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Microbiome-mediated regulation of anti-fungal immunity

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Anti-fungal immunity is characterized by the continuous interplay between immune activation and immune regulation processes. These processes have now been clearly shown not only in animal pre-clinical models but also in humans. To create and maintain this immune homeostasis, reciprocal interactions among the host immune system, fungal pathogens, and the microbiome are crucial. Notably, the microbiome exerts multiple direct and indirect antifungal effects that are particularly aimed at minimizing host tissue damage. Thus, in this microbiome era, the architecture of 3D culture system or 'tissue organoids' might finally represent a simple but effective *in vitro* 'holobiont' to unravel the diverse interactions and adaptations that evolve to overcome fungal infections.

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Introduction

Emerging fungal pathogens have recently had devastating effects on several plant and animal species [1]. Consequently, much research has focused on the condition of certain plant groups or wild animals that are not able to develop immunological resistance and mount "herd immunity" to protect the group population. In humans, the rapid increase of opportunistic fungal infections over the past four decades directly follows the global intensification in prevalence of immune-related diseases and medical therapy, mainly antibiotic use or invasive surgical procedures. A balance in the interactions between immune function and opportunistic fungal pathogen biology seem to be key to ensuring host survival in the fight against fungi.

Clinical reports show that manifestations of fungal infection can range from asymptomatic-mild mucocutaneous to life-threatening infections. However, deep epidemiological investigations clearly show that fungal infections mainly affect patients with a severely compromised immune system, for example, lung transplant [2,3] and hematopoietic stem cell recipients, and patients with hematological diseases [4,5], cancer, or undergoing chronic corticosteroid treatment. The fungi that seem to cause severe human fungal diseases include the airborne fungus *Aspergillus fumigatus*, which induces chronic pulmonary Aspergillosis, invasive aspergillosis, fungal asthma and keratitis; the encapsulated fungus *Cryptococcus neoformans* that induces cryptococcal meningitis complicating HIV/AIDS; the human commensal yeast *Candida albicans*, which induces invasive candidiasis; *Pneumocystis jirovecii* that leads to severe pneumonia; and the dimorphic fungus *Histoplasma capsulatum* that causes histoplasmosis [6,7].

As described in plant pathology, the pathogenesis of fungal infections involves the interaction of two partners (the host and the fungal pathogen) and a contribution from the environment. This conception has been coined as the 'disease triangle' [8]. Recently, two additional concepts have been developed for the way, in which animal fungal pathogens interact with the host. One concept is the 'damage-response' framework, which emphasizes that the outcome of a pathogen-host interaction is determined by the amount of damage incurred by the host, suggesting that immunoregulation is strictly related to resistance to infections [9]. The other concept is related to the role of the microbiome and its metabolic products, which might impact on the host immune regulatory network [10•,11,12]. The microbiome might also have direct control on fungal fitness by direct/indirect interactions with the pathogen. In this review, we focus on how the host, fungal pathogens and the microbiome may reciprocally interact via direct or remote mechanisms, using selected metabolic products.

The holobiont

The paradigm recently described as the 'metaphysics of biology' defines host–microbiome multispecies systems as 'holobionts' [13,14]. In the holobiont, inter-kingdom interactions evolve to ensure general homeostasis. To understand how holobionts might control the constant exposure of human hosts to fungal pathogens, we must consider 'hologenomic' adaptations at different levels: between the host and the fungal pathogens, between the host and the microbiome and between the microbiome and fungi (Figure 1).

Host–fungal interactions: from innate sensing to adaptive tolerance

During the initial interactions between the host and fungi, the host immune system detects fungal particulates that are enriched with bioactive carbohydrates (such as β-glucans, mannan, and chitin) via pattern recognition receptors, including Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) [15]. The architecture of the fungal cell wall regulates complex communications with the host, as the fungus can alter the cell wall composition and change its ability to interact with host CLRs [16••]. The intracellular signaling that occurs upon CLR activation promotes the maturation and migration of dendritic cells (DCs) to lymph nodes. This effect occurs via intracellular signaling mediated by ITAM or FcRγ chains and CARD9/MALT1/Bcl10 complex recruitment. Transcription factors activated by the complex, as NF-κB, NFAT, and AP1 promote inflammation but also regulatory mechanisms, including determining the transcription of several co-stimulatory molecules and the release of polarizing cytokines from DCs [17]. A collaboration between CLRs exerts tight control over fungal burden [18]. Indeed, mice lacking CLRs are dramatically susceptible to systemic *Candida* infection compared to control mice, due to defective early innate immune responses. These mice develop hyper-inflammation to try to control excessive fungal growth, which likely results in multi-organ failure [18]. Thus, the absence of early sensing seems to delete the regulatory networks necessary to limit host tissue damage.

