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Review

Two fundamentals of mammalian defense in fungal infections: Endothermy and innate antifungal immunity

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Abstract

The environment of animals is inhabited by enormous fungal species, but only a few hundreds are pathogenic for mammals. Most of potentially pathogenic fungal species, excluding dermatophytes, seldom cause the disease in immunocompetent hosts. Data from literature indicate, that an immune system and endothermy are foundations for this mammalian relative resistance to fungal systemic infections. Stable and high temperature of the body restricts invasion and growth of potentially pathogenic fungi. Together with elevated metabolism it supports the effectiveness of mammalian immunity. The innate immunity is assigned to prevent the invasion of various microbes (including fungi) to the hosts' organism. It consists of cellular receptors and several humoral factors as the Antimicrobial Peptides. If the physical barriers fail in stopping the invader, it is recognized as "alien" by multiple Pattern Recognition Receptors (PRRs) like Toll Like Receptors (TLRs) expressed by cells of innate immunity and/ or C-type lectins. At the same time innate inflammation begins and the complement cascade is activated. These mechanisms are able to stop and clear some fungal infections. During existing infection the adaptive immunity is induced. This review aims to show the role of mammalian endothermy and to point the most important elements of innate antifungal immunity.

Key words: fungal infections, mammals, PAMPs, PRRs, innate immunity, endothermy

Introduction

Fungi are highly diversified organisms, classified as a separate taxonomic kingdom. They colonize a number of various habitats. The quantity of fungal species was estimated at 1.5 million, but some predictions, based on the effects of modern sequencing methods, anticipate the number of 5.1 million species existing on the Earth (Blackwell 2011). Among them only a few hundreds are considered to be pathogenic for humans and other mammals (Taylor et al. 2001, Casadevall 2005, Robert and Casadevall 2009). The list of human and mammalian fungal pathogens encloses: *Candida* spp., *Cryptococcus neoformans*, *Aspergillus* spp., *Histoplasma capsulatum, Coccdioides immitis, C. posadsii, Blastomyces dermatitidis, Paracoccidioides brasiliensis*, dermatophytes, some representatives of *Hyphomycetes* (e.g. *Penicilium, Paeciliomy-*

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ces, Scopulariopsis, Fusarium or *Exophiala*) and some *Zygomycetes* (e.g. *Mucor* or *Rhizopus*) (Burgeois and Kuchler 2012). The majority of fungal species, excluding dermatophytes, rarely cause the disease in hosts with efficient mechanisms of defense. The main factors predisposing humans and other mammals to severe fungal diseases are primary and secondary immunodeficiences, as indicate multiple cases of life-threatening mycoses, observed among immunocompromised hosts (Batura-Gabryel 2003, Sobol et al. 2009). Thus complex innate and adaptive mechanisms of the mammalian immune system are fundamentals in resistance to fungal infections.

The vast majority of publications about fungal diseases in animals concerns cold-blooded vertebrates, like *Amphibia* and Pisces. Papers referring to the cases of severe amphibian mycoses caused by *Batrachochytrium dendrobatidis* may serve as the example. These infections have resulted in large decline or extinction of about 200 of frogs' populations and species (Skerratt et al. 2007, Rollins-Smith et al. 2011). Moreover, phaeohyphomycoses and saprolegnioses of fish and their eggs are noted quite frequently (Hussein et al. 2001, Faisal et al. 2007, Cao et al. 2012). Numerous data may lead to the conclusion, that poikilothermic animals are more susceptible to fungal infections. Additionally, the list of mammalian and human fungal pathogens mentioned above, seems to be quite short, when compared to the thousands of species pathogenic to lower vertebrates. The main difference between these groups of animals is their immunity and mammalian endothermy. Some experiments points that high body temperature is the second foundation of mammalian resistance to fungal infections (Robert and Casadevall 2009, Bergman and Casadevall 2010).

Interaction between the host and pathogenic fungus

To use a mammalian organism as the source of nutrients, potentially pathogenic microbes have to colonize the host, invade the hosts' tissues and establish themselves inside its organism. Thus some fungal species have evolved and formed many virulence factors e.g. polysaccharide capsule of *Cryptococcus neoformans* inhibits phagocytosis and enhances the dissemination of the fungus. Also fungal enzymes such as phospholipases and keratinases are used to overcome the hosts' immunity and to gain the access to the tissues and cells (Steenbergen and Casadevall 2003, Dworecka-Kaszak 2008, Krutkiewicz 2010). Some of them like SAP1, SAP2, SAP3 proteases of *C. albicans* serve as adhesins (Monod et al. 2002, Krutkiewicz 2010) or may contribute with fungal cell integrity, also inside the phagocyte (e.g. *C. neoformans'* laccase) (Steenbergen and Casadevall 2003). Furthermore, adherence abilities, molecular mimicry and thermal tolerance are important for various fungi. The crucial virulence factor for fungal opportunists like *C. albicans* and *Penicilium marneffei* is their ability to switch the morphology during the transition from a commensal or environmental organism into pathogen (Cooper and Haycocks 2000, Andrianopoulos 2002, Cooney and Klein 2008, Krutkiewicz 2010). Dimorphism is also an important virulence factor of *B. dermatitidis, H. capsulatum* and *C. immitis*. Besides changes in the enzymatic activity, this transformation modifies also fungal cell wall and aggravate the recognition of fungus (Cooney and Klein 2008, LeibundGut-Landmann et al. 2012).

