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Effects of Intestinal Fungi and Viruses on Immune Responses and Inflammatory Bowel Diseases

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Abstract

The intestinal microbiota comprises diverse fungal and viral components, in addition to bacteria. These microbes interact with the immune system and affect human physiology. Advances in metagenomics have associated inflammatory and autoimmune diseases with alterations in fungal and viral species in the gut. Studies of animal models have found that commensal fungi and viruses can activate host-protective immune pathways related to epithelial barrier integrity, but can also induce reactions that contribute to events associated with inflammatory bowel disease. Changes in our environment associated with modernization and the COVID-19 pandemic have exposed humans to new fungi and viruses, with unknown consequences. We review the lessons

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learned from studies of animal viruses and fungi commonly detected in the human gut and how these might affect health and intestinal disease.

Keywords

mycobiome; virome; Microbiome; virus; fungi; mucosal immunity; inflammatory bowel disease

The intestinal microbiota contains bacteria, archaea, fungi, protozoans, and viruses. Among these, bacteria have received the most attention. The human intestine contains hundreds of bacterial species, reaching a density of 10^{12} cells per gram of intestinal content. Disruptions to microbial communities (dysbiosis) have been associated with diseases, and strategies to restore a composition associated with health are being developed as therapies. As demonstrated in mice, intestinal colonization by individual and groups of bacteria affect physiology via production of metabolites and/or direct effects on the immune system. However, bacteria are not the only microbes in the gut, and are not sufficient to mediate all the functions attributed to the intestinal microbiota.

We review viruses and fungi of the gut microbiota and their effects on the immune system (Figure 1). Increasing evidence suggest that viruses and fungi regulate physiology via multiple interactions with host cells during symbiosis with the human gastrointestinal tract, despite their relative low abundance compared with bacteria. Studies of the total genetic content of stool specimens have found that viral and fungal genomes each account for 1% or less of the total. Nevertheless, the absolute number of fungi and viruses are much more impressive (Figure 1). Each gram of stool contains 10^8–10^9 virus-like particles (VLPs), most of which are from phages that infect bacteria[1]. Phages shape the functions and composition of bacteria by lysing them or integrating into their genomes. Only few examples in the current literature demonstrate phages directly interacting with immune cells[2,3], thus this review will focus on viruses that infect animal cells.

The gastrointestinal tract within the first few months after birth is exposed to animal RNA and DNA viruses from at least 16 families, including anelloviruses, picornaviruses (a large family that includes enteroviruses and coxsackieviruses), caliciviruses (noroviruses and sapoviruses), parvoviruses, adenoviruses, astroviruses, circoviruses, polyomaviruses, and papillomaviruses[4,5]. Although the degree to which these viruses establish long-term infections and affect human health is an active area of investigation, experiments in animal models indicate these viruses are not silent passengers. Shotgun sequencing studies of VLPs (to enrich for viruses) in feces have shown that individuals with inflammatory bowel diseases (IBD), celiac disease, colorectal cancer, or enteric graft vs host disease have alterations in viromes associated with increased phage diversity and the presence of certain animal viruses[6–11]. One study identified a new family of eukaryotic small circular DNA viruses of oro-respiratory viromes linked with periodontal and respiratory disease severity[12]. However, methodology hurdles have made it difficult to define a normal virome in a way that is quantitatively rigorous and inclusive of all RNA and DNA animal viruses. HIV-positive individuals with low $CD4^+$ T-cell counts have increases in adenoviruses and

anelloviruses in fecal samples[13]; it is likely that the immune system normally suppresses these viruses to levels below the threshold of detection by metagenomics.

Like bacteria and viruses, fungi are members of the normal microbiota. Metagenomic studies have found fungi to constitute 0.01%–0.1% of the human gut microbiome[1415]. There has been no accurate estimate of the fungal biomass in the gastrointestinal tract—such an endeavor would be confounded by differences in genome sizes, the lack of many annotated genomes, and different sizes of fungal cells. Exposure to fungal commensals, pathobionts, or environmental and food-associated species has effects on the immune system that should be studied, regardless of the absolute numbers of fungi in the gut or their degree of stable colonization[16,17].

A systematic review of gut mycobiota studies found only 15 of the 267 identified species were detected in multiple studies[18]. Only 13 of those species, belonging to *Candida, Saccharomyces, Aspergillus, Cryptococcus, Malassezia, Cladosporium, Galactomyces,* and *Trichosporon*, can grow at 37°C and thereby potentially inhabit the gastrointestinal tract[18,19]. Studies that aimed to broadly define the human intestinal mycobiome analyzed fecal samples from 317 healthy donors from the Human Microbiome Project[20] or 98 healthy volunteers[21]. Both studies found a high prevalence of *Candida* and *Saccharomyces*, followed by *Malassezia* or *Cladosporium*[14,21]. In addition to these most prevalent taxa, the studies reported another 177 and 63 fungal genera, respectively, that could be environmental and dietary transients[22]. These findings indicate that healthy humans might have a core mycobiome, with a highly diverse and variable remainder of less-represented fungi.

Effects of viruses on the immune response

When disease is absent, the presence of eukaryotic viruses in humans is frequently described as an asymptotic infection. However, viruses are intracellular parasites, so active viral replication can affect the immune system. Experiments with murine norovirus (MNV) indicate that enteric viruses can engage the immune system without causing diarrhea, with outcomes that are beneficial or adverse, depending on the circumstances. MNV is a positive-strand RNA virus related to human noroviruses and was discovered in an animal facility in 2003 as a transmissible virus that killed immunocompromised mice[23]. Related strains of MNV, many of which establish robust persistent infection without causing overt disease, were subsequently identified in a multitude of institutions and vendors worldwide. Observations made in microbiota-deficient mice support the concept that MNV is a viral member of the gut microbiota. Inoculating germ-free mice or mice that have been given antibiotics with a persistent strain of MNV reverses many of the intestinal defects observed in mice without microbiota, including aberrant morphology of the small intestinal villi, reduced numbers of local T cells, and susceptibility to chemical injury by dextran sodium sulfate (DSS)[24].

The effects of intestinal viral infections are frequently mediated by interferons. Type I interferons (IFNs), such as IFNAs and IFNB, and type III IFNs, such as IFNLs, are produced in response to viral nucleic acids. Although best known as cytokines that induce expression of antiviral genes, IFNs have functions other than reducing viral burden. As an

example, production of type I IFNs in response to chronic infection by the herpesvirus murine cytomegalovirus (CMV) induces production of APOL9 by macrophages, which stimulates epithelial proliferation and wound repair in multiple organs, including the colon[25]. In vulnerable humans, CMV in the gut is associated with colitis. CMV infection of blood monocytes that are precursors of gut macrophages leads to upregulation of SMAD7 and makes these cells resistant to TGFB signaling, promoting inflammation and differentiation[26]. It is possible that the IFN response is necessary to counteract such an inflammatory response for viruses that establish symbiotic relationships. Consistent with this possibility, the ability of MNV to compensate for the absence of intestinal bacteria is mediated by interleukin 22 (IL22)-producing innate lymphoid cells (ILCs), which are indirectly activated by type I IFN to protect epithelial cells[27].

