment that promotes disease. Conversely, the same pathway may affect different mechanisms in distinct cell types leading to development of disease. For example, defects in autophagy in epithelial cells lead to impaired Paneth cell function while a similar autophagy defect in macrophages impairs IL-1β secretion, resulting in a milieu that favors development of IBD. In addition, the synergy may be between a “first hit” represented by a defect in a pathway and a “second hit” from a microbial trigger. Variants in the *HNF4A* locus have consistently demonstrated strong and specific associations UC, which along with the *CDH1* variants, suggest a key role for epithelial barrier integrity in the pathogenesis of UC. Several genetic variants within the human leukocyte antigen complex on chromosome 6p21 have been strongly associated with UC. The most consistent association with increased risk of IBD has been the DRB1*01:03 allele, which is associated more strongly with UC than CD, whereas the DRB1*04:01 allele is found less commonly in UC (Jostins et al., 2012). These variations impact microbial recognition at the level of antigen presentation, and are clue to the involvement of the microbiota in IBD.

Impaired NOD2 function has also been implicated in a potentially distinct subtype of microbial dysbiosis. This is supported by a number of factors, including the dichotomous role of NOD2 in the pathogenesis of CD: around one third of all patients with CD have a deleterious mutation in *NOD2* on at least one allele, compared to around one tenth in the healthy population (Hugot et al., 2001; Ogura et al., 2001). *NOD2* status alone is thus a very strong predictor of CD incidence, and the gene likely plays a key role in pathogenesis in a subset of patients. However, this also means that there are other independent host factors with strong combined effects. *NOD2* mutations are further associated with clinical subtypes including ileal stenosis and surgical intervention (Ananthakrishnan et al., 2014). IBD patients with *NOD2* mutations have an increase in adherent microbes to the intestinal mucosa (Swidsinski et al., 2002) and significant shifts in the relative abundances of *Faecalibacterium* and *Escherichia* species (Frank et al., 2011). Thus, although the number and complexity of genetic and microbial mechanisms involved in IBD pathogenesis appears to be large, resources and data are now available to characterize them in human patient

populations. If the route to IBD pathogenesis is imagined as an energy diagram, then the *NOD2* risk allele, and other predisposing genetic alterations, lower the "activation energy" required to bring the microbial composition of the gut towards the disease state (Fig. 1).

Gut Microbiome Observation and Association in Patient Populations

Although the rise of culture-independent techniques for microbial community characterization can be largely attributed to the development of "high-throughput" sequencing platforms, the earliest studies bridging this gap relied on gel electrophoresis or Sanger sequencing to identify alterations to the microbiome in human IBD. This work continued to refine the insights begun by culture-based techniques and set the stage for subsequent deep metagenomic studies of microbiological and ecological shifts during disease. For example, temporal temperature gradient electrophoresis (TTGE) of 16S rDNA targeting a limited number of large phylogenetic groups identified what is now a typical increase in the enterobacteria (Martinez-Medina et al., 2006; Seksik et al., 2003) and the reduction of anaerobes including the Clostridia and Veillonellaceae (Joossens et al., 2011; Ott et al., 2004), but (also like later studies) produced conflicting results on whether microbial diversity in IBD was increased (Seksik et al., 2003) or decreased (Bibiloni et al., 2006; Ott et al., 2004). Terminal restriction fragment length polymorphism (T-RFLP) characterization, including twin studies (Dicksved et al., 2008; Willing et al., 2009), identified decreased diversity in both CD and UC (Andoh et al., 2007). The technical difficulty of these techniques initially restricted patient numbers to a few dozen cases and controls, however, making their interpretation particularly challenging.

Soon after, though, a number of groups undertook Sanger sequencing, initially using 16S rRNA hybridization followed by fosmid cloning to compare CD, UC, and healthy individuals from biopsies (Baumgart et al., 2007; Gophna et al., 2006; Prindiville et al., 2004) and in stool samples (Manichanh et al., 2006). These studies settled on more consistent trends, including the enrichment of the Enterobacteriaceae (Baumgart et al., 2007; Gophna et al., 2006) and a decrease in community diversity in CD (Baumgart et al., 2007; Manichanh et al., 2006). The earliest such investigations remained limited by sample numbers (generally under 10 per group), with the first large-scale sequencing-based study of the microbiota in IBD appearing in 2007 (Frank et al., 2007). This used amplicon sequencing on a total of 190 biopsies from CD, UC, and control individuals. IBD samples were depleted for Lachnospiraceae and Bacteroidetes and enriched for Proteobacteria and Actinobacteria, and CD cases in particular were more readily separable from control subjects (and from UC) by means of gut microbial composition.

During the transition to high-throughput sequencing techniques, an early pyrosequencing study on the 454 platform that examined 40 twin pairs discordant for CD and UC identified an increase in the Enterobacteriaceae and a decrease in *Faecalibacterium*, consistent with previous studies (Willing et al., 2010). A small number of array hybridization-based studies were performed in a similar timeframe (Michail et al., 2012), again echoing these enrichments for Proteobacteria and reduction in Clostridia and overall diversity. The degree of agreement with earlier work, especially with regard to a general decrease in diversity in CD and UC and shared shifts in abundance of specific organisms, is remarkable. However,

differences that do exist can be attributed to differences in community profiling methods, differences in protocols between laboratories, and differences between the patient populations studied (including geographic, genetic, and age-related differences). It is unclear whether the changes to the microbiota described in these studies are a causal factor or merely a consequence of intestinal inflammation and IBD. However, several studies indicate that these microbial shifts occur prior to, or in conjunction with, the onset of inflammation and clinical phenotypes. A recent study by Gevers et al. has examined a cohort of new-onset CD patients which includes samples taken at the time of diagnosis, before the initiation of treatment, and from multiple sections of the gut including ileal tissue, rectal tissue, and stool samples (Gevers et al., 2014). This study, the largest study of the IBD microbiome to date, has added support to the most consistent subset of these results: enrichment for the Enterobacteriaceae and depletion of Clades IV and XIVa Clostridia during disease-associated inflammation (Gevers et al., 2014) (Fig. 2). Furthermore, the study finds that these shifts in microbial abundance are even more pronounced in patients who received antibiotics at the time of sample collection, suggesting that antibiotics may exacerbate CD-associated dysbiosis.

