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Immune and genetic gardening of the intestinal microbiome

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Abstract

The mucosal immune system – consisting of adaptive and innate immune cells as well as the epithelium – is profoundly influenced by its microbial environment. There is now growing evidence that the converse is also true, that the immune system shapes the composition of the intestinal microbiome. During conditions of health, this bidirectional interaction achieves a homeostasis in which inappropriate immune responses to nonpathogenic microbes are averted and immune activity suppresses blooms of potentially pathogenic microbes (pathobionts). Genetic alteration in immune/epithelial function can affect host gardening of the intestinal microbiome, contributing to the diversity of intestinal microbiota within a population and in some cases allowing for unfavorable microbial ecologies (dysbiosis) that confer disease susceptibility.

Introduction

The small intestine and colon house a complex bacterial ecosystem consisting of an estimated 10^{14} cells in humans, 10 fold greater than the number of human cells [1]. Many have beneficial functions such as harvesting energy from otherwise indigestible plant polysaccharides (transferred to the host via short chain fatty acids), triggering formation of an intestinal mucus barrier, promoting intestinal vascularization, metabolizing xenobiotics, and preventing colonization by pathogens [2–6]. The microbiome also has profound effects on the immune system, the subject of other reviews in this issue. The beneficial properties of the intestinal microbiome confer an evolutionary advantage to animals that can regulate the microbial communities in their digestive tracts. Intestinal microbes also benefit from the survival and reproduction of their hosts. It has been hypothesized that in response to evolutionary pressure favoring mutual survival, hosts and intestinal microbes have co-evolved traits that foster symbiosis [7,8].

The existence of genetically encoded mechanisms for regulating microbial composition is suggested by the observation that across mammals, the intestinal microbiome clusters by

taxonomic order rather than geography [9]. This could also reflect similarities in diet, which has been shown across mammals and within human populations to strongly influence the intestinal microbiome [10–12]. However, the microbiome of taxonomic groups such as bears/pandas with highly variable diets (carnivore, omnivore, herbivore) most resembles the microbiome of other members of the same taxonomic group rather than taxonomically unrelated mammals with a similar diet [9]. An alternate non-genetic explanation for taxonomic clustering of microbiota is a founder effect due to initial colonization by microbes of parental origin, allowing for stable transmission of a species-specific microbiome without active host involvement. Human neonates are known to be initially colonized by maternal vaginal or skin bacteria depending on mode of delivery and founder effects have been demonstrated for specific microbes such as H. pylori [13,14]. However, longitudinal studies of human infants and ex-germ-free mice have demonstrated wide fluctuations in microbial community structure – possibly reflecting environmental or stochastic factors – before stabilization into an adult microbiota distinct from (though still influenced by) the founding microbiota [15–17].

Since diet and founder effects, while important, do not alone explain taxonomic specificity of microbial composition, it is apparent that genetically encoded mechanisms exist to ensure that early fluctuations in intestinal microbiota result in the emergence of a stable, species-appropriate microbiota. Host factors that could potentially be utilized to regulate the microbiota include immune activity, intestinal motility, attachment surfaces, and secreted products that are metabolized by bacteria. The existence of host gardening of the intestinal microbiome has largely been probed by comparing the microbiota of mice deficient in a putative gardening gene to littermate controls using 16S ribosomal RNA sequencing. The vast majority of gardening genes identified in this manner are involved in innate (including epithelial) and adaptive mucosal immunity (Table 1).

Microbial sensing via pattern recognition receptors

The immune system is equipped with an intricate system of germline encoded pattern recognition receptors (PRRs) that recognize microbial molecular patterns. Many of the genes found to affect the microbiome are PRRs, suggesting that gardening is an active process in which mucosal cells adjust their gardening activity in response to microbial composition.

Toll-like receptors (TLRs)

TLRs are transmembrane PRRs that exist either on the cell surface - where they recognize external components of bacteria, mycoplasma, fungi, and viruses - or in the endolysosomal compartment, where they recognize microbial nucleic acids [18]. The cell surface TLRs include TLR1 (which recognizes lipoproteins), TLR2 (lipoproteins), TLR4 (lipopolysaccharide), TLR5 (flagellin), and TLR6 (lipoproteins). The endolysosomal TLRs include TLR3 (double-stranded RNA), TLR7 (single stranded RNA), TLR 8 (single stranded RNA), and TLR9 (unmethylated DNA with CpG repeats). TLR signaling pathways are dependent upon two adaptor molecules, MyD88 (all TLRs except TLR3) and TRIF (TLR3, TLR4). The critical role of TLRs in recognizing microbial pathogens is revealed by

the phenotype of MyD88-deficient humans, who develop recurrent pyogenic bacterial infections [19].

A number of studies have suggested a role for TLRs in immune gardening of the microbiome. An early study found that MyD88−/− mice had increased Rikenellaceae and Porphyromonadaceae in cecal contents [20]. Deletion of MyD88 specifically in intestinal epithelial cells resulted in increased TM7 and decreased *Lactobacillus* in feces [21]. These mice also developed overgrowth of colonic and ileal mucosa-associated bacteria, which was associated with invasion of bacteria such as *Klebsiella pneumoniae* into mesenteric lymph nodes [21,22]. This indicates that epithelial MyD88-dependent gardening mechanisms exist that modulate microbial composition and restrain invasive pathobionts. The specific TLRs involved are largely unknown, but strong evidence exists that TLR5 has a gardening effect. TLR5−/− mice developed an altered microbiome which caused spontaneous colitis or, after rederivation into a new facility, obesity that could be transmitted to co-housed wild-type mice [23,24]. Obese TLR5−/− mice had altered abundance of 116 phylotypes and colitic TLR5−/− mice were found to have a bloom of Enterobacteriaceae, in particular *Escherichia coli*, penetrating the colonic mucus layer [25]. This suggests that colitis can be precipitated by deficient gardening of invasive pathobionts. TLR2 has also been reported to affect microbial composition, with 22 differentially abundant phylotypes in knockout (KO) mice [26].