Much of our knowledge about how viral nucleic acid is sensed through pattern recognition receptors in the intestine comes from studies of noroviruses and rotaviruses as pathogens rather than commensals. Rotaviruses are double-stranded RNA viruses that were a leading cause of viral gastroenteritis and death in children until the introduction of the vaccine; although rotaviruses remain a major health concern, noroviruses have become the main threat in many regions of the world. Virus RNA activates toll-like receptor 3 (TLR3) and TLR7 signaling via MYD88 and TRIF, or RIG-I and MDA5, which interact with MAVS, to induce production of IFNs by infected cells[28,29]. However, rotaviruses and noroviruses encode virulence factors that block IFN signaling[30–32]. IFN-independent effector mechanisms potentially compensate, such as the NOD-like receptor (NLR) NLRP9b, which induces death of epithelial cells to prevent rotaviruses from completing replication[33]. Other cytokines can mediate mutually beneficial outcomes for viruses and their hosts. IL1A recruits monocytes and neutrophils that serve as new targets for propagation of the virus[34]. IL22 is produced as a consequence of a complex immune response involving multiple cell types coordinated by MAVS and chemotactic monocytes during MNV infection, which protects intestinal epithelial cells from intestinal injury[27]. Further studies of these innate immune pathways are needed to elucidate the symbiotic effects of intestinal viruses.

The mechanisms that inform vaccination strategy and whether it is possible to launch an effective adaptive immune response against enteric viruses is a subject of intense research (for a review, see[35]). Norovirus infections can persist and cause serious illness in immunocompromised individuals, but less is known about how prolonged shedding occurs in asymptomatic people with presumably intact adaptive immune responses[36]. In contrast to a strain that establishes an acute infection in myeloid cells, persistent strains of MNV infect intestinal epithelial tuft cells and evoke weak responses by $CD8^+$ T cells[37,38]. Studies of the encephalomyocarditis virus have found that NLRP6 and its binding partner DHX15 are intestinal epithelium-specific sensors of RNA that might also detect noroviruses[39,40]. It will be important to elucidate how cell tropism, such as infection of goblet cells by enteroviruses, astroviruses, or adenoviruses[41–43], affects innate and adaptive immune responses in the gut and long-term outcomes of colonization.

Small DNA viruses, such as adenoviruses, circoviruses, and anelloviruses, are ubiquitous in the gut[4,5,44]. It will be important to determine how DNA-sensing pathways downstream of TLR9 and cGAS and STING mediate the effects of the gut virome. The finding that α-

defensins produced by Paneth cells, which are secretory epithelial cells in the small intestine with bactericidal activity, promote responses of neutralizing antibodies against 1 strain of mouse adenovirus while promoting infectivity of another strain indicates that we have much to learn about the interactions between the intestinal barrier and these ubiquitous viruses[45,46].

Effects of fungi on the immune response

Gut fungi, their metabolites and toxins, and their effects on bacterial populations can directly or indirectly influence host immunity[16,17]. Pattern recognition receptors interact with carbohydrates on the surface of the fungal cell wall (Figure 2). C-type lectin receptors (CLRs) are mainly involved, but TLRs are considered to have secondary or additive effects. CLRs such as dectin 1, dectin 2, dectin 3, mincle, and the mannose receptor, recognize fungal cell wall components including β-glucan, mannans, and mannose-associated complexes. With the exception of the mannose receptor and MelLec, these CLRs signal via spleen associated tyrosine kinase (SYK), a CARD9–BCL10–MALT1 complex, and/or RAF1[47]. Activation of SYK signaling requires an immunoreceptor tyrosine-based activation motif (ITAM) or ITAM-like motif within the receptor tail (dectin 1) or an associated FcRγ adapter molecule (dectin 1, dectin 2, dectin 3, mincle).

Dectin1 binds fungi in the intestines[48]. Mice lacking this receptor or dectin 3 develop more severe colitis following administration of DSS than wild-type mice, accompanied by expansion of opportunistic fungal genera such as *Candida* and *Trichosporon*; the antifungal drug fluconazole reduces the severity of colitis[48,49]. Dectin 1 deficiency in humans is associated with increased colonization of the gut by *Candida* and graft vs host disease following bone marrow transplantation[50,51]. Experiments with multi-CLR knockout mice revealed combined functions of dectin 1, dectin 2, and mincle in the immune response to *Candida albicans*, compared with any single receptor[52]. Activated dectin 3 induces the expression of mincle or forms heterodimers with dectin 2, which signals through FcRγ[53]. Mincle, alternatively, can affect dectin 1-mediated production of IL-12 by promoting degradation of interferon regulatory factor 1 (IRF1) via PI3K, AKT, and E3 ubiquitin ligase activation[54]. The E3 ubiquitin ligase CBLB mediates degradation of dectin 1 and dectin 2 to serve as a negative regulator of antifungal immunity[55,56]. There are therefore synergistic and antagonistic interactions among CLRs that regulate the antifungal immune response in the gut.

CLR signaling pathways are not redundant, but all include CARD9, based on studies of *Card9*$^{-/-}$ mice[47]. Gut fungal and bacterial communities are altered in *Card9*$^{-/-}$ mice, with notable loss of *Lactobacillus* spp[57]. Tryptophan-metabolizing bacteria, including lactobacilli, produce ligands for the aryl hydrocarbon receptor, which stimulate release of IL22 by group 3 ILCs and T-helper (Th) cells[57,58]. These abnormalities in the intestines of CARD9-deficient mice partially explain the decreased levels of aryl hydrocarbon receptor ligands, reduced expression of IL22, REG3G, and REG3B in colon. These phenotypes are partially rescued by supplementation with 3 strains of Lactobacilli[57]. CARD9 deficiency also affects immune modulation by gut fungi, which can affect colon cancer development[59–61].

Inflammasomes are cytosolic oligomers that mediate processing of IL1B and are part of the mucosal immune response to fungal infections. NLRP3 and NLRC4 inflammasomes protect mice from vaginal *Candida* infection via activation of IL1B by caspase 1[62,63]. Detection by NLRP3 seems to depend on morphologic features of the fungi (yeast vs hyphae)—NLRP3 is activated by *Candida* hyphae and hyphae-produced secretory molecules[64,65].

CLRs are expressed by and active in phagocytic cells, including macrophages, dendritic cells (DCs), and neutrophils. Among these, a population of mononuclear phagocytes (MNPs), marked by the fractalkine receptor CX3CR1, expresses the highest levels of dectin 1, dectin 2, and mincle[66] (Figure 2). $CX3CR1^+$ MNPs phagocytose fungal species in the intestines, and then activate T cells.[66,67]. Intestinal colonization with *Candida* induces T-cell production of IL17 and IL22 and these Th17 cells control of specific commensal bacteria and fungi in mice and humans[47,66,68–71]. Activation of Th17 cells by $CX3CR1^+$MNPs in response to mycobiota might help maintain intestinal homeostasis.

In addition to direct interaction with phagocytic cells, fungi alter phagocyte function via their secreted products. *C albicans* hyphae secrete the toxin canadidalysin, which induces NLRP3 inflammasome-dependent IL1B maturation via K^+ efflux in macrophages and DCs[65]. Furthermore, *Candida* secrete aspartic proteases such as SAP3 and SAP6, which activate CLR-independent responses by monocytes, macrophages, and DCs via induction of K^+ efflux and reactive oxygen species[72]. Fungal toxins and other soluble factors are absorbed through extension of “balloon-like” protrusions by a population of $CX3CR1^+$ macrophages in the distal colon that protect the epithelium[73].

Receptors expressed on cells outside of the immune system, such as ERBB2, EGFR, EPHA2, FIBCD1, and MelLec contribute to the antifungal immune response at mucosal surfaces, with less clear roles in the gut (Figure 2)[74–78]. In addition to receptor-mediated antifungal responses, *C albicans* can directly damage of oral and vaginal epithelium via the toxin candidalysin, resulting in production of inflammatory cytokines[79,80]. Candidalysin has been reported to damage human epithelial colorectal adenocarcinoma cells[81], justifying further exploration of the effects of this toxin in the gut.

