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Fungal dysbiosis: immunity and interactions at mucosal barriers

Iliyan D. Iliev^{1,2,3} and Irina Leonardi^{1,3}

¹Gastroenterology and Hepatology Division, Joan and Sanford I. Weill Department of Medicine, Weill Cornell Medicine, New York, NY 10021

²Department of Microbiology and Immunology, Weill Cornell Medicine, New York, NY 10065

³The Jill Roberts Institute for Research in Inflammatory Bowel Disease, Weill Cornell Medicine, New York, NY 10021

Abstract

Fungi and mammals share a co-evolutionary history and are involved in a complex web of interactions. Studies focused on commensal bacteria suggest that pathologic changes in the microbiota, historically termed as ‘dysbiosis’, are at the root of many inflammatory diseases of non-infectious origin. Yet, the importance of dysbiosis in the fungal community—the mycobiota—was only recently acknowledged to have a pathological role as novel findings suggest that mycobiota disruption can have detrimental effects on host immunity. Fungal dysbiosis and homeostasis are dynamic processes that are probably more common than actual fungal infections, and therefore constantly shape the immune response. In this Review, we summarize specific patterns associated with fungal dysbiosis, and discuss how mucosal immunity has evolved to distinguish fungal infection from dysbiosis and how it responds to these different conditions. We propose that gut microbiota dysbiosis is a collective feature of complex interactions between prokaryotic and eukaryotic microbial communities that can affect immunity and influence health and disease.

Introduction

Fungi are omnipresent in our living environment and are an integral part of all ecosystems¹. They have played a central role in the evolution of life². Molecular evidence suggests that animals and fungi have coevolved since diverging from plants over 1 billion years ago³. This has provided plentiful opportunities for fungi to strongly influence the evolution of animals³ and their immune system^{4,5}. The immune system has been crucial in establishing a close relationship between host, bacteria and fungi, by keeping the peace at the highly colonized barrier surfaces. Although this relationship often results in **sympiosis**^{6–8} (BOX 1), it can lead to diseases that have devastating socioeconomic impact^{9–11}.

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Box 1**Ecological relationships between fungi and their host**

There are numerous examples of oppositional and symbiotic relationships between fungi and animals. While some fungi such as members of *Pleurotaceae* family have become predators using elaborated strategies to catch and digest nematodes living in the soil, other fungi such *Harposporium* spp. and *Ophiocordyceps* spp. have adapted a parasitic lifestyle¹²⁸. *Ophiocordyceps* fungal species have gone as far as manipulating the host behaviour to ensure their own survival and dispersal¹²⁸. Other fungi such as *Blastomyces dermatitidis* and some *Aspergillus* spp. have developed mechanisms to mimic mammalian regulatory molecules and suppress host immunity^{129,130}. The skin and the mucosal surfaces of animals and humans are rich on nutrients and provide temperate environment for commensal fungal symbionts to thrive. *Malassezia* spp. are well adapted to sebaceous microenvironments and are abundantly present on the skin^{9,60}. The feet are home of a diverse fungal community dominated by saprophytic fungi that feed from the dead cells, which are continuously shed at this skin site⁹. Symbiotic relationships between fungi and their host are well established in the gastrointestinal tract of different orders of animals from insects to mammals^{10,11}. Whereas some fungal symbionts are crucial for processing indigestible lignocellulose associated with the herbivores diet, other symbionts produce enzymes necessary for the neutralization of dietary toxic compounds¹¹. The host, on the other hand, provides its commensal mycobiota with an optimal environment to grow, feed and procreate. In some cases the fungal symbiont are even vertically transmitted through generations¹³¹, suggesting that similar to bacterial symbiosis, some fungal commensals might be inherited from the mother. Recently, high throughput sequencing approaches revealed that mouse and human intestines are home to diverse fungal community which might play an important role in host physiology and immunity^{13,15–17,21}.

The rapid development of deep sequencing and computational technologies has provided great opportunities to explore the structure and functionality of microbial communities associated with our body surfaces. Extensive microbial data generated worldwide has enabled the scientific community to uncover the functions of the human microbiota¹². The bacterial communities have been the primary focus of these sequencing efforts and less data is currently available characterizing the fungal microbiota — the mycobiota. Nevertheless, recent studies reveal that host-associated fungal populations are also dynamic and responsive to environmental and pathophysiological changes^{9,13–17}.

A century of studies focused on commensal bacteria in the gut suggest that ‘**dysbiosis**’¹⁸ is in the root of several diseases of complex etiology involving genetic polymorphisms, immune mechanisms and the microbiota^{19,20,21}. Although the term dysbiosis is imperfect, it describes conditions that are distinct from and is widely used to describe altered bacterial communities as both a cause and a consequence of pathologies (BOX 2).. Nonetheless, infections and dysbiosis can prelude to each other (homeostasis-dysbiosis-infection vs homeostasis-infection-dysbiosis) in a dynamic continuum. Several studies indicate that a similar process involving fungal communities — ‘fungal dysbiosis’ — could affect the host

mycobiota (Figure 1)^{9,16,17,21–24}. Since fungi are common inhabitants of all barrier surfaces, changes in fungal communities might have substantial effects on the host immune responses.

Box 2

Uncoupling infection from dysbiosis

Early in the 16th century Girolamo Fracastoro proposed that minute invisible seeds — ‘*seminaria*’ — transmit diseases from person to person¹³². This revolutionary theory of the microbial origin of diseases was proven about 100 years later with the discovery of the microscope by Antony van Leeuwenhoek¹³³. It took another 200 years for the theory to fully ripen. In the second half of the 19th century Luis Pasteur and Friedrich Jakob Henle independently published their ideas of the microbial origin of diseases^{134,135}. The idea was further developed by Robert Koch¹¹², who eventually developed guidelines known as “Koch’s postulates” intending to move the field forward from observations to evidence proved causative relationship between microbes and disease¹³⁶. Over the years Koch’s postulates underwent several modifications incorporating application to acute and chronic diseases with diverse etiologies¹³⁷ and comprising the advances that genomics-based approaches have had on clinical microbiology and virology^{138,139}. Although Koch’s postulates framed the idea of the microbial origin of infectious diseases, they cannot explain situations where microbes indirectly shape a pro-inflammatory or anti-inflammatory environment to affect immunity. During the second half of the 19th century, while working on causes of longevity, Ilya Metchnikoff theorized that intestinal microbes can “auto-intoxicate and auto-infect” their host¹⁸. He and his colleagues Stamen Grigoroff and Leon Massol proposed that lactic acid producing bacteria, which they called *Bulgarian bacillus*, can decelerate this process¹⁸. Together with the concept of beneficial bacteria, Metchnikoff coined the term ‘dysbiosis’ (initially dysbacteriosis) to define pathologic changes of intestinal microflora¹⁸. However, dysbiosis is a generalizing and wide definition, which can be applied to either cause or effect. Some have criticized these shortcomings¹⁴⁰ or have attempted to define dysbiosis within several conditions¹⁴¹. Similar to the modifications that Koch’s postulates underwent over time, the concept of ‘dysbiosis’ will probably evolve to reflect the modern advances in microbiome science.