A recent systematic study of the microbiome in TLR-deficient mice raised questions about these findings [27]. No statistically significant differences in microbial composition were observed between individually caged TLR2, TLR4, TRL5, TLR9, and MyD88 KO mice compared to littermate controls. The reason for the discrepancy with prior studies is unknown. The earlier TLR2 data may have been misleading as the TLR2−/− and control mice were bred separately, making it possible that differences in microbial composition reflected environmental factors [26]. However, the earlier MyD88 and TLR5 studies used heterozygote breeders and the MyD88 study employed individual caging [20,23]. The lack of effect of TLR genotype in the recent study may have resulted from the mice being housed in a facility with autoclaved cages, irradiated food, and acidified water whereas other studies utilized standard SPF facilities [27]. This raises the point that gardening, while genetically encoded, acts on environmental input and so could potentially be mitigated in a clean environment.

NOD1/2

The NOD-like receptor (NLR) family consists of at least 23 cytoplasmic proteins, many with specificity for bacterial products [28]. The best studied are NOD1, NOD2, and the NLR components of the inflammasome. NOD1 and NOD2 recognize moieties from peptidoglycan – meso-diaminopimelic acid (DAP) and muramyl dipeptide (MDP), respectively – which are found on many bacterial and mycobacterial pathogens. NOD1 is expressed widely while NOD2 is expressed primarily in immune cells and epithelial cells. Following microbial sensing, NOD1 and NOD2 translocate to the plasma membrane and initiate downstream signaling pathways via RIP2 (also known as RICK) [29]. NOD1 and

NOD2 are also able to induce the formation of autophagosomes by recruiting Atg16L1 to the plasma membrane, resulting in engulfment of invading bacteria [30].

NOD2 has drawn considerable attention due to the strong link between NOD2 genetic polymorphisms and Crohn's disease. NOD2−/− mice had altered microbial composition in the ileum, colon, and feces as well as an increased number of ileal mucosa-associated microbes [31–33]. NOD2−/− mice treated with DSS (dextran sulfate sodium) and AOM (azoxymethane) to induce colitis-associated malignancy had increased colonic *Rikenella* and *Paludibacter* compared to controls [31]. The microbiome of NOD2−/− and RIP2−/− mice conferred susceptibility to DSS colitis that was transmissible to co-housed wild-type mice [31]. In humans, a study of the ileal microbiota of normal, colitis, and Crohn's disease patients demonstrated that Crohn's-associated NOD2 polymorphisms are associated with altered ileal composition independently of disease phenotype [34]. Specifically, NOD2 was found to influence the abundance of Proteobacteria, Bacillus, and Clostridium group IV by 16S sequencing and the *Clostridium Coccoides – Eubacterium Rectales* group by quantitative RT-PCR. Human patients homozygous for NOD2 polymorphisms have increased levels of both Firmicutes and Bacteroides in ileal tissue, paralleling results in mice [32].

We are unaware of any studies that have used 16S sequencing to evaluate the intestinal microbiome in NOD1−/− mice, though one paper using quantitative RT-PCR did not demonstrate any changes in abundance of a selected panel of bacterial groups [35].

Inflammasome

Inflammasomes are cytosolic protein complexes consisting of a pattern recognition receptor (generally a NLR), the adaptor protein ASC, and procaspase 1. The primary function of an inflammasome is to cleave caspase 1 into its active form, which can then activate the inflammatory cytokines IL-1β and IL-18 by proteolysis. NLRP1, NLRP3, NLRP6, and NLRC4 have been documented to form inflammasomes in response to diverse stimuli including MDP, flagellin, PrgJ-like proteins from Gram negative bacteria, and reactive oxygen species generated by mitochondrial stress [36,37]. NLRP6 is found predominantly in epithelial cells, but its ligand remains unknown [38]. Mice deficient in NLRP6, ASC, or caspase-1 developed an abnormal microbiota that conferred transmissible susceptibility to DSS colitis and fatty liver disease [38,39]. These mice were found to have increased *Prevotellaceae* and TM7 in feces and expansion of a crypt base microbe containing electron dense intracellular material (a feature seen in some Prevotella). This demonstrates that intracellular sensing of invasive pathobionts such as Prevotella by NLRP6 can activate gardening to prevent disease-promoting dysbiosis. Interestingly, stress was recently shown to downregulate NLRP6 via corticotropin-releasing hormone, causing alteration of the small intestinal microbiome and enteritis that was transmissible to co-housed mice [40].

Epithelial gardening

IL-18

The intestinal epithelium exists in close proximity to the intestinal microbiota and is a likely site of microbial sensing and gardening. The role of the epithelium in gardening is supported

by the phenotype of mice with epithelial specific MyD88 deletion and further characterization of NLRP6-mediated gardening [21]. An analysis of IL-1β, IL-1R, and IL-18 KO mice revealed that only IL-18−/− mice had an altered microbiome conferring increased DSS colitis susceptibility [38]. While the microbiome of IL-18−/− mice was not identical to that of NLRP6 and ASC KO mice, it was characterized by the same increased abundance of *Prevotellaceae* and TM7. Bone marrow chimera experiments revealed that IL-18 expression was required in non-hematopoietic cells to prevent dysbiosis, strongly suggesting that the epithelium was the source of IL-18 processed by the NLRP6 inflammasome. The mechanism of IL-18 gardening is not known but is likely multifactorial as IL-18 has diverse effects on the immune system including promoting T helper responses (Th1, Th2, and Th17 depending on the cytokine milieu), increasing cytotoxic CD8+ T cell activity, activating NK cells, inducing macrophage inflammatory cytokine production, and recruiting neutrophils [41].