Balancing the viral and fungal communities

In the absence of an underlying condition or genetic deficiency, intestinal colonization by viruses and fungi might have immune-protective effects. Although it remains to be determined whether a healthy or normal virome exists, recognition of viral nucleic acid clearly has homeostatic effects that promote intestinal barrier function (Figure 3). Mimicking viral infection of the epithelium through administration of a RIG-I ligand offsets the intestinal barrier damage caused by radiation and chemotherapy[20], consistent with a previous finding showing that MAVS signaling protects against DSS-mediated colitis[82]. Based on in vitro experiments, RIG-I recognition of RNA from the bacterial microbiota was originally suggested to protect against DSS[82]. However, providing mice with a cocktail of antivirals increases susceptibility to DSS[83,84]. According to one model, enteric viruses are essential for inducing IL15 downstream of RIG-I and MAVS, a cytokine necessary to maintain intra-epithelial lymphocytes with tissue regenerative properties[83] (Figure 3).

TLR3 and TLR7 agonists that mimic viral double-stranded and single-stranded RNA, respectively, induce production of type I IFNs by colonic plasmacytoid DCs (pDCs), which also protects against DSS[84]. This same study demonstrated a similar protective effect of inactivated rotavirus, and that *TLR3* and *TLR7* gene variants are associated with IBD susceptibility in humans. Also, a TLR7 agonist recreates the beneficial effect of MNV infection by enhancing resistance against vancomycin-resistant *Enterococcus* through IL22 producing ILCs[85] (Figure 3). IFNL has an anti-inflammatory effect in the gut by inhibiting reactive oxygen species production and degranulation by neutrophils[86]. In this study, an antiviral cocktail was used to implicate viral members of the microbiota in IFNL production (Figure 3). As with the other studies that rely on antivirals, it will be important to rule out non-specific effects of the drugs by identifying specific viruses that serve these functions. Astroviruses require consideration. In a recent study, immunocompromised mice were found to be surprisingly resistant to MNV and rotavirus infections, which was attributed to presence of a murine astrovirus strain that provides cross-protection against other viruses through production of IFNL in the gut[87] (Figure 3). Murine astrovirus can also protect neonates from enteropathogenic *E. coli* colonization[43]. The oral polio vaccine (an attenuated strain of poliovirus) reduces the incidence of diarrhea caused by rotavirus and other agents[88], indicating that similar interactions between viruses occur in human gut. So, much like the gut bacterial microbiota increases resistance to colonization by bacterial pathogens, the gut virome promotes resistance against exogenous viral infections.

Virus-induced production of IL22 by ILCs might be important for intestinal barrier function in the presence of pathogenic bacteria. Persistent and non-persistent strains of MNV protect mice given antibiotics from induction of diarrhea by *Citrobacter rodentium* and vancomycin-resistant *Enterococcus*[24,85] (Figure 3). MNV-induced IL22 led to the survival of newly weaned mice, which otherwise died during *C rodentium* infection. Young children have multiple viral infections, which might help them from infections with pathogenic bacteria 27. MNV also increases resistance to lung infection by *Pseudomonas aeruginosa*, although it is not clear how an enteric virus affects other organs[89].

Anti-fungal agents promote expansion of filamentous fungi such as *Wallemia mellicola*, *Aspergillus amstelodami*, and *Epicoccum nigrum*, which aggravate intestinal inflammation and allergic airway disease[61,67,90,91]. The systemic effects of intestinal fungi are not known. Depletion of CX3CR1^{+} MNPs from the gut but not the lung revealed that these cells detect fungal dysbiosis and increase numbers of Th2 cells and eosinophils to promote lung allergic inflammation[67]. Furthermore, mono-colonization with *C albicans* or *S cerevisiae*, which are recognized by CX3CR1^{+} MNPs[66], supports the establishment of intestinal homeostasis and protects against virus-induced lung inflammation and DSS-induced gut barrier damage[92]. However, administration of a single antibiotic did not protect mice with intestinal *C albicans*[70]. Similarly *S cerevisiae* was not protective (and even detrimental) in SPF mice[93], indicating the pre-existing gut microbiota composition influences intestinal inflammation or protective immunity by intestinal fungi.

Gut-adapted *C albicans* protected mice from systemic challenge with fungal and bacterial pathogens, including *C albicans* itself[94]. This innate immune memory response[95,96] was of short duration, required IL6, and was observed in lymphocyte-deficient mice[94]. However, in

the same mice, a *C albicans* isolate that had not been adapted to the gut protected against subsequent infections with *C albicans* or *Staphylococcus aureus*, by inducing a Th17 cell-mediated responses[70]. These findings indicate that *C albicans* can induce innate and adaptive immune memory responses, possibly to specific strains of fungi. Altogether, these studies have shown that a balanced gut mycobiota contributes to immune homeostasis, and that intestinal colonization with specific fungi can induce immune memory for specific enteric viruses and bacteria.

Laboratory mice are raised in artificial housing conditions that limit the diversity of microbes they encounter. Releasing laboratory mice into a confined outdoor enclosure that aims to replicate their natural environment increases the diversity and load of intestinal fungi, especially *Aspergillus* species[97,98]. This expansion of fungi was associated with increases in peripheral granulocytes, activated T cells, and other signs of immune maturation. Similar observations concerning the gut mycobiota were made in wild mice, which are also infected with viruses that are excluded from institutional vivaria[99,100]. Deficiencies in gut fungi or microbes, along with other variables relating to exposure to infectious agents[100], might account for the inability of the immune system of laboratory mice to fully recapitulate that of adult humans. Mouse colonies with natural mycobiomes or viromes can be maintained in a laboratory environment, allowing researchers to overcome some of these shortcomings[99]. Findings from studies in which intestinal fungi and viruses have been altered, or specific fungi and viruses have been introduced (such as *C albicans* and MNV), have revealed unexpected benefits to mice, such as improved resistance to barrier disruption by chemicals and pathogens as mentioned earlier.

IBD

IBD, colorectal cancer, and other gastrointestinal and hepatologic disorders have been associated with altered communities of fungi and viruses[6–11,16,17,101–105] . Most sequencing and experimental studies have focused on IBD, for which data from multiple cohorts and experimental models are available. IBD, including Crohn's disease (CD) and ulcerative colitis (UC), are associated with altered richness and diversity in the gut mycobiota[102,103,106,107]. *Candida* (mainly *C albicans)* are the predominant genera reproducibly identified in fecal or mucosal samples from IBD cohorts[18,101–105] (Table 1). *Candida* species can be intestinal symbionts or pathobionts, but the effects of other fungi are under investigation. These studies are complicated by their abundance in food sources, on skin, or in the surrounding environment[18,22,94,108]. There have been numerous studies of mycobiota in fecal or mucosal samples from patients with CD or mixed populations of patients with CD and UC, but only few of studies from patients with UC (Table 1).

Despite confounding factors related to methodology and technology, which have hindered direct comparisons among studies, *C albicans* and *C parapsilosis* appear to be consistently increased in fecal or mucosal samples from patients with CD, whereas changes in proportions of *C tropicalis*, *Saccharomyces,* and *Malassezia spp*. vary among cohorts[16,18,101−10549] (Figure 4A, B). *C albicans* is also expanded in fecal samples from patients with *C difficile* infection (CDI), compared to patients without CDI[138] (Figure 4C). Based on limited information, *S cerevisiae* appears to be reduced in feces of patients with

IBD—particularly those with active inflammation[102,109]. Oral administration of *S cerevisiae* to mice produced effects ranging from detrimental to neutral to protective during DSS-induced colitis[92,93,110].