Interactions between the host and potential pathogen are modified by the hosts' immunity. Animal organisms had evolved complex mechanisms of defense, which are able to prevent the infection or to terminate the infection before the disease occurs. Some of them e.g. the antimicrobial innate immunity were established very early during evolution and are present also in the invertebrates. The mammalian immune system is more sophisticated and more effective. The entire protection against most of fungal pathogens, requires the cooperation between the innate response provided by phagocytes (macrophages and neutrophils) and dendritic cells, and adaptive mechanisms directed by immunocompetent cells (Brown 2011). The advantage of almost instantly acting innate defense mechanisms is detection and elimination of several various pathogens. These mechanisms are also involved in activation of the specific, adaptive (acquired) response, involving cell mediated immunity (CMI) and humoral immunity (HI) (Weinberg et al. 1998, Zelante et al. 2007, Blanco and Garcia 2008). That immunity may be also protective during reinfection. If innate mechanisms are overcome by pathogenic fungus, CMI enclosing the Th1 type of the immune response is the main mechanism of defense. Moreover, the production of certain types of antibodies is important in antifungal protection and in pathogen clearing (Blanco and Garcia 2008). However, Th2 type of the immune response may cause the hypersensitivity, frequently observed during fungal diseases. Also, certain immunoglobulins in some fungal infections may contribute to the pathogenesis (e.g. in *C. neoformans* or *C. albicans* infections) (Casadevall 1995, Blanco and Garcia 2008). In both CMI and HI, the role of particular elements depends on the host and on the invading fungus. Although many of the pathways of the adaptive immunity has already been explained, the

complexity of all adaptive mechanisms and differences observed among various hosts does not allow for generalization.

The basis of the relative mammalian resistance to fungal infections

During their life all animals have permanent contact with various hostile microorganisms. The effective resistance to their constant attacks is essential for organisms' survival. It begins with physical "obstacles" which eliminate some of microbes. The next step is proper recognition of attacking fungi followed by activation of defense mechanisms capable of destroying the invader. All of this is provided by the innate immunity enclosing the abilities of phagocytic cells (neutrophils and macrophages), the activity of the complement system and inflammatory response. Additional advantage of mammals is their endothermy. This achievement enables proper action of the immune system and eliminates many of the fungal pathogens, unable to grow in elevated, stable temperature of the mammalian body. If the complete elimination of pathogens is not possible, the disease occurs.

Innate defense mechanisms

Innate defense mechanisms were traditionally classified as constitutive and inducible mechanisms. While the constitutive mechanisms are inherent barrier present in the "natural doorways" of the body, constantly confronted with the fungi existing in an environment, inducible pathways are activated during the recognition of fungi (Zelante et al. 2007). The physico-chemical barriers of the skin and mucosal membranes of the gastrointestinal, genitourinary and respiratory tracts are the first line of defense against fungi, able to stop many of them (Tizard 2004, Blanco and Garcia 2008). The intact skin epidermis covered with sebum rich in short-chain fatty acids and high concentration of NaCl is the physical barrier difficult to cross. Excluding the dermatophytes, able to penetrate it with keratinolytic enzymes, other fungi (e.g. *Zygomycetes*) to cause the clinical mycosis have to be implemented into deeper skin layers or to the tissues during the trauma. Sometimes the fungal infection develops in the skin areas debilitated due to extensive burns or in diabetic lesions. Frequently, mycoses are the consequences of other disorders affecting the skin e.g. atopic dermatitis or lethal acrodermatitis in dogs (McEwan 2001).

Besides the skin, other entry to the mammalian body are the mucous membranes. Their continuity is supported by the presence of antimicrobial peptides, IgA and carbohydrate-digesting lysozyme. Lysozyme has been found in secretions such as tears and saliva and in some body fluids. It is also present in granulocytes, macrophages and monocytes. This enzyme has well known antibacterial activity, but it is also able to kill or to impede the growth of various fungi e.g. *Candida, Cryptococcus, Histoplasma, Aspergillus* and *Paracoccidioides* (Brown 2011). Mucosal membranes are also "self-cleaned" by their natural functionality e.g. by the GI peristalsis, urine flow or by congenital reactions like coughing and sneezing.

As it is known, some fungi (e.g. *Candida* yeasts) participate in the oral, gastrointestinal and vaginal biota of healthy individuals (Jouault et al. 2006). This well adapted biota is following defensive barrier, playing the supportive role in innate immunity, because it impedes the settlement of pathogens and counteracts the infection (Blanco and Garcia 2008). The fungal disease develops, if some of mentioned mechanisms are disturbed or debilitated. For example, vulvovaginal candydosis frequently occurs when the natural biota is disrupted after antibiotic therapy. The efficiency of physico-chemical barriers, supported with immunity seems to be quite high, considering how many fungal species encounter an animal during its' life and how many fungal elements it inhales or ingests.

Simultaneously with stopping many of encountering microbes the innate response is capable of more sophisticated reactions. The important assignment of innate immunity is the protection against the microbes breaking through the physico-chemical barriers. This kind of response is almost immediate because the first reactions are observed within minutes. Innate defense mechanisms detect and recognize unfamiliar, chemical structures common to microbes and absent in animals (Zelante et al. 2007). Generally, they identify "invaders" on molecular level and react with specialized sentinel and defensive cells. During inflammation the innate response is mediated by the phagocytes mainly macrophages, neutrophils and monocytes. It is effective in controlling of some fungal infections, when those specialized cells, are able to phagocytose and destroy fungal elements. The multifactorial inflammatory response and the activated complement cascade are additional, accompanying processes involved (Tizard 2004, Zelante et al. 2007, Brown 2011, Santamaria et al. 2011).

The first element of defense – the recognition of pathogen: PAMPs and PRRs

Activation of innate antifungal defense mechanisms depends on the recognition of the **PAMP** structures (Pathogen-Associated Molecular Patterns),