Antimicrobial products: defensins, cathelicidin, RegIIIγ

Intestinal epithelial cells are able to produce a variety of cationic peptides with broad antimicrobial activity including beta defensins, alpha defensins, and cathelicidins [42]. Beta defensins are both constitutively expressed by epithelial cells and induced by stimuli such as TLR ligands [43]. They act as direct antimicrobial agents and as chemoattracts for immune cells (including Th17 cells) via CCR6 [42,44]. While beta defensins are attractive candidates to garden the microbiota, this has not yet been studied to our knowledge. Alpha defensins are expressed by neutrophils and Paneth cells, a specialized epithelial cell type in the small intestine that releases granules containing defensins and other antimicrobial products in response to microbial stimuli [45]. Mice lacking an enzyme required to process alpha defensins have an altered small intestinal microbiome characterized by reduced *Bacteroides* [46]. Conversely, transgenic expression of an alpha defensin in Paneth cells resulted in increased *Bacteroides* and loss of segmented filamentous bacteria (SFB) colonization in the small intestine. Transgenic mice had fewer Th17 cells in the small intestine, consistent with reports that intestinal SFB colonization is critical for induction of Th17 responses in mice [46,47]. Alpha defensin-mediated gardening may be regulated by the nutrient composition of the diet. It was recently shown that dietary tryptophan deficiency or deletion of ACE2, an enzyme required for tryptophan transport into intestinal cells, caused reduced production of antimicrobial products including alpha defensins [48]. ACE2−/− mice had an altered ileal microbiome (including increased abundance of several members of the *Limibacter* and *Paludibacter* genera) and increased DSS colitis susceptibility that was transmissible to germ-free mice. The altered microbiome was reversible by treatment with nicotinamide (a product of tryptophan metabolism) or treatment with a dipeptide form of tryptophan which could be absorbed without ACE2.

Cathelicidin in its active peptide form, LL-37, has broad antimicrobial activity and acts as a chemoattract for neutrophils, monocytes, and T cells via the formyl peptide receptor-like 1 [42,49]. Expression of cathelicidin by epithelial cells and innate immune cells can be induced by short chain fatty acids derived from bacterial metabolism and by vitamin D [50–52]. Indeed, vitamin D deficiency resulting in inadequate cathelicidin production has been proposed as a mechanism of tuberculosis susceptibility in dark-skinned populations [52].

While we are unaware of any direct studies on microbial composition in cathelicidin deficient mice, one study has evaluated the microbiome of mice deficient in vitamin D receptor and Cyp27B1 (the enzyme that generates the active form of vitamin D, 1,25-dihydroxycholecalciferol) [53]. These mice had an altered microbiome characterized at the family level by higher abundance of Desulfovibrionaceae, Bacteroidaceae, and Erysipelotrichaceae and lower abundance of Lactobacillaceae, Lachnospiraceae, Ruminococcaceae, Streptococcaceae, and Deferribacteraceae. Intestinal expression of Cyp27B1 has recently been shown to be regulated by the microbiota, suggesting that vitamin D mediated gardening is modulated by PRR signaling [54]. Interestingly, deficiency of vitamin D receptor (VDR) or Cyp27B1 exacerbates DSS colitis [55,56]. This can be rescued by epithelial-specific transgenic expression of VDR, demonstrating the physiologic importance of vitamin D signaling for epithelial homeostasis [57]. However, vitamin D can enhance tight junctions and is involved in many immune processes including T cell homing to the intestine, maintenance of intraepithelial T lymphocytes, and development of natural killer T cells, so it is unclear if the microbial phenotype of these mice or their susceptibility to colitis is due to reduced cathelicidin expression [56,58–60].

Paneth cells and enterocytes also secrete RegIIIγ, an antimicrobial lectin produced predominantly in the ileum that has preferential activity against Gram positive bacteria [61]. It is minimally expressed in germ-free mice but rapidly induced upon conventionalization. This likely occurs through TLR-mediated microbial sensing by epithelial cells, as epithelial cell specific deletion of MyD88 greatly reduces RegIIIγ [22]. Loss of RegIIIγ expression after antibiotic treatment allowed for expansion of an antibiotic-resistant Gram positive pathogen, vancomycin-resistant *Enterococcus* [62]. RegIIIγ−/− mice have been found to have increased mucosa-associated microbiota in the ileum, characterized by overgrowth of at least two groups of Gram positive organisms - segmented filamentous bacteria (SFB) and *Eubacterium rectale* [22]. This corresponded to increased Th1 activity and IgA production in the small intestine, suggesting that the altered microbiome provoked adaptive immune responses. Increased intestinal Th17 activity was not observed in RegIIIγ−/− mice, but systemic Th17 responses were not assessed [22].