Malassezia sequences were increased in intestinal washings from patients with CD; the presence of *M restricta* was associated with S12N mutation in CARD9, which increases risk for CD and UC[104]. Phagocytes from these patients had increased production of inflammatory cytokines upon fungal stimulation[111]. Alternatively, the S12NΔ11 truncation mutation in CARD9 disrupts its interaction with the ubiquitin ligase TRIM62; this prevents production of inflammatory cytokines by DCs exposed to ligands from fungi[112] (Figure 4A).

Most mechanistic studies have been performed with a limited set of model fungi strains. In contrast, researchers have made progress elucidating strain-specific properties of disease-associated bacterial species[113,114]. Strain-specific features of IBD-associated fungi might affect development of inflammation. Altogether these studies indicate that patients with IBD have changes in the intestinal mycobiota, and that *Candida* are consistently associated with an inflamed gut. The roles of specific strains of *Candida*, other fungal species, and their metabolites contribution to development of bowel inflammation requires further investigation.

Consistent with findings from studies of fecal mycobiota from patients with CD, blood samples from these patients have higher frequencies of *C albicans*-reactive T cells compared with healthy controls[71]. So intestinal *C albicans* might promote inflammation by inducing development of fungal antigen-reactive Th17 cells (Figure 4A). However, patients with CD given the IL17A antibody secukinumab have a higher rate of fungal infections than patients with CD who do not take this drug[115]. This adverse outcome correlated with pathology features, indicating that IL17 affects fungal communities, possibly in the gut.

Antibodies against *S cerevisiae* mannan (ASCA) are increased in blood samples from patients with CD or other diseases with a gastrointestinal component, such as autoimmune liver diseases, primary biliary cirrhosis, alcoholic liver disease, and primary sclerosing cholangitis[16,116]. Although the role of ASCAs in pathogenesis is unclear, their detection identifies individuals who will develop CD within 5 years with high accuracy[117]. A loss of function mutation (T280M) in CX3CR1 is associated with impaired production of ASCA in patients with CD, and decreased titers of serum IgG against *C albicans, C parapsilosis, S cerevisiae,* and *P kudriavzevii*, but not *Wallemia* or *Malassezia spp.,* which have low presence in the gut[66], indicating a link between $CX3CR1^{+}$ MNPs and ASCA (Figure 4A).

Fecal microbiota transplantation (FMT) was reported to be effective in a subset of patients with UC[118]. A high abundance of *C albicans* in feces before FMT was associated with a clinical response (Table 1). Stable titers of IgG against *C albicans* and decreased *Candida* abundance in fecal samples after FMT was associated with reduced severity of UC. This observation indicates that FMT acts in part by reducing *Candida* abundance and containing proinflammatory immune responses by fungi during intestinal inflammation[119] (Figure 5A, B).

Although we have focused on viruses that infect eukaryotic cells, fecal samples from patients with IBD have alterations in phage communities, including increases in the Caudovirales family in samples from CD or UC patients compared to healthy controls[7,11,120,121] (Table 1). Siphoviridae of the order Caudovirales were more likely to be transferred to patients with UC during FMT compared with other phages[122]. Fecal samples from patients with CDI have a high abundance and low diversity of Caudovirales. FMT treatment of CDI alters the phage composition of fecal samples, and response to treatment is associated with increased abundance of donor-derived Caudovirales taxa in recipients[123] (Figure 5C). Fecal filtrates from healthy donors were effective in treatment of CDI in 5 patients, suggesting that phage or other components of feces contribute towards the effects of FMT[124]. It is not clear whether phage infection of bacteria occurs before or after dysbiosis, but nucleic acids from phages induce production of type I IFNs by phagocytes[2]. Oral inoculation of germ-free mice with phages induced a Th1 cell-mediated immune response, via activation of TLR9 on antigen-presenting cells that internalized viral DNA. This response did not involve type I IFNs, and exacerbated DSS-induced colitis[3]. Expansion of phages in microbiomes of patients with IBD therefore may contribute to intestinal inflammation.

Viruses that infect eukaryotic cells, such as anelloviruses, are more prevalent in fecal samples from patients with IBD than individuals without IBD[7,125]. Intestinal mucosal samples from patients with UC have an increased abundance of Hepadnaviridae, whereas samples from patients with CD have an increased abundance of Hepeviridae. Intestinal mucosal samples from patients with UC also have lower levels of Polydnaviridae and Tymoviridae than controls, whereas samples from patients with CD have reduced Virgaviridae abundance[126]. Some of these viruses are better known for infecting plants and insects, and may have originated from the diet, similar to fungi identified in sequencing experiments. Antecedent norovirus infection is associated with CD in the Swedish National Register, and a separate study demonstrated association between norovirus infection and flares of CD[127,128] (Table 1). These finding raise important questions about whether patients with IBD are more susceptible to viral infections or to adverse consequences of infections, such as flares.

Eukaryotic viruses contribute to disease in animal models of IBD. Mice with variants of *Atg16l1* associated with increased risk of CD have structural abnormalities in Paneth cells upon persistent infection by MNV[129,130]. This defect, in which Paneth cells lack their characteristic antimicrobial granules, was observed in patients with CD homozygous for the *ATG16L1* variant encoding the T300A substitution[129]. ATG16L1 is required for autophagy, in which cytosolic material such as damaged mitochondria are sequestered in double-membrane vesicles and targeted to the lysosome for degradation[131]. MNV-infected mice with this variant of *Atg16l1* develop more severe intestinal inflammation and have increased mortality following chemical injury with DSS than wild-type mice[130,132].

In the intestinal epithelium, ATG16L1 is required for organelle homeostasis and prevents necroptosis in response to virus-induced tumor necrosis factor (TNF)[132]. Intestinal organoids generated from individuals homozygous for the *ATG16L1* T300A variant are also susceptible to necroptosis in the presence of TNF or cytokines produced by allogeneic Th1

cells[133]. MNV also exacerbates Th1-associated colitis in *Mdr1a*$^{-/-}$, *Stat1*$^{-/-}$, and *Il10*$^{-/-}$ mice[134–136]. MNV infection therefore induces production of cytokines that normally protect the intestine from bacterial pathogens, but cause damage in tissues that are genetically susceptible. The observation that MNV triggers or exacerbates disease in animal models of IBD lends support to the concept that animal viruses are a relevant component of the gut microbiota.

The induction of IFN and Th1 cell-mediated responses could be a common mechanism by which intestinal viruses contribute to inflammatory diseases. Reovirus infection of mice induces production of IL12B by CD103^{+}CD11B^{-}CD8A^{+} DCs, which promotes Th1 polarization in the mesenteric lymph nodes (MLNs) and increases reactivity towards dietary antigens, together with a type I IFN response that interferes with differentiation of T-regulatory cells[137]. A non-persistent strain of MNV induces a similar break in tolerance, and with both viruses, the Th1 cell-mediated response requires IRF1[137,138]. The observation that the titer of antibodies against reovirus correlates with expression of IRF1 in small intestinal biopsies from patients with celiac disease is consistent with findings from mice[137]. Frequent rotavirus infections have also been associated with development of celiac disease[139].

In mouse models of CD and celiac disease, viral infections induce features associated with human disease (such as Paneth cell defects and gluten-reactive lymphocytes). However, viral infections are insufficient to induce overt disease. A requirement for additional environmental triggers could explain how common viruses are associated with diseases in 1% or less of the population. Also, effects of viruses are strain-specific in mouse models of CD and of celiac disease[130,137,138]. If these observations provide any indication of what happens in humans, it would be a challenge for epidemiology studies to identify viral causes of these diseases. Few studies are equipped to distinguish the presence of a virus at the level of point mutations.