present on the microbes invading the animal. These structures are evolutionary conserved and invariant in many groups of pathogens, including fungi. The majority of data about fungal PAMPs refers to opportunistic or pathogenic species, frequently isolated from severe human mycoses. These structures in other fungi are less known. In general, the recognition of fungi is based on the detection of cell wall components, mostly carbohydrate polymers and mannosylated proteins, by specialized corresponding receptors. In *C. albicans* glucuronoxylomannan (GXM), mannan, β1,3- and β1,6-glucan and phospholipomannan (PLM) of cell wall were identified as PAMPs. Also, GXM of *C. neoformans, C. gattii* and *A. fumigatus* were recognized as PAMPs (Fonseca et al. 2010, Brown 2011, Burgeois and Kuchler 2012). Additionally, some fungal proteins can be sensed e.g. one of adhesins – BAD-1 in *B. dermatitidis* or one of heat shock proteins – HSP60, present in the cell wall of *H. capsulatum* (Long et al. 2003, Brown 2011). Pathogenic *C. albicans* is recognized by proteins glycosylated with O- or N- linked mannosyl chains, present in outer layer of cell wall (Netea et al. 2006b). Some publications indicate that fungal PAMPs are also nucleic acids e.g. DNA and RNA of *Candida* spp. and *A. fumigatus* alike DNA of *C. neoformans* and *Malassezia furfur* (Fonseca et al. 2010, Brown 2011, Burgeois and Kuchler 2012). However, nucleic acids are exposed for recognition only after the fungus is destroyed or phagocytosed. They trigger alternative pathways of the immune response through mechanisms activated by the PAMPs of fungal cell wall (Brown 2011, LeibundGut-Landmann et al. 2012). It is also presumed, that chitin similarly to other surface polysaccharides may be fungal PAMP, but neither corresponding receptor nor its location is established (Brown 2011).

To identify PAMPs, the pattern recognition receptors (PRRs) are used by multiple hosts' cells, mainly macrophages, monocytes, dendritic cells and endothelial cells (Zelante et al. 2007, Blanco and Garcia 2008). PRRs activated by equivalent PAMPs initiate multiple cellular processes, leading to the specific immune response against attacking fungus. These receptors may be bounded with the phagocytes membrane and directly recognize the fungi (e.g. Toll-like receptors – TLRs, dectin-1). Some of them are present in cytoplasm or endosomal vesicle (TLR9), other like collectins are secreted by both phagocytic and non-phagocytic cells as soluble PRRs. They attach to the pathogen, opsonize it and allow to identify the fungus by membrane-bounded opsonic receptors e.g. complement receptors (CRs) (Brown 2011, LeibundGut-Landmann et al. 2012). Among PRRs responsible for the identification of fungal PAMPs are the receptors for complement components (CRs), soluble Galectin-3, membrane scavenger receptors (CD5, CD36) and the family of C-type lectin receptors (CLRs), such as dectin-1, dectin-2 and Mincle (Brown 2011, Burgeois and Kuchler 2012, LeibundGut-Landmann et al. 2012). Other PRRs are the receptors for mannosyl/fucosyl glycoconjugate ligands (MRs) (Zelante et al. 2007, Brown 2011). Because of multiple PAMPs associated with various pathogenic fungi, to start the specific immune response against different fungi numerous PRRs are activated simultaneously or sequentially.

The main group of PRRs involved in recognition of fungi are Toll-like receptors, but distinct hosts can use different TLR to identify the same fungus. Moreover, the engagement of single TLR varies depending on fungal morphotype and the route of infection (Bourgeois et al. 2010, Santamaria et al. 2011). Sometimes single TLR is more "universal" and binds different PAMPs of various fungi (Netea et al. 2006a, Burgeois and Kuchler 2012). For example, TLR2 identifies phospholipomannan (PLM) of *C. albicans* (Jouault et al. 2006) and unknown ligands of hyphae or conidia of *A. fumigatus* (Netea et al. 2003). TLR2 also recognizes β-glucan of *Coccidioides posadsii* (Viriyakosol et al. 2005), *C. albicans* (Netea et al. 2006a) and *H. capsulatum* (Sorgi et al. 2009). Next, TLR4 is stimulated by mannanosylated proteins (O-linked mannosylated proteins) of *C. albicans* (Netea et al. 2006a), by GXM of *C. neoformans* (Shoham et al. 2001) and by ligands present on conidia of *A. fumigatus* (Netea et al. 2003). The ligand for TLR3 is double-stranded RNA, released within the phagosome from conidia of *A. fumigatus*, while TLR7 is activated by single-stranded RNA from *C. albicans*. The recognition of genomic DNA by endosomal or cytoplasmic TLR9 is common for several fungal species, but the identified motifs are different (Burgeois and Kuchler 2012). In some cases TLRs may form heterodimers to enhance the possibility of fungal recognition in different hosts. Heterodimers of TLR2/TLR6 and TLR2/TLR1 are receptors for cryptococcal GXM and some ligands of *A. fumigatus,* but the first one recognizes *A. fumigatus* only in mice, whereas the second can identify that fungus both in mice and in humans (Burgeois and Kuchler 2012, Rubino et al. 2012).

The second important group of PRRs is the family of C-type lectins (CRLs) enclosing dectin-1, dectin-2, Mincle, pentraxin-3, DC-SIGN (CD209) and mannose receptors (MRs, CD206, CD280) (Bourgeois et al. 2010, LeibundGut-Landmann et al. 2012). CRL are present in soluble form in plasma (collectins) or on the surface of endothelial cells and leukocytes (selectins). Generally, mammalian lectins are proteins able to identify bacterial and fungal carbohydrates like mannans, glucans, lipophosphoglycans or

Table 1. The occurrence of Pathogen-Associated Molecular Patterns (PAMPs) in chosen fungi and their recognition by Pattern Recognition Receptors (PRRs).

Fungal species	Fungal PAMPs	Recognition of fungal PAMPs by PRRs and co-receptors	PRRs co-receptors with confirmed physical interactions
Candida albicans	GXM – glucuronoxyloman	TLR4	Galectin-3; Dectin-1; SIGNR1
	N-linked mannan	TLR4	
	O-linked mannan	TLR4	
	PLM – phospholipomannan	TLR2; TLR4	
	β-glucan	TLR2/Dectin-1	
	genomic DNA	TLR9	
	ssRNA	TLR7	
Aspergillus fumigatus	GXM – glucuronoxyloman	TLR4; TLR1/TLR2; TLR2/TLR6 (only in mice)	
	PLM – phospholipomannan	TLR2/TLR1; TLR2/TLR6	
	Undefined ligands on hyphae and conidia	TLR2	
	dsRNA	TLR3	
Histoplasma capsulatum	β-glucan	TLR2/ Dectin-1	Dectin-1
Coccidioides posadsii	β-glucan	TLR2/ Dectin-1	Dectin-1
Cryptococcus gattii	GXM - glucuronoxyloman	TLR2/TLR1; TLR2/TLR6	
Cryptococcus neoformans	GXM – glucuronoxyloman	TLR2/TLR1; TLR2/TLR6; TLR4	
	genomic DNA	TLR9	
	PLM – phospholipomannan	TLR2/TLR1; TLR2/TLR6	