RELMβ

Intestinal goblet cells are capable of secreting not only mucus but also bioactive molecules including RELMβ, a product specific to goblet cells that is upregulated during inflammatory processes and parasite infection [63–65]. RELMβ is undetectable in germ-free mice but is induced within 48 hours of conventionalization, indicating that its expression is controlled by microbial factors [66]. It stimulates macrophages to secrete inflammatory cytokines including TNFα and IL-12, which then promotes IFNγ production by T cells [63,67]. KO mice show reduced DSS colitis severity, impaired responses to helminths, and resistance to diet induced obesity [63,65,67,68]. These mice were found to have an altered microbiome characterized by differential abundance of 15 Bacteriodetes, 15 Firmicutes, and 1 Proteobacteria lineages, indicating that RELMβ has a gardening effect [68]. However, it is unknown whether the phenotypes of these mice were due to alteration of the intestinal microbiota.

FUT2

Epithelial gardening involves not only antimicrobial and immunomodulatory molecules but also mucus glycoproteins, which serve as attachment sites and energy sources for microbes. Considerable evidence now exists that the intestinal microbiome is influenced by fucosylation – the addition of L-fucose to the terminal β-D-galactose residue of mucus glycoproteins by α1–2 fucosyltransferase (encoded by the FUT2 gene). Fucose is utilized as a nutrient source by some bacteria, most notably *B. thetaiotaomicron*, and can be scavenged by pathogens such as *E. coli* and *Salmonella typhimurium* early during infection [69–71]. Some intestinal microbes utilize fucose to synthesize capsular polysaccharides [72]. In addition, the H antigen created by FUT2 has been shown to serve as an attachment site by pathogens such as norovirus and *Campylobacter jejuni* [73,74]. Fucosylation is a microbiota-dependent process. In germ-free mice, fucosylation occurs immediately after birth but is lost over time [75]. Fucosylation was restored by conventionalization or colonization with *B. thetaiotaomicron*, which expresses an unidentified product in response to low fucose levels that induces fucosylation [76]. Germ-free FUT2−/− mice colonized with human feces had increased abundance of *Parabacteroides*, *Eubacterium*, *Parasutterella*, *Bacteroides*, and Lachnospiraceae and decreased abundance of an unclassified Clostridiales genus in their feces [77]. Interestingly, the microbiome of FUT2−/− mice fed a polysaccharide-deficient diet was unchanged from controls, demonstrating the interplay that exists between diet and host gardening. In humans, deficiency of FUT2 – which occurs in around 20% of individuals, termed “non-secretors – was associated with shifts in microbial composition in the colonic mucosa (increased *Coprococcus* and an unclassified Lachnospiraceae), feces (reduced *Bifidobacteria*), and bile (reduced Proteobacteria) [78–80]. The altered intestinal microbiome in non-secretors is suspected to account for their increased risk for inflammatory/autoimmune diseases including Crohn’s disease, primary sclerosing cholangitis, psoriasis, and type 1 diabetes [80–83].

FUT2 is not the only reported example of non-immune genetic gardening. Sialyltransferase is an enzyme that synthesizes sialyllactose in milk, which may act as a nutrient source for some microbes and affect bacterial adhesion. In its absence, knockout and WT co-fostered mice developed an altered microbiome characterized by decreased susceptibility to DSS colitis [84]. Apolipoprotein A1 is a component of high-density lipoprotein. Knockout mice have features of metabolic syndrome, in particular impaired glucose tolerance, and alterations in microbial composition [85]. In addition, other glycosylation enzymes besides FUT2 involved in mucus formation may also affect microbial composition. For instance, deficiency of enzymes necessary for the synthesis of core 1, core 2, and core 3 O-glycans present in mucus have been reported to increase DSS colitis susceptibility or induce a microbiota-dependent spontaneous colitis [86–88]. It is not yet known if these phenotypes were related to alterations in microbial composition, but this is a plausible mechanism.

Innate lymphoid cells (ILCs)

In recent years, groups of cells have been characterized that resemble subsets of T cells but lack a rearranged antigen receptor. These innate lymphoid cells include natural killer (NK) cells, GATA3-dependent cells with a Th2-like cytokine profile, and RORγt-dependent cells

[89]. RORγt-dependent ILCs are the primary intestinal source of IL-22, a cytokine induced by IL-23 which is critical for epithelial production of antimicrobial proteins including RegIIIβ, RegIIIγ, S100A8, and S100A9 [90–92]. Mice lacking ILCs have minimal RegIIIγ expression by the intestinal epithelium [90,93]. IL-22 production appears to be regulated by the microbiota in a manner that depends on the route of exposure to microbial ligands. Colonization of germ-free mice reduced IL-22 production by ILCs, possibly via induction of epithelial IL-25 by microbial products [90]. In contrast, systemic administration of a bacterial product, flagellin, increased IL-22 production by triggering IL-23 release from CD103+ DCs via TLR5 [94].

The ability of ILCs to regulate antimicrobial production by epithelial cells has made them an attractive candidate gardening cell type in the mucosa. Indeed, antibody-mediated depletion of ILCs and anti-IL-22 treatment resulted in increased SFB levels in the feces [95]. It has not yet been reported whether ILCs influence the composition of the remainder of the microbiome in the intestinal lumen. ILC depletion and anti-IL-22 treatment has also been found to permit systemic dissemination of *Alcaligenes xylosoxidians*, an invasive Proteobacteria species that normally is contained to mouse Peyer's patches and mesenteric lymph nodes [92]. This suggests that in addition to gardening luminal SFB, possibly via regulation of IL-22, ILCs also guard against invasive species that have penetrated the epithelial barrier.