In this Review article, we discuss the mycobiota in homeostasis and dysbiosis, and how mucosal immunity distinguishes between fungal infections and fungal dysbiosis and responds to these conditions. We define specific patterns associated with fungal dysbiosis and changes in mycobiota diversity at several body sites. Finally, we discuss the impact of fungal dysbiosis on commensal bacteria and how these two fundamentally different communities influence each other to affect the outcome of the immune response.

Mycobiota at steady state and in dysbiosis

Fungi have been traditionally studied using culture-dependent methods. Only recently, advances in deep sequencing technologies have provided opportunities to unveil the complexity of the fungal communities colonizing the mammalian barrier surfaces. Most approaches have relayed on sequencing the fungal **internal transcribed spacer 1** (ITS1)

and ITS2 regions and using computational algorithms to align **operational taxonomic units** (OTUs) to respective fungal databases^{9,16,25,26}. Whereas fungal identification remains challenging in environmental and soil samples due to the enormous fungal diversity and the presence of many unknown fungal species²⁷, the development of several databases designed to specifically target host-associated fungal communities have made it possible to identify over 80% of the fungal ITS sequences derived from fecal or mucosal samples^{9,28–30}. Despite this progress, each step of this identification approach can contribute to biases^{26,28} and complicate the comparison of mycobiota sequencing results from different studies. Additional bias might be introduced by therapeutic and dietary interventions that can contribute to further changes in fungal communities¹⁵. Despite these confounding factors, several studies have revealed similarities in how fungal communities adapt to environmental perturbations and these will be summarized in this section. Keeping in mind the limitations that the term “dysbiosis” imposes (BOX 2), we will further define specific conditions associated with fungal community disruption in the context of inflammatory diseases, host immunity and drug-triggered microbial alterations.

Mycobiota in the oral cavity

The oral cavity is one of the first mucosal sites where asymptomatic carriage of fungi was described³¹. Diversity in the oral mycobiota is low and dominated by members of the *Ascomycota* phylum, mainly *Candida* spp.³². Culture independent methods have revealed the presence of several members of the *Saccharomycetaceae* family (*Candida albicans*, *Candida dubliniensis*, *Candida rugosa*, *Candida pararugosa*, *Saccharomyces cerevisiae*, *Hanseniaspora uvarum*, and *Pichia* spp.), and *Fusarium* spp. that represent the majority of fungal species found in the oral cavity³². Dysbiosis in the oral mucosa is poorly characterized and most studies have focused on the overgrowth of *Candida albicans* upon immunosuppression and diabetes³² (Figure 1B). The first culture-independent assessment of the oral mycobiota in patients with HIV revealed that in addition to overgrowth of *C. albicans*, the presence of other species belonging to the *Ascomycota* phylum, including *Dothideomycetes* spp. and *Leotiomycetes* spp., was also increased whereas *Tremellomycetes* spp. were reduced³². Notably, this study did not observe any alteration of the bacterial microbiota, indicating that the observed fungal dysbiosis is probably mediated by a direct effect of the compromised immune system and not by an indirect effect mediated by changes in the bacterial microbiota.

Mycobiota in the gastrointestinal tract

The fungal community in the lower gastrointestinal tract is more rich and diverse than in the upper gastrointestinal tract. A recent review of 36 published gut mycobiota articles concluded that only 15 out of the 267 identified species were detected in more than 5 studies³³. Among these 15 species, 13 can grow at 37°C and thus have the potential to permanently inhabit the intestinal niche^{22,34}. These species belong to the genera *Candida*, *Saccharomyces*, *Aspergillus*, *Cryptococcus*, *Malassezia*, *Cladosporium*, *Galactomyces* and *Trichosporon*³³. Nevertheless, commensalism is not the only way fungi can influence host immunity. For instance, although *Histoplasma* spp., *Blastomyces* spp. and *Coccidioides* spp. cannot colonize the mucosal surfaces they can cause severe infections in the lung³⁵.

Similarly, non-commensal fungi present in the diet, the air or other environmental sources, might also activate immune responses in the gut^{15,24} (Figure 1B).

The high fungal variability observed in different studies has been interpreted as a sign of the temporal instability of the intestinal mycobiota^{13,34}. Yet, most of the studies rely on the sampling of fecal material. Although additional data are needed to make definitive conclusions, a recent study of the mucosa-associated fungi suggests that a more stable community is associated to the gut mucosa³⁶ and this can be perturbed during intestinal disease³⁷. These results are interesting since fungi that are in close proximity to the intestinal mucosa might have increased potential to interact locally with the intestinal epithelium and the mucosal immune system.

Inflammatory conditions that target the lower gastrointestinal tract have been known to promote bacterial dysbiosis for a long time and this has highlighted the important contribution of bacteria in the etiology of **inflammatory bowel disease** (IBD)^{38,39}. In light of these findings, several studies have investigated the fungal dysbiosis in patients with IBD and revealed a general increase of fungal burden in the colonic mucosa during both Crohn's disease^{21,22} and ulcerative colitis⁴⁰. Consistent with these results, the fungal burden increases and pronounced fungal translocation occurs in the mucosa during the chronic stage of colitis⁴¹ and during the acute stage of colitis in mice deficient in the fungal receptor dectin-1 (*clec7a*, see below)¹⁶. Together, these studies suggest that genetic factors, extensive tissue damage and the presence of inflammatory environment can cause fungal dysbiosis in the gut.

Several fungal taxa appear to be consistently altered during chronic intestinal inflammation throughout multiple studies. Patients with IBD have an increased occurrence and abundance of cultivable *C. albicans* in the feces, which was further confirmed by culture-independent studies^{17,21–23}. In patients with Crohn's disease, the presence of *Candida albicans* and *Candida parapsilosis* increased in the inflamed mucosa³⁷. Increased abundance of *Candida tropicalis* in Crohn's disease correlated with inflammation and increased levels of anti-*S. cerevisiae* antibody (ASCA)²³. Consistently, intestinal inflammation is sufficient to promote *Candida* spp. colonization and increased abundance in the mouse gut^{16,41}. Although it is clear that *Candida* spp. expand during intestinal disease (Figure 1), its effect on other gut fungi remains unclear. Some studies suggest that the presence of the *Ascomycota* phylum decrease in favour of expansion of *Basydiomycota* spp.^{17,37}, whereas another study report a general increase of five *Ascomycota* species in IBD²¹.

Clone libraries approaches based on 18S rDNA denaturing gradient gel electrophoresis (DGGE) show that patients with Crohn's disease have increased fungal diversity and richness in the faeces and the mucosa, compared with healthy individuals or individuals with other forms of intestinal inflammation such as patients with infectious colitis^{37,40}. By contrast, ITS-sequencing approaches showed either no changes in the mycobiota composition in fecal samples¹⁷ or colonic mucosa²² during Crohn's disease, or a reduction in fungal diversity in a paediatric IBD cohort that mainly included patients with Crohn's disease⁴². A further study showed a decreased species richness in the feces from patients with Crohn's disease and their healthy relatives when compared with unrelated healthy

controls, but no change in fungal diversity was reported²³. Interestingly, one study shows that patients with active ulcerative colitis have a clear reduction in fungal diversity¹⁷. The differences in the gut mycobiota observed among different studies suggest that in addition to inflammation, other factors, such as immunosuppressive therapy, disease type (Crohn's disease or ulcerative colitis) or disease-specific diet might influence the structure of the mycobiota in patients with IBD.