Perhaps the strongest evidence that intestinal viruses promote chronic immune diseases in humans comes from studies of patients with type 1 diabetes. Group B coxsackieviruses and other viruses with fecal to oral transmission have been suspected to contribute to this autoimmune disease, in which T cells attack insulin-producing pancreatic β cells[140]. In one of the few prospective, longitudinal virome studies, chronic enterovirus B infection and lower incidence of mastadenovirus C infection were associated with development of autoantibodies against insulin[141]. Episodic enteroviral infection was not associated with disease, indicating that prolonged infections might contribute to development of chronic disorders. Other prospective studies have also associated an altered enteric virome with development of type 1 diabetes, such as lack of early-life infection with small, single-stranded DNA viruses belonging to the *Circoviridae* family and development of serum antibodies associated with type 1 diabetes[44,142]. Experiments with the non-obese diabetic (NOD) mouse model of type 1 diabetes found that infection with group B coxsackievirus reduced disease in younger mice, but accelerated disease if introduced into older mice, at a time at which insulitis had already initiated, indicating that infection affects ongoing autoimmune reactions[143,144].

Rotaviruses and reoviruses are not considered pancreatropic, but have a similar time-dependent effect on diabetes[145–147]. In NOD mice, rotavirus accelerates type 1 diabetes by spreading from the gut to the lymph nodes that drain the pancreas, where type I IFNs are produced by pDCs to activate autoreactive lymphocytes[147]. MNV infection also protects against development of diabetes in NOD mice, which is associated with increases in T-regulatory cells and an altered bacterial microbiota[148]. Thus, studies of patients with type 1 diabetes and NOD mice have shown that gut viruses can affect extra-intestinal immune responses. Intestinal fungi also have extra-intestinal effects, modulating systemic immune responses as well as immunity in the lung and liver[16,61,67,70,71,105,149].

Many patients with COVID-19 have gastrointestinal symptoms. An increasing number of published and unpublished findings are reporting the presence of SARSCoV-2 in stool or intestinal tissue, consistent with the observation that the virus readily replicates in intestinal organoids[150–152]. The aforementioned experiments in mice predict the presence of the virus, or viral RNA, in the gut would induce an immune response with a broad range of consequences for the host. Additionally, prospective studies are necessary to understand the long-term consequences of SARS-CoV-2 infection for individuals with IBD or other immune-mediated disorders. As our understanding of SARS-CoV-2 pathogenesis improves, we are confident that the relationship between this virus and the gastrointestinal tract will be clarified.

Conclusions

Decades of infectious disease research have shown that fungi and viruses activate innate and adaptive immunity at barrier surfaces. Fungi and viruses are indispensable parts of the human gut microbiota fully integrated within the bacterial community and affect the immune response, health, and development of inflammatory diseases. Despite advances, our understanding on the interactions between these components of the gut microbiota and the mucosal immune system is limited. Deep sequencing-based analysis of gut viruses and fungi indicate the composition of the mycobiome and virome vary considerably among cohorts, but that specific viruses and fungal species are associated with multiple cohorts—these warrant further investigation.

Intestinal viruses and fungi modulate the immune response and can contribute to intestinal inflammation, making them targets of microbiome-based therapies. Broad spectrum antifungal or antiviral drugs have been consistently reported to have detrimental effects on patients. Diets and FMT alter the fungal and viral components of the gut[16,123,153], but have other broad effects, making it a challenge to identify any virus-mediated effects on health or disease. Identifying specific fungi or viruses in the intestinal microbiota that contribute to or prevent disease, and any strain-specific targets, would be required to develop therapeutic agents that modify these microbes. Testing pharmacologic agents that alter immune responses to fungi or viruses might also help identify their targets. Nevertheless, considerably more basic research is needed to inform therapeutic approaches in this new area of microbiome science.

Another emerging area of research not covered by this review is interactions among different microbes in the gut. Studies of the oral, vaginal, and lung mucosa provide evidence for viruses-bacteria and fungi-bacteria interactions at these sites. Several studies have begun to explore such interactions in the gut. Many enteric viruses that infect eukaryotic cells require the bacterial microbiota for infection and transmission. Mechanisms include direct effects in which bacteria bind virions to enhance stability and attachment to target animal cells, or indirect effects of bacteria on the mucosal immune system[154–158]. In contrast to these mechanisms in which bacteria promote viral infection, in mice that are spontaneously resistant to rotavirus infection, segmented filamentous bacteria were found to promote epithelial cell proliferation and expulsion to block virus infectivity[159]. This shows that the presence of an individual bacterial species can affect the course of an enteric viral infection.

Most studies of interactions between fungi and bacteria in the gut have focused on the detrimental effects of bacteria on fungi, although positive or nutritional relationships have been documented[17,22]. Lactobacilli in the intestine promote restructuring of the *C albicans* cell wall and mask β-glucan by producing lactic acid[160]. *Serratia marcescens* releases unique anti-fungal effector proteins with activity against *Candida* species and *S cerevisiae*[161]. However, entirely new approaches must be developed to assess trans-kingdom and intra-kingdom interactions among microbes in the intestinal community.

Although intestinal fungi and viruses might be the dark matter of the microbiota, they are a highly immunoreactive component with unrecognized potential. It will be important to learn how these microbes affect each other, and the immune response, to promote health and development of disease.

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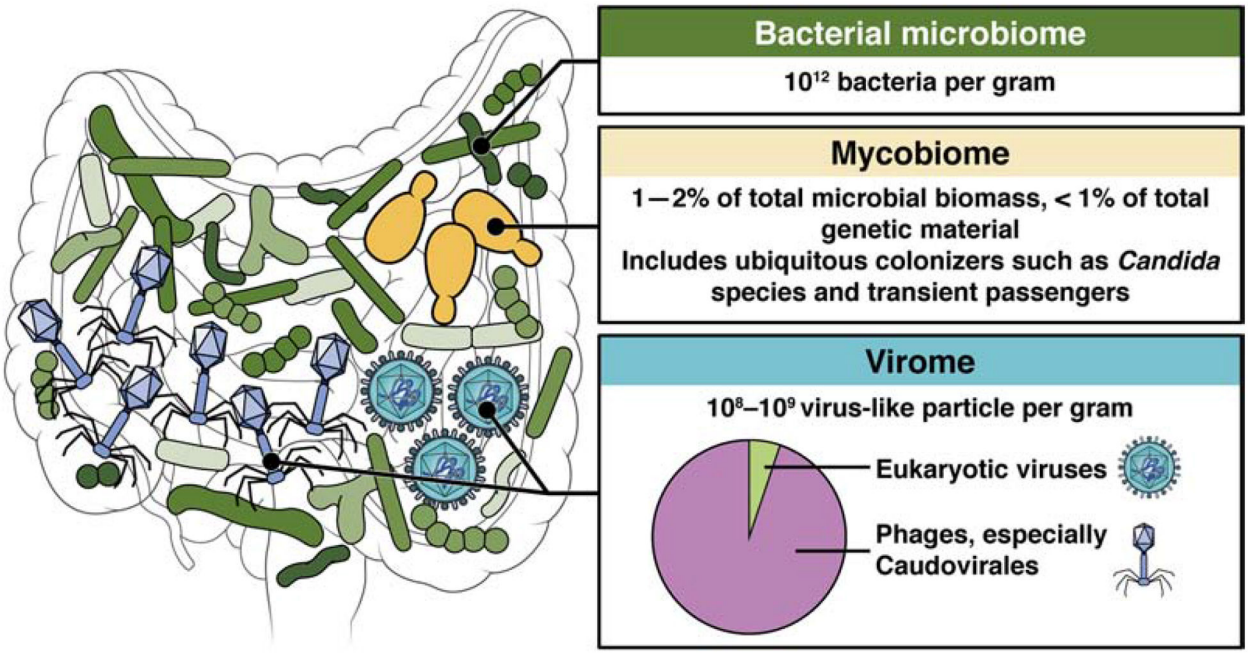

Figure 1. Fungi and viruses in the intestinal microbiota.