A subset of RORγt-dependent ILCs has recently been described that expresses T-bet, lacks CCR6 expression, and differentiates into NKp46+ RORγt-dependent ILCs [96]. These cells are reduced in number in germ-free mice. A similar reduction is seen in MyD88−/−TRIF−/− mice, suggesting that TLR signaling controls this population. IFNγ-producing T-bet+CCR6- RORγt-dependent ILCs played a critical role in the intestinal response to *Salmonella* and may also be involved in microbial gardening. This ILC subset is absent in Rag−/−Tbet−/− mice, which develop an altered microbiota causing transmissible colitis [97,98]. These mice had differential abundance of 69 phylotypes in feces by 16S sequencing and increased abundance of *Klebsiella pneumoniae* and *Proteus mirabilis* (both Proteobacteria) by culture [99]. It was subsequently discovered that the colitis phenotype was attributable to colonization with *Helicobacter typhlonius* [98]. This invasive Proteobacteria triggered a response by an IL-17-producing CCR6+ subset of RORγt-dependent ILCs, previously shown to mediate colitis induced by *Helicobacter hepaticus* [98,100]. Of note, the emergence of a colitogenic microbiota in Rag−/−Tbet−/− mice was initially attributed to increased production of TNFα by dendritic cells (DCs) although it now appears to be most likely due to a deficit in T-bet-dependent ILCs [97]. Current evidence argues against a role for DCs in gardening, as mice deficient in the two CD103+ lamina propria DC populations had no alteration in intestinal microbiota [98]. However, the CD103-CD11b+ DC subset which expresses TNFα in Rag−/−Tbet−/− mice has not been studied [101].

Adaptive immune cells

B cells

Evidence for a role of the adaptive immune system in microbial gardening has come largely from studies of B cells. Intestinal B cells are present in the lamina propria, isolated lymphoid

follicles (ILF), and Peyer's patches (PP). They develop into IgA-producing plasma cells after undergoing class switching and affinity maturation in a T-cell-dependent manner in PP germinal centers or a T-cell-independent manner in ILFs and the lamina propria [102,103]. This process requires activation-induced cytidine deaminase (AID) and microbial triggers. Germ-free mice have few IgA-secreting intestinal B cells despite normal numbers of B cells [104]. IgA is rapidly induced upon bacterial colonization and persists even after disappearance of bacteria from the gut, suggesting that IgA-secreting B cells are imprinted by microbial exposure rather than requiring ongoing stimulation [105].

Mice deficient in all B cells were found to have alterations in the jejunal microbiome including increased *Paracoccus*, increased *Lactococcus*, and decreased Clostridiaceae [106]. The absence of non-IgM antibodies and somatic hypermutation due to AID deficiency caused increased culturable anaerobes and SFB in the small intestine [107,108]. This could be reversed by surgically connecting the circulatory system of AID knockouts to wild-type mice, indicating that serum factors (presumably immunoglobins) mediated the phenotype [108]. Similar microbial changes were observed in Rag−/− mice (i.e. T and B cell deficient), which could be reversed by bone marrow transplantation from WT but not AID−/− mice. The importance of somatic hypermutation in IgA gardening was recently shown using AID knock-in mice carrying a mutation that allowed for normal class switching but disrupted somatic hypermutation [109]. These mice had alterations in their small intestinal microbiome characterized by increased culturable anaerobes and increased abundance of *Lachnospiraceae*, Bacteroidales, and *Lactobacillus*. Recently, a population of small intestinal IgA-producing plasma cells was discovered that expresses inducible nitric oxide synthase (iNOS) and TNFα in a microbiota-dependent manner [110]. Absence of expression of both iNOS and TNFα in B cells resulted in deficiency of IgA-producing cells in the lamina propria. Interestingly, this was accompanied by reduced small intestinal SFB, suggesting that B cells can garden in a TNFα/iNOS manner that favors SFB whereas IgA gardens in a manner that limits SFB.

T cells

T cells, in particular T follicular helper (T_{FH}) cells, have been implicated in microbial gardening through their regulation of B cell class switching and affinity maturation in PP germinal centers [111]. T_{FH} cells have characteristically high expression of the inhibitory receptor, programmed cell death-1 (PD1). In the absence of PD1, mice develop antibody-mediated arthritis, glomerulonephritis, and cardiomyopathy [112,113]. PD1−/− mice also have an altered microbiome, including increased culturable anaerobic bacteria in the small intestine and increased Erysipelotrichaceae, Prevotellaceae, Alcaligenaceae, and TM7 in the cecum [114]. This was attributed to poor affinity maturation of the IgA repertoire due to aberrant B cell selection in PP germinal centers [115]. Possible additional evidence for T cell regulation of B-cell-mediated gardening comes from a study of mice lacking TGFβ-induced FoxP3-expressing regulatory T cells due to CNS1 deficiency. These mice had increased numbers of germinal center B cells and produced autoantibodies reacting against intestinal antigens [116]. They developed a B-cell-mediated enteritis characterized by plasma cell expansion and Th2 cytokine expression. These mice had increased TM7 and *Alistipes* in their feces. However, it is unclear if this was due to altered gardening (perhaps

secondary to heightened B cell activity) or was a secondary consequence of intestinal inflammation.

CD1d-restricted T cells

Natural killer T (NKT) cells are a class of T cells that express NK markers and respond to lipid antigens presented by CD1d as well as to cytokine stimulation by IL-12, IL-18, and type 1 interferons [117]. They have potent effector activity through their release of cytokines including IFNγ, IL-4, IL-17, and TNFα. A subset of these cells, termed invariant NKT (iNKT) cells, has a restricted TCR repertoire and has been shown to be strongly regulated by microbial signals. In germ-free mice, iNKTs are increased in the colonic lamina propria in a CXCL16 dependent manner but decreased in the liver and spleen, which could be reversed by colonization with *Sphingomonas* (which expresses ligands recognized by iNKT cells) [118–120]. iNKTs respond to a wide range of pathogens and may also be involved in gardening [121]. One study of CD1d−/− mice observed alterations in the fecal microbiota, increased ileal SFB, and increased small intestinal colonization by pathogens including *E. coli* and *Pseudomonas aeruginosa* [122]. NKTs may be involved in gardening downstream of NLRP6 and IL-18, but this has not yet been evaluated.

