Cryptococcal Pathogenicity and Morphogenesis

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Abstract
Purpose of Review The aim of this review is to give an overall idea of *Cryptococcus* biology paying special attention to its capacity to adapt through its morphogenetic program to the hostile host environment. This morphogenetic program consists of a significant increase in capsule size and the formation of Titan cells.
Recent Findings Research on Titan cells had been hampered by the need of obtaining these cells from the lungs of infected mice. The production of Titan cells in vitro has supposed a major step in understanding the role of this morphotype in the virulence of *Cryptococcus*.
Summary In this regard, *Cryptococcus* has acquired the capacity of inducing a heterogeneous population during infection that allows it to evade the host immune system attack, proliferate, and disseminate to the CNS where it produces meningoencephalitis which is fatal if not treated properly.

Keywords *Cryptococcus* · Polysaccharide capsule · Melanin · Morphogenesis and Titan cells

Introduction

Cryptococcosis is a systemic disease caused by yeasts that belong to the genus *Cryptococcus*. Despite its high prevalence in immunocompromised patients especially in HIV-positive individuals in sub-Saharan Africa, and its high associated mortality rate, it is somehow a forgotten disease. In this review, we aim to offer the reader an overview of *Cryptococcus* biology, focusing on its main phenotypic characteristics and paying special attention to its unique morphological program during interaction with hosts.

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Cryptococcus and Cryptococcosis

Cryptococcus is included in the Basidiomycota division and contains at least 40 species, out of which, *Cryptococcus neoformans* and *Cryptococcus gattii* are the most frequent causative agents of cryptococcosis [1]. The most common clinical presentation of the disease is a pulmonary infection and further dissemination to the central nervous system producing meningitis. The most important phenotypic characteristic in *C. neoformans* and *C. gattii* is the presence of a capsule that surrounds the cell body.

Initially, *C. neoformans* and *C. gattii* were classified as one species and were distinguished by their antigenic differences. However, the discovery of two different teleomorphs together with whole-genome sequence data lead to the recognition of *C. neoformans* and *C. gattii* as two different species. More recently, it was proposed to classify *C. neoformans* in two different species, and *C. gattii* in five different species [2]. Although no consensus among the scientific community has been yet achieved [3].

Cryptococcus neoformans and *C. gattii* share many characteristics but the type of infection and their epidemiology are different. In this section, we will briefly review the main characteristics of each species. In the rest of this review, we will focus on *C. neoformans*, unless otherwise is stated.

Cryptococcus neoformans

Cryptococcus neoformans infects mainly immunosuppressed individuals. The principal risk factor associated with this pathogen is advanced HIV. Other risk factors are organ transplantation and receipt of immunosuppressive medications (e.g., corticosteroids). This yeast is cosmopolitan, with worldwide distribution, and can be isolated from multiple sources including pigeon excreta and soil [1].

Cryptococcus neoformans is typically acquired by inhalation of spores, and therefore, the lungs are usually the first organs to be colonized. In immunocompetent individuals, the infection is almost always controlled by the host immune system. However, in immunosuppressed individuals, especially those with reduced numbers of T cells such as those with advanced HIV, *C. neoformans* can induce an invasive infection with dissemination through the blood vessels to preferentially the central nervous system, where it causes meningoencephalitis with a high associated mortality rate if not treated properly.

The incidence of cryptococcal meningoencephalitis has decreased thanks to the availability of the effective HIV therapy, but continues being high with an estimated > 223,100 new cases per year that resulted in 181,100 deaths in 2014 [4••]. Cryptococcal disease remains a leading cause of mortality in developing countries, where access to antiretroviral therapy is limited and HIV prevalence is high [5].

Cryptococcus gattii

Initial studies suggested that *C. gattii* was predominantly found in tropical and subtropical areas [6]. However, more recent studies show that *C. gattii* has a much more widespread distribution and is often associated with eucalyptus trees [7].

Similar to *C. neoformans*, *C. gattii* is acquired by inhalation. However, *C. gattii* and *C. neoformans* are genetically and biochemically different. *Cryptococcus gattii* infects both immunocompromised and immunocompetent individuals [8]. In some countries, such as in Brazil, *C. gattii* is endemic and responsible for the 60% of all cryptococcosis infections [9].