In addition to investigations of microbial ecology and composition during IBD, recent work has also begun to examine the underlying molecular and metabolic mechanisms using culture-independent techniques. The earliest shotgun metagenomic study of the disease was included in the MetaHIT cohort (Qin et al., 2010), which comprised mainly healthy individuals with a subset of 21 UC and 4 CD patients. Although this provided a wealth of raw data, minimal analysis was performed for the small subpopulation of IBD patients, confirming mainly reduced diversity (including both ecological and gene diversity) and an overall separation from healthy microbiomes. The first investigation of functional dysbiosis during IBD (Morgan et al., 2012) built on this by including inferred microbial gene content from 231 subjects and an additional 11 metagenomes. This study identified enrichment in microbial pathways for oxidative stress tolerance, immune evasion, and host metabolite uptake, with corresponding depletions in SCFA biosynthesis and typical gut carbohydrate metabolism and amino acid biosynthetic processes. Intriguingly, similar microbial metabolic shifts have been observed in other inflammatory conditions such as type 2 diabetes (Qin et al., 2012), suggesting a common core gut microbial response to chronic inflammation and immune activation.

Host-microbial interactions in disease require molecular mechanisms from the host as well, of course, and IBD has a history of deep investigation of human genetics that is detailed below. Despite the strong suggestion of gut microbial interactions in IBD-associated genetic loci (Jostins et al., 2012), few studies have yet assessed any of these compositional or functional responses in the microbiome in tandem with host genetics. Modest effects have been observed for *Eubacterium* relative to *NOD2* risk alleles (Li et al., 2012) and *Faecalibacterium* with *FUT2* (Rausch et al., 2011), but the challenge of associating many risk alleles with many microbial community members can easily be underpowered in small cohorts. Mouse models of these alleles (Kashyap et al., 2013; Spor et al., 2011) and others described above tend to exhibit much stronger dysbioses, but these have yet to be investigated in large human populations.

Finally, systems-level views of gut microbial involvement in IBD have only very recently and minimally begun to be explored. Few analysis approaches yet exist that integrate transcriptional, metabolic, and other ‘omics perspectives on the gut microbiome with disease phenotype. Gut metatranscriptomes have not yet been assessed during IBD, although during health approximately half of microbial genes appear to be transcribed basally, the remaining half showing context-specific up- or down-regulation (Franzosa et al., 2014). The extensive contribution of even the healthy gut microbiota to metabolite production and consumption makes stool metabolomics a particularly promising route (McHardy et al., 2013), with early results showing amino acid levels, SCFAs, and additional tricarboxylic acid (TCA) cycle products perturbed in the IBD gut (and to a lesser degree circulating) metabolome (Marchesi et al., 2007; Ooi et al., 2011). Most current untargeted metabolite profiles of the gut are derived from genetic (Sayin et al., 2013) or infectious (Lu et al., 2012) mouse models of inflammation, which in such extreme conditions tend to result in downregulation of typical metabolites ranging from amino acids through lipids and bile acids. One of the closest looks at the humanized (gnotobiotic) murine gut microbiome and metabolome (Marcobal et al., 2013) showed that fluctuations in metabolite levels are often mirrored in microbiota composition, that they vary consistently both in response to dietary shifts and inter-individually, and that these changes are somewhat preserved between human donors and humanized murine hosts.

Metabolomic and metaproteomic assays of stool assess both host and microbial products; while the origin of metabolites can be difficult to determine, proteins from this mixed population can typically be assigned to particular microbial or host (e.g., human or mouse) sources. Peptide sequence specificity has been used to separate host from microbial secreted proteins (Lichtman et al., 2013), for example, showing extensive immune and metabolic remodeling in response to varying microbial community composition. Similarly in mice, the (primarily microbial) metaproteome appears to mirror the metatranscriptome quite closely (McNulty et al., 2013), with most variability in an artificially colonized system arising in response to the optimization of microbial responses to nutrient availability. During IBD, the only metaproteomic studies to date have included either just six monozygotic twin pairs concordant or discordant for CD (Erickson et al., 2012) or a set of 9 UC and control subjects (Presley et al., 2012). Half of the former subjects represented ileal CD patients with a particularly extreme phenotype accounting for most of the variation in microbiome structure and protein expression, including depletion of most typical carbohydrate utilization and amino acid metabolism pathways in correspondence with overall reduced microbial diversity. The latter study found protein levels to better distinguish colitis subjects from controls than did microbial membership alone, and associated protein fluctuations with 14 particularly informative taxa, but only 87 proteins were surveyed in depth. Both studies found substantial disruption of typical host-microbial cross-talk during this extreme phenotype, and much deeper investigations of the gut ecosystem will be necessary to understand the community-wide molecular mechanisms affected during IBD.