γδ intraepithelial lymphocytes (IELs)

A diverse collection of T cells reside within the intestinal epithelium, of which about 60% express TCRγδ. This IEL subset secretes growth factors that promote epithelial growth/repair and can combat enteric pathogens through IFNγ production and direct cytotoxic activity [123]. After epithelial injury, γδ IELs prevent mucosal penetration of bacteria by secreting growth factors, chemokines, and antimicrobial products in a partially MyD88-dependent manner [124]. γδ IELs may also play a role in gardening invasive pathobionts in the absence of epithelial injury. Colonization of germ-free mice induces expression of antimicrobials by γδ IELs including RegIIIγ and RegIIIβ [125]. This could be reproduced by colonization with a single endogenous mucosa-invasive *E. coli* species or wild-type *Salmonella* (but not a non-invasive mutant), suggesting that γδ T cells respond to microbes that penetrate the mucus barrier. Absence of γδ T cells resulted in an inability to control new invasive species early after exposure. WT mice co-housed with TCRγδ−/− mice showed increased RegIIIγ expression by γδ T IELs, suggesting that these mice were exposed to mucosa invasive bacteria which arose in TCRγδ−/− mice due to deficient gardening. However, it has not yet been directly demonstrated to our knowledge that loss of γδ T cells alters the composition of the microbiome.

Inter-individual variation in microbial composition due to genetic variation in gardening

The existence of multiple mechanisms for gardening the intestinal microbiome raises the possibility that variation in gardening genes can contribute to microbial diversity within populations. This has been supported by multiple mouse studies. Comparison of 8 inbred strains revealed that each had a distinct microbial composition which was not eliminated by cohabitation, though founder effects could not be excluded [126]. Two quantitative trait loci (QTL) analyses of interbred mouse lines have identified multiple genetic factors influencing

the abundance of microbes at various taxonomic levels [127,128]. The first study identified thirteen QTLs affecting microbial composition, including one mapping to a region containing IL-22, IFNγ, and Irak3 (an inhibitor of TLR signaling) that affected *Lactococcus* and Coriobacteriaceae abundance [127]. The second study identified five QTLs, of which three contained candidate genes affecting immune activity (consistent with the immune system playing a major role in gardening) [128]. This included type 1 interferons, Irak4 (a signaling molecule utilized by some TLRs), and Tgfb3, which influenced the abundance of *Bacteroides*, Rikenellaceae, and Prevotellaceae, respectively. Host genetics can also interact with diet, as was shown in a study reporting wide variation among 52 strains of mice in the effect of a high-fat, high-sucrose diet on the microbiome [129]. The authors identified a single nucleotide polymorphism (SNP) near 3 amylase genes which was associated with enrichment of *Enterobacteriaceae* on a high fat, high-sucrose diet.

In humans, twin studies have been used to evaluate the global impact of genetic variation on microbial composition. The intestinal microbiome has consistently been shown to be more similar between pairs of monozygotic twins than between pairs of unrelated individuals [130–134]. However, monozygotic twins share many environmental factors including maternal colonizers, early life environment, and childhood diet. This can be controlled by comparing monozygotic twins to dizygotic twins. Significantly increased microbial similarity between monozygotic twins compared to dizygotic twins has been reported in two studies using the Sorensen index to analyze data from 16S Sanger sequencing and temporal temperature gradient gel electrophoresis [130,133]. In contrast, two studies using high throughput 16S sequencing and a phylogenetic similarity measure, Unifrac, reported no difference in microbial similarity between monozygotic twins compared to dizygotic twins [131,132]. However, even if this is correct, the global similarity analysis used in these studies may not reveal shifts in the abundance of specific microbial groups due to genetic variation.

This review has already mentioned two genes, FUT2 and NOD2, with genetic variants that affect microbial composition [34,78–80]. Recently, a microbiome association study using 30 Crohn's associated SNPs identified a genetic variant, the rs11747270a SNP, that correlates with *Prevotella* abundance in sigmoid colon biopsies [135]. This SNP maps to the locus for immunity-related GTPase family M (IRGM) [136]. This protein regulates autophagy, a process triggered by PRRs such as NOD2 that is important for clearance of intracellular bacteria including *Salmonella*, *Mycobacterium*, and invasive *Escherichia coli* [137]. A second Crohn's associated IRGM polymorphism has been reported to reduce the effectiveness of autophagy in response to intracellular bacteria [138]. Interestingly, *Prevotella* species including *P. intermedia* and *P. bivia* have been reported to invade epithelial cell lines [139,140]. It is conceivable that the elevated abundance of *Prevotella* in colonic biopsies was due to increased survival of invasive organisms due to deficient IRGM activity. As further genome wide association studies are performed for microbial composition, it is likely that many additional examples of genetic polymorphism in gardening genes will be identified in human populations.