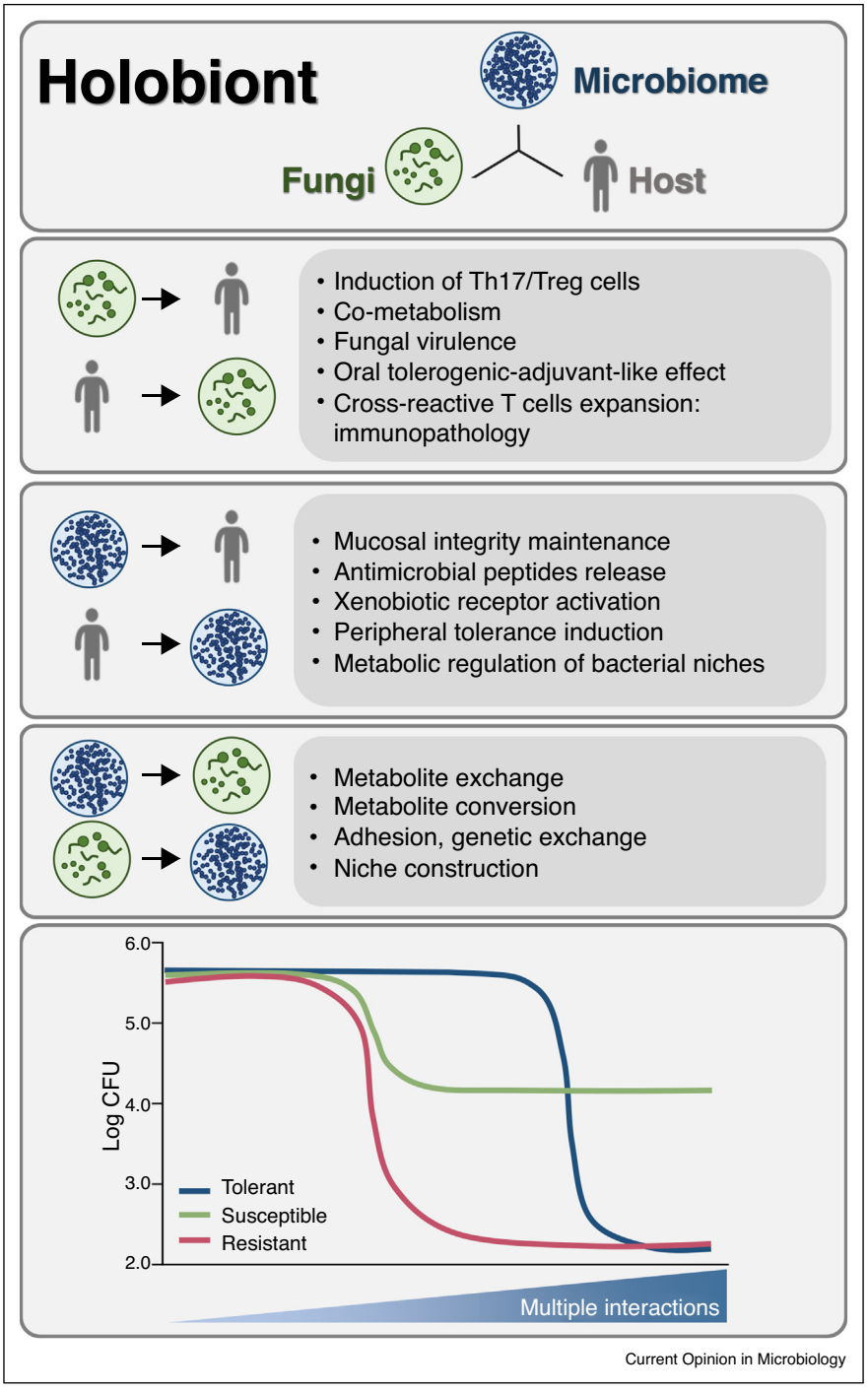
In terms of adaptive immunity, innate recognition leads to fungal-specific T-cell immunity against fungal infections in the blood, lungs, gut, and skin in animals and humans [19,20•,21••,22]. In the case of *C. albicans* infections, unrestricted Th17 activation may lead to host tissue damage and can predispose the host to airway respiratory diseases [20•]. Under steady-state conditions, Th17 protects the integrity of the cutaneous barrier while in the disrupted skin, such as in *Malassezia* skin infections, IL-23 and IL-17AF support *Malassezia*-induced inflammation [19]. Th17 cells seem to have a high vaccination potential. For example, in the case of *Cryptococcus gattii* infection, lung-resident memory T cells producing IL-17 confer protection upon second exposure to the fungus. A vaccination based on DC administration suppressed the fungal burden in the lungs and improved the survival of mice infected with *C. gattii* [23]. Thus, during the interaction between the host and fungal pathogens, inflammatory cell/pathway activation is often balanced by regulatory networks.