The intestine contains microbes that include bacteria, archaea, fungi, protozoans, and viruses. Most of these microbes are bacteria, which interact with the mucosal immune system. Fungi account for less than 1% of the total genetic material in fecal samples, but they are larger organisms that might occupy different niches along the gastrointestinal tract. Certain genera, such as *Candida*, are ubiquitous and establish long-term colonization. Viruses include phages, which infect bacteria, and viruses that infect eukaryotic cells. Phages can affect immune and other cell types via effects in bacteria they infect. Although viruses account for a small proportion of the mammalian gut microbiota, and the degree to which they establish long-term colonization is unclear, they could have large effects in that they directly invade and replicate in host cells. There is evidence fungal and viral members of the intestinal microbiota affect differentiation and function of the immune system. In this way, intestinal fungi and viruses might increase or decrease risks of diseases ranging from nosocomial infections to autoimmune diseases or IBD.

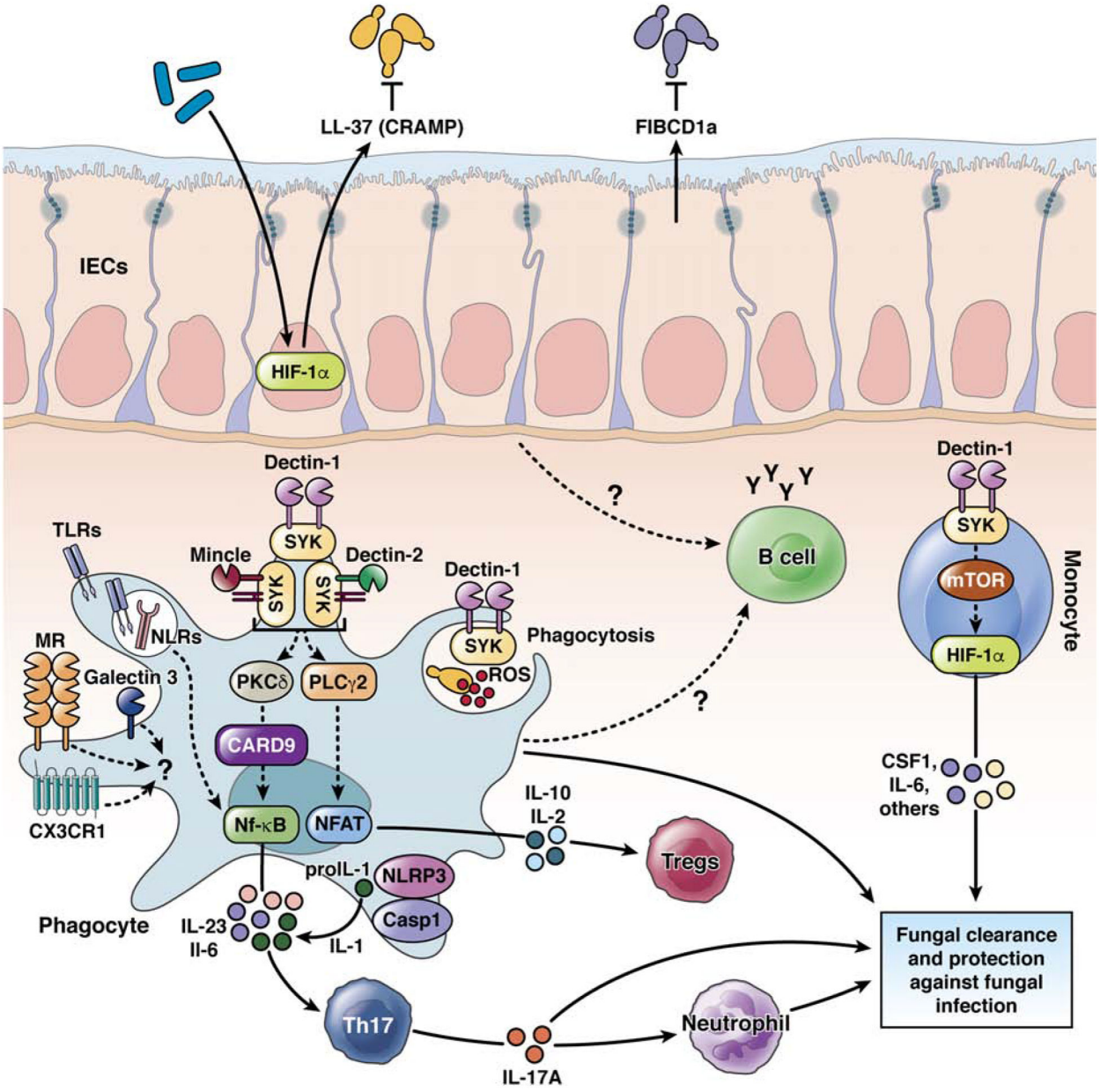

Figure 2. Innate and adaptive immune responses to fungi in the gut.

At the mucosal surface of the intestines, CLRs (such as dectin 1, dectin 2, and mincle) interact with fungal cell wall components, resulting in activation of SYK. SYK activates protein kinase C delta (PKCD) to signal via CARD9 and activate nuclear factor (NF)-kB, phospholipase C gamma 2 (PLCG2) to activate NFAT, production of reactive oxygen species (ROS), or MTOR to activate hypoxia inducible factor 1 subunit alpha (HIF1A). These signaling pathways activate production of IL23, IL6, IL10, IL2, IL1 by phagocytes (macrophages, monocytes, DCs, and neutrophils) and CSF1 and IL6 by monocytes ("trained immunity"). The interaction with fungi-charged phagocytes results in the development of Th17 cells and recruitment of neutrophils to the intestinal lamina propria. Receptor-independent functions of phagocytes supports eradication of specific fungal products and

cells. TLRs, NLRs, inflammasome components, and galectin 3 also recognize fungal components. These combined effects result in killing of fungi or tolerance to them and a balanced gut fungal community or disease development.