Causality and Mechanism in Mouse Studies

Mouse models have provided great insight into the role of the microbiota in IBD because of a key advantage they hold over patient cohorts: they can be easily perturbed both

environmentally and genetically to determine the causal mechanisms of disease initiation and progression. Most patient cohort studies examining the association of the microbiota with IBD are retrospective. By definition, these studies examine the composition of the microbiota after disease onset rather than addressing shifts in microbial composition that may have contributed to disease, as a prospective study would more directly address.

In some cases, the same alterations observed after disease onset may also be present prior to disease or during progression. This is true, for example, in the *Tbx21*$^{-/-}$ × *Rag2*$^{-/-}$ UC (TRUC) mouse model of colitis, in which *Klebsiella pneumoniae* and *Proteus mirabilis* both correlate with outcome and elicit colitis in *Rag2*$^{-/-}$ and even wild-type adults (Garrett et al., 2010; Garrett et al., 2007). Conversely, this assumption does not hold true in “dnKO” (*Il10rb*$^{-/-}$ and T cell-restricted dominant negative *Tgfbr2*) mice, where colonization of antibiotic-pretreated IBD-susceptible animals with isolates of *Bacteroides thetaiotaomicron*, but not commensal Enterobacteriaceae, resulted in robust colitis (Bloom et al., 2011). Surprisingly, there was no difference in the fecal abundance of *B. thetaiotaomicron* between colonized dnKO (colitic) versus colonized *Il10rb*$^{+/-}$(non-colitic) mice, although the Enterobacteriaceae were more than 100-fold enriched in the dnKO compared to *Il10rb*$^{+/-}$ mice (Bloom et al., 2011), consistent with previous results seen in studies of both IBD patients and mouse models (Dicksved et al., 2008; Frank et al., 2007). Compositional changes to the gut microbiota can thus be either causal, responsive, or a mixture of the two in experimental models of IBD, underscoring the importance of addressing the role of the microbiota, and more specifically the question of causation, in the context of murine intervention studies.

To establish the mechanisms of microbial influence in IBD, and thereby causality, microbial molecular functions that alter host physiology must be identified. The studies above fulfill Koch’s postulates, for example, and demonstrate microbial causality in an animal model, but they remain a few steps removed from demonstrating causation in human IBD (Fig. 3). One of the lessons from the recent renaissance in microbiome research has been to adopt an ecological model of microbiology, rather than the “one-microbe, one-disease” model that has been a mainstay of infectious disease and microbiology research in past decades. Although a collection of organisms within the microbiome can have an additive or indeed synergistic effect on physiology (Kim et al., 2007), these effects can be subdivided into strain-level effects (and numerous models have been developed which establish a role for individual strains or species in IBD (Elinav et al., 2011; Garrett et al., 2010; Kim et al., 2005; Powell et al., 2012)), and further subdivided into individual molecular effects imparted by those organisms. Therefore, an important recent step forward in understanding the function of the microbiome in IBD has been to “drill down” into human microbiome-wide association studies with detailed molecular and biochemical analyses in mouse models to decipher the cellular and molecular circuitry that connects microbial products to host signaling pathways and physiological responses.

The concept of reducing the effect of a complex microbial community to an individual molecule with a defined host physiological response is well illustrated by a recent collection of studies examining the effects of SCFAs on colonic T cell populations. Individual bacterial strains isolated from healthy human stool were assayed for the induction of CD4^{+}FOXP3^{+}

Treg cells, identifying a collection of 17 strains with strong activity that belonged to Clades IV, XIVa, and XVIII of the Clostridia (Atarashi et al., 2013). As these groups of Clostridia are potent SCFA producers, it was argued that the Treg cell induction was imparted primarily by this group of metabolites (Narushima et al., 2014), and indeed independent studies found G protein-coupled receptor 43 (Gpr43, now known as Ffar2)-mediated (Maslowski et al., 2009) proliferation of Treg cells in response to SCFAs (Arpaia et al., 2013; Furusawa et al., 2013; Smith et al., 2013), resulting in reduced colitis (Furusawa et al., 2013; Smith et al., 2013) (see Fig. 2). Furthermore, the gut microbial product niacin enables colonic macrophages and dendritic cells to induce the differentiation of Treg cells and suppress colitis and intestinal cancer in a manner dependent on Gpr109a, now known as Hcar2 (Singh et al., 2014).

To highlight the need for additional mechanistic studies on the microbiome, prior to the recent work on SCFAs and Treg cell function, the only other microbial product that has been directly linked to a beneficial effect on host immune function has been polysaccharide A (PSA) produced by the commensal bacterium *Bacteroides fragilis* (Mazmanian et al., 2005). PSA interacts with Toll-like receptor 2 primarily on antigen-presenting cells, and is processed and presented by the major histocompatibility class II complex to activate $CD4^+$ T cells (Round et al., 2011), which can result in both immunoprotection through IL-10 secretion, or pro-inflammatory responses via interferon-γ secretion (Dasgupta and Kasper, 2013). *B. fragilis* protects mice from *Helicobacter hepaticus*-induced colitis in a PSA-dependent manner (Mazmanian et al., 2008). A recent study by Kasper and colleagues suggests that the immunomodulatory action of *B. fragilis* may not be a function of PSA alone, but also sphingolipids (An et al., 2014). Sphingolipids produced by *B. fragilis* negatively regulate intestinal natural killer T cells and prevent their excessive activation during oxazolone-induced experimental colitis, thus contributing to the maintenance of host immune balance (An et al., 2014) (see Fig. 2).