Excessive inflammatory responses can also alter fungal virulence or survival, affecting the outcome of the infection. For example, we demonstrated that high IL-17 levels impact on *Candida* gene expression and virulence via a direct interaction with the pathogen [24]. On the other side of the coin, *Candida* has been shown to recruit IL-17$^+$ cells via the release of candidalysin — a hyphal-associated peptide that damages oral epithelial cells [25]. Therefore, the reciprocal interactions between the host and the fungus may explain the immunopathology generated at the mucosal levels by dysregulated immunity. In human T cell physiological repertoires, particulate aereoantigens such as *Aspergillus* spores or *Candida*, induce specific circulating, stable Tregs and conventional T cells (Tcon), where the percentage of Tregs where dominants on Tcon. This effect is not induced by soluble antigens, as these do not occur in the form of particulates in fungi [22]. This induction of dominant Tregs supports the concept of a co-expansion of effector T cells and Tregs during the immune reaction against fungal pathogens to limit immunopathology. Strikingly, Th17 cells specific against *C. albicans* also drive immunopathology in the lung against *Aspergillus*. Thus, fungus–host interactions are cross-reactive but also protective where the fungus normally interacts with the host [20•,21••]. The ability of oral β-glucan-based prebiotics to induce Foxp3$^+$, IL-10$^+$, and IL-17$^+$ T cells *ex vivo*, underlines that the interactions between fungi and the host can have a direct impact on host tollerogenic responses [26]. Mutual host–fungal interactions are also mediated by nutrient availability, host-fungus co-metabolism and the delivery of secondary metabolites from the fungus [27,28]. In summary, reciprocal interactions exist between fungi and the host immune system. Inflammatory pathways and regulatory networks co-exist to control both the fungal load and host tissue damage. Mostly, in the environment, interactions between bacteria and fungi are essential for improving ecosystem performance [29]. Evidence supports that also in humans, as holobionts, multiple interactions among the host, fungi, and bacteria may increase the efficiency of the immune response against fungi (Figure 1).

Microbiome–host interactions: what we expect in case of fungal infections?

The last decade of research focused on the microbiome has shown that the evolving microbial–host associations are essential for ensuring human life on earth. This finding has been demonstrated in several physiological contexts. Indeed, dysbiosis might represent a key factor for the risk of developing obesity, autoimmunity, cancer and infections [30]. The Integrative Human Microbiome Project emphasized the importance of inter-kingdom associations in health and diseases [31]. We have demonstrated that in conditions of unrestricted availability of the essential amino acid Tryptophan, Lactobacilli increase host resistance against mucosal *Candida* infections, via the activation of the xenobiotic receptor Aryl hydrocarbon receptor (AhR) and type 3 Innate Lymphocytes (ILCs) [32]. In this context, we have highlighted a perfect 'triangle' of interactions among the host, the pathogen, and commensal microbes, where resistance against *Candida* is achieved by increasing the levels of the

Figure 1



The holobiont concept. Multispecies organisms evolve several biological adaptations together as a unique biological individual. In the upper panel, multiple type of interactions in the host-pathogen-microbiome triangle are shown. In the lower panel, it is plotted the outcome of fungal infection in three different human phenotypes, depending on the amount of multiple interactions occurring in the host.

commensal-driven cytokine IL-22 [32]. Direct interactions between *Candida* and Lactobacilli also show that they are natural competitors in the mucosal environment, where Lactobacilli exert fungistatic effects by using several metabolic products, such as lactic acid, acetate, or biosurfactants [33,34]. In contrast, fungi can have an 'antibiosis' effect against bacteria, by releasing antibiotics to restrain bacterial growth. In addition, bacteria and