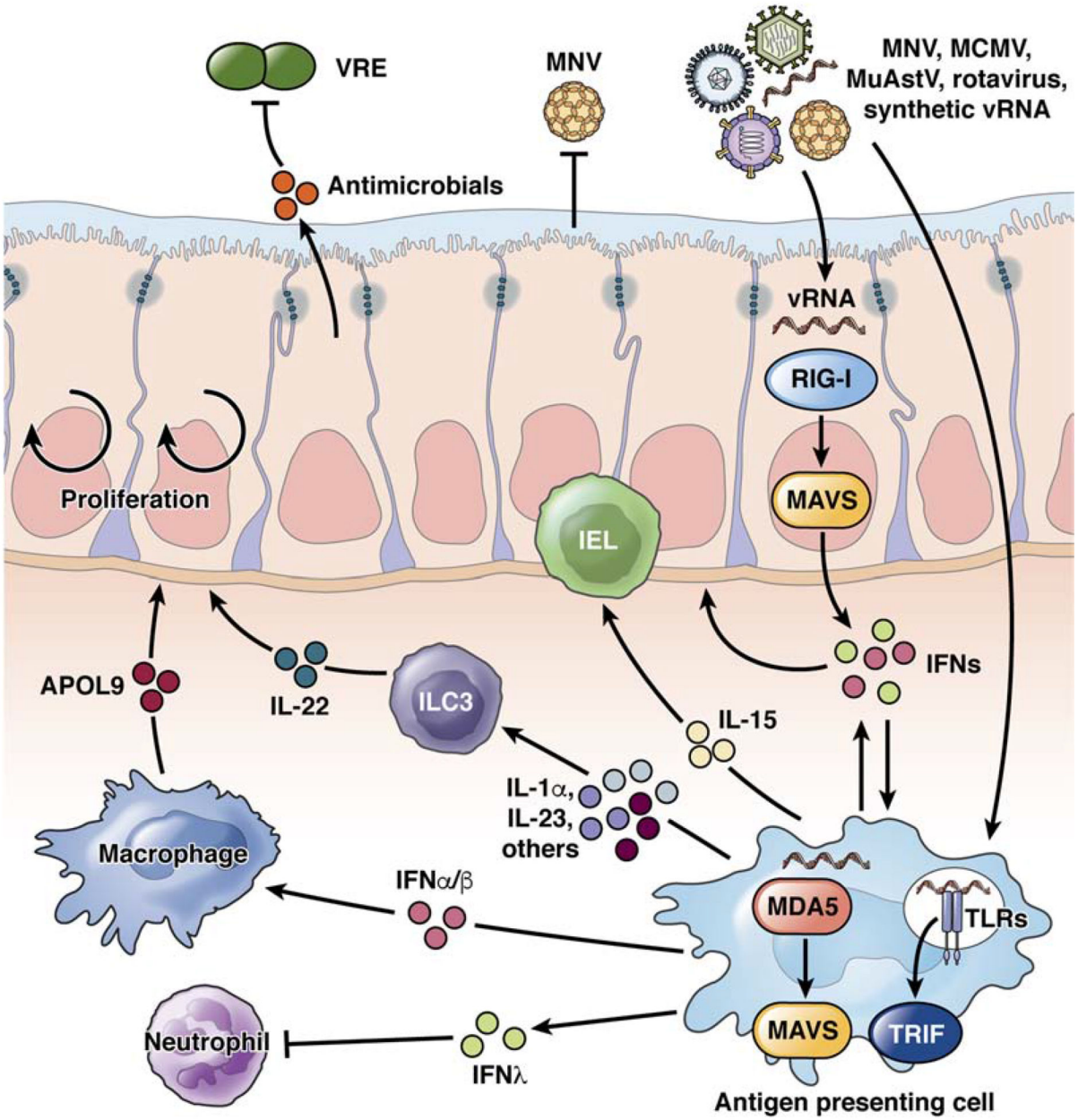

Figure 3. Recognition of viruses by immune cells fortifies the intestinal barrier.

MNV, cytomegalovirus (MCMV), astrovirus (MuAstV) infect myeloid, lymphoid, or epithelial cells, depending on strain. These viruses, heat-killed rotavirus, or synthetic viral RNAs (vRNAs) activate RIG-I or MDA5 in cytosol or TLRs on endosomes of epithelial cells and antigen-presenting cells, which signal through MAVS and TRIF, respectively. These signaling pathways trigger production of type I IFN (IFNA and IFNB), which mediate bi-directional communication between myeloid cells with the epithelium; IFNL, which inhibits neutrophils; IL15, which promotes IEL function; and IL23, which induces ILC3s to produce IL22, increasing resistance to injury and bacteria such as vancomycin resistant *Enterococci* (VRE). IFNA and IFNB also activate macrophages to produce APOL9, which promotes epithelial cell proliferation and protection from injury.

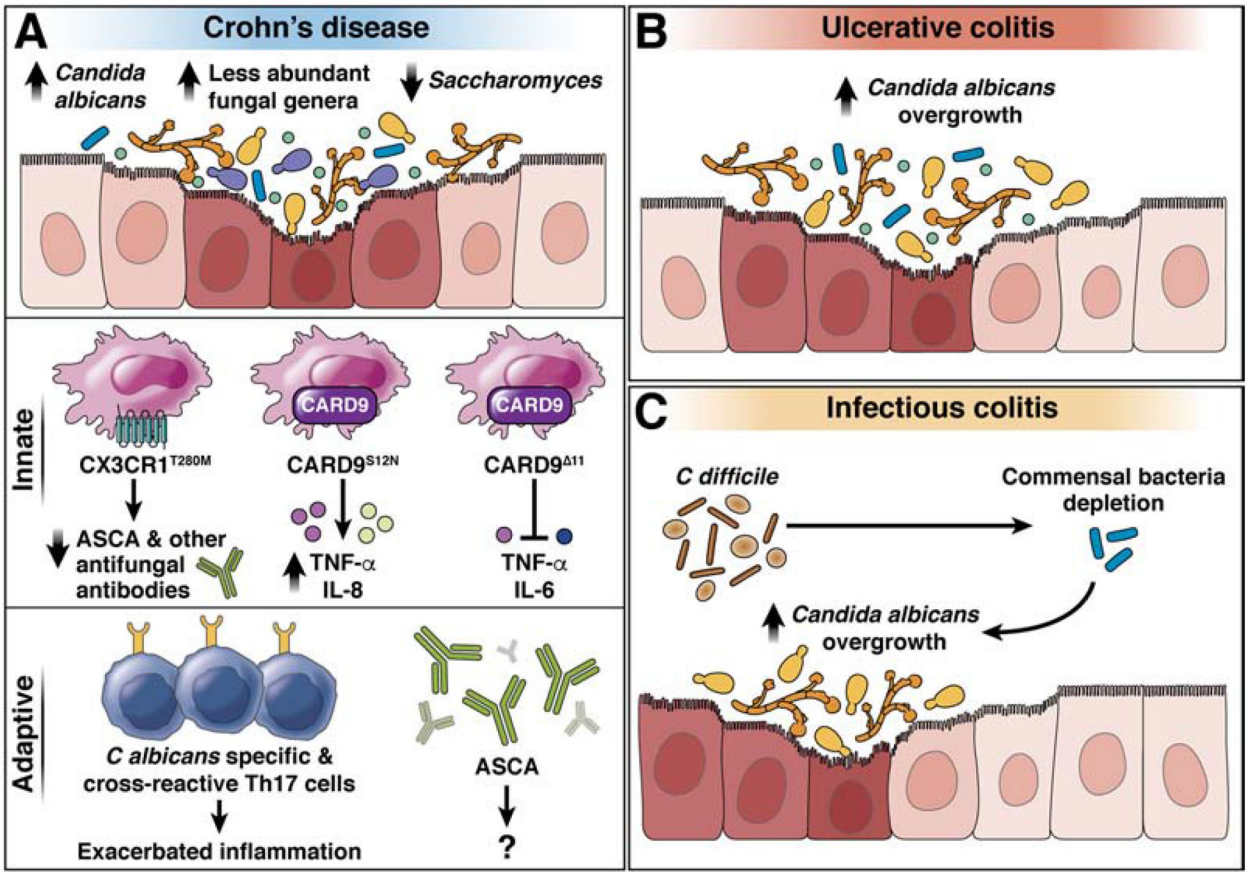

Figure 4. Gut fungi in patients with IBD

A) Gut fungal dysbiosis, characterized by the overgrowth of opportunistic fungal species, in patients with CD. CD is associated with *Candida* spp. overgrowth, expansion of less abundant fungal genera such as *Malassezia* spp., and decrease of *Saccharomyces* spp. in fecal samples. The T280M mutation in CX3CR1 is associated with decreased ASCA and other antifungal antibodies' production; the S12N mutation in CARD9 increases production of inflammatory cytokines (TNF, IL8,); the delta 11 deletion in CARD block production of TNF and IL6 to prevent development of CD. Expansion of *C albicans*-specific and *Aspergillus* cross-reactive Th17 cells and increase of ASCA antibodies is associated with Crohn's Disease. *Candida albicans* overgrowth has been observed in patients with UC (B) and CDI (C).