The most characteristic clinical outcome is pneumonia. Only a minority of cases develop meningitis, and often as a result of some immunodeficiency [10, 11].

Despite *C. gattii* infections constitute only 1% of the worldwide cryptococcosis cases, this species gained importance for the scientific community and health professionals starting around 1999 due to an outbreak of infection in Vancouver Island, Canada [12].

Host Interaction

The interaction of *Cryptococcus* with the host immune system is very complex and has been widely studied [13].

Cryptococcus has developed different strategies that allow him to survive within the host, produce damage, and disseminate preferentially to the central nervous system [14••].

Intracellular Lifestyle

The first site of infection is the lungs where cryptocococcal cells encounter resident macrophages. The outcome of this interaction determines the course of infection [15, 16].

Macrophages and neutrophils are the frontline of the immune system defense of the host. The phagocytosis process depends on the activation of phagocytic receptors that can be divided in opsonic and non-opsonic.

Early studies proved that phagocytosis was inhibited by the capsule, and that acapsular mutants were easily phagocytosed [17]. The phagocytosis can be mediated through the union of phagocytic receptors located on macrophages' surface and the epitopes located at cryptococcal cell wall (non-opsonic mechanisms). Therefore, the capsule impairs the recognition of these epitopes and thus, inhibits the phagocytosis [18]. Interestingly, the C-type lectin Dectin-2 recognizes the mannans at the cell wall and induces a non-protective Th2 response [19]. Besides, other antiphagocytic mechanisms that do not depend on the capsule have been described, such as the secretion of the antiphagocytic protein App1 [20, 21] and the transcription regulator Gat201 [22].

Phagocytosis can also be antibody/complement mediated (opsonic mechanism) and it depends on capsule size and on the location of the complement binding protein [23]. Despite all these mechanisms, *Cryptococcus* is phagocytosed in vivo. *Cryptococcus* is found predominantly within macrophages few hours after the infection [24]. After phagocytosis, an antimicrobial environment is produced in the phagosomes, which includes the production of reactive oxygen (ROS) and nitrogen species (NOS), alteration of pH, production of antimicrobial peptides, and limitation of nutrients in an attempt to kill *Cryptococcus* [25, 26]. The killing of the fungus is one of the possible outcomes; however, *Cryptococcus* can survive within the macrophage, proliferate, and eventually lyse the macrophages [27].

Macrophage activation occurs through distinct types of immune response: pro-inflammatory Th1 and Th17 responses and anti-inflammatory Th2 response. During *Cryptococcus* infection, the Th1 and Th17 responses lead to a protective response while the Th2 is associated with exacerbation of disease, supporting intracellular survival and cryptococcal proliferation [28••, 29•, 30]. Interestingly, *Cryptococcus* virulence factors such as the polysaccharide capsule, melanin and urease production, and the formation of Titan cells promote a dominant anti-inflammatory Th2 response, decreasing significantly the production of inflammatory cytokines and facilitating the intracellular survival and proliferation of the fungus [31, 32•, 33, 34].

If macrophage attack is evaded, *Cryptococcus* is able to proliferate within the phagosome where nutrient uptake is of great importance. Iron, for example, is an extremely important nutrient to the fungus. Indeed, low levels of iron within macrophages induce morphological changes in *Cryptococcus* such as capsule enlargement, Titan cells formation, and melanin production. Besides, *Cryptococcus* increases the expression of a siderophore iron transporter (SIT1) and of an iron permease (FTR1) that allow the fungus to increase its iron storage and survive [35, 36].

Cryptococcus can lyse the macrophages and evade killing. The mechanisms involved in this process remain poorly known. It is hypothesized that *Cryptococcus* can mechanically lyse the cells by simple proliferation or by an exaggerated production of polysaccharide. Besides, it is known that *Cryptococcus* can induce the apoptosis of the host cells via the alternative NF-kB pathway [37].

Interestingly, *Cryptococcus* has developed different ways to exit the macrophages without lysing them, such as lateral transfer to another macrophage and "vomocytosis" that is a non-lytic process of expulsion of the yeast from macrophages, allowing *Cryptococcus* to disseminate without inducing local inflammation [16, 38].

Dissemination to the Central Nervous System

Dissemination to the central nervous system (CNS) causing cryptococcal meningitis is a major cause of HIV-related deaths worldwide [39].