glycoinositol-phospholipids with mannose, glucose and N-acetyloglucosamine domains (Ramkumar et al. 2003, Tizard 2004). One of important lectins is soluble mannose binding lectin (MBL) (Brown 2011). It recognizes mannose and N-acetylglucosamine on many microorganisms, what enables its binding to multiple bacteria and yeasts, including *C. albicans* and *C. neoformans*. MBL is an important factor of innate immunity because it may serve as opsonin and activate the complement alternate pathway (Tizard 2004). Mannose receptor is also present in the cell membrane of mature macrophages and immature myeloid dendritic cells. Besides tissue macrophages the Macrophage Mannose Receptor (MMR), also known as CD206 and MRC1 (mannose receptor C, type 1) is expressed on lymphatic endothelial and on liver cells (Ramkumar et al. 2003, Santamaria et al. 2011). Besides N-mannan of *C. albicans* it identifies also N-acetyl-D-glucosamine and glycoprotein A of *Pneumocystis carinii* (Brown 2011).

Other receptors from C-type lectin family are dectin-1 and dectin-2 (dendritic cells-associated C-type lectins). Dectin-1 – the main receptor for β-glucan is expressed in myeloid dendritic cells. It initiates their endocytosis. Recognition of β-1-3 glucan by this receptor starts the response against various fungi including *Aspergillus*, *Candida*, *Pneymocystis* and *Coccidioides* (Burgeois et al. 2010, LeibundGut-Landman 2012). Sometimes the recognition of fungal β-glucans via dectin-1 is limited, because the morphology of fungus has been changed during phase transformation. A good example is *C. albicans*. During its' yeast phase, β-glucan is exposed only in budding scars, but on the surface of hyphal form of *C. albicans* this structure is covered by the mannan layer (Brown 2011, LeibundGut-Landman 2012). If β-glucan is masked by other components like α-glucan layer of *H. capsulatum* or by external hydrophobin of *A. fumigatus'* resting conidia, to recognize the fungus dectin-1 cooperates with TLRs or with other CLRs e.g. DC-SIGN (Burgeois et al. 2010, Brown 2011).

Dectin-2 identifies the mannose rich motifs of various pathogenic and opportunistic fungi including *H. capsulatum, P. brasiliensis, A. fumigatus, C. albicans* and *Trichophyton rubrum*. Together with dectin-1 it influences the activation of dendritic cells by fungal pathogens (Burgeois et al. 2010).

The third C type lectin – Mincle is typical for macrophages. It identifies α-mannose. Although, this receptor recognizes *C. albicans* and initiates the potent

inflammatory response, it is not necessary for the phagocytosis of this yeast. Mincle is also involved in *Malassezia* identification. Together with the FcγR (the receptor for Fc fragment of Igs) it also activates the response to *Fonsecaea pedrosi*, although this reaction results in chronic infection (Burgeois et al. 2010, Brown 2011, LeibundGut-Landmann et al. 2012).

Cooperation between several PRRs (also those from different families) enables the adjustment of the host response. It also improves the recognition of PAMPs and increases the specific pathogen identification (Santamaria et al. 2011, Burgeois and Kuchler 2012). For example, pathogenic *C. albicans* is detected by at least three PRRs identifying three surface-associated PAMPs. At first mannose receptor binds to N-linked, branched, long mannose chains of cell wall proteins, but TLR4 recognizes short, O-linked mannose residues of mentioned proteins. Additionally, complex of Dectin-1/TLR2 identifies β-glucan. First two PAMPs are present in the outer part of the cell wall, but β1,3-glucan is situated in its' inner layer. It is accessible only when surface mannoproteins are damaged or in the budding scars of *C. albicans* (Netea et al. 2006b). Also, TLR2 can cooperate with galectin-3 to differentiate *C. albicans* from non-pathogenic *Saccharomyces cerevisiae* (Burgeois et al. 2010).

All of mentioned pathogen recognition receptors activate innate effector cells, although the response induced by them may involve many different pathways. The result of PRRs stimulation by fungal PAMPs also leads to the production of various cytokines, including those triggering the adaptive immunity and T cells differentiation or influencing the antibody response (Burgeois et al. 2010, Brown 2011, LeibundGut-Landmann et al. 2012). The occurrence of various fungal PAMPs in chosen species of pathogenic and opportunistic fungi and their recognition by different PRRs is shown in Table 1.

The second step – neutralization of pathogen: complement activation, the inflammatory response and the phagocytic cells

In order to protect the host, fungal detection and recognition must be followed by neutralization of the invader. One of the most potent host-protecting systems is the complement system able to neutralize some pathogens. In uninfected hosts this system remains inactive, but during the infection complement cascade is instantly activated to destroy the invading microorganisms. Complement components – enzymatic and receptor proteins are produced by hepatocytes and macrophages. Some of them are stored in neutrophils. The accumulation of complement components in neutrophils, macrophages and in body fluids makes them easily accessible for the quick reaction to invaders (Tizard 2004).

Regardless of its innate nature the complement system is activated by both innate and acquired immune mechanisms. The classical activation pathway is initiated during fungal infection by immune complexes made by antibodies bound to fungal antigens. The second way of complement activation is alternative pathway, induced by direct contact of complement component C3 with the carbohydrates and lipopolysaccharides in microbial cell wall. The third way of complement activation is the lectin pathway. This innate mechanism is mediated by soluble mannose binding lectin (MBL) – the PRR mentioned above. All of three complement activation pathways lead to formation of membrane attack complex (MAC) and initiate the pathogen killing (Tizard 2004). In fungal infections the complement may be activated by various PAMPs like exposed β-glucan. Moreover, the recognition of fungal PAMPs may result in opsonization by soluble PRRs e.g. complement components like C3 or MBL. Subsequent recognition and phagocytosis is mediated by appropriate phagocyte receptors including complement receptor 3 (CR3). One of soluble PRRs is respiratory collectin – the pentraxin-3 present in the respiratory portals of entry. It opsonize the inhaled conidia of *A. fumigatus*, activating the complement deposition. The final result of this process is the neutrophilic phagocytosis of conidia (LeibundGut-Landmann et al. 2012). Among the end-effect of complement activation is also the production of chemotactic peptides including C5a and the complex C5b67. The first one attracts the neutrophils and eosinophils, while the second factor is chemotactic for mentioned cells and also for macrophages and basophils (Tizard 2004). Both neutrophils and macrophages are the main phagocytic cells included in the innate immune response for fungal infections (Brown 2011).

The complement activation is also strongly connected with acute inflammatory response, because some of its components degranulate mast cells and influence the release of vasoactive molecules from platelets (Tizard 2004). As it is known one of the clinical signs of dermatophytosis is strong inflammatory response observed as "the ring worm". In all fungal infections inflammatory response is controlled by various PRRs, which may either stimulate the inflammation processes or initiate the anti-inflammatory response. One result of activation of TLR2/TLR1 and TLR2/TLR6 heterodimers is the cytokine production e.g. pro-inflammatory tumor necrosis factor α (TNF-α) and Interleukin-12 (IL-12), that stimulates

adaptive response involving Th-1 cell. This reaction is required for clearance of some fungal infection (Blanco and Garcia 2008, Burgeois and and Kuchler 2012). Additionally, the same heterodimers may activate slightly different signaling pathway, using other intracellular adaptors or transcription factors and resulting in production of IL-10 and TGF-β (Transforming growth factor-β) (Burgeois and and Kuchler 2012). Both of mentioned cytokines are immunosuppressive. IL-10 suppresses the production of IL-2, IFN-γ and TNF-β and inhibits the secretion of TNF-α, IL-1 and IL-6. It also regulates the function of macrophages and Th-1 cells and activity of B and NK cells. TGF-β influences the differentiation of B and T cells, macrophages and dendritic cells (Tizard 2004). Other PRRs activate the synthesis of different cytokines, chemokines and other factors promoting the antigens processing by antigen presenting cells (APCs), including dendritic cells (DCs) (Bourgeois et al. 2010, LeibundGut-Landmann et al. 2012). Although TLRs are essential for both innate and adaptive immunity, activation of some of these receptors may be also harmful for the host, because they enhance the hyperproduction of pro-inflammatory cytokines in autoimmune or chronic inflammatory diseases (Zelante et al. 2007).

Besides the detection and recognition of fungal pathogens the innate immunity is also involved in neutralization of attacking fungi. The fungus entering the hosts' tissues activates the synthesis of chemotactic factors able to attract the leukocytes and all other effector immune cells. Among chemotactic factors are complement activation products mentioned above, then APs – antimicrobial peptides (described below) and leukotriens. All these factors attract the cells involved in host defense mechanisms e.g. neutrophils, monocytes, macrophages, dendritic cells, natural killer cells and T-γδ cells. After the recognition of fungus by various PRRs phagocytic cells can ingest and destroy fungal elements. Main cells involved in the phagocytosis and killing are macrophages and neutrophils, whereas dendritic cells internalize, process and present fungal antigens (Brown 2011). In different infections various primary effector cells are activated: neutrophils are involved in killing of *C. albicans* and *A. fumigatus,* while macrophages are the primary phagocytic cells during *Cryptococcus* or *Pneumocystis* infections (Blanco and Garcia 2008).

During the phagocytosis, the cellular membrane encloses the fungus in the intracellular phagosome. This process depends on actin, but is also conditioned by phagocyte type, the chemistry of fungal cell wall and the presence of serum opsonins, which facilitates phagocytosis. Moreover, different mechanisms are employed in phagocytosis of different morphological forms of fungus and in their uptake by dendritic cells. DCs can internalize conidia of *Aspergillus* and *Candida* yeasts by coiling mechanism, while hyphal forms of both fungi are phagocytosed by zipper-type mechanism. Additionally, phagocytes and dendritic cells can use special structures based on actin to internalize the fungus. Macrophages ingest conidia of *Aspergillus* by "ruffle-like" structure, while *C. parapsilosis* can be internalized by DCs and macrophages with special structures called "fungipods". After the uptake of fungal elements dendritic cells induce the adaptive immunity, because similarly to other APCs they process and present fungal antigens (Brown 2011).

Killing of the invading fungus may proceed either outside the phagocyte, when the fungal elements cannot be phagocytosed or within the phagolysosome inside the phagocytic cell. The whole process of phagosome maturation and phagolysosome formation is controlled by some TLRs, opsonic and non-opsonic receptors (Brown 2011). The major mechanism of fungal destruction by phagocytes is the respiratory (oxidative) burst, mediated by reactive oxygen intermediates (ROI). The protein complex of phagocyte component NOX2 ($gp91^{phox}$) forming NADPH oxidase is essential in this process. It assembles at the membrane of phagosome. If the fungal particles were not ingested, it is formed at the plasma membrane of the phagocyte. NADPH oxidase produces the superoxide, subsequently converted into toxic, reactive oxygen intermediates e.g. hydrogen peroxide or hydroxyl radicals. Other intermediates are hypoiodous and hypochlorous acids, produced by myeloperoxidase (MPO) of monocytes and neutrophils. Macrophages are deficient in MPO, but they may acquire this enzyme through mannose receptors and transport it into phagolysosome (Brown 2011). Genetic deficiency of phagocytic NADPH oxidase (e.g. humans with Chronic Granulomatous Disease – CGD) or the loss of MPO significantly increases the risk of severe mycoses including invasive aspergilosis or candydosis (Brown 2011). Production of reactive oxygen intermediates is activated by FcγR and by multiple PRRs including TLRs, soluble galectin-3 or membrane dectin-1 (Brown 2011).

Likewise ROI, the antifungal effect have also Reactive Nitrogen Intermediates (RNI), produced by inducible nitric oxide synthase (iNOS, NOS2). This enzyme removes the amino group from L-arginine and produces nitric oxide (NO), having a small antimicrobial activity of its own. Further reaction of this molecule with superoxide leads to peroxynitrite, which is the potent fungicide. The role of RNI is still uncertain and many aspects of this process remain to be explained. However, the data obtained from experiments on iNOS-deficient mice suggest, that RNI are

important in the outcome of *C. neoformans* infections. At the same time, mice deficient in both iNOS and in NOX2 (gp91^{phox}) show extreme susceptibility to candydosis, but *in vitro* their phagocytes maintain the ability to kill *Candida*. It suggests that another mechanism is involved in killing of pathogenic fungi in phagocytes (Brown 2011).

Phagocytic cells have also other, non-oxidative antifungal mechanisms to kill extracellular fungal particles as well as phagocytosed fungi. Among them are gelatinase-associated lipocalin found in neutrophils or special hydrolases – serprocidins. This group of serine proteases encloses cathepsin G, proteinase-3 and elastase. All of them are considered the important antimicrobial factors. These enzymes have antifungal activity e.g. against *Histoplasma, Candida* and *Aspergillus* (Brown 2011). Other elements of phagocyte-mediated antifungal immunity are Antimicrobial Peptides (AMPs) produced by phagocytes and epithelial cells, described below.

Humoral factors of the innate antimicrobial and antifungal immunity

As it was mentioned the first barriers for fungi are skin and mucous membranes. Their role as the physical obstacle for pathogens was described above, but there are some additional factors enhancing the first line of immunity: the antimicrobial peptides (APs; AMPs). APs are gathered in these sites, where the microbial invaders encounter preferably and in the natural "doorways" like the gastrointestinal tract or the airways. Various mammalian cells e.g. epithelial cells of mucosal tissues, leukocytes, circulating phagocytes and other cells can produce some AMPs, varying with chemical structures, antimicrobial potentials and effects on the host cells (Marshal and Arenas 2003, Brandenburg et al. 2012). Antimicrobial peptides are present in cytoplasmic granules inside some cells like neutrophils and lymphocytes, including NK-cells and cytotoxic T-cells (Tizard 2004).

In mammals AMPs were found in humans, pigs, cattle, horses (Brown 2011, Brandenburg et al. 2012) and in rhesus macaques, mice, rats, hamsters, guinea pigs, rabbits. Genes encoding AMPs were also identified in elephant, opossum and hedgehogs (Bruhn et al. 2009). Basing on their structural differences APs were divided into three families: defensins, cathelicidins and histatins. Defensins are cationic, cysteine-rich antimicrobial peptides observed in plants, invertebrates and vertebrates. Six cysteine residues, forming disulfide bonds are characteristic elements of their chemical structure (Bruhn et al. 2009). Mammalian defensins were classified in three families: aα-, β- and θ-defensins. Alfa-defensins were discovered in some mammalian species like humans, mice, rhesus macaques and horses, but they were not found in dogs and cattle. They are produced in neutrophils and in secretory, epithelial Paneth cells of the small intestine. The protective role of four different α-defensins RED1, 2, 3 and RED4, secreted in the gastrointestinal tract of the rhesus macaque has been observed. In horses thirty eight transcripts of intestinal h-defensins have been found, and at least twenty of them may code functional peptides (Bruhn et al. 2009). In humans six α-defensins have been described as the part of the systemic innate defense: four Human Neutrophil Peptides (HNP) and two Human Defensins – HD5 and HD6. They are contributing to the innate immunity of gastrointestinal mucosal surfaces (Cunliffe 2003). Human neutrophilic α-defensins HNP1-HNP4 have antifungal effect against *A. fumigatus, C. albicans, C. neoformans* and *H. capsulatum*, but the mechanism of this fungicidal activity is not explained (Brown 2011). Well-studied mice h-defensins are the cryptdins and cryptdins-related enteric peptides. Twenty three different peptides have been described in the small intestine, cecum, colon and rectum of these animals. It is presumed, that these defensins regulate local bacterial colonization and play an important role in the protection against the infection of given GI sections (Bruhn et al. 2009). In guinea pigs defensins called guinea pig neutrophil peptides (GPNPs) have revealed fungicidal effect against yeasts – *C. parapsilosis* and *C. neoformans* (Selsted and Harwig 1987). Defensins obtained from hamsters' neutrophils HaNP-1, 2, 3 and HaNP-4 (Hamster Neutrophil Peptides) have various antimicrobial activity depending on the peptide and its amount (Mak et al. 1996).

Beta-defensins being a part of humoral innate mechanisms have been found in mucus, in several tissues of different mammals and also in the udder. It is believed, that they are involved in maintaining the natural biota associated with multiple, different niches such as skin, the airways, the GI tract starting from the oral cavity and genitourinary tract (Weinberg et al. 1998, Bruhn et al. 2009, Brandenburg et al. 2012). *In vivo* β-defensins show anti-microbial activity against fungi e.g. *C. albicans,* some enveloped viruses and G+ and G- bacteria (Bruhn et al. 2009, Brandenburg et al. 2012).

Theta-defensins were isolated from leukocytes and from the bone marrow of rhesus macaques. In non-human Primates they display antifungal, antibacterial and antiviral activity. In humans mRNAs encoding two θ-defensins are expressed in the bone marrow, but functional θ-defensins are missing (Selsted 2004). It is presumed that it may influence the humans' susceptibility to HIV infections (Nguyen et al. 2003).

Table 2. The occurrence of various antimicrobial peptides (APs) and their activities in different mammals.