Patients with Crohn's disease have increased levels of antifungal antibodies, which show that an immune response to intestinal fungi is elicited during IBD⁴³. These systemic ASCA IgG and IgA are widely recognized as reliable diagnostic markers for Crohn's disease and are good predictors of the disease course⁴³. Recent studies show that *C. albicans* can act as immunogen for ASCA production⁴⁴ and that ASCA are cross-reactive to cell wall mannans isolated from other yeasts including commensal *Candida* species⁴⁵. Despite this link with intestinal fungi, the exact mechanism behind ASCA generation and their role in antifungal immunity is yet to be understood.

The frequent co-occurrence of gastrointestinal symptoms and neurodevelopmental disorders has uncovered the presence of bacterial dysbiosis in autism-spectrum disorders (ASD), schizophrenia and other neurodevelopmental disorders^{46,47}. Patients with ASD and Rett syndrome were recently reported to have altered intestinal mycobiota^{47,48}; however whether fungi also contribute to the development of gastrointestinal symptoms in these patient groups is currently unknown.

Mycobiota in the lung, vagina- and skin

Disease-mediated fungal dysbiosis has been described at other barrier sites with lower fungal burden than the gut. The healthy lung has long been considered an aseptic organ and bacterial colonization was thought to occur only during disease²⁰. Similarly, the fungal burden is generally low in healthy individuals whose lung mycobiota appears to be largely composed of environmental fungi or fungi disseminating from the oral cavity, including species belonging to the *Cladosporium*, *Aspergillus*, *Penicillium* and *Candida* genera^{25,32,49}. By contrast, more stable fungal communities can colonize the lung when its physiology is altered (Figure 1B). In the majority of patients with cystic fibrosis, for example, the fungal burden in the lung appears increased, whereas **alpha diversity** is reduced and correlate with disease severity^{50,51}. Despite the high inter-individual variability of mycobiota composition, fungal genera such as *Candida* and *Aspergillus*, have been linked to morbidity in individuals with cystic fibrosis^{50,51}. Notably, once established, lung dysbiotic fungal communities seem to stabilize and persist even in the presence of antibiotic or immunosuppressant therapy^{51,52}. Fungi have also been associated with the severity of asthma^{20,53}, however it is unclear whether the effect is local or influenced by the intestinal mycobiota as suggested by experimental studies^{24,54,55}.

Vaginal candidiasis is by far one of the most common mucosal fungal infections in humans. Yet, fungal communities are found in healthy women without active infections¹¹⁰. Similar to the oral mucosa, the vaginal mycobiota is less diverse and is mainly colonized by *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis* and *S. cerevisiae*^{56,57,58}. Several factors, such as antibiotic use, pregnancy, HIV infection and recurrent vulvovaginal candidiasis have been

associated with alterations of the vaginal fungal community structure (Figure 1B)^{56,58,59}. Vaginal *Candida* spp. colonization increases up to 40% during pregnancy, due to the increased estrogen levels and glycogen production by vaginal epithelial cells, and this might have important consequences on pregnant women, including preterm delivery⁵⁶.

In contrast to the other surfaces of the body, the skin is cold, dry and has higher availability of lipids and proteins (such as keratin), which constitute the major nutrients for the colonizing fungal flora including lipophilic species such as *Propionibacterium* spp. and *Malassezia* spp.^{9,60}. The skin microbiota shows temporal stability and can be influenced by primary immunodeficiencies^{60,61,62}. When skin integrity is compromised, fungi rapidly populate the wound site. A recent study demonstrated that fungi are present in 80% of the chronic wounds in patients with type 1 and type 2 diabetes (Figure 1B)⁶³. Despite the high interpersonal variability of the wound mycobiota the study showed an association between *Candida* spp., *Trichosporon* spp. and *Rhodotorula* spp. with non-healing wounds and amputation while temporally unstable fungal and bacterial communities were associated with a positive healing outcome⁶³. Therefore, in addition to bacteria, a specific signature of wound fungal community can have a negative outcome of the skin healing process.

Altogether, these studies suggest that disease-induced dysbiotic fungal communities are often well adapted to the changes induced by pathological conditions and can contribute to the disease course. Further studies are required in treatment-naive individuals, including paediatric patients and newly diagnosed patients, to make critical insight into a purely disease-driven dysbiosis. Mouse studies are an essential tool to analyze the effect of fungal dysbiosis under such conditions and have already provided some evidence for fungal involvement in several pathologies as discussed below.

Influence of age, sex, diet and environment

Several studies show a high variability of the mycobiota between different individuals, which complicates the distinction between healthy and dysbiotic fungal communities. This variability highlights the need for a more strict definition of dysbiosis (BOX 2) and the need of experimental studies that define the consequences of an altered mycobiota. The mycobiota composition is substantially influenced by diet, gender, age and geographical locations⁶⁴. *Aspergillus* spp. and *Tremellomyces* spp. are more abundant in male individuals whereas *Candida* spp. are more abundant in females possibly due to spreading from the vagina⁶⁴. Genera such as *Aspergillus*, *Tremellomyces* and *Penicillium* are abundant in infants whereas the overall diversity of fungal community decreases with age^{42,64}. Similarly, the diversity of the skin mycobiota decreases with age, probably due to the increasingly sebaceous skin that favours colonization by *Malassezia* species⁶⁵.

The diet can influence not only bacterial but also fungal communities, mainly as a constant source of fungal species that are associated with vegetables, fruits and dairy products¹⁵. Individuals eating a controlled animal-derived diet that was rich in cheese were associated with an increased fungal burden and fungal species detected in their feces corresponded to those isolated from the food they had eaten^{15,34}. Diet might be one of the factors contributing to the differences observed in the fungal community across different ethnicities²¹. Altogether, these studies suggest that dietary fungal intake contributes

substantially to the gut mycobiome and that specific dietary nutrients can sustain specific fungal community members (Figure 1B). In support for this, a recent study reported that *Candida* spp. associated positively with carbohydrates and negatively with saturated fatty acids, whereas *Aspergillus* spp. correlated negatively with dietary short chain fatty acids¹⁴.

Mucosal immunity to fungi

The immune system has evolved to tolerate fungi and respond to them upon injury or infection, and the presence of fungi at mammalian barrier surfaces might constantly shape immunity. Mucosal immunity to fungi has been explored largely in the context of fungal infections^{8,35,66–72} (Figure 2A). Yet very little is known about the role of fungi in influencing immunity during steady state or during dysbiosis (Figure 2B). In this section, we discuss mechanisms of antifungal immunity using the lower gastrointestinal tract as a model barrier surface, where despite their high abundance, fungi rarely cause infection whereas fungal dysbiosis occurs frequently^{16,17,21–24}.

The role of cell-mediated immunity

Cell types involved in innate and adaptive immune responses to fungi during infection have been well characterized at several barrier surfaces such as the oral mucosa, the lungs, the vaginal mucosa and the skin (Figure 2A)^{66,68,69,72–76}. Innate immune cells are important for the clearance of fungi and for the initiation of adaptive immune responses during fungal infections^{8,35,66–72}. Neutrophils, CCR2⁺Ly6C⁺ monocytes, CX3CR1⁺ mononuclear phagocytes, CD11b⁺CD103⁺ dendritic cells, natural killer cells and epithelial cells have important functions in antifungal immunity by having a role in recognition, phagocytosis and killing of fungi as well as through their indirect activation by cell-cell crosstalk^{66,68,69,72–78}. The activation of specific cell types or cell-cell crosstalk is further dependent on the specific barrier surface affected by fungal infection (Figure 2A). Whereas some fungi, such as *Candida* spp., are widely present and have the ability to infect several barrier sites, the distribution of other fungi is tissue-dependent²⁹. As a central hub of mucosal immunity, the gastrointestinal tract is naturally equipped with a cellular machinery to recognize and interact with the microbiota⁷⁹. The intestine harbours several subsets of phagocytes, which are known to respond to bacterial infections or to fluctuations in the bacterial communities⁷⁹. Although phagocytic cell populations probably sense mycobiota in the intestine, their role during fungal dysbiosis remains unknown.

In addition to cellular innate and adaptive immunity, humoral immune mechanisms such as the complement and antifungal antibodies play an important role in antifungal immunity⁸⁰ and might be involved in shaping the mycobiota at the mucosal surfaces. Innate immune cells are further equipped with an array of receptors necessary to sense and interact with fungi⁸.

The role of C type lectin receptors

Genetic evidence and experimental studies have underlined a central role for C-type lectin receptors (CLRs) in antifungal immunity, whereas Toll-like receptors (TLRs) and NOD-like receptors (NLRs) mostly play a secondary role^{5,81}. CLRs, such as dectin-1, dectin-2,

dectin-3, mincle and the mannose receptor, recognize several molecules present in the fungal cell wall^{5,81}. Spleen tyrosine kinase (syk) is the primary signal transduction molecule used by several of these receptors, and activation requires an immunoreceptor tyrosine-based activation motif (ITAM) or ITAM-like motif present within the receptor tail (in the case of dectin-1) or within an associated FcR γ adapter molecule (in the case of dectin-2, dectin-3, mincle)^{5,81,62}. Upon receptor engagement, ITAM motifs are phosphorylated by Src family kinases, leading to the recruitment and phosphorilation of syk and activation of a downstream signalling cascade including phospholipase C γ 2 (PLC γ 2), protein kinase C δ (PKC δ), the CARD9-Bcl10-MALT1 complex and the I κ B kinase (Figure 3). The mannose receptor is an exception and does not activate this pathway^{5,81}.

Dectin-1

The fungal β -glucan receptor dectin-1 has been widely studied as a crucial receptor for fungal phagocytosis, killing and cytokine response⁸². Mouse studies have revealed a protective role of dectin-1 in systemic candidiasis⁸² and in the control of fungi at several mucosal surfaces, such as the skin⁶⁹, the oral mucosa⁶⁷ and the lungs⁷². The role of dectin-1 in protection against *Candida* spp. infection appears to depend on specific fungal strains⁸³, suggesting that some fungi can mask their cell wall β -glucans and escape recognition. In humans, a mutation in *CLEC7A*, the gene encoding dectin-1, leads to poor dectin-1 expression and impaired cytokine production upon fungal re-stimulation⁷⁰. As a result, patients carrying this mutation suffer from recurrent vulvovaginal infections and **onychomycosis**, partially due to defective IL-17 production in response to fungi⁷⁰.

Dectin-1 might also control antifungal immunity during fungal dysbiosis. We have shown that dectin-1-deficient mice developed more severe colitis accompanied by mycobiota changes and overgrowth of opportunistic genera such as *Candida* and *Trichosporon*¹⁶. This phenotype was dependent on the inability of *Clec7a*^{-/-} mice to control *Candida* spp. during intestinal inflammation and treatment with the antifungal drug fluconazole ameliorated colitis^{16,84}. In situ examination of mouse colons revealed absence of hyphal growth in the lower gastrointestinal tract during *Candida* spp. colonization of wild-type mice and mice with defects in antifungal immunity¹⁶ (and unpublished observations; I.L., I.D.I.). By contrast, hyphal growth is commonly observed during *Candida* spp. infections at other mucosal sites^{66,69} and factors involved in hyphal elongation or yeast to hyphae transition have been shown to either aid fungal invasion (which is mediated by cell elongation 1 gene (*ECE1*))⁸⁵ or commensalism (which is mediated by Efg1p)⁸⁶. This suggests that colitis in *Clec7a*^{-/-} mice is not aggravated by a typical fungal infection, but that other aspects of host-fungal interactions affecting the mycobiota might be involved. Consistent with the mouse models, patients carrying a two-marker *CLEC7A* haplotype developed more severe refractory ulcerative colitis supporting a possible role of dectin-1 in severity¹⁶, but not susceptibility to intestinal disease⁸⁷. Furthermore, dectin-1 deficiency is also associated with increased colonization of *Candida* spp. in the gut and the development of graft-versus-host disease in patients who have received transplants^{88,89}. Altogether, these studies suggest that impaired antifungal immunity, *Candida* spp. overgrowth and fungal dysbiosis might be involved in several intestinal diseases.

The role of dectin-2

Dectin-2 has been implicated in the induction of T helper 17 (Th17) responses and protection from systemic candidiasis⁹⁰; however, its role in mucosal immunity is less explored. Dectin-2 mediated immunity might be important for the generation of fungi-specific Th17 responses to *Fonsecaea pedrosoi* and resolution of skin infection in a mouse model of **chromoblastomycosis**⁹¹. Immunity to the skin commensal *Malassezia* spp. is also partially controlled by dectin-2⁹². In the lung, dectin-2 might be involved in recognition of fungal and mite allergens⁹³, yet dectin-2 knockout mice do not develop more severe lung inflammation upon infection with fungi such as *Cryptococcus neoformans*⁹⁴. The role of dectin-2 in human antifungal mucosal defense is even less clear. A recent study described a polymorphism in *CLEC6A* (encoding dectin-2) that is associated with susceptibility to pulmonary cryptococcosis⁹⁵. However, it is uncertain whether dectin-2 is directly responsible for this phenotype.

The role of other CLRs

Other CLRs, such as mincle and dectin-3, that induce Syk-CARD9-Bcl10-MALT1-dependent signaling are also involved in mucosal immunity. Dectin-3 induces the expression of mincle upon specific stimulation⁹⁶ and forms heterodimers with dectin-2 to induce signalling presumably through Fc γ coupling (Figure 3)⁹⁷, suggesting that these receptors are expressed in an interdependent manner. Dectin-3- and mincle-deficient mice are all susceptible to *Candida* spp. systemic infections^{81,90,97}; however it is less clear whether these receptors are directly involved in mucosal immunity to fungi. Mincle, for instance, can recognize a lipophilic component of *Malassezia* spp. cell wall *in vitro*⁹², but whether such recognition of commensal *Malassezia* spp. occurs in the skin has not been determined.

Dectin-3, which is encoded by *Clec4d*, was recently shown to control intestinal inflammation in mice. *Clec4d*^{-/-} mice developed more severe colitis, which was associated with increased *C. tropicalis* burden and efficiently treated with fluconazole⁹⁸. Decreased numbers of Th17 cells, impaired phagocytosis by dectin-3-deficient macrophages and defective intestinal epithelial cell barrier in these mice can be partially responsible for *C. tropicalis* overgrowth. However, the mechanisms behind increased susceptibility to colitis remain unclear as several pro-inflammatory cytokines (including tumour necrosis factor) were reduced in the colons of *Clec4d*^{-/-} mice⁹⁸. Although the structure of the microbiota was not assessed in this study, augmented fungal burden and the increased relative abundance of *C. tropicalis* in the intestines of *Clec4d*^{-/-} mice indicate changes in the fungal community. Finally, the relevance of dectin-3 to human intestinal disease remains uncertain due to the lack of reports on genetic associations between *CLEC4D* polymorphisms and IBD.

The role of CARD9

CARD9 holds a key downstream position to several antifungal receptors such as the CLRs discussed above (Figure 3)⁸¹. As such, CARD9 is crucial for the induction of cytokine production and triggering of cellular immunity to fungi at several mucosal sites^{8,71,99,100}. Thus, biallelic mutations in *CARD9* have important consequences on the control of mucosal and systemic fungal infections^{8,71,101}. Although CARD9 might be involved in signalling via

receptors that sense stimuli other than fungi, mice and humans deficient in CARD9 appear to be specifically susceptible to fungal infections^{71,99–101}. CARD9-deficient patients often develop chronic mucocutaneous candidiasis characterized by persistent and recurrent *Candida* spp. infections of the mouth, skin, vagina and other mucosal surfaces^{8,71}. *CARD9* mutations can also lead to severe onychomycosis, **phaeohyphomycosis** and **deep-seated dermatophytosis**, affecting deeper subcutaneous tissue^{8,102}. Fungal infection in these patients are caused by *Trichophyton* spp. and *Phialophora* spp. and can expand to affect lymph nodes and bones^{8,102,103}. These rare, but severe effects of CARD9 deficiency suggest a central role for this adaptor protein in controlling mucosal and systemic immunity to fungal infections in humans.

Additionally, CARD9 has been implicated in IBD. A nonsynonymous single nucleotide polymorphism in *CARD9* has been detected with high frequency in both Caucasian (at a frequency of 53%) and African (at a frequency of 25%) populations^{104,105}. This polymorphism is strongly associated with the risk of developing both Crohn's disease and ulcerative colitis¹⁰⁶. Using experimental models of colitis, it was shown that CARD9 signaling can be protective against fungi that come in contact with the intestinal mucosa during intestinal barrier damage¹⁰⁷. *CARD9*^{-/-} mice had increased antifungal antibodies in serum and colitis could be partially ameliorated by antifungal treatment, suggesting fungal involvement in the observed phenotype¹⁰⁷. Furthermore, both the fungal and bacterial gut communities in *CARD9*^{-/-} mice were altered¹⁰⁸. Interestingly, *Candida* spp. and *Aspergillus* spp. were hardly detectable in *CARD9*^{-/-} mice, whereas *Sporobolomyces* genus was the most prevalent group of fungi¹⁰⁸, suggesting that *C. tropicalis*, which expands in dectin-1 and dectin-3 deficient animals^{16,98}, was not the main driver of dysbiosis during CARD9 deficiency. Consistently, human mycobiota studies failed to establish a positive correlation between CARD9 polymorphism and *Candida* spp. overgrowth, which are otherwise present in patients with IBD¹⁰⁷. Finally, besides the CARD9 polymorphism reported above^{104,105} other CARD9 deficiencies in humans have not been associated with predisposition to IBD.

While some CARD9 mutations are accompanied with a defect in the production of interleukin-17 (IL-17)⁷¹, other mutations do not affect IL-17 mediated immunity¹⁰⁹. Consistently, mechanistic studies in mice demonstrated that a subtle tuning and timing of CLR-CARD9-IL-17 mediated response to fungal infections might be taking place^{100,74}. Although CARD9 has a clear role in adaptive Th17 cell responses to oral *C. albicans* infection, it was not required for IL-17-dependent innate immunity in this model¹⁰⁰. In addition, the role of CARD9 in the late stages of mucosal immunity to fungi has been described during *Aspergillus fumigatus* lung infection⁷⁴. Of note, CARD9 has a redundant role in the induction of Th17 immunity during systemic infection with other *Candida* species, such as *C. tropicalis*¹¹⁰. Whether CARD9 can influence Th17 cell responses during mucosal infection with other non-*Candida* opportunistic fungi remains to be elucidated.

The role of IL-17 and IL-22

While more studies are needed to define CARD9-dependent and CARD9-independent regulation of mucosal IL-17 responses to fungi, the role of this cytokine during mucosal fungal infections has been evidenced by multiple human genetic and mouse experimental

studies summarized in several recent reviews^{80,81,111}. Despite the excellent body of experimental work, it is still unclear whether the IL-17 pathway has an effect on commensal fungal communities. A proof-of-concept clinical study exploring IL-17A blockage with **secukinumab** in patients with Crohn's disease identified an unexpected higher rate of fungal infections among the treated individuals¹¹². This adverse outcome correlated with augmented intestinal pathology and suggests a possible role of IL-17 in controlling fungal communities; IL-17 could mediate the production of antimicrobial peptides (AMPs) by epithelial cells, which has been shown to clear *Candida* spp. infection in the oral mucosa⁷⁸. Similar to IL-17, IL-22 acts on epithelial cells as a potent inducer of AMPs and is protective against mucosal *Candida* spp. and *Aspergillus* spp. infections^{66,68,72,73}. These barrier enhancing properties might explain the indispensable role of IL-17 and IL-22 in the protection against fungal infection at the mucosal surfaces and provide strong rationale to explore the role of these pathways in relation to fungal communities in the gut.

Other contributors

Several other molecules, including the cellular machineries such as NLRP3, NLRC4, and Caspase 1 — which are involved in the processing of pro-IL-1 β into its biologically active form — and IL-1 β , have been explored in the context of mucosal immunity to fungal infections^{67,80,113}. NLRP3 and NLRC4 inflammasomes are protective in mouse models of vaginal *Candida* spp. infection^{67,80,114}. Furthermore, genetic polymorphisms in NLRP3 are associated with susceptibility to recurrent vulvovaginal candidiasis, increased IL-1 β production and hyper-inflammation^{115,116}. Fungal sensing by NLRP3 inflammasomes seems to be morphotype dependent with a preferential activation by *Candida* spp. hyphae⁸⁰. However, whether any of these molecules is involved in control of the mycobiota during steady state or upon dysbiosis remains unclear.