Despite these successes in unraveling the molecular mechanisms of IBD induction in mice, it is important to remember that nearly all mouse IBD studies are performed in the context of the complete deletion of a gene, a strong chemical colitogen, a highly controlled and uniform microbial environment, and in many cases a combination of all three of these non-physiological factors. Human IBD does not involve such strong genetic and environmental perturbations, and these systems thus may or may not be appropriate models for particular components of the human disease. Recently, a mouse model of the T300A mutation in the autophagy gene *ATG16L1* showed defective clearance of the ileal pathogen *Yersinia enterocolitica* and sustained inflammatory cytokine response (Lassen et al., 2014; Murthy et al., 2014). The future use of mouse models that replicate precise human genetic alterations discovered from IBD genome-wide association studies in the context of IBD-relevant alterations to the microbiota will be an important step forward in modeling human IBD.

Gaps, Challenges, and Next Steps

The gut microbiome's potential as a candidate biomarker for host health in IBD has not yet been fully explored. As a cross-sectional diagnostic indicator for IBD, colonic microbial profiles provide only modest delineation between patients and asymptomatic controls

(Dicksved et al., 2008; Frank et al., 2007; Morgan et al., 2012), ruling out a simple, universal “magic bullet” for early diagnosis, although optimistically this need not be the case in all complex inflammatory conditions. Instead, heterogeneity among CD, UC, and subtypes within them (e.g., ileal CD) suggests that carefully chosen microbial samples could, for example, be used to improve classification under high-risk conditions such as ileal pouch surgery (Tyler et al., 2013).

Similarly, little work has yet been done on using gut microbial readouts to classify patients with respect to expected response to treatment. In CD, for example, 5-ASAs are a relatively mild first-line therapy to which some patients respond well and others only minimally; a clear microbial predictor of patient response would eliminate the need for a lengthy step-up process (Kamm, 2004; Peyrin-Biroulet et al., 2013). Longitudinal studies have not yet been carried out with appropriate designs to discover prognostic microbial biomarkers of treatment response, flare onset and recovery, or disease severity, however. Likewise, it is not yet clear for IBD or for other inflammatory conditions whether the “right” biomarkers, if any exist, will be derivable from stool, mucosal biopsies, or other sample types, or whether they will consist of one or a few microbial abundances, overall ecological profiles, gene expression, peptides, metabolites, or other biomolecular markers.

Likewise, ecological therapies leveraging the microbiome are still in their infancy in IBD, as in nearly all complex, chronic conditions. Successes such as fecal microbiome transplants for *Clostridium difficile* infection (Borody and Khoruts, 2012) highlight the great potential of microbial modulation under such circumstances, but early results for this broad-brush approach in IBD have been mixed (Aroniadis and Brandt, 2013; Sha et al., 2014; Smits et al., 2013). Similarly, “untargeted” probiotics tend to have significant but small beneficial effects in IBD (Leone et al., 2013; Whelan and Quigley, 2013), suggesting that microbial community modulation has the potential to improve outcome if the correct, specific pressures can be discovered and applied. Some of these may be positive, in the sense of probiotically restoring one or more microbial taxa, or providing a selective growth advantage to underrepresented clades by means of diet, pharmaceuticals, or prebiotics (DuPont and DuPont, 2011). Others may be negative, selecting against community members by the same mechanisms or through the development of particularly narrow-spectrum antibiotics (Lemon et al., 2012). Some of the most successful current molecular treatments in cancer – another complex, multifactorial disease – were developed to inhibit or promote extremely specific molecular components of dysregulated pathways (Deininger et al., 2005; Perez and Baweja, 2008). A comparable level of specificity may be needed to treat complex dysbioses, either targeting pathway components intracellularly in one or more host or microbial cell types, or extracellularly by depleting, competing with, or supplementing native metabolites or signaling molecules. The first steps toward these goals have already been taken by investigating the bioactivity of unique natural products in the gut (Holmes et al., 2012; Lemon et al., 2012), but none have as yet been developed with specific therapeutic potential in IBD.

The development of study designs for diagnostic or therapeutic applications of the gut microbiome in IBD serves as a model by which other microbially-linked conditions can be investigated (Fig. 4). Most study design considerations in the microbiome vary along several

axes, each of which must be considered to identify experimental components appropriate for the conditions and hypotheses at hand.

1. *Clinical relevance vs. experimental control*. Any translational study is, at best, more variable in a patient population relative to a laboratory environment, but typically more likely to provide results that are applicable clinically.
2. *"Top down" vs. "bottom up" information*. Many observational studies of the microbiome are driven by 16S profiling alone, resulting in descriptive information. This can be complemented by assays that are typically more expensive and/or time-consuming, but which provide molecular or mechanistic detail.
3. *Host vs. microbial profiling*. Assays such as 16S profiling or metagenomic and metatranscriptomic sequencing of stool samples yield information primarily on microbial activity. Conversely, transcriptional profiling of mucosal biopsies describes the human host's activity. Other assays, such as stool metabolomics and proteomics, can capture molecular activities originating from either source.
4. *Sample availability*. In patient populations, stool samples are more readily accessible than biopsies, but are still difficult to collect in bulk from very large cohorts.
5. *Cost*. Staged study designs are particularly appropriate for the microbiome (Tickle et al., 2013), as they allow the benefits of large cross-sectional profiles combined with molecular detail derived from a subset of individuals, conditions, or time points.

In addition to these microbiome-focused choices, other study design considerations are shared with any high-throughput, translational biology. The care that must be taken both during study design and analysis to ensure reproducibility cannot be overemphasized (Peng, 2011). Microbiome assays are currently particularly sensitive to batch effects and inter-population differences (Koren et al., 2013; Leek et al., 2010), in addition to the multiple hypothesis testing issues common to any high-dimensional analysis (Sandve et al., 2013). Additionally, like other complex, polygenic, personalized conditions such as cancer, the generalizability of any finding to other populations must be considered (Yatsunenko et al., 2012), as should heterogeneity among disease subtypes (Cancer Genome Atlas, 2012; Morgan et al., 2012; Willing et al., 2009).

Studies of the gut microbiome's role in complex disease must also contend with a wide range of time scales, in contrast to studies focusing solely on human genetics or transcriptional responses. Several of these are due to distinct molecular processes: host genetics are fixed over the course of a lifetime, host epigenetic modifications can occur over days to months (Sasai et al., 2013), changes in microbial populations due to doubling times can likewise take anywhere from days to months (Dethlefsen and Relman, 2011; Gajer et al., 2012), and host or microbial transcription, metabolism, or protein expression can change in seconds to minutes.

However, other temporal considerations are dictated by larger-scale processes and phenotypes. Transit times in response to dietary shifts occur over hours to days, for example

(David et al., 2014), and in IBD evidence suggests differences both between pediatric and adult cases and between new-onset and established disease (Gevers et al., 2014). The microbiome also changes rapidly during early development, particularly during infant colonization (Koenig et al., 2011; Sharon et al., 2013; Yatsunenko et al., 2012). Microbiome studies must thus identify the correct temporal scale(s) at which to operate for any particular hypothesis.

Finally, although stool samples provide one of the most readily accessible windows into the gut microbiome for most studies, the snapshot they offer is distinct from that provided by mucosal biopsies, lavages, or other sample types (Li et al., 2011; Morgan et al., 2012; Stearns et al., 2011; Swidsinski et al., 2002). Stool is regularly characterized by an expansion of Firmicutes relative to Proteobacteria and Bacteroidetes at the mucosal surface, and the heterogeneity of the microbiome within individual stool samples is not yet well-understood. Additionally, the biogeography of the intestinal tract itself influences microbial community structure and function, with dramatic differences in pH, microbial density, and composition between the small and large intestine and in a more gradual continuum across the colon (Eckburg et al., 2005; Morgan et al., 2012; Peterson et al., 2008; Stearns et al., 2011). Diffusion rates of microbes, microbial metabolites, and host biomolecules through the mucosa and lumen are generally not well characterized, making their biogeographical sources and sinks somewhat unclear. The fecal microbiome may thus be viewed as a somewhat noisy function of the mucosal microbiome (Gevers et al., 2014), providing a more clinically convenient but sometimes less interpretable picture of microbial activity *in situ* at the mucosa.

Mouse models of IBD, although coming with the slate of drawbacks typical of any model system, were one of the first indicators of the gut microbiome's crucial role in the disease and continue to be critical for understanding its molecular mechanisms. In mouse models, control of gut community composition allows the membership and molecular function of gut microbes to be closely linked and studied in tandem. It also allows for perturbations that affect immune function to be examined in the context of defined shifts in the microbial community. Focusing on well-defined microbial gene functions and metabolites under such conditions is more experimentally reproducible, particularly when gnotobiotic transfer from individual human donors can be replicated multiple times, for example. Finally, microbial gene functions and pathways, and their effects on mucosal immune function, shown to contribute to IBD in such models can be brought back to the clinic as potential therapeutic molecules or molecular targets.

Though it is a field with a long history, studies of the role of the gut microbiota in IBD have made large strides within the past half-decade. The field has moved from shallow 16S sequence-based community profiling studies to "high resolution" deep metagenomic characterizations at the strain and gene level, both in mouse models and in increasingly larger and more sophisticated human cohort studies. The focus has moved from taxonomic descriptive studies to detailed, metagenomics-based functional characterizations that have been related to its effects on the host immune response. In the coming half-decade, the focal point of gut microbiota studies in IBD will shift from broad functional characterizations towards the identification of specific effector molecules including microbial structural

components, intracellular and secreted metabolites and proteins, and other regulatory and signaling components produced from the dialog that is transpiring between the gut microbiota and the host immune system. Adopting a molecular-centric perspective in assessing the role of the microbiome in IBD will provide a fine-grained resolution of this dialog, and has the potential to enable numerous therapeutic avenues to tackle this disease.