Cryptococcus has developed different strategies that allow it to cross the blood-brain barrier (BBB). One of these strategies is the so-called "Trojan horse" mechanism, by which *Cryptococcus* can cross the BBB inside macrophages [40, 41]. *Cryptococcus* can also directly invade the BBB and enter in the brain tissue by a mechanism called transcytosis [42, 43]. In this process, *Cryptococcus* induces the formation of microvillus-like membrane protrusions in the BBB cells, where it adheres and gets internalized reaching the brain parenchyma without affecting the integrity of the host cells [44]. Recently, Aaron et al. found that the EPH-EphrinA1 (EphA2) tyrosine kinase receptor-signaling pathway mediates the transcytosis across the BBB in a CD44 host receptor dependent manner [45]. Therefore, silencing or inhibiting EphA2 prevents *Cryptococcus* from crossing the BBB.

However, *Cryptococcus* can reach the CNS producing damage to the endothelial cells that compose the BBB by altering their cytoskeleton, which has been named paracellular penetration [46–48]. Indeed, *Cryptococcus* urease enzyme also plays a role in its dissemination, since urease-deficient strains are less efficient in transmigrating the BBB. It is hypothesized that urease degradation produces ammonia that can damage the endothelium and increase the permeability of *Cryptococcus* through the BBB or that cryptococcal urease

possesses substrate specificity that facilitates the transcytosis [49, 50].

Interestingly, none of these mechanisms are mutually exclusive, and indeed there are increasing evidences that all of them contribute to the invasion and colonization of the central nervous system.

Latency and Reactivation

Besides causing disseminated infection in immunocompromised patients, *Cryptococcus* can also cause a latent asymptomatic infection and stay in the host for long periods of time. Epidemiological studies have revealed patients in which these latent cells could restart infection under adequate stimuli [51].

Little is known about the mechanisms involved in the latency/reactivation of *Cryptococcus*. When in experimental latency, *Cryptococcus* displays low or almost undetectable metabolism being able to enhance its metabolic activity under favored conditions. In vivo, rats are the best models to study latency of *Cryptococcus*, since they are intrinsically resistant to cryptococcosis. In this model, yeasts are not eliminated and a chronic infection is developed with the formation of granulomas where *Cryptococcus* is found mostly inside macrophages [52, 53].

Virulence Determinants

Cryptococcus spp. are yeasts of special interest to study fungal pathogenesis since they have developed different adaptation mechanisms to the host but also virulence factors, which are defined as those that produce damage to the host.

Melanin

Production of melanin is one of the most characteristic features of *Cryptococcus*. Melanin is a negatively charged dark pigment, hydrophobic, and extensively spread in the environment. There are different types of melanin: eumelanins, pheomelanin, alomelanins, and piomelanins. In particular, *Cryptococcus* synthesizes eumelanin using diphenol, aminophenol, and diaminobenzene compounds as substrates [54]. Some of these compounds, such as L-DOPA, are present in the central nervous system, and therefore, it is thought that melanization confers an adaptative advantage to infect that organ [55].

Melanin is synthesized by a phenoloxidase enzyme called laccase encoded by *LAC1* gene [56]. The expression of this gene is regulated by the ion, cupper, and glucose concentration [57, 58]. Laccase enzyme is located at the cell wall and it is important for *Cryptococcus* survival since it contributes to keep its cell wall integrity.

Melanin protects *Cryptococcus* from stress factors such as UV, free radicals, and high temperature, which facilitate its

survival in the host. Furthermore, in mice, infection of melanin particles induces an inflammatory response, suggesting that this pigment could modulate the immune response and therefore it could have an important role in virulence.

Growth at 37 °C, Adaptation to Alkaline pH, and Expression of Urease and Phospholipases Enzymes

The capacity to grow at 37 °C is essential for any human pathogen. *Cryptococcus* is able to grow at a wide range of temperatures, from 25 until 37 °C. Therefore, it can infect both environmental hosts and mammals.

In the environment, *Cryptococcus* is found in pigeon excreta. However, birds do not get infected, probably because their body temperature is around 40–42 °C.

In the last years, many proteins required for growing at 37 °C have been identified. These proteins are involved in stress response, cell wall assembly, plasma membrane integrity, basal metabolism, and polarized growth [59–62].