Acquired immune deficits can influence the microbiota

The critical role of mucosal immunity in shaping the intestinal microbiome suggests that not only genetic but also acquired deficits in immune function could affect the microbiota. A common cause of acquired immunodeficiency in humans in recent decades has been HIV. It is now appreciated that HIV transmission, replication, and persistence occur largely in mucosal tissues [141]. Rapid depletion of mucosal memory CD4+ T cells occurs during acute infection and is sustained throughout the chronic phase of disease [142]. This is accompanied by a decrease in the ratio of Th17:Tregs, increased CD8+ T cells, and decreased NK cells. As a consequence of these immune disturbances, HIV patients have increased epithelial permeability, systemic microbial translocation, and elevated serum inflammatory markers consistent with a chronic inflammatory state [143]. In most patients, these mucosal immune disturbances are not reversed completely by highly active anti-retroviral therapy [144]. In light of changes in mucosal immunity induced by HIV, it has been predicted that HIV infection would impact the intestinal microbiome. A longitudinal study of chimpanzees before and after SIV infection observed increased abundance of *Sarcina*, *Staphylococcus*, and *Selenomonas* in the feces [145]. Three recent papers evaluating the mucosal and fecal microbiome of untreated and treated HIV patients have confirmed altered microbial communities in HIV [146–148]. All three identified a reduction in Alistepes and two identified a reduction in Bacteroides. However, they each reported enrichment of different microbial groups in HIV patients. One identified enrichment of multiple members of the Proteobacteria phylum (including pathobionts such as *Pseudomonas*), another *Prevotella*, and the third *Fusobacterium* (a genus which includes pathobionts that can invade epithelial cells) [149]. The discrepancies may reflect different sampling sites in each of the studies or stochastic overgrowth of pathobionts in the face of disrupted immune gardening. Acquired deficits in immune function can also be seen in patients treated with immunosuppressive medication to prevent rejection of solid organ transplants. While we are not aware of published reports on the intestinal microbiome of this population, at least one study has examined the oral microbiome. Transplant patients were found to have altered microbial composition including increased abundance of pathobionts such as *Klebsiella*, *Acenitobacter*, and *Pseudomonas* [150]. More broadly, medications that affect the immune system – such as immunomodulators and biologics (not including antibiotics) used to treat inflammatory bowel disease, rheumatic diseases, and psoriasis – could also potentially affect microbial gardening and result in disease-promoting dysbiosis. There is minimal data at present on this topic, though the existence of medication-specific microbial profiles was suggested in the oral microbiome study and one of the HIV studies [148,150].

Conclusion

There is now extensive evidence that genetically encoded mechanisms exist for gardening the intestinal microbiome. These pathways pair microbial sensing by PRRs to antimicrobial effector mechanisms such as defensins, RegIIIγ, and IgA (Figure 1). However, many questions remain. At present, the gardening activity of specific PRRs has been characterized in terms of shifts at the genus or higher taxonomic level in knockout mice. It is unknown what changes occur at the species or strain level, whether PRRs have overlapping activity,

and which microbial features trigger gardening pathways dependent upon a single PRR (e.g. NLRP6-mediated gardening of Prevotella, which was unaffected by loss of any other inflammasome-forming NLR) [38]. The effector mechanisms that allow for targeted gardening of specific microbes are also unknown (other than IgA), as host antimicrobial products generally have a wide spectrum of activity. While many immune cell types have been implicated in gardening, the relationships between each immune cell type and specific microbial taxa are largely uncharacterized with exceptions such as SFB gardening by B cells and NK T cells.

Despite the many unknowns, it is clear that gardening of the intestinal microbiome is critical for health. In many studies, disruption of gardening resulted in dysbiosis which conferred susceptibility to diseases including colitis, obesity, and fatty liver disease. This often corresponded to a bloom of pathobionts, especially Proteobacteria such as *Escherichia coli* in TLR5−/− mice, *Prevotella* in NLRP6−/− mice, *Helicobacter* in Rag−/−Tbet−/− mice, and *Alcaligenes* in ILC-deficient mice. The three human genetic variants associated with gardening are all also risk factors for Crohn's disease, possibly due to dysbiosis. Gardening can be affected not only by genetic variation but also by diet (as shown for tryptophan/defensins), stress (as shown for NLRP6), and acquired immune deficits (including, potentially, due to immunosuppresive medication). Disease-promoting dysbiosis arising from aberrant microbial gardening could prove to be a common theme in medicine.

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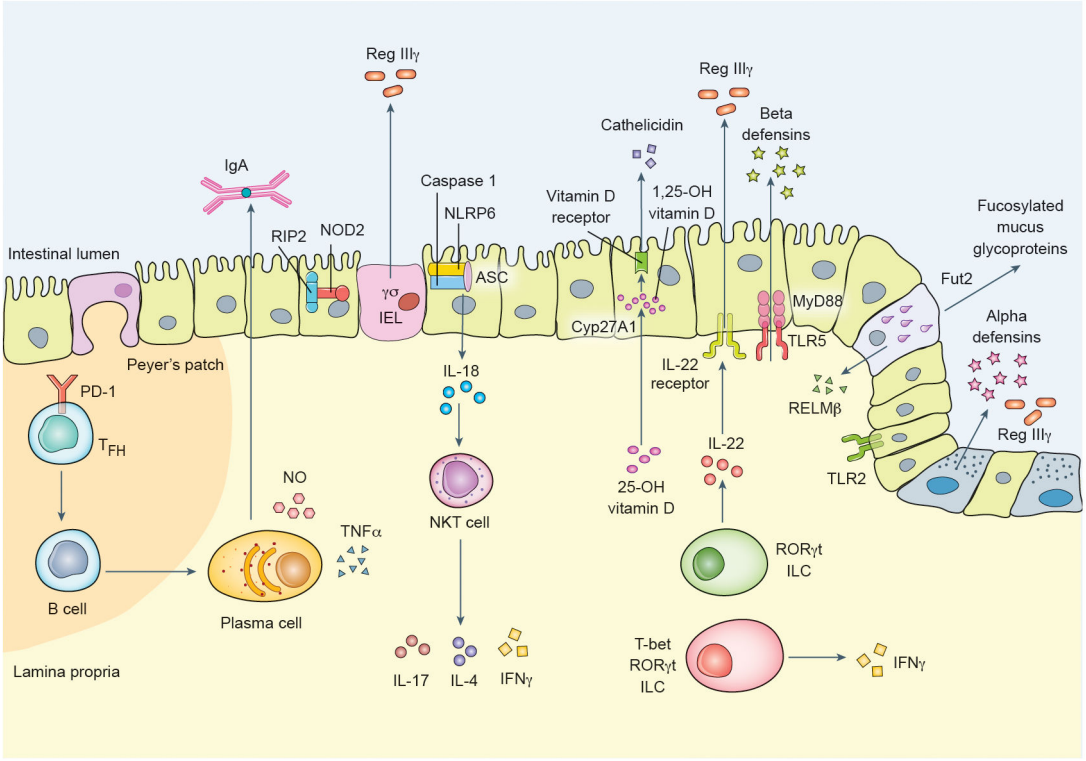