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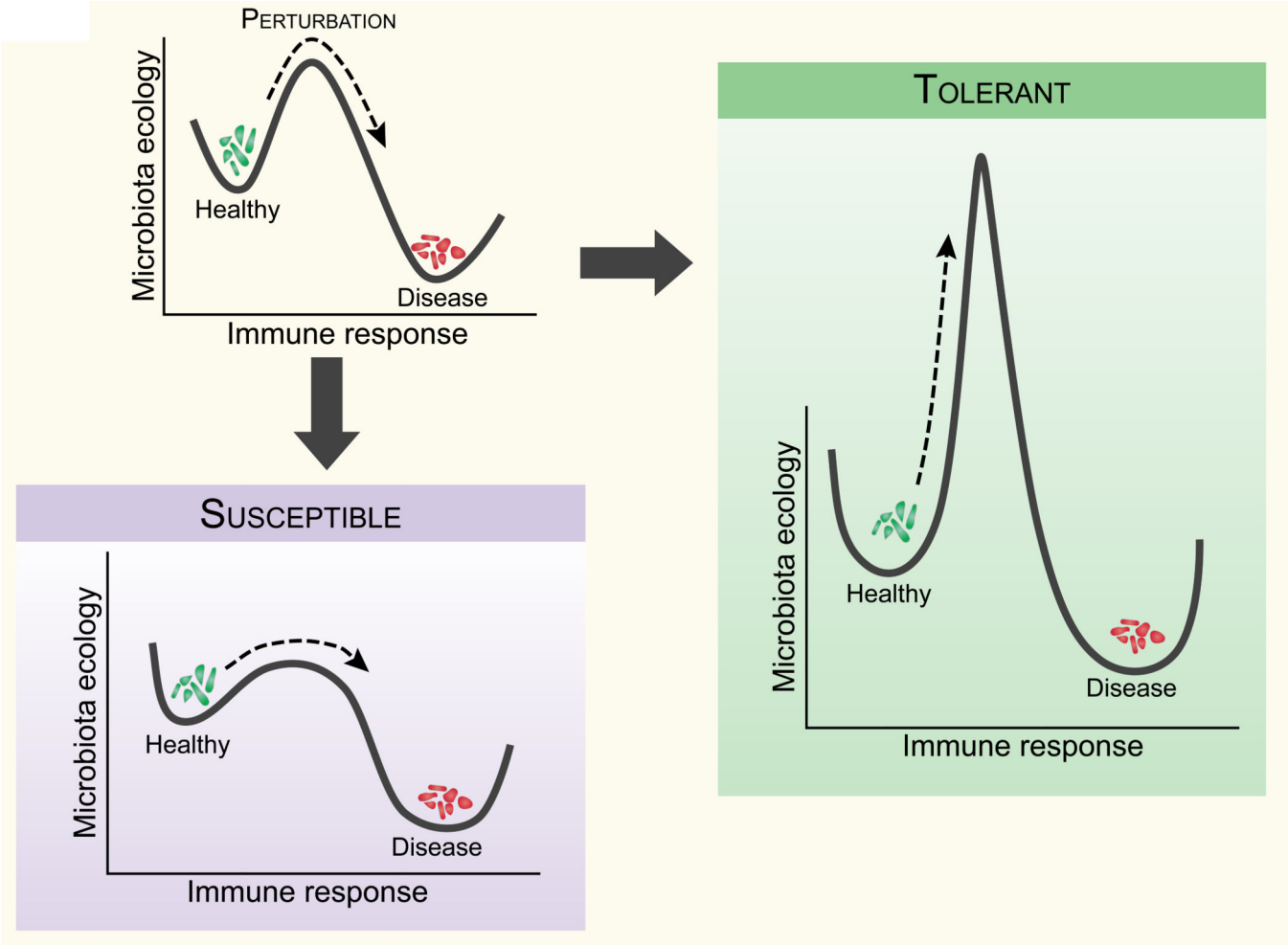

Figure 1. Disease progression can be modeled as the dynamical response of a multi-stable, multi-factorial system

A systems-level perspective on health and disease states in IBD and other microbiome-associated conditions can be illustrated using an energy landscape model. The contours of the landscape (i.e., the depth of the healthy vs. disease state) determine how likely it is for an individual to progress from one state to another. Individuals with a lower "activation energy" to this landscape, for example those carrying a *NOD2* mutation, may be predisposed to a shift in the microbial community that correlates with IBD (see Section "Human Genetic Mechanisms of Microbial Interaction in IBD"). Both healthy and disease states are characterized by distinct microbial configurations and immune responses. A disturbance or perturbation, such as the introduction of an inflammation-promoting pathobiont or treatment with antibiotics, might cause the system to transition to a new "disease" state. The underlying contours of the landscape are determined by host genotype as well as environmental and physiological factors. A susceptible individual (e.g., carrying disease risk alleles; below, in purple) would be more sensitive to minor perturbations, while a tolerant individual (bearing protective regulatory variants; right, in green) would exhibit robust behavior against strong stimuli.