Once in the lungs, *Cryptococcus* has to adapt to an alkaline environment. To accomplish that, the Rim alkaline response pathway is activated which leads to expression of Rim101 that directly regulates genes required for various stress responses including low iron, high salt concentration, and proper cell wall maintenance [63–65].

Another characteristic of *Cryptococcus* is the expression of a urease enzyme, which allows it to use exogenous sources of urea. Urease is important for *Cryptococcus* virulence because it promotes dissemination to the central nervous system [49, 66] and a Th2 response, impairing the elimination of the yeasts. More recently, it has been described how urease alters phagolysosomes, delaying intracellular replication and thus facilitating *Cryptococcus* dissemination when transported within macrophages to the central nervous system [67].

Finally, two phospholipases B (Plb1 and Plb2) and a phospholipase C have been associated with survival, maintenance of the homeostasis, and intracellular replication of *Cryptococcus* within macrophages in vitro [68–70]. In parallel, another phospholipase with only lysophospholipase activity has been characterized in *C. gattii*, which could help to understand the differences in virulence in these two species [71].

Capsule

The polysaccharide capsule is the main phenotypic characteristic of *Cryptococcus*. This structure can be easily observed when yeasts are suspended in India ink as refrigent white halo. The capsule confers *Cryptococcus* protection against phagocytic cells and also interferes with the host immune system. Indeed, the capsule is its principal virulence factor and therefore it is the most studied structure of this pathogen. Apart from its contribution to virulence, the capsule is important medically since its polysaccharide (PS) is the cryptococcal antigen used in diagnosis [72].

When observed in India ink suspension, the capsule looks a homogenous structure. However, it exhibits different densities in different regions being the inner layer denser, more rigid, and less permeable than the outer layer [73–75].

The polysaccharide is composed approximately 90% of glucuronoxylomannan (GXM) formed by a (1,3) mannose backbone with ß(1,2) and ß(1,4) xylose and ß(1,2) glucuronic acid substitutions [76]. The other two minor components of the capsule are GalXM and mannoproteins [77, 78]. In addition to the PS components, the capsule contains lipid structures whose function is unknown [79, 80].

Cryptococcus capsule is a key component for its atypical morphogenetic program, which will be extensively reviewed in the following section.

Morphogenesis

Unlike other well-known fungi that transition from a yeast form to an invasive filament form, such as *Candida albicans*, *Cryptococcus* does not change its shape, but its size. This morphological transition is important regarding adhesion, invasion, dissemination, and evasion of the immune system [81••].

Cryptococcus only forms filaments during sexual reproduction although pseudohyphae have occasionally been observed in tissues [82]. However, *Cryptococcus* is characterized by inducing other types of changes during infection that involve exclusively a change in size. *Cryptococcus* can increase its size in two different ways: by increasing only the size of the capsule or by increasing both capsule and the cell body sizes. These changes produce a very heterogeneous yeast population in the lungs, which contribute to evade the immune response.

Changes in Capsule Size

The increase in capsule size of *Cryptococcus* is one of the most characteristic features and can be observed in vitro in different media [83, 84]. In rich media, the size of the capsule is around 1–2 μm but there are conditions that induce a drastic increase in size. This phenomenon was first described in the 50s. Afterwards, it was shown that CO_2 and iron limitation induce the growth of the capsule [85, 86]. More recently, other factors that induce this process have been described, such as mammalian serum, medium with low concentration of nutrients at neutral pH, or mannitol [83, 84, 87]. Conversely, other factors such as osmotic pressure or high glucose concentrations reduce the size of the capsule. Little is known about the regulation and the molecular mechanisms responsible for this process [88]. However, there is a correlation between ex vivo capsule size and the intracranial pressure of patients [89].

The growth of the capsule occurs mainly in the G1 phase of the cell cycle [90]. It has been shown that the increase in size of the capsule requires the accumulation of a significant amount of new polysaccharide [91, 92]. It has been estimated that the weight increase of the cells due to the growth of the capsule is about 20% of the total weight of the cells and this process occurs in a few hours being an energy-cost process for the cell [74, 93], which is particularly interesting because it occurs in nutrient-limiting conditions. The capsule growth is one of the first responses of *Cryptococcus* after reaching the lungs, and thus, it is considered an early morphological response [94]. Cells with larger capsules are more resistant to stress factors, such as free radicals or antimicrobial peptides [26], so it has been postulated that it is a mechanism that allows the survival of *Cryptococcus* within macrophages [23].