AP family/sub-family			Characteristic element of structure	Mamalian species	Antimicrobial activity/other activity
Defensins	α-defensins	cryptdins and cryptdins-related enteric peptides (23 peptides)	6 cysteine residues, forming disulfide bonds, position of the cysteins vary in sub-families	mice	regulation of local bacterial colonization; protection against the infection of selected gastrointestinal tract (GI) sections
		Human Neutrophil Peptides – HNP (4 peptides)		humans	induction of chemokine synthesis;
		Human Defensins – HD5 and HD6		humans	induction of chemokine synthesis
		4 α-defensins: RED1, RED2, RED 3 and RED4		rhesus macaques	protection of gastrointestinal tract (GI);
		DEFA I and ~20 other peptides		horses	probable protection of intestines; antimicrobial activity
				rats, rabbits, guinea pigs[1], hamsters[2]	[1]GPNPs – microbicidal for selected bacteria, antifungal against *C. albicans* and *C. neoformans* [2]HaNP-1, 2, 3, 4 – antibacterial activity depending on peptide and its concentration
	β-defensins			various species	anti-microbial activity against fungi e.g. C. albicans, G+ and G- bacteria and some enveloped viruses; maintaining the natural biota of different niches (e.g. skin, oral cavity and airways, the intestines, genito-urinary tract)
	θ-defensins			rhesus macaques, primates	antifungal, antibacterial and antiviral
Cathelicidins			N-terminal cathelin domain	humans, rodents, horses, pigs and cattle.	activity against parasites, bacteria or fungi; cytotoxic effect on eukaryotic cells; induction of chemokine synthesis
Histatins			cationic peptides, rich in histidine	humans, primates	maintaining of oral health; antibacterial and antifungal activity (e.g. Histatin 5 activity against *C. albicans, C. neoformans* and *A. fumigatus*)

The second group of AMPs are cathelicidins secreted by leukocytes. They have been identified in humans, rodents, horses, pigs and cattle. Their expression/presence is not restricted only to lymphoid organs, because they have been also found in the skin, intestinal epithelium and in the brain. Cytotoxic effect on eukaryotic cells and activity against parasites, bacteria or fungi has been demonstrated for cathelicidins. In humans there is the only one cathelicidin – hCAP-18. It has been found in monocytes, lymphocytes and NK cells, in neutrophil granules and in epithelial cells. The hCAP-18 has been also detected in plasma and in the secretions such as seminal fluid or sweat. This cathelicidin is able to disrupt cellular membranes of *Cryptococcus* and *Candida*, but it has no effect on filamentous fungi. Both cathelicidins and defensins induce chemokine synthesis (Brown 2011, Brandenburg et al. 2012).

Histatins, the third group of mammalian APs, reveal antibacterial and antifungal activity and maintain oral health. These peptides are secreted by the parotid, sublingual and submandibular salivary glands. The strong activity of histatin 5 against *C. albicans, C. neoformans* and *A. fumigatus* has been already proven. Antifungal activity of histatins encloses disruption of outer cellular membrane followed by cell lysis, dysregulation of the cell wall synthesis and depolymerysation of actin cytoskeleton (Brandenburg

et al. 2012). Table 2 shows the occurrence of mammalian APs and their known activity in chosen animal species.

Epithelial antimicrobial peptides, called small Cationic Antimicrobial Peptides (CAPs) have been found in various mammals, birds and other animals – insects and amphibians. They have strong cationic charge, because of multiple arginine and lysine residues. Among them are CAPs found in pigs – cecropins and protegrins (Weinberg et al. 1998).

Besides antimicrobial activity important feature of AMPs is their influence on the immune system. These peptides are chemotactic factors for immune and non-immune cells (e.g. macrophages, neutrophils, dendritic cells, T-cells). They act like adjuvants in the inflammatory response. Also it has been proven, that AMPs can modify the expression of pro-inflammatory cytokines such as TNF-α or IL-1β and anti-inflammatory cytokines like IL-10, at the same time. It indicates, that APs maintain the balance between the induction of inflammation and the protection of host from harmful effects of the excessive inflammatory response (Brandenburg et al. 2012). During infection their activity intensify innate and adaptive responses. Their impact on the host defense involves the enhancement of phagocytosis. Also, AMPs play a role in the recruitment and maturation of antigen presenting cells e.g. dendritic cells (Brandenburg et al. 2012). All collected data demonstrate, that antimicrobial peptides are significant elements of host-pathogen interactions and may determine their outcome.

Endothermy

Besides the complexity of their immunity, the mammalian resistance to pathogenic fungi is strongly connected with the endothermy of these animals (Robert and Casadeval 2009, Bergman and Casadevall 2010). This is an evolutionary achievement of warm-blooded mammals and birds. Despite all metabolic costs elevated rates of resting metabolism determine high and stable body temperature and ensure high, prolonged activity of these animals. Also, endothermic animals are independent of ambient temperature and capable of inhabiting various environments, including habitats with low or highly variable temperatures (Hillenius and Ruben 2004). Additionally, it is suggested that these physiological adaptations were selected during evolution, because they had provided better protection against many pathogens (Casadevall 2005). Data confirming this were presented in some experiments, done by Casadevall's groups (Robert and Casadevall 2009, Bergman and Casadevall 2010). The tests were based on the comparison of mammalian temperature with thermal growth tolerances of chosen ascomycetous and basidiomycetous species, examined for growth in temperatures ranging from +4°C to +45°C. Statistical analysis revealed that most of the investigated fungi can grow at temperature range 12-30°C, but their thermal tolerance decreases rapidly if the temperatures exceeds 35°C. Moreover, the authors noticed that every 1°C increase of the cultivation temperature, excluded the growth of further ~6% of strains. Additionally, the proportion of strains growing at 25°C was higher among the isolates from living hosts, than among those recovered from soil. At 37°C the proportion of growing strains was significantly higher for isolates from endothermic animals (Robert and Casadevall 2009). The authors have suggested, that temperatures in the 30-40°C range generate "the thermal exclusionary zone", which can protect mammals against many environmental pathogens. According to presented opinion, the inability of most fungal species to grow at the range of mammalian body temperature, reflects the relative paucity of fungal diseases in the immunocompetent mammals. Moreover, fever occurring as the systemic reaction during infection, adds further 1-3°C to the normal body temperature, what can also reduce the proportion of fungi inhabiting and surviving in the host (Robert and Casadevall 2009, Bergman and Casadevall 2010).