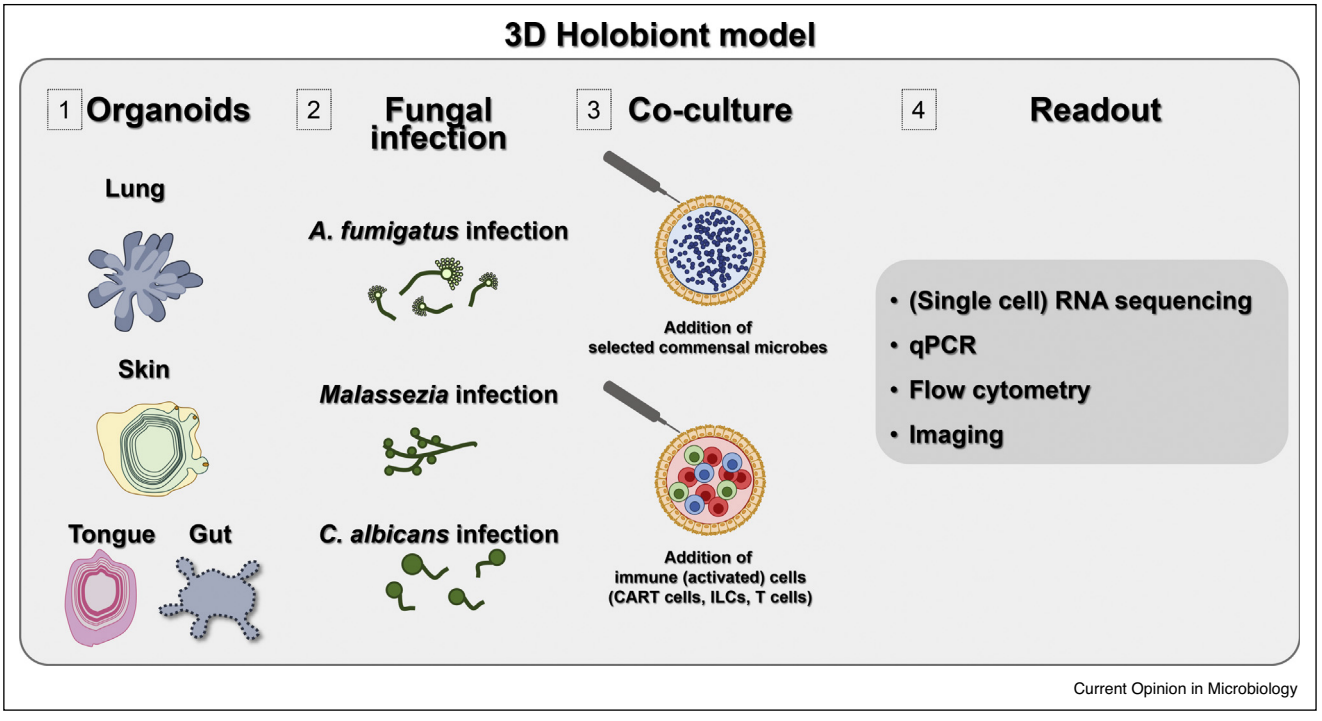
fungi interact by exchanging genetic fragments, metabolites and nutrients [10•]. Another demonstration that bacteria can form an axis of barrier immunity is that fecal microbiota transplantation prevents *Candida* gut colonization. These findings highlight the potential clinical applications of microbiota in preventing pathogenic fungal infections [35•]. In mice, complete gut resistance to *Candida* mucosal colonization is due to the commensal *Bacteroides thetaiotamicron*, which triggers barrier immunity against the fungus that is mediated by HIF-1α and LL-37 [36]. Interestingly, *Streptococcus oralis* induces *Candida* dissemination in a TLR2-dependent manner [37]. Thus, microbiota may also exacerbate *Candida* colonization and immunopathology when hyperinflammation at the infection site is induced.

Of note, the gut microbiota may also affect antifungal immunity in the lung. In a mouse model of invasive Aspergillosis, antibiotic vancomycin treatment demonstrated that gut dysbiosis reduces Th17 polarization in the lung, affecting host resistance [38]. As for gut Th17 polarization [39], segmented filamentous bacteria were responsible for Th17 polarization here. The microbiota might also affect the delay of onset of antifungal inflammation, predisposing the host to systemic infection. This effect has been observed in the case of pulmonary *C. gattii* infection [40]. Germ-free mice were infected with *C. gattii*. and antifungal immunity was compared to conventional mice. Under these circumstances, cytokine production and lung tissue damage were reduced [40].

A previously neglected niche of our microbiome is the respiratory tissue. Only recently, deep sequencing advanced technology revealed that the healthy lung is not sterile. The lung microbiota is enriched in bacteria, fungi, and viruses coming from mucosal secretions, aspiration of the nasopharynx and from the oropharynx. In the healthy lung, bacteria of the genera *Propionibacterium*, *Streptococcus*, *Haemophilus* and *Veillonella* coexist with the fungi *Aspergillus*, *Penicillium*, and *Candida*, without establishing an infection [41]. This paradigm is now not surprising if we consider the multiple known interkingdom interactions in other tissues. A gut and respiratory microbiome axis has also been deeply investigated over the past few years. Both niches interact via immune and metabolic pathways and affect lung pathogenesis of asthma, cystic fibrosis and lung cancer [42–44].