Despite our growing knowledge of antifungal immunity and available antifungal drugs, a rapid rise of fungal infections has prompted the search for novel immunomodulatory strategies to induce immunity to fungi. A recent study demonstrated that inhibition of JNK1 promotes expression of CD23 (a recently discovered CLR), production of nitric oxide and a potent antifungal effect both in vitro and in vivo¹¹⁷. E3 ubiquitin ligase CBLB that ubiquitinates SYK, was shown to regulate antifungal immune responses downstream of dectin-1 and dectin-2 (Figure 3)^{118,119}. Genetic deletion or peptide-based inhibition of CBLB promoted strong antifungal response, enhanced fungal killing and protected mice during systemic and cutaneous *C. albicans* infections¹¹⁸. Because of their downstream position of several antifungal receptors, CBLB or JNK1 blockage might be a therapeutic avenue to pursue in individuals with increased risk of fungal infections and gastrointestinal complications, such as patients who are immunosuppressed or are infected with HIV. Finally, several other CLRs, chemokine receptors and pathways have been studied in the context of antifungal immunity, however their role in mucosal immunity to fungi has not been explored.

Interplay between bacterial and fungal communities

Fungi and bacteria share similar niches on the mucosal surfaces and can undoubtedly influence each other. Interactions between fungi and bacteria can occur directly by physical contact and through secreted molecules, or indirectly through alteration of the host immune response. Several studies have attempted to identify inter- and intra-kingdom correlations between fungi and bacteria using next generation sequencing^{14,17,23}. However, this approach needs to be validated experimentally since correlations between relative abundances are at high risk of identifying false associations¹²⁰.

Experimental studies suggest that fungal colonization is modulated by bacterial communities, both clinically²¹ and in mouse models^{13,54}. Short term antibiotic treatment is sufficient to promote a persistent *Candida* spp. colonization in the mouse gut^{13,54} with immunological effects at distant sites such as the lung^{54,55}. Although antibiotic treatment rarely promotes the outgrowth of *Candida* spp. in the oral mucosa¹²¹, antibiotic-induced vaginal candidiasis is relatively common, suggesting a mucosal site-specific nature of bacterial–fungal interplay. Lactobacilli-induced reduction of pH or production of bacteriocin-like compound can inhibit the growth of *Candida* spp. and are both an essential barrier for the colonization of several fungal species in the vaginal mucosa^{31,122}. In addition to lactobacilli, other bacterial species including *Pseudomonas* and *Burkholderia*, as well as bacterial metabolites such as butyrate, can inhibit *Candida* yeast-to-hypha transition; a process needed for the formation of resistant biofilms of fungi and bacteria^{123,124}. However, fungal communities can adapt to the selective pressure exerted by the host and their bacterial neighbours. Analysis of the relatively stable fungal communities in the cystic fibrosis lung, suggests that *Candida* spp. clones were resistant to the filamentation-repressive effects of *Pseudomonas aeruginosa* through a mutation in the repressive gene NRG1⁵¹. Although the formation of biofilms might contribute to the morbidity of both oral and vaginal candidiasis^{32,124} such biofilms have not been detected in the gut^{16,125}, which is probably due to inability of *Candida* to switch to hyphal growth in this environment. According to a recent study, *C. albicans* adopts a third ‘GUT’ (gastrointestinally induced transition) morphology in the intestines that cannot induce systemic disease and is metabolically accustomed to the intestinal environment¹²⁵.

In addition to *Candida* spp., other fungal species can compete for the establishment in an environmental niche. Compounds secreted by *Pichia* spp. can inhibit the *in vitro* growth of *Candida* spp., *Aspergillus* spp. and *Fusarium* spp., the formation of biofilms by *Candida* spp. and reduce the fungal burden in a mouse model of oral candidiasis³². Similarly, bacteria can affect fungal colonization indirectly through the modulation of the host immune response. For example, in mice, *Bacteroidetes thetaiotamicron* prevents *C. albicans* colonization of the gut via the HIF-1 α mediated production of the antimicrobial peptide CRAMP¹²⁶.

The host immune response to fungi might in turn indirectly influence bacteria in the gut. A recent study demonstrated that in the absence of commensal *Candida* spp., *Clec7a*^{-/-} mice are protected from colitis through a mechanism involving intestinal bacteria⁸⁴. The authors found that in the absence of *Candida* spp., antimicrobial peptides targeting gram-positive bacteria were reduced in the colons of *Clec7a*^{-/-} mice, leading to an increase in commensal

Lactobacillus murinus that induced regulatory T cell expansion and protection from colitis. However, upon colonization with *C. tropicalis*, this potentially beneficial effect was lost and *Clec7a*^{-/-} mice developed more severe intestinal inflammation⁸⁴. Similarly, *CARD9*^{-/-} mice that are susceptible to fungi^{99,107}, also carry altered populations of tryptophan metabolizing bacteria including reduced lactobacilli¹⁰⁸. This correlated with decreased aryl hydrocarbon receptor (AHR) ligands and defective *Il22*, *Reg3g*, and *Reg3b* expression in the colons of *CARD9*^{-/-} mice that could be rescued by supplementation with lactobacilli¹⁰⁸. The protective role of lactobacilli through the production of AHR ligands and stimulation of IL-22 release by ILCs has been previously described as a mechanism promoting *Candida* spp. colonization resistance in the gut¹²⁷. Whether fungal and bacterial communities influence each other during these cases of apparent interkingdom dysbiosis in genetically susceptible host remains unclear. Nevertheless, such studies suggest a more complex microbial interkingdom relationship and possibly a dual role of antifungal immunity pathways in the control of bacterial and fungal populations in the gut.

Finally, specific disruption of the healthy gut fungal community can also have an adverse effect to the host health. We and others have recently demonstrated that prolonged antifungal treatment of healthy wild-type mice exacerbated immune-mediated disease in several experimental models of colitis and lung allergy, leading to the expansion of neutrophils, monocytes, Th1 cells, Th17 cells (which was shown in a colitis model) or eosinophils, Th2 cells and IgE-producing B cells (which was shown in a lung allergy model, Figure 2B)^{24,55}. Analysis of the mycobiota from mice treated with fluconazole and amphotericin B revealed dramatic changes in the composition and diversity of the gut mycobiota, suggesting the induction of gut fungal community dysbiosis²⁴. Oral supplementation with *Aspergillus amstelodami*, *Epicoccum nigrum* and *Wallemia sebi*, which expanded during fungal dysbiosis, was sufficient to recapitulate the detrimental effects of antifungal drugs on inflammatory disease. In addition to the mycobiota, drug-induced fungal dysbiosis may affect bacterial community structure and genera such as *Bacteroides*, *Clostridium* and *Lactobacillus*, which are also decreased during fungal dysbiosis²⁵.

Concluding remarks

A fast growing number of studies are detailing the complexity of antifungal immune responses, yet most evidence focuses on fungal infections. Increasing evidence indicates that human barrier surfaces harbour diverse communities of fungi that cohabit with the host throughout its life. Although opportunistic fungi within those communities can cause pathologies in susceptible individuals, the majority of host–fungal interactions at the body surfaces are non-infectious. This raises several questions: Do commensal fungi, similar to bacteria, contribute to shaping and maintaining immune homeostasis? Which pathways and intercellular networks are involved in sensing commensal fungi during the steady state? The characterization of immune responses in presence of dysbiotic and steady state fungal communities might provide key answers. Future studies on the crosstalk between the mycobiota, fungal metabolites, and epithelium and mucosa resident immune cells will propel the field toward understanding how mycobiota interacts with host immunity at the barrier surfaces. Understanding the mechanisms behind fungal dysbiosis might provide

diagnostic and therapeutic targets by identifying fungi that could or should not be targeted during inflammatory conditions.

Interkingdom microbiome interactions involving fungi can also modulate immunity indirectly by affecting microbiome function and metabolism. The application of metagenomics, metatranscriptomics and metabolomics holds promise to begin identifying the mediators of interkingdom interactions; however, the use of experimental approaches to validate the mediators remains crucial. The fast growing field of antifungal immunity has entered a new age in which a focus on complex microbial communities rather than single fungal species will shed light on the role of mycobiota in homeostasis and inflammation. Collectively, an increasing body of evidence suggests a crosstalk between gut fungi, bacteria and the host mucosal immunity. Disruption of this network can contribute to interkingdom microbial community alterations with detrimental consequences to the host and thus position fungi in the crossfire between mucosal immunity and commensal bacteria.

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Glossary

Mycorrhiza

a symbiotic relationship between a mycorrhizal fungus and the root of a plant.

Symbiosis

an ecological relationship between different species that persistently live in close contact. Symbiosis include relationships such as mutualism, parasitism and commensalism.

Dysbiosis

a generalizing term initially coined by Ilya Metchnikoff indicating a disruption of a microbial community resulting in a shift from its composition at steady state. Dysbiosis can be caused by multiple factors and can be either a cause or a consequence of disease. The term is undergoing revision in light of the recent advances of microbiome science.

Internal transcribed spacer (ITS)

a sequence in the fungal genome positioned between 18S and 5.8S (ITS1) or 5.8S and 28S (ITS2) fungal rDNA widely used in mycobiome next generation sequencing. Because of their variability, the ITS regions are often sufficiently different to classify fungal genera and species.

Onychomycosis

a fungal infection of the fingernails or toenails.

Chromoblastomycosis

a chronic, localized, infection of the skin and subcutaneous tissue caused by pigmented fungi containing sclerotic bodies.

Secukinumab

a human blocking monoclonal antibody that binds to interleukin-17A.

Inflammatory Bowel Disease

a relapsing and remitting condition of complex etiology characterized by inflammation of the lower digestive tract with possible extraintestinal manifestations. The most common types are Crohn's Disease and Ulcerative Colitis.

Phaeohyphomycosis

a heterogeneous group of fungal infections characterized by the presence of pigmented fungal cells.

Deep-seated dermatophytosis

a fungal infection of deep keratinized tissue (including skin, hair, and claws).

Alpha diversity

a biodiversity measure of the mean species diversity within an individual environmental habitat.

Operational taxonomic units (OTUs)

clusters of marker gene sequences (e.g 16S or ITS) based upon sequence similarity used for taxonomy-independent community analysis.

Commensalism

a relationship between two species where one organism benefits from the other without affecting it.

Richness

the number of different species represented in an ecological community.

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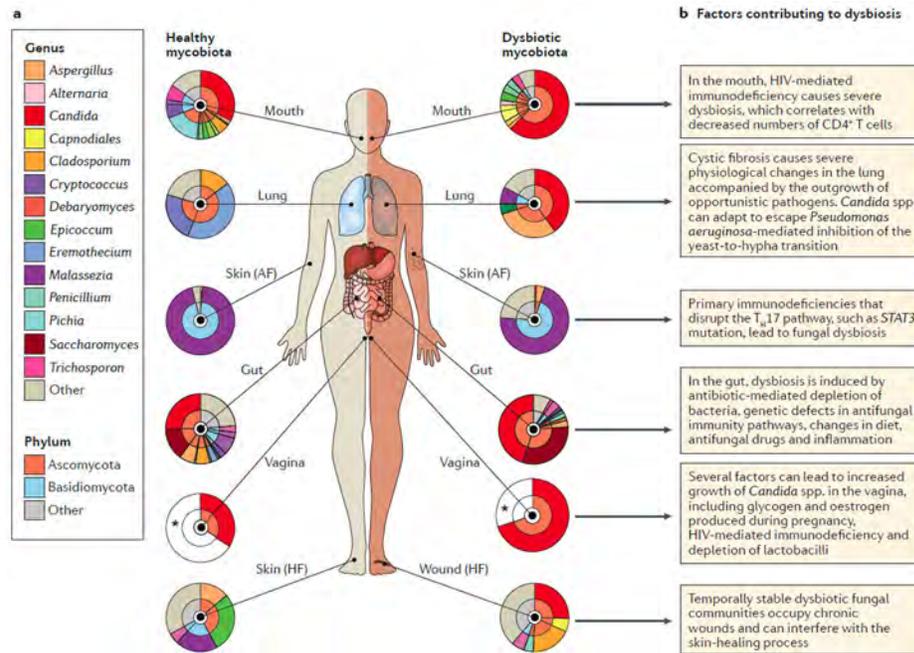


Figure 1. The mycobiota during health and in dysbiosis

A) During homeostasis, diverse fungal communities reside all human barrier surfaces during homeostasis, such as the mouth³²; lung⁵³; skin⁹, gut²² and vagina⁵⁷ (left hand side). The pie charts represent the relative abundance of the observed taxa at the phylum and genus levels (inner and outer circles, respectively). Of note, the data for the vagina are estimates that are based on culture-dependent studies, due to a lack of sequencing-based studies related to disease conditions (indicated with an asterisk). ‘Other’ refers to sequences with <5% relative abundance. During diseases, those communities are perturbed (right hand side). Dysbiotic fungal communities are observed in the oral cavity³² and the vagina⁵⁸ of HIV patients; in the lung of cystic fibrosis patients⁵¹; on the skin of primary immunodeficiency⁶¹ and chronic wounds⁶³ patients; in the gut of Crohn’s disease²² patients. **B)** Factors contributing to fungal dysbiosis at different barrier surfaces. AF, antecubital fossa; HF, hind foot; STAT3, signal transducer and activator of transcription 3.