Formation of Titan Cells

Another characteristic change of *Cryptococcus* occurs when there is massive growth of the capsule and the cell body. In this way, blastoconidia of a total size greater than 30 μm can be formed and those are called "Titan cells," although these cells can reach up to 70 μm in vivo [94–97]. This change is considered a late morphological response, since these cells are observed several days after the infection. These cells were described in clinical samples [98] but had not been extensively characterized until 2010 [95, 96]. Titan cells are polyploid, have a thickened cell wall, and have a denser capsule than normal-sized cryptococcal cells. In addition, these cells can proliferate and have a progeny of normal size [99, 100]. Both, Titan cells and their progeny, are more resistant to stress factors, which provide a mechanism of adaptation to the host and evasion of the immune response. Recent studies have shown differences in mannose content and the distribution of specific epitopes in *Cryptcoccus neoformans* capsule [101, 102].

The main pathway that has been linked to the formation of Titan cells is signaling through cAMP since mutants of adenylate cyclase do not form these cells [96]. In this way, two receptors coupled to G proteins that are important for the induction of Titan cells have been described: Ste3a (pheromone receptor α) and Grp5 protein. Both receptors control the formation of Titan cells through the elements of the cAMP pathway. The signaling by this metabolite regulates the activation of the PKA-regulated Rim101 transcription factor that is necessary to induce Titan cell formation [103].
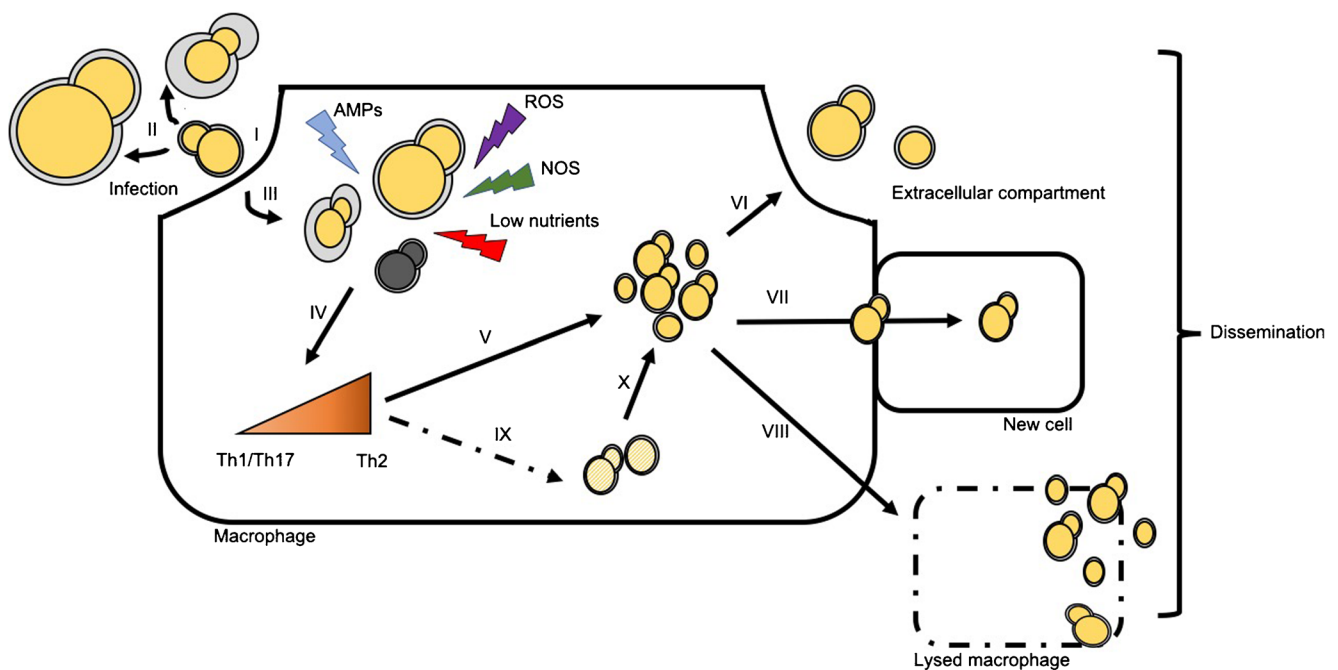