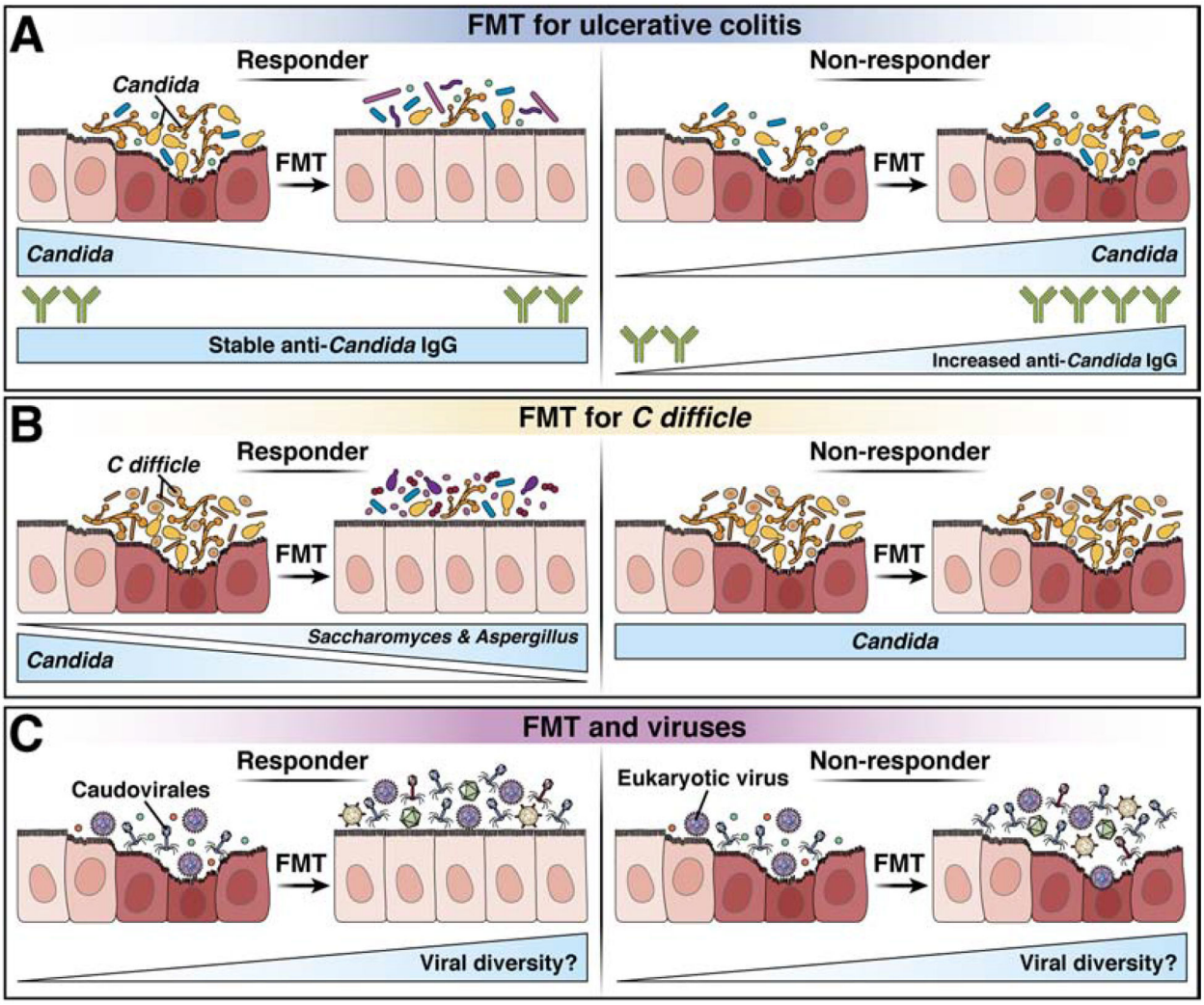

Figure 5. Gut fungi and viruses in FMT.

A) In patients with UC, increased *C albicans* abundance before FMT is associated with a clinical response (top), whereas stable titers of IgG against *C albicans* in blood samples, and decreased *Candida* abundance in fecal samples, associate with reduced disease severity. B) In patients with CDI, a high relative abundance of *Saccharomyces* and *Aspergillus* in fecal samples after FMT is associated with response to the treatment (top), whereas overrepresentation of *C albicans* and decreased fungal diversity after FMT associate with non-responsiveness (bottom). C) In patients with CDI, FMT modulates gut phage composition; response to FMT is associated with an increased abundance of *Caudovirales* taxa. Little is known about the effects of viruses that infect eukaryotic cells. It will be important to determine which viruses are included in donor sample and how FMT affects the viruses already present in the recipient.

Table 1.

Viral and Fungal Microbes in the Intestine

Microbe	Disease or potential therapy	Alterations in fungi or viruses	Immune Responses	References
Fungi				
	CD	*Candida* expansion and decreased *S cerevisiae* in several cohorts Increased abundance of *M restricta* in 1 cohort *Malassezia* and *Aspergillus* presence linked to CARD9 S12N in 1 cohort Fungal dysbiosis characterized by decreased *Ascomycota* and increased *Basidiomycota* diversity in several cohorts	Increased *C albicans*-reactive T cells. Increased ASCA Decreased ASCA in persons with T280M in CX3CR1	65,100–102,104,107,162
	UC	*Candida* expansion in several cohorts	None reported	100,105,163
	FMT for patients with UC	High gut *Candida* before FMT associated with clinical response in 1 cohort Decreased *Candida* abundance after FMT associated with reduced severity in 1 cohort	Increased anti-*Candida* antibodies in placebo controls are abrogated in FMT recipients	119
	FMT for patients with CDI	Gut fungal dysbiosis and expansion of *C albicans* in patients with CDI. Decreased *Candida* abundance after FMT, increased *Candida* abundance after FMT non-responders Increased *Saccharomyces* and *Aspergillus* in responders in 1 cohort	None reported	153
Viruses				
	CD	Expansion of Caudovirales phages in several cohorts Increased frequency of anellovirus in a combined cohort Exclusive presence of enteroviruses in 1 cohort Norovirus increases odds of disease in the Swedish National Registry and flares	None reported	7,11 6,126–128
	UC	Other eukaryotic viruses display altered presence but potentially cohort-specific or associated with diet Expansion of Caudovirales phages in several cohorts. Increased frequency of anellovirus in several cohorts. Other eukaryotic viruses display altered presence but potentially cohort-specific or associated with diet	None reported	7,11,121,125,126
	FMT for pediatric patients with UC	Siphoviridae phages belonging to Caudovirales displayed enhanced engraftment.	None reported	122
	FMT for patients with CDI	FMT responsiveness associated with engraftment of donor-derived Caudovirales phages, consistent with observation that filtered feces contains therapeutic benefit	None reported	123,124
	Celiac disease	Patients more likely to have high titers of anti-reovirus antibody Frequent rotavirus infection associated with disease development in a longitudinal study	*IRF1* expression in intestinal mucosa of patients with high anti-reovirus titer	137,139

ASCA, Anti-saccharomyces cerevisiae antibodies; FMT, Fecal microbiota transplantation; CDI, Clostridium difficile infection; IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis