Studying fungal pathogens of humans and fungal infections: fungal diversity and diversity of approaches

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Guilhem Janbon[1], Jessica Quintin[2], Fanny Lanternier[3], and Christophe d'Enfert[4]

[1]Unité Biologie des ARN des Pathogènes Fongiques, Département de Mycologie, Institut Pasteur, Paris, 75015, France

[2]Groupe à 5 ans Immunologie des Infections Fongiques, Département de Mycologie, Institut Pasteur, Paris, 75015, France

[3]Unité de Mycologie Moléculaire, Département de Mycologie, Institut Pasteur, UMR2000 Paris, 75015, France

[4] Unité Biologie et Pathogénicité Fongiques, Département de Mycologie, Institut Pasteur, USC2019 INRA, Paris, 75015, France

Abstract

Seminal work by Louis Pasteur revealed the contribution of fungi – yeasts and microsporidia to agroindustry and disease in animals, respectively. More than 150 years later, the impact of fungi on human health and beyond is an ever-increasing issue, although often underestimated. Recent studies estimate that fungal infections, especially those caused by Candida, Cryptococcus and Aspergillus species, kill more than one million people annually. Indeed, these neglected infections are in general very difficult to cure and the associated mortality remains very high even when antifungal treatments exist. The development of new antifungals and diagnostic tools that are both necessary to fight fungal diseases efficiently, requires greater insights in the biology of the fungal pathogens of humans in the context of the infection, on their epidemiology, and on their

role in the human mycobiota. We also need a better understanding of the host immune responses to fungal pathogens as well as the genetic basis for the increased sensitivity of some individuals to fungal infections. Here, we highlight some recent progress made in these different areas of research, in particular based on work conducted in our own laboratories. These progresses should lay the ground for better management of fungal infections, as they provide opportunities for better diagnostic, vaccination, the development of classical antifungals but also strategies for targeting virulence factors or the host.

In 1860, Louis Pasteur is solicited by Napoleon III to cure a “disease” affecting wines exported to England. His work will eventually result in the identification of a yeast (*Mycoderma vini*) as the agent responsible for the transformation of the sugar of the grape in alcohol and of a bacterium (*Mycoderma aceti*) as the agent responsible for the acidification and thus the wine “disease”. He then proposed to heat the wine to “cure” the disease setting the bases of the pasteurization principle. Some years later, he demonstrated the implication of the microsporidia *Nosema bombycis* in the Silkworm disease that was then devastating the silk industry in France and he found a way to limit contagion. These founding discoveries lifted for the first time the veil on microscopic fungi and resulted some years later in the spectacular extension of the fungal kingdom way beyond the well-known mushrooms. Nowadays, about 120,000 species of fungi have been described in varying degrees of details using classical botanical description (1) but recent advances in sequencing technologies and computer sciences associated with the analysis of a large set of very diverse biotopes spectacularly changed our vision on fungal diversity. A recent estimate of nearly 4 million predicted fungal species makes this kingdom the most diverse of the domain Eukarya (1, 2).

Some of these fungi have been used for the good of the human kinds. For ages, fungi have been used by humans to transform their nutriments mostly through fermentation. For instance, yeasts are used in the traditional and modern food industry and can also be exploited for the degradation of waste products or to produce industrially relevant products (3, 4). For centuries, traditional Chinese medicine uses fungi for healing and currently, interests have focused on polysaccharides, that are a crucial component of fungi cell walls (5). Within the multitude of polysaccharides present, β-glucans are a key reason fungi are used in cosmetics, as food additives or as medicinal purposes (6); they have also shown beneficial effects in the outcome of various diseases (7). Other fungi are threats for the crop industry and for the

environment. One can cite *Magnaporthe oryzae* that causes rice blast disease which is responsible for approximately 30% of rice production losses globally (8) or *Fusarium oxysporum* that might destroy the entire banana industry in the world in the coming years (9). Climate change and human driven extensive continental fauna exchanges are triggering sudden changes in fungal ecology sometimes associated with spectacular decline of some animal species. Thus, *Batrachochytrium dendrobatidis* and *B. salamandrivorans* are responsible for the stunning decline of frog and salamander species in different parts of the world including Europe and *Pseudogymnoascus destructans* causes the White-Nose Syndrome which killed millions of bats in the United States and Canada in 2018 (10, 11).

Pathogenic fungi also affect human beings. Whereas superficial fungal infections are usually benign, invasive infections are much harder to treat and they have an astonishing impact on human health, being responsible for major mortality rate. Although human fungal diseases have been neglected, the most recent studies estimate that they kill more than 1.6 million people every year (12). The impact of fungi on human health is an ever-increasing issue. Invasive or chronic fungal infections affect 4.9 million persons every year in the word (13). The financial impact is proportionally high. For instance, fungal diseases have been reported to have costed more than $7.2 billion dollars in the United States in 2017 (14). The epidemiology of fungal infections is highly dependent on the type of patients affected and on the ecology of the pathogenic fungi. As such, for opportunistic fungal infections, the prevalence of fungal diseases can depend on the underlying associated diseases. Thus, *Pneumocystis* and *Cryptococcus* infections, which are associated with the AIDS outbreak, are prevalent in poor countries that do not have a general access to anti-retroviral therapy (15, 16). In contrast, invasive candidiasis and allergic bronchopulmonary or invasive aspergillosis, which are associated with cancer and surgery, are more often diagnosed in rich countries (17). The

epidemiology of primary fungal pathogens is mainly dependant on the natural prevalence of the fungi in the environment. For instance, Paracoccidioidomycosis is one of the most prevalent systemic mycoses in Latin america and its epidemiology is mainly restricted by the geographical distribution of different *Paracoccidioide*s species in these regions (18). For similar reasons, the burden of histoplasmosis is reaching that of tuberculosis in Latin America (19).

Fungal infections are in general very difficult to cure and the mortality remains very high even when validated antifungal treatments can be used (20). The first reason is that no efficient vaccine is yet available and the arsenal of antifungal molecules available is limited and not available in most countries. Actually, very few classes of antifungal molecules are used to treat fungal infections and there is concern about their toxicity. Notably, echinochandins are the only class of antifungal molecules that has been developed over the last 15 years. The underlying disease (AIDS, cancer, …) weakening the host immunity and type of patients (neonates, elderlies..) with altered immune responses and mostly affected by opportunistic fungal pathogens also explain therapeutic failures. Drug resistance is also an emerging issue. First, species naturally resistant to some antifungal drugs are emerging or show increased incidence in recent years due to large use of antifungal prophylaxis and use of more acute fungal identification tools (21). Second, prolonged antifungal treatments in clinics or intensive usage of antifungal drugs of the same classes in agriculture are associated with the emergence of resistance isolates (21, 22). The last reason is economical. As said above a large part of fungal infections affect poor people with limited access to antifungal drugs. For instance, the current guidelines for treatment of cryptococcocal meningitis published by the World Health Organization in 2018, are short-course induction regimen with amphotericin B deoxycholate and flucytosine, followed by long term fluconazole treatment. However, the

price and the absence of license strongly limit the access of these drugs in the countries the most impacted by this disease. Amphotericin B is thus not available in more than 25% of the countries and flucytosine cannot be obtained in more than 75% of them (23).

Lastly, the high mortality and morbidity associated with fungal diseases is due to the difficulties we have to perform early diagnostic. The failure exists in part because sensitive molecular tools to diagnose early and specifically this type of diseases are often lacking. In that sense, the fact that some commensal fungi of the human body such as *Candida albicans*, *Malassezia* sp. and very common environmental fungi such as *Aspergillus fumigatus* can also be responsible for invasive life threatening fungal infections renders these diagnostics challenging.

In order to fight fungal diseases, new diagnostic tools and antifungal drugs with improved efficiency are needed. In this context, we need a greater understanding of the virulence factors used by fungal pathogens and of the biology of pathogenic fungi during the infection, including their responses to variations in their environment. As said above a large number of fungi are present in the environment, some of them being natural components of the human microbiota, but only a very small subset of them can induce a disease (24). We still poorly know which specific modifications in their metabolism help them colonize and harm the human host. We need to improve our knowledge of the natural ecology of these fungi and the epidemiology of these infections. The development of an infectious disease also depends on the status and efficacy of the immune response of the host. Adaptive and innate immune responses have been both shown to play major roles to fight fungal infections but the mechanisms of their activation and their modulation by the fungal pathogens is a very active area of research. Finally, although recent progresses have been made in the understanding of the genetic bases of the variable susceptibility of the patients, number of questions remain.

In recent years, the genetics and the genomics of fungal pathogens as well as the immunology of the fungal infections have made spectacular progresses. Here, we provide some insights on these progresses, in particular based on work conducted in our own laboratories.

Fungal genomics: a revolution in the study of fungal pathogens

The turn of the twenty first century has seen the advent of fungal genomics with genome sequencing of selected isolates of the main fungal pathogen species: *C. albicans, Candida glabrata, C. neoformans* and *A. fumigatus* (25-28). Exploration of these genomes and specifically their gene content has provided significant understanding on their evolution and biology. Moreover, knowledge of these genomes has paved the way for functional genomics approaches in the main fungal pathogens of humans, with the development of approaches based on transcript profiling (microarrays and now RNA-Seq), proteomics as well as the establishment of collections of knock-out mutants (29-31). Examples of these approaches will be presented below.

While early investigation of genome sequences focused on the identification of coding sequences (CDS) mainly based on bioinformatics criteria, recent studies have now revealed that there is more complexity than originally anticipated and this should be taken into consideration when investigating fungal pathogens. Indeed, the multiplication of high throughput sequencing strategies has uncovered an astonishing potential of transcript diversity in fungal cells. The coding genome is not the only source of this diversity. Long and small ncRNAs of different origins have been widely identified in fungi although their exact number and qualities remain mostly to be described in fungal pathogens (27, 32-34). Moreover, some of them such as the circular RNAs (35) have not been yet identified in fungal pathogens and should add to the complexity of their transcriptome. Besides mRNAs, very little

information is available concerning the role of these different types of RNAs. Actually, only one lncRNA has been investigated in *Cryptococcus* (36) and although few papers report the central roles of siRNA in drug resistance regulation and genome homeostasis in different fungi (37-40) their roles is most probably much wider.

The diversity of the RNA molecules is further increased through transcriptional and post transcriptional mechanisms. For instance, alternative splicing has been described to be very common in *Cryptococcus* (41). One might predict that this mechanism is also prevalent in other fungi and more specifically fungal pathogens as their genes are known to be generally intron-rich (42); *Candida* genes being notable exceptions in that sense. Alternative transcription start and polyadenylation site usage have been shown to be prevalent in model yeasts (43, 44) and more recently in *Cryptococcus* (Janbon et al, unpublished data). Similar to alternative splicing, alternative transcription start and polyadenylation sites represent prominent sources of transcript diversity and they can regulate both coding and non-coding transcripts. Finally, RNAs have recently been shown to be affected by additional posttranscriptional modifications (45) and editing (46). Yet, these modifications have not been studied in fungal pathogens and the list of all potential transcripts present in a pathogenic fungal cell is far to be completely written.

Besides the known coding transcripts, the impact of expression of the other features on the biology of fungal pathogens has been poorly studied. Yet alternative splicing has been shown to poorly influence proteome diversity and appears to be more a means to finely tune gene expression in response to environmental cues (41). In contrast, several examples of regulated alternative start sites have been described suggesting that this mechanism might represent a major means to diversify the proteome in fungi (47, 48). This suggests that an important part of the proteome is still not described in this kingdom. A more complete description of these

peptides and proteins might have a major impact in our understanding of fungal virulence but also in term of biological active compounds and in diagnostic tools identification.

From one genome to many genomes

As mentioned above, pioneering work in fungal genomics focused on selected isolates, mainly those used in the laboratory as models. The introduction in the late 2000s of next generation sequencing, especially short-read technologies, has allowed cost-effective whole genome sequencing (WGS) of multiple isolates with consequences in epidemiology and our understanding of population diversity in the different fungal pathogens, which will be illustrated from work in our own laboratories.

Whole genome sequencing is becoming the method of choice in molecular epidemiology, progressively replacing other typing methods such as microsatellite analysis or multi-locus sequence typing (MLST). Indeed, WGS does not require the specific identification of molecular markers and an assessment of their suitability as markers of intra-species diversity. This approach is facilitated by the availability of reference genome sequences, whose number is increasing thanks to large-scale projects such as the 1000 fungal genomes project (http://1000.fungalgenomes.org/home/), the Y1000+ project (https://y1000plus.wei.wisc.edu/) or the Global Catalogue of Microorganisms (GCM) program, which aims to produce 2500 complete fungal genomes in the next 5 years (49). Yet, de novo genome assemblies are possible even using short read sequencing thus allowing genome-based molecular epidemiology to be conducted in the absence of a reference genome. An example of the impact of WGS in molecular epidemiology is provided by the recent work of Garcia-Hermoso et al. (50) who leveraged WGS in order to investigate small outbreaks of invasive wound mucormycosis in the hospital setting. These authors investigated

outbreak and non-outbreak isolates of *Mucor circinelloides,* an environmental mold responsible for mucormycosis in diabetic, immunocompromised and severely burned patients. Their sequencing of multiple isolates collected from a number of patients could reveal significant diversity at the genome level across these isolates, even within patients. Only occasional identity was observed that could correspond to direct transmission between patients or contamination with the same environmental source. Taken together, in this specific case, it appeared that infection mainly originates from a heterogeneous pool of strains from a cryptic environmental reservoir even though cross-transmission between patients cannot be excluded. In contrast to this study, WGS-based epidemiology can reveal a common source of infection in patients. For instance, Vaux et al. (51) could observe that an outbreak of fatal cases of invasive fungal infections due to *Saprochaete clavata* was due to closely related cases, possibly originating from a common source of contamination, most likely a medical device used for storage and infusion of blood products. These and other observations indicating that *Saprochaete clavata* can be associated with dairy products is highly relevant for the management of future outbreaks due to this newly recognized pathogen. These two examples clearly underline the potential of WGS for investigation of outbreaks due to fungal pathogens.

WGS is also revolutionizing our understanding of population structure in fungal pathogens. Large collections of isolates collected worldwide have been assembled for several fungal pathogens and analyzed by WGS. For instance, genome characterization of 387 *C. neoformans* isolates has provided insights in the mechanisms underlying speciation in this species complex, with cases of differential selective pressure between environmental and clinical isolates and loss of genetic diversity in some lineages (52). In another study, genome sequencing of 186 isolates revealed a relationship, between genetic lineage and clinical

outcome, with patients infected with the *C. neoformans* VNB lineage having significantly worse survival (53). Recent genome characterization of 182 *C. albicans* isolates (54) has shed new light on the population structure in this species and the mechanisms that underlie the diversity seen across isolates. Notably, *C. albicans* has long been proposed to reproduce clonally, maintaining a diploid state and the identification of a sexual stage in this species is lacking. The population genomics study conducted by Ropars *et al*. (54) is consistent with clonal reproduction in this species, as was already inferred from MLST and other molecular typing methods (55, 56). Indeed, the *C. albicans* population is divided in a number of genetic clusters (also referred to as clades) that are identified using a variety of molecular markers (55). Yet, population genomics has also revealed examples of recombination between these genetic clusters (54). This is consistent with the occurrence in the human host of a parasexual cycle that had been demonstrated in vitro and in animal models following the identification in the *C. albicans* genome of a mating-type-like locus that controls sexual identity (57). Parasexuality in *C. albicans* occurs by mating of sexually-compatible diploids, yielding tetraploids, and subsequent return to the diploid stage through random loss of chromosomes rather than meiosis. Interestingly, this second phase of the parasexual cycle, when conducted *in vitro*, results in the frequent formation of aneuploids, which are rare in the *C. albicans* population, suggesting that the parasexual cycle is a rare event or aneuploids have strongly reduced fitness compared to diploids. *C. albicans* haploids have also been described that are thought to arise from diploids through random chromosome loss events (57). These haploids have very low fitness and a tendency to autodiploidize. No example of natural autodiploids have been reported in population studies suggesting that, in the host, haploids and their autodiploids are rare or rapidly counter-selected.

Being mainly diploid, *C. albicans* isolates display heterozygosity. WGS has revealed that loss-of-heterozygosity is a prevalent mode of diversification within genetic clusters, as hypothesized earlier from MLST studies (54, 55, 58, 59). Indeed, all sequenced isolates show examples of loss-of-heterozygosity (LOH) that can encompass short regions (the consequence of gene conversion events), larger regions (the consequence of mitotic recombination or break-induced replication) or the entirety of a chromosome. Notably, recent work has revealed that each human individual harbors closely-related isolates that differ by a large number of short LOH events, suggesting that we are not colonized by a single clone but rather a pseudo-clone (60). While a necessity to cope with the adverse effects of DNA double strand breaks that result from different forms of stress, LOH can have positive effects on the fitness of *C. albicans* in some cases. For instance, LOH can contribute to increase the copy number of resistant alleles that are responsible for resistance to antifungals and hence lead to increased antifungal resistance (61). Such events can be followed by additional genome rearrangements, such as the formation of isochromosomes, leading to an additional elevation of antifungal resistance (62). Hence, genome rearrangements such as LOH and aneuploidies, appear as key amplifiers of antifungal resistance in *C. albicans* and this observation has been extended to other fungal pathogens (63-65). Alternatively, LOH can lead to homozygosity of loss-of-function mutations that contribute to adaptation of *C. albicans* to specific niches. For instance, accumulation of loss-of-function mutations in the *NRG1* gene that encodes a negative regulator of hyphal formation has been shown to accumulate in *C. albicans* isolates found in the lung of cystic fibrosis patients, suggesting that constitutive filamentation is favorable in this unusual niche (66). Inversely, accumulation of loss-of-function mutations in the *FLO8* gene that encodes a positive regulator of hyphal differentiation have been shown to contribute to increased fitness in the gastro-intestinal tract of mice lacking a functional microbiota,

suggesting that the yeast form is favored in these conditions (67). Of note, WGS has revealed that a specific cluster of *C. albicans* isolates, also known as *C. africana,* has emerged through major LOH events which resulted in the accumulation of homozygous loss-of-function mutations, some of which affecting genes known for their contribution to *C. albicans* virulence (54). *C. africana* isolates are restricted to the genital niche and only cause genital infections, which might be explained by the loss of key functions underlying *C. albicans* ability to occupy multiple niches following ancestral LOH events.

In summary, WGS has gained unparalleled importance in both epidemiology and our understanding of population structure and evolution of fungal pathogens. The example of *C. albicans* that we have developed here highlights major contributions of population genomics to our understanding of the mechanisms of diversification in this species and how these mechanisms can lead to specific phenotypes and adaptation to specific niches. Similar examples have arisen from population genomics studies of other fungal pathogens. As the number of genome-sequenced isolates increases, it is becoming possible to perform genotype-phenotype association studies that are deemed to reveal new genes that contribute to important virulence traits of fungal pathogens.

Functional genomics: the case of fungal morphogenesis and virulence

As mentioned above, characterization of the genomes of fungal pathogens has paved the way for the implementation of functional genomics in these organisms. Besides transcriptomics and proteomics, genome-wide collections of genetically-engineered mutants are now available for the main fungal pathogen species, among which *C. albicans, C. neoformans, A. fumigatus* (29-31, 68). The combination of these different approaches is shedding new light on many important biological processes in these species, including processes that are relevant

to their success as pathogens. Here we will provide examples on how these approaches have indeed provided insights on one of the most striking properties of fungal pathogens, *i.e.* their ability to undergo morphogenetic switches. Indeed, most fungal pathogens are able to adopt various morphologies that have different contributions in pathogenesis. For instance, *C. albicans* alternates between yeast and hyphal forms, the former contributing to attachment to surfaces and dissemination through the bloodstream while the latter is important for the invasion of tissues and escape from phagocytic cells (69). *C. albicans* can also adopt other morphologies such as the opaque cells dedicated to mating (see above), the grey cells and the chlamydospores, the latter being produced in nutrient-limiting, oxygen-limited, low temperature environments and possibly constituting a resistance form of *C. albicans* (69, 70). Transcript profiling of *C. albicans* cells undergoing the yeast-to-hypha transition has rapidly revealed that this transition is associated with the expression of a specific subset of genes, so-called hypha-induced genes, several of which encode cell wall proteins that play important roles in the interaction of *C. albicans* hyphae with host cells (71, 72)]. For instance, the *ALS3* gene encodes an adhesin that can interact with a variety of substrates including host cell cadherins (73, 74). This interaction triggers internalization of hyphae by host cells in a clathrin-dependent manner (75). Of note, *ALS3* is also a key player in the formation of biofilms contributing to intercellular interactions (60) and Als3 is currently used for the development of a vaccine preventing *C. albicans* infections (76). Another hypha-induced gene, *ECE1*, encodes for several secreted peptides among which candidalysin, a pore-forming toxin that contributes to damaging host cells and triggering host immune responses (77). Other hypha-induced genes encode cell cycle regulators such as the hypha-specific cyclin Hgc1 whose interaction with the cyclin-dependent protein kinase Cdc28 drives polarized growth in hyphae (78, 79). A large number of additional genes have now been involved in the yeast-to-hypha

transition. Of note, while it has long been thought that this morphogenetic switch was correlated to the ability of *C. albicans* to cause systemic infections, systematic analysis of 674 knock-out mutants by Noble *et al.* (80) has revealed that some mutants with in vitro defects in hyphal morphogenesis could remain unaffected for pathogenesis while other mutants fully competent for hyphal formation were defective for pathogenesis. This has led to the conclusion that, besides morphogenesis, *C. albicans* requires additional functions for pathogenesis such as a functional DNA damage response, metal ion homeostasis, and the ability to acquire nutriments such as lipids. While hyphal morphogenesis remains a central process in the establishment and progression of *C. albicans* disseminated infections (and generally other infections), its role in commensalism is more controversial. Indeed, it has been shown that mutants with defects in hyphal formation have increased fitness in the gastro-intestinal tract suggesting that hyphal morphogenesis is disadvantageous for commensalism (67). Yet, this is only observed in dysbiosed mice and hyphal morphogenesis appears important for GI colonization in eubiosed mice. Hence, hyphal formation may have evolved to facilitate commensalism in the eubiosed GI tract in the first place.

Characterization of *C. albicans* mutants has revealed a large number of genes that control positively or negatively the expression of hypha-induced genes (71, 72). It is remarkable that multiple environmental signals, signaling cascades and transcription factors are important for hyphal morphogenesis. Strikingly, many of the identified positive and negative transcriptional regulators of hypha-induced genes bind at the same promoters. For instance, we have shown that Efg1, arguably the transcription factors that is the most central to the yeast-to-hypha transition, Sfl1, Sfl2, Skn7, and Ndt80 all bind at the promoter regions of several hypha-induced genes, questioning how the regulation of these genes is achieved (81, 82). A similar phenomenon has been observed in the biofilm regulatory circuitry whereby 10 transcription

factors show cross-regulation and often bind at the same promoters of biofilm-induced genes (83, 84). Again, why expression of such genes requires the cooperation of so many transcription factors is an open question. It has been proposed that this may allow robustness of the transcriptional circuitry or fine-tuning of the response (83).