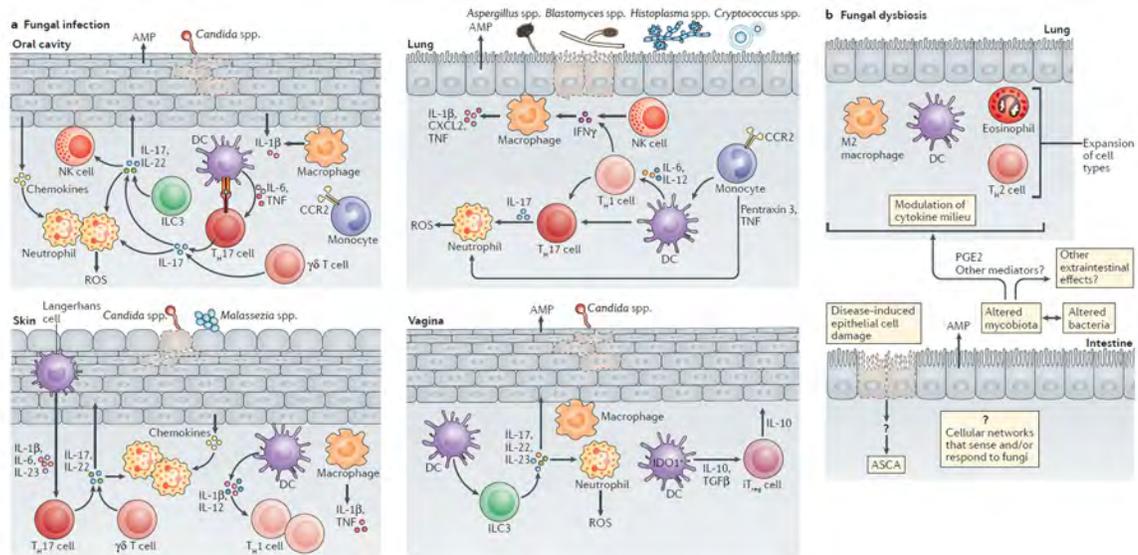


Figure 2. Examples of mucosal immune responses to fungal infection and dysbiosis

The host barrier surfaces are inhabited by mycobiota. Several sites, such as the skin, lung, oral cavity and the vagina are prone to fungal infections and fungal dysbiosis upon compromised host immunity, whereas the gastrointestinal tract is resistant to fungal infections, but susceptible to fungal dysbiosis. Host mucosal immunity has evolved to distinguish fungal infection from fungal dysbiosis. **A)** Fungal infection leads to breaches in the epithelial surfaces due to fungal activity, which in turn leads to rapid infiltration of neutrophils and monocytes at the site of infection. The production of cytokines and chemokines by epithelial and innate immune cells leads to further influx and recruitment monocytes, neutrophils and T cells among others. Resident phagocytes such as macrophages and dendritic cells (DCs) recognize and process fungal antigens and promote fungal specific T cell responses and polarization. Innate lymphoid cells (ILC3) can respond directly to fungi or to the inflammatory environment during infection with production of IL-22 and IL-17. Epithelial cells produce antimicrobial peptides that directly affect fungal survival. **B)** By contrast, fungal dysbiosis is characterized by alterations in the mycobiota composition that might be both the cause and/or consequence of changes in the tissue environment or changes in the intestinal lumen. Fungal dysbiosis can influence both local and systemic immunity through several mechanisms including the modulation of cytokine milieu, the activation of different cell types and the release of metabolites. Fungal dysbiosis in the gut can influence immunity at distant sites such as the lung and contribute to allergy. Intestinal inflammation and breaches of the intestinal epithelial barrier caused by non-fungal triggers can lead to direct exposure to fungal antigens coming from the intestinal lumen and development of systemic IgG and IgA anti-*Saccharomyces cerevisiae* antibodies (ASCA). Mucosal immunity to gut fungi during dysbiosis is poorly explored and only a few molecules have been studied in this context. At the barrier surfaces, fungi and bacteria can affect each other; thus, microbiota dysbiosis is probably a collective feature of the complex crosstalk between the fungal and bacterial microbiota, and the host. CCR2, CC-chemokine receptor 2; CXCL2, CXC-chemokine ligand 2; IDO1, indoleamine 2,3-dioxygenase 1; IFN γ , interferon- γ ; iTreg,

induced T regulatory; NK, natural killer; PGE2, prostaglandin E2; TNF, tumour necrosis factor; TH, T helper.

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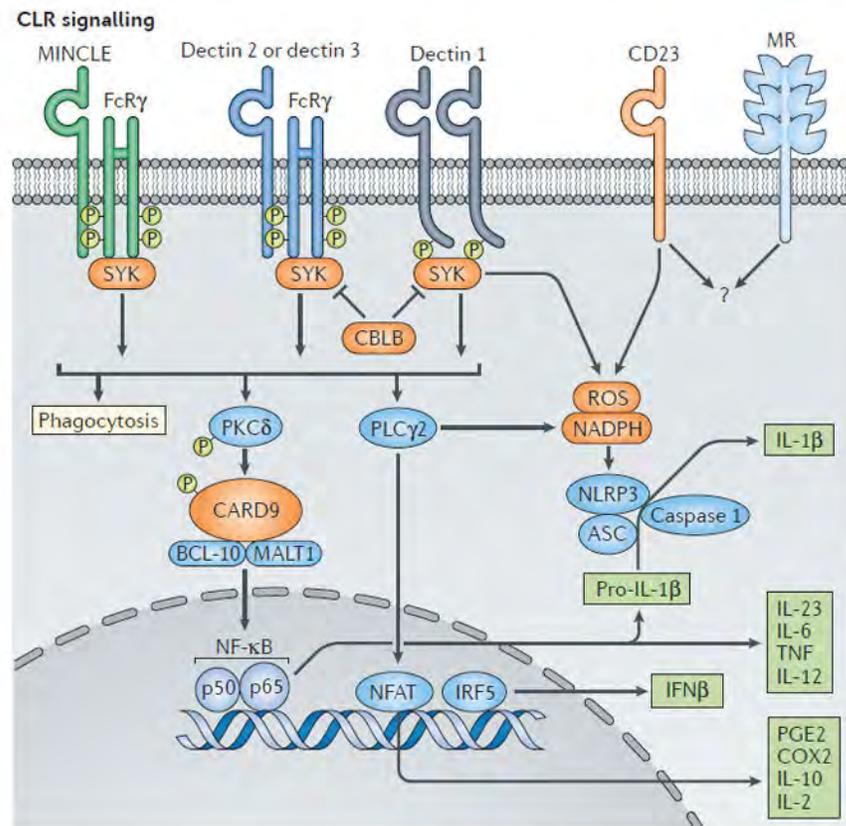


Figure 3. C-type lectin receptor (CLR) recognition and signaling

Fungal polysaccharides are recognized by C type lectin receptors (CLRs) such as macrophage-inducible C-type lectin (MINCLE), dectin 1, dectin 2 and dectin 2, resulting in the activation of spleen tyrosine kinase (SYK). The E3 ubiquitin ligase CBLB, which ubiquitylates SYK, regulates antifungal immune responses downstream of dectin 1 and dectin 2. CLRs trigger phagocytosis and respiratory burst (reactive oxygen species production, ROS) through the SYK-dependent phospholipase C γ 2 (PLC γ 2) activation of NADPH phagocyte oxidase and killing of fungi. Engagement of protein kinase C δ (PKC δ) followed by activation of the CARD9–BCL-10–MALT1 complex leads to nuclear factor- κ B (NF κ B) activation, caspase 1 activity and the production of cytokines and other mediators that are crucial for host defence against fungi. Dectin-SYK induction promotes IRF5-dependent IFN- γ production. CD23 is a newly identified CLR that is upregulated upon dectin 1 activation and that leads to the production of ROS. The signaling events downstream of CD23 and the mannose receptor (MR) remain unknown (indicated by a question mark). ASC, apoptosis-associated speck-like protein containing a CARD; CARD9, caspase recruitment domain-containing protein 9; CBLB, casitas B-lineage lymphoma-b; COX2, cyclooxygenase 2; FcR γ , Fc receptor common γ -chain; IFN β , interferon- β ; IRF5, interferon-regulatory factor 5; MALT1, mucosa-associated lymphoid tissue lymphoma translocation protein 1; NFAT, nuclear factor of activated T cells; NLRP3, NOD, LRR and Pyrin domain-containing protein 3; PGE2, prostaglandin E2; TNF, tumor necrosis factor.