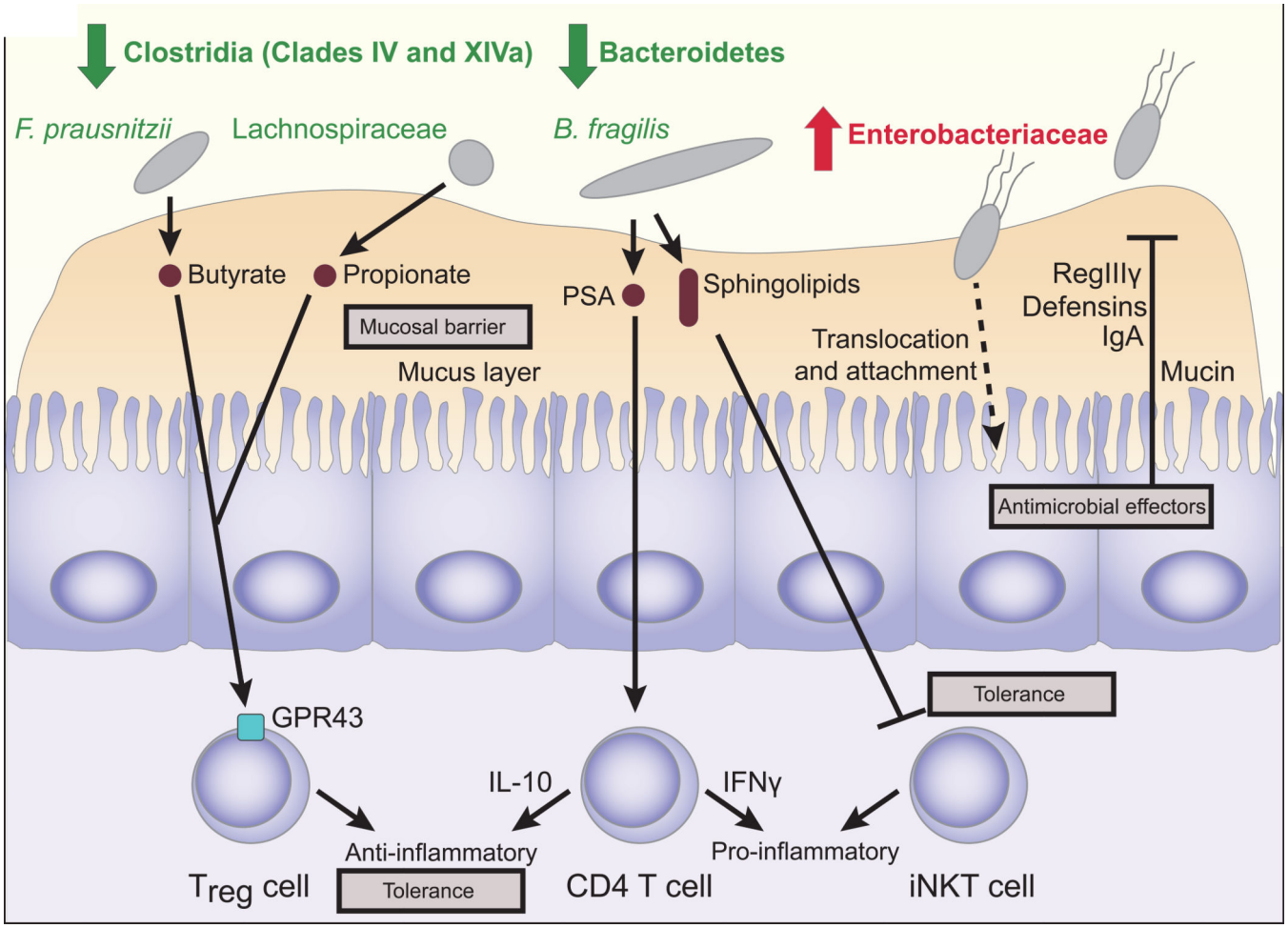

Figure 2. Interactions between the gut microbiota and the intestinal mucosa in IBD

This illustration depicts the major alterations to the composition of the gut microbiota in IBD, host mechanisms to correct this dysbiosis, and their functional consequences on the host mucosa. The lumen (yellow), mucus layer (brown), epithelium (purple brush-border-containing cells), and lamina propria (bottom purple section) are shown. The most consistent observations from microbial profiling studies are shown (clades with decreased abundance in IBD are in green, increased abundance in red). Specific microbial mechanisms supported by strong experimental evidence are included. These mechanisms include the expansion of the Treg cell compartment by microbially produced butyrate and the inhibition of intestinal natural killer T cell function by microbially produced sphingolipids to promote tolerance, as well as epithelially secreted antimicrobial factors and Goblet cell production of mucin to expand the mucus layer and limit microbial activity at the epithelium. These mechanisms are grouped into themes, displayed in gray boxes.

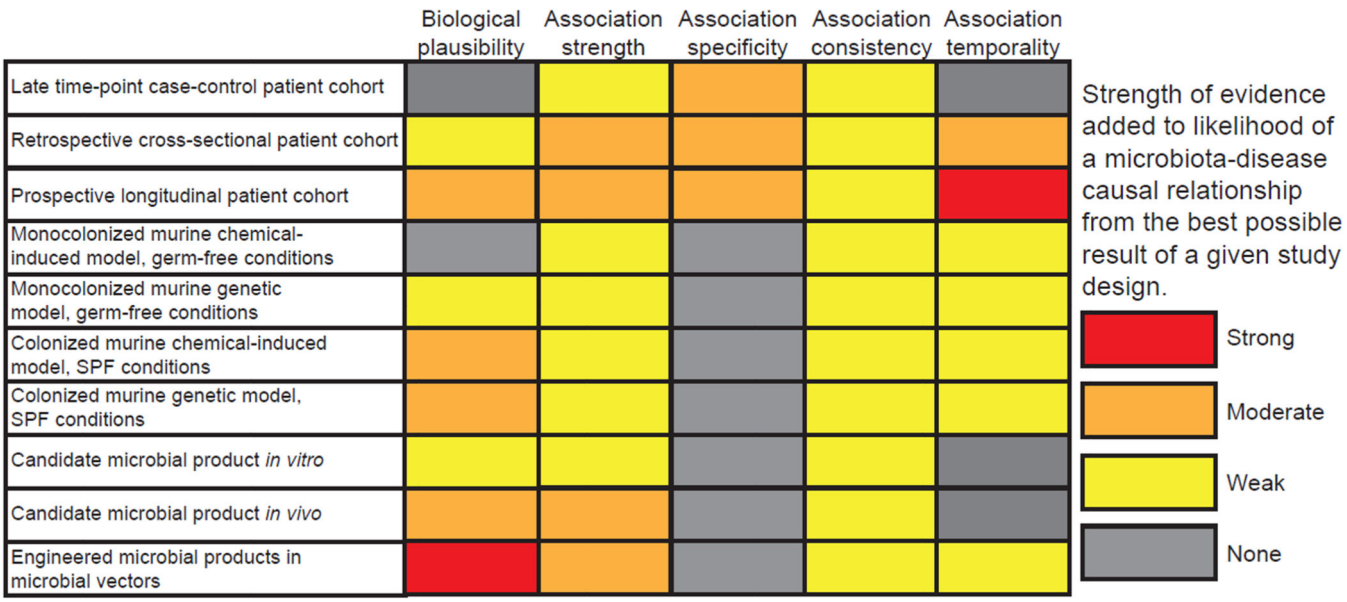

Figure 3. Modified Hill's criteria for assessing a causal role for the gut microbiota in IBD