Similarly, the *C. neoformans* cell morphology is very dynamic which influences the pathogenicity of the cells. For instance, each *Cryptococcus* cells is surrounded by a polysaccharide capsule that constitutes its main virulence factor. Indeed, the presence of a capsule is absolutely required for virulence and acapsular mutants are unable to induce an infection. Capsule size and structure is highly variable both *in vitro* and *in vivo* (85). Over the past years, several elements have been identified as inducers, such as serum or low glucose concentration, or repressors, such as high iron or glucose concentration, of capsule formation (86). The recent advance in genomics and the usage of genome-wide screening have identified genes implicated in capsule biosynthesis (87) as well as signal transduction pathways implicated in these regulations (88). Interestingly, the screening of large collection of tagged mutants in a mouse model of cryptococcosis has also identified genes necessary for infection but without any identified capsule defect paving the way for the discovery of new virulence factors in this yeast (29, 89). In addition to the capsule, cell size is very dependent on the conditions of growth including the different organs in the host. The most spectacular example of this cell size dynamics in *C. neoformans* are Titan cells (90). These extremely large cells (up to 100 µm) are produced during the infection in the lung. They are polyploid and they possess a modified cell wall and capsule structure. Although identified some years ago, the production of Titan cells was restricted to in vivo conditions up to recently, thus preventing most of the genetic analysis of this morphological change. Recently, three groups of authors have reported the identification of in vitro conditions able to mimic partially the titanization process

(91-93) and the description of some genes implicated. Notably, Hommel and colleagues used a whole genome sequencing and comparison approach together with the screening of a gene deletion mutant collection to identify the cAMP/PKA/Rim101 pathway as a major regulator of Titan cell formation in *C. neoformans* (91).

The Host - Pathogen equilibrium

Invasive fungal infections mainly occur in immunocompromised patients. Invasive aspergillosis is associated with haematological malignancies, neutropenia, stem cell transplant, solid organ transplant, and intensive care unit patients. Targeted therapy as ibrutinib was also recently reported to be associated with invasive aspergillosis (94). Invasive candidiasis occurs in patients with neutropenia, preterm neonates but also with invasive procedures including surgery and central catheter. Cryptococcosis and pneumocystosis are associated with HIV infection. However, susceptible non-HIV population is rising including solid organ transplant patients, cancer and patients treated with corticosteroids or immunosuppressive drugs. By contrast with these acquired immune deficiencies, patients with inherited immunodeficiencies are also prone to develop invasive fungal infections (95). Severe combined immune deficiencies predispose to mixed infections including invasive fungal infections with pneumocystosis. Chronic granulomatous disease is due to a defect in polymorphonuclear oxidative burst and associated with an important risk to develop invasive aspergillosis. STAT3 deficiency is also associated with predisposition to aspergillosis (96). Autoantibodies directed against interferon gamma and GMCSF are associated with development of cryptococcosis advocating for a potential role of GMCSF and IFN gamma in anti cryptococcal immunity (97, 98).

A few patients will develop invasive fungal infections, sometimes very severe without known risk factors. Host genetic predisposition has to be studied in these patients. As an example, patients from North Africa were reported for years to develop a disease called “Maladie dermatophytique” with very atypical and devastating invasive dermatophyte infections in patients without any known risk factors. A CARD9 deficiency could be defined to be the genetic etiology of this infectious disease (99). CARD9 deficiency is also described to predispose to invasive candidiasis, aspergillosis and phaeohyphomycosis (100). The description of Mendelian predisposition to invasive fungal infections can help to better decipher antifungal immunity and to develop new therapeutic tools to treat invasive fungal infections (101).

Host genetic background can also play a role in patients with acquired immunodeficiency. As an example, gene polymorphism in genes encoding soluble Pattern Recognition Receptor as pentraxin-3 were reported to be associated with a higher risk to develop invasive aspergillosis in hematopoietic stem cell transplant (102) and solid organ transplant (103).