Interestingly, on the basis of the type of lung microbiome, humans can be divided into different pneumotypes [45••]. One pneumotype is characterized by a high bacterial load and supraglottic predominant taxa, such as the

Figure 2



A 3D organoid holobiont. Different tissue organoids may be obtained from human iPSCs or adult stem cells and infected with fungal pathogens to closely resemble the *in vivo* situation where infection may occur (1-2). A mixed co-culture may be obtained by using microinjection of immune cells with potential anti-fungal activity and selected taxa obtained by metagenomics studies on the target tissue of the infection (3). Immune cells may be innate cells as ILCs, monocytes, neutrophils as well as bioengineered CAR T cells or previously activated T cells (Th17, Treg). Possible readout on the 3D organoid-coculture is shown (4).

anaerobes *Prevotella* and *Veillonella*. Another pneumotype is characterized by a low bacterial burden and environmental taxa. In a multi-center study, the UniFrac distance to the upper airway showed a significant inverse correlation with the percentage of Th17 cells and a positive correlation in the predominant supraglottic taxa pneumotype. Thus, Th17 cell polarization and expansion associates with a lung microbiome particularly enriched of oral taxa [45••]. It remains now to be determined how the different human pneumotypes respond to pathogen challenges, particularly those inducing lung chronic inflammation, such as *Aspergillus* infection.

We recently built a multi-center, prospective study where we recruited patients diagnosed with hematological malignancies. We collected nasal and pharyngeal swabs and as expected from previous studies, we found a higher level of microbial richness in the pharyngeal compared to nasal microbiome. In addition, patients with a high risk of developing fungal infections showed a lower level of microbial richness with a decreased abundance of strict anaerobes, such as Clostridiales, and a relative expansion of the genera *Veillonella*, *Enterococcus*, and *Lactobacillus* (*paper submitted*).

Overall, studies focused on microbiome-host interactions highlight the idea that both resistance or tolerogenic immune responses may be activated while the fungal infection occurs. Therefore, a possible scenario may be that multiple interactions among fungi, bacteria, and the host will determine a tolerant holobiont, while altered or reduced amount of interactions as in case of dysbiosis or immune disorders may result in host susceptibility to pathogens. We have shown that when tollerogenic immune responses are missing as in *Ido1*-deficient mice, the host acquires a resistant phenotype characterized by antifungal barrier immunity supported by increased amount of Lactobacilli in the gut and the release of host IL-22 (Figure 1, lower panel) [32].

A 3D holobiont model

Nowadays, different *in vitro* models using novel and advanced biotechnology approaches may be used to study multiple interactions among the host, pathogens, and microbiome. Epithelial organoid cultures, whether derived from iPSCs or adult stem cells, constitute one platform for immunological research for numerous applications [46]. 3D organoid systems may be used as a mini-holobiont (Figure 2), where it is possible to readily dissect the different functions of the key points of the triangle: the host, the microbiome and the pathogen(s). Indeed, epithelial composition changes upon infection, as does immune-cell activation under the influence of the microbiome. We have already proposed the use of human 3D lung and intestinal organoids originated from iPSCs to model mucosal tissue inflammatory processes by adding primary human monocytes. Here, organoids have been infected with *Aspergillus* and respond to PRR stimulation by expressing and releasing several cytokines (Clinical and Translational Immunology, PMID: 32377340). In the future, optimization of triple culture organoids will be fundamental to the study of these interactions in infections and the development of new therapeutic approaches.

Conclusions

Even though there is a broad scientific interest in understanding the power of the Earth's microbiomes, knowledge gaps delay their effective use for addressing urgent medical and environmental challenges. Further studies are warranted to determine the dynamics of the microbiome following pathogen challenge. How the microbiome changes local replication and how the stability of the enterotypes or pneumotypes may vary within individuals during an infection it is still a matter of debate. A key point will be to decipher the functional meaning of microbiome-dependent asymptomatic local inflammation and how this might contribute to long-term tissue health.

Conflict of interest statement

Nothing declared.

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CRediT authorship contribution statement

Teresa Zelante: Conceptualization, Writing - original draft, Writing - review & editing. **Claudio Costantini:** Investigation. **Luigina Romani:** Project administration, Resources, Funding acquisition.

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- • of special interest
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