Figure 1.
Putative mechanisms for immune gardening of the intestinal microbiome.

Table 1

Genes that have been implicated in microbial gardening in mouse and/or human studies. Targets of microbial gardening are more abundant in KO mice except where noted.

Gardening gene	Putative microbial targets of gardening	Phenotype of Knockout Mice	Disease Association (for human genetic variants)
Pattern recognition receptors			
MyD88 [20]	Rikenellaceae, Porphyromonadaceae		
MyD88, epithelium [21]	TM7, *Lactobacillus* (D), *Klebsiella pneumonia* (I)	Exacerbated DSS colitis	
TLR5 [23,25]	*Escherichia coli* (I), Prevotellaceae (A), Lachnospiraceae (A), *Alistipes* (A), *Bacteroides* (A)	* Obesity, spontaneous colitis	
# TLR2 [26]	*Alistipes*, *Lactobacillus*, *Clostridium*, *Eubacterium*	Protection from diet-induced metabolic syndrome	
NOD2 [31–34]	Proteobacteria, Bacillus, Clostridium group IV	* Exacerbated DSS colitis	Crohn's disease, Blau syndrome
# RIP2 [31]		* Exacerbated DSS colitis	
# NLRP6 [38]	*Prevotellaceae*, TM7	* Exacerbated DSS colitis	
# ASC [38]	*Prevotellaceae*, TM7	* Exacerbated DSS colitis	
# Caspase-1 [38]	*Prevotellaceae*, TM7	* Exacerbated DSS colitis	
Epithelium			
# IL-18 [38]	*Prevotellaceae*, TM7	* Exacerbated DSS colitis	
Alpha defensins [46]	SFB, *Bacteroides* (D)		
ACE2 [48]	*Limibacter*, *Paludibacter*	* Exacerbated DSS colitis	
Vitamin D receptor [53]	Desulfovibrionaceae, Bacteroidaceae, Erysipelotrichaceae	Exacerbated DSS colitis	
Cyp27B1 [53]	Desulfovibrionaceae, Bacteroidaceae, Erysipelotrichaceae	Exacerbated DSS colitis	
RegIIIγ [22]	SFB, *Eubacterium rectale*	Increased intestinal Th1 cells and IgA production	
RELMβ [68]	Bacteriodetes (A), Firmicutes (A), Proteobacteria (A)	Resistance to diet- induced obesity	
FUT2 [77–80]	Various (see text)		Crohn's disease, primary sclerosing cholangitis, psoriasis, type 1 diabetes
Innate lymphoid cells			
IL-22 [92,95]	SFB, *Alcaligenes* (I)		
# T-bet, with Rag [98,99]	*Klebsiella pneumonia*, *Proteus mirabilis*, *Helicobacter typhlonius*	* Spontaneous colitis	
Adaptive immunity			
μMT (i.e. no B cells in KO) [106]	*Paracoccus*, *Lactococcus*, Clostridiaceae (D)	Lipid malabsorption	
AID [107–109]	SFB, culturable anaerobes	Susceptibility to *Y. enterocolitica*	

Gardening gene	**Putative microbial targets of gardening**	**Phenotype of Knockout Mice**	**Disease Association (for human genetic variants)**
iNOS and TNFα, B cells [110]	SFB (D)	Susceptibility to *C. rodentium*	
# PD1 [114]	Erysipelotrichaceae, Prevotellaceae, Alcaligenaceae, TM7	Arthritis, glomerulonephritis, cardiomyopathy	
CNS1 [116]	TM7, *Alistipes*	Enteritis, gastritis	
CD1d [122]	SFB, *E. coli*, *Pseudomonas aeruginosa*		
Other			
IRGM [135]	*Prevotella*		Crohn's disease
# Sialyltransferase [84]	Ruminococcaceae (D)	* Attenuated DSS colitis	
# Apolipoprotein A1 [85]	Rikenellaceae, Lachnospiraceae, Erysipelotrichaceae (D)	Impaired glucose tolerance, increased body fat	

D=depleted, I=mucosal invasion, A=altered abundance that was not further described (or phylotypes within the taxonomic group were both increased and decreased).

#Denotes genes implicated in gardening by knockout mouse studies that did not use littermate controls from heterozygote breeding.

*Denotes phenotypes that can be attributed to the altered intestinal microbiota based upon co-housing experiments or reconstitution of germ-free mice.

SFB=segmented filamentous bacteria