Humans are daily exposed to numerous potential fungal pathogens that are commensals or present in the environment, through inhalation, ingestion and contact. Hence, these fungi cause diseases only occasionally and our ability to prevent infection relies on our immune system. Although adaptive immunity activation is part of the immune responses triggered upon fungal infection, we will focus our review on the innate immunity system and the recent advances related to its modulation. As said, the adaptive immune system is very potent and remembers previous encounters with specific pathogens, destroys them upon additional attacks. Adaptive immune responses, however, require to be activated; several days can pass before the responses are effective. Meanwhile, microbes will rapidly proliferate and spread to an advanced infection. During these first critical hours and days of exposure to a new

pathogen, the host relies on the innate immune system to protect itself from infection. Although innate immune responses are not antigen-specific to a particular pathogen as the adaptive immune responses are, they recognize highly conserved molecular patterns of pathogens through specific circulating or cellular receptors. Upon recognition, effective innate immune responses are mounted to fight and eradicate the invaders. Indeed, although innate immune responses in vertebrate are also able to activate the adaptive immune responses if necessary, most of the invading microorganisms are detected and destroyed within minutes or hours by the innate immune defence mechanisms.

The classical and long-time accepted description of the immune system involves that the innate immune system and associated effectors cells (monocytes, macrophages, neutrophils, and NK cells) is rapid, relatively non-specific, and unable to build immunological memory. On the other hand, the adaptive immune system takes longer to develop, is antigen-specific, and capable of immunological memory. Accumulating amount of past and recent studies highlights that innate immune cells can learn from previous encounter and alter their function (104). This challenge in the classical paradigm is supported by a wide variety of reported data in plants, invertebrates, and mammals (105, 106). In mammals, cells of the innate immune compartments such as monocytes or natural killer (NK) cells, build up an innate memory upon a first challenge with certain microbes, pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs). Once these cells encounter a secondary stimulus, either similar or unrelated to the first insult, their response is altered, which could result both in a stronger or attenuated response. The overall capacity of innate immune cells to remember and alter their responses is referred as innate immune memory (107) and the induction of a non-specific memory resulting in an enhanced immune status is termed “trained immunity” (106).

Historically, trained immunity was demonstrated in mice in which T-/B-cell deficient animals are protected against a lethal fungal infection, with a significant reduction in mortality, when primed with a low dose of the human fungal pathogen *C. albicans*. Similar protection is mediated by the fungal cell wall component of the yeast, β-glucan.

C. albicans/ β-glucan mediated innate immune memory improve cellular host defence, which ultimately leads to a better survival of the host, a phenomenon requiring functional circulating monocytes (108). Using an *in vitro* assay that recapitulate the protocol used *in vivo* in mice, and human primary monocytes, we gained consequent insights on the mechanistic behind β-glucan-mediated innate immune memory. The functional reprogramming of monocytes is induced through the β-glucan receptor dectin-1 and the non-canonical Raf-1 pathway, but not the Syk pathway. β-glucan immune training of monocytes decreases ROS production-induced by zymosan, enhances microbicidal activities, and cytokine production *in vivo* and *in vitro* (108, 109).

In addition, a complex interplay between immunological, metabolic and epigenetic changes drives the characteristics of innate immune memory. As such, β-glucan mediated innate immune memory deeply modifies the epigenetic landscape of numerous genes in monocytes with stable increased and decreased enrichements of the active H3K4me1, H3K4me3, and H3K9ac, as well as of the repressive histone H3K9me3 through the transcription factor ATF7 (108, 110, 111). The durability of the epigenetic imprinting is highlighted by the presence of latent enhancers. In addition, β-glucan training generates a metabolic shift in monocyte from oxidative phosphorylation to aerobic glycolysis (the Warburg effect). The decrease in oxygen consumption and increase in lactate production and NAD+/NADH ratio require the Akt/mTOR/HIF-1α (112). Besides glucose metabolism, further analysis of the transcriptional signature of β-glucan trained monocytes demonstrated major differences observed in TCA

cycle metabolites and fatty acid metabolism (113). These alterations in cellular metabolism influence the epigenetic program of innate immune monocytes (113) placing metabolic pathways as crucial for maintenance and induction of innate immune memory. In addition, cholesterol synthesis pathway, but not the synthesis of cholesterol itself, also modulates β-glucan trained immunity and epigenetic reprogramming (114).

Although since the first mention of trained immunity (108) numerous seminal studies have been published on the in vitro mechanism of β-glucan innate immune memory, very little is known on the *in vivo* mediated increased inflammatory response and even less on the protection mechanism in mice against secondary challenges. This protection requires functional circulating monocytes, mediated by the chemokine receptor CCR2, despite their short lifespan in circulation. One proposed mechanism explaining the discrepancy between the lifespan of monocytes and the duration of protection in mice is the progenitors of the myeloid lineage as β-glucan induces their expansion (115).