Fig. 1 Infection, intracellular lifestyle, and dissemination of *Cryptococcus*. The lungs are the first site of infection, where alveolar macrophages phagocytose yeast cells (I). However, *Cryptococcus* can also become a Titan cell, increasing its body and/or capsule size, outside the macrophage and impair its phagocytosis (II). If phagocytosis does occur, then *Cryptococcus* cells undergo morphological changes such as capsule enlargement, transition to Titan cell, and melanin production that protect against different stresses elicited by the macrophage (III). These morphological changes lead to a dominant anti-inflammatory non-protective Th2 immune response in the macrophages (IV). In this permissive environment, *Cryptococcus* can replicate intracellularly (V) and different outcomes are possible: Yeast cells can exit the macrophage without lysing it, a process named "vomocytosis" (VI), *Cryptococcus* can be transferred to a second non-infected macrophage by lateral transfer (VII) or lyse the macrophage and exit to the extracellular environment (VIII). All of these outcomes contribute not only to the establishment of the infection but also to the dissemination. Furthermore, depending the immunological response achieved by the host, *Cryptococcus* can reduce its metabolism and enter a state of latency (IX), from which infection can be reactivated under more favorable conditions for *Cryptococcus* proliferation (X)

Interestingly, mutants of phospholipase B1 that show a clear defect in intracellular proliferation can form Titan cells within macrophages, which could be a strategy to survive within the host [104].

Host Factors Involved in Titan Cell Formation Little is known about the function of Titan cells during infection and which host factors regulate this morphological transition. Titan cells contribute to the permanence of the yeast in the lungs since they cannot be phagocytosed nor be eliminated easily. In addition, they inhibit the phagocytosis of other cells of regular size and induce a Th2 type immune response [105, 106]. It has been shown that the proportion of this type of cells is particularly high in models of asymptomatic infection, which suggests that these cells participate in the latency phase of the infection [96]. Interestingly, it has been observed that a Th2-polarization of the immune response correlates with a higher proportion of Titan cells in the lungs [33]. Furthermore, the proportion of these cells increases when co-infections are made with strains of MATa and MATα sexual alleles, which indicates that the sexual type plays an important role in the induction of Titan cells [95].

What Happens In Vitro? One of the major limitations to investigate Titan cells is the difficulty of reproducing this phenomenon in vitro. However, last year, three independent groups published in the same journal different in vitro conditions that result in the appearance of Titan cells [107•, 108•, 109•]. Despite that the in vitro Titan cells do not reach the size of the titan cells found in the lungs of infected mice, they have an average cell body size of 15 μm and share many phenotypic characteristics with the in vivo Titan cells. Briefly, Trevijano-Contador et al and Dambuza et al agree on the importance of incubation in limited nutrient conditions containing serum for an overnight at 37 °C in a CO_2 enriched environment and at a low cell density. However, Hommel et al describe a protocol where longer incubations up to 120 h are required and no presence of serum nor of CO_2-enriched environment is necessary [107•, 108•, 109•], showing that multiple pathways may be involved in this morphological change during *Cryptococcus* infection. For a more detailed comparison and analysis of the three protocols to induce Titan cells in vitro, we suggest the reading of this very recent review [110••].

Conclusions

Cryptococcus has developed different strategies to survive and proliferate within the host. Its intracellular lifestyle together with its capacity to undergo a complex morphological program constitutes the main characteristics of this fungal pathogen (as represented in Fig. 1). Little is known about the molecular mechanisms involved in some of the processes described above. Research on this field has been complicated due to the difficulties of performing molecular modifications on *Cryptococcus*. However, in the last years there have been several important additions to the molecular toolbox of *Cryptococcus* that for sure will enable deeper studies at the molecular and cellular level. Furthermore, the recent discovery of the conditions for producing Titan cells in vitro will enlarge the knowledge of this peculiar morphogenetic transition and their full consequences for the development of the infection and the immune response of the host.

Compliance with Ethical Standards

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Papers of particular interest, published recently, have been highlighted as:
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