Experimental design considerations for a study of the role of the gut microbiota in IBD should include factors that directly address causation. Shown at the top are 5 of the Hill's criteria that are most applicable to gut microbiota studies in IBD, including biological plausibility (Does the study provide evidence of a mechanistic link between the microbe or community of microbes and disease?), association strength (Is there a strong statistical association between the microbe or community of microbes and disease?), association specificity (Is the microbe or community of microbes also known to be involved in other unrelated diseases or is there high specificity for the disease in question?), association consistency (Do independent laboratories report the same association?), and association temporality (Does the putative disease-causing microbe or community of microbes appear at the anticipated time prior to the onset of disease?).

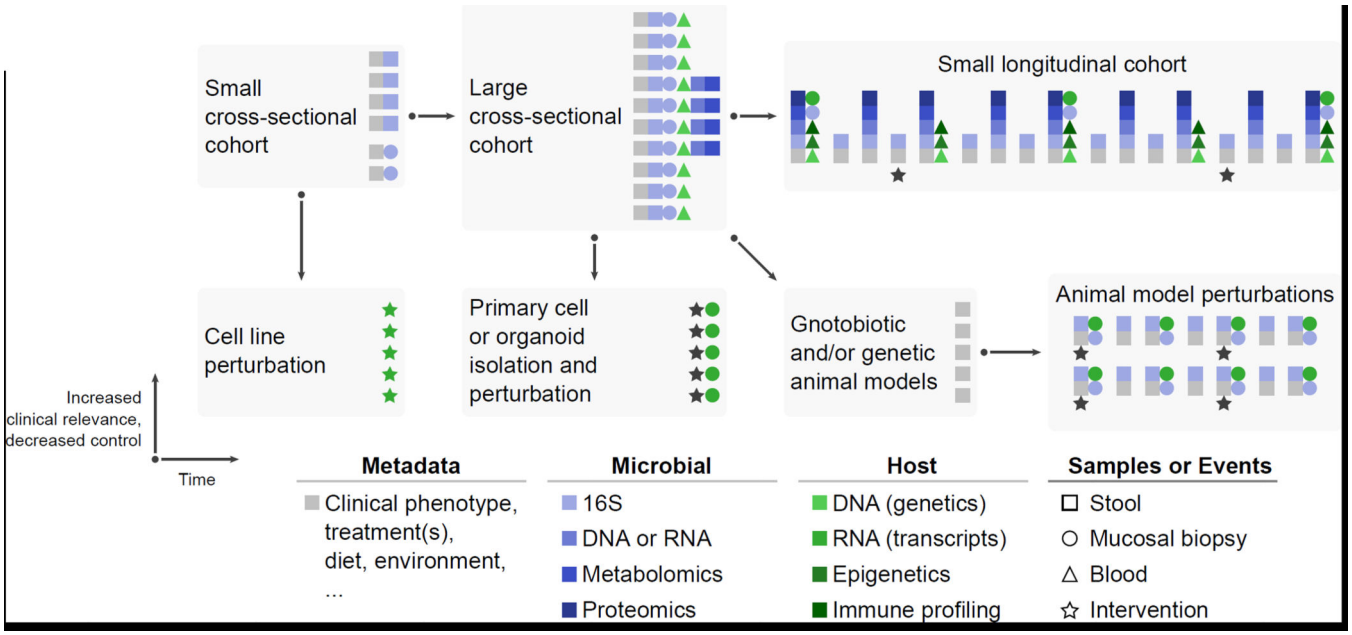

Figure 4. Experimental design considerations in gut microbiome studies, using IBD as an example

The development of gut microbiome study designs in IBD can serve as an example for other gut microbial dysbioses, as they demonstrate the interplay of biology, sample availability, and financial constraints. Observations typically start with “top down,” descriptive studies of the stool or (less often due to availability) mucosal microbiome in modestly sized populations. “Bottom up” molecular detail can be added efficiently by perturbation studies in cell lines, but these are limited in relatability to primary populations. Two-stage study designs offer cost-effective scalability to larger cohorts (Tickle et al., 2013), which are also better powered for genetic profiling. Patient samples can be used to derive primary cell lines (Miyoshi and Stappenbeck, 2013) or organoids (Sato et al., 2011) for controlled perturbations that more closely recapitulate *in vivo* conditions. Likewise stool samples can be transferred to gnotobiotic animal models (Goodman et al., 2011) to determine the causal contribution of the microbiome to phenotype, leading in more complex designs to longitudinal perturbations in multiple genetic models or environmental conditions. Finally, in-depth profiling of moderately sized patient populations over time can provide multiple views on gut microbial contributions to phenotype. This remains cost-effective when combined with staged study design (i.e., not all assays are run on all samples), and sample availability and perturbations such as treatment changes are typically contingent on clinical care.