The Mycobiota

As said previously, fungi represent major actors in most ecosystems. Not only they are well known players in the genesis of the soils but they can be symbiotic of plants in mycorrhiza (116) or as endophytes. They are also part of composite organisms such as lichens (117). Some are aquatic. Overall, they are components of very diverse biotopes; some being extreme and spectacular, the other being as common as dust behind the door (107, 118). In the last ten years, thanks to the development of NGS, fungi have been shown to be important components of our microbiota constituting the mycobiota (119). With 10^6 fungal cells per gram in the colon, the fungi are much less numerous than the bacteria (10^{11} per gram) but they are much bigger organisms and are thought to have individually a larger influence than a bacteria (120).

Analyses of the mycobiota from different areas of the human body revealed the high diversity of the fungal flora depending on the place of the sampling (120). For instance, commensal fungi represent more than 50 genera of fungi (121) but *Candida*, *Saccharomyces* and *Cladosporium* are prominent in the gut whereas *Malassezia*, *Aspergillus* and *Penicillium* are the major ones on the skin. The mycobiota like the microbiota can also vary from one person to another and its composition is dependent on the way of living and on diet (122). Interestingly, fungi and bacteria composition do not have the same dynamics suggesting a large range of microbe-to-microbe interactions. Some of these interspecies interactions have been described *in vitro*. For instance, *C. albicans* can promote the growth of the anaerobic bacteria *Clostridium difficile* in aerobic conditions (123). Similarly, Briard and colleagues reported that *Pseudomonas aeruginosa* produce phenazines and volatile compounds that alter positively and negatively the growth of its lung microbiota partner *A. fumigatus* (124, 125). These analyses suggest a wide and complex equilibrium of microbe-to-microbe interactions within the different human microbiota where fungi and bacteria compete for nutrients and secrete a wide range of molecules to promote or restrict the growth of the others.

In recent years, commensal fungi have been also shown to play important role in influencing local and peripheral immune responses (119). For instance, a prolonged oral treatment of mice with antifungal drugs result in a profound modification of the intestinal mycobiota which is associated with increased sensitivity to colitis and this sensitivity can be reverted to a normal level through artificial restoration of the normal intestinal fungi flora (126). Compelling evidences also strongly suggest a role of commensal fungi in the development of some gastrointestinal diseases such as Inflammatory Bowel Disease (IBD). For instance, genetic studies in IBD patients identified mutations in genes coding for proteins

implicated in fungal recognition and immune response such as CARD9 and dectin-1 (127-129). More, the analysis of the gut mycobiota in some cohorts of IBD patients clearly demonstrated fungal dysbiosis with a trend of *Candida* sp over-representation as compared to healthy volunteers (130). More recently, the gut commensal *C. albicans* has been shown to be a unique and broad regulator of Th17 immunity in humans, modulating the immune response against other fungi including *Aspergillus fumigatus* during acute allergic bronchopulmonary aspergillosis (131).

Future challenges

Fungal pathogens associated diseases are still linked with very high mortality. Hence, the identification of new families of safer antifungal drugs and the development of vaccines associated with new tools to perform early diagnostic represent the main goals of the field. Early diagnostic is still difficult as many of these pathogenic fungi are natural commensal of the human body and some others are very common in the environment. The ideal diagnostic tool should not only detect the pathogenic microorganism but also the stage at which the commensal or the environmental fungus is switching to become pathogenic and invasive. The identification of new families of antifungal drugs is also limited by the fact that these pathogens are eukaryotes. Most molecules that can inhibit fungal growth are also highly toxic for humans. We still need to improve our knowledge on the biology of these fungi during the infection and identify the key factors implicated in their virulence. The implementation of new or recently improved technologies in sequencing, metabolomics or microscopy should help us to get better insights on the cell biology, biochemistry and genetics of pathogenic fungi. Together with progresses in the understanding of the immune response during fungal infections, and on the potential of innate immune system boosting in protecting adaptive-

immune deprived patients, this knowledge will be translated in the identification of better diagnostic tools and specific drug targets. In that sense, the usage of fungus-specific CD4 T cells as specific sensors for the diagnostic of fungal infections is very promising (132).

The other main challenge is societal. Fungal diseases are mostly neglected and their impact on human health is not widely appreciated. This under-appreciation results in a limited amount of resources specifically dedicated by funding agencies to this field, thus limiting its development. We thus need to obtain better knowledge on their epidemiology and their impact on human health in developed countries but also in the poorest ones. In that sense, the type of survey done by the National Center of Mycosis and Antifungal of the Institut Pasteur is instrumental. New fungal pathogens like *Candida aureis* and *Mucor* sp. have been recently identified as emerging and will represent a challenge for the physicians and the researchers in the coming years. Fungal research initiated by Louis Pasteur in the middle of the 19th century has known fantastic development in the recent years but still needs to be further developed to better fight these deadly pathogens.

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