More data confirming mentioned hypothesis can be found in the literature. Normal body temperature of rabbits is 39-40°C. In these animals the systemic infection with*C. neoformans* is difficult to obtain even by direct injection of fungus. Probably their high body temperature makes them relatively resistant to the infection. However, the disease may be induced experimentally under two conditions: firstly, the fungus has to be inoculated directly to the testes, where the temperature is lower and secondly, the animals' immunity should be suppressed e.g. with administration of corticosteroids (Casadevall 2005). The influence of body temperature may also be observed in White Nose Syndrome (WNS) of bats, caused by *Geomyces destructans,* a psychrophilic fungus, colonizing the skin. The disease occurs in different species, but exclusively in hibernating animals, when their body temperature is the lowest (Casadevall 2005, Blehert et al. 2009). The recovery from WNS is possible when bats are awoken from hibernation, properly treated and kept in the temperature range 18-23°C (Meteyer et al. 2011, Casadevall 2012). Also, it was observed, that raising of the temperature in terrarium to the 37°C, along with the body temperature of frogs infected by *Batarachochytrium dendrobatidis*_enables the cure (Casadevall 2005). In general, it can be stated, that the increase of temperature may influence the positive outcome of the fungal disease.

Conclusion

The biological significance of fungi existing in the environment can hardly be ignored. In human and veterinary medicine the most important are fungi capable to infect the host and to develop clinical mycoses – the primary pathogens. Also, in recent years the major medical attention has been focused on saprophytic or commensal fungal species (like *Aspergillus, Cryptococcus* or *Candida*), causing severe, opportunistic infections in hosts with primary and secondary immunodeficiencies. However, the list of pathogenic fungi seems to be quite short when compared to numerous species prevailing in our environment. In the vast majority of cases, this constant contact with fungi does not lead to the infection, because mechanisms of innate immunity, followed by the more specific adaptive response are suitable for defense against the majority of fungi inhabiting our environment. If innate mechanisms are suppressed or disturbed or they are unable to avoid the fungal infection, critical role plays the cell mediated immunity with the dominance of Th1 type of the immune response (Blanco and Garcia 2008). In some cases innate mechanisms play a role in commensal host-fungal interaction, as it is with *C. albicans* participating in the oral, gastrointestinal or vaginal biota of healthy individuals (Jouault et al. 2006). The homeostasis between commensal yeasts and their host is conditioned by the presence of other members of biota and by hosts' defense mechanisms including physical barriers and immunity. Additionaly it is aided by special Antimicrobial Peptides (APs, AMPs) secreted by various cells, including epithelial cells of mucosal tissues and phagocytes. AMPs maintain the natural biota and together with it, prevent the colonization of pathogens in the gastrointestinal tract and in the genitourinary tract (Bruhn et al. 2009, Brandenburg et al. 2012). Antimicrobial peptides also influence both type of hosts' immunity by activation of various phagocytes and enhancing their phagocytic activity. Also, APs control the inflammatory response and take part in antigen presentation and differentiation of B- and T-cells (Brandenburg et al. 2012).

Innate immunity mechanisms have been programmed to detect all encountering microbes, including fungi, but they have not been designed for specific identification of particular fungus. The innate immunity provides the general reaction and quickly distinguishes the invaders on the basis of alien PAMP structures present on all microbes. The recognition is done by the set of various pathogen recognition receptors, expressed on host cells, including sentinel macrophages, neutrophils and dendritic cells or secreted in soluble form. PRRs like Toll-like receptors and C-type lectins, are activated by elements of the fungal cell wall (e.g. β-glucan, mannan, carbohydrate polymers and mannosylated proteins) or by fungal nucleic acids (Brown 2011, Burgeois and Kuchler 2012, LeibundGut-Landmann et al. 2012). After detection of fungal PAMPs, pathogen recognition receptors stimulate the inflammatory response including the migration of innate phagocytic cells and production of various cytokines. Some of them influence the acquired immunity either increasing the antifungal effect or suppressing it. In fungal infections macrophages and neutrophils are the main effector cells capable of destroying fungi. The internalization of fungus by phagocyte leads to the formation of phagosome and destruction of fungal elements by oxidative and non- oxidative mechanisms (Brown 2011).

The second great advantage of mammals in defense against fungal pathogens is edothermy, supporting mammalian immunity. Achieved during evolution high and stable body temperature makes "the thermal exclusion zone" for most of the fungi. The limit of mammalian temperature range 35-40°C is the boundary, which can hardly be explored by potentially pathogenic, environmental fungi (Robert and Casadeval 2009, Bergman and Casadevall 2010). Most of known primary fungal pathogens and opportunists such as *C. neoformans, P. marneffei* or *C. albicans* are thermo-tolerant, because only these fungi are able to grow in the temperature of mammalian body (Cooper and Haycocks 2000, Casadevall 2005, Casadevall 2007). Moreover, all pathogenic fungi have special adaptations allowing for evasion of innate defense mechanisms. They either inhibit the recognition by concealing of their PAMPs with extracellular capsule or "atypical" external layer or they are able to avoid phagocytosis, escape from phagosome or resist the oxidative burst with anti-oxidant enzymes. Usually, in clinical mycoses additional factors exist, increasing the susceptibility of host e.g. disorders causing the loss of phagocytic cells or the defects in their antifungal functions (Brown 2011). Moreover, the risk of opportunistic fungal infections increases enormously in all cases of primary/secondary immunodeficiences, including AIDS and oncological diseases and in endocrine disorders (e.g. diabetes) (Batura-Gabryel 2003, Sobol et al. 2009). In the majority of fungal infections in humans, observed in recent years, either the innate mechanisms of defense were debilitated or the invasive medical procedures helped the invading fungi to pass over the immunity (Nawrot and Karpiewska 2002, Pasqualotto et al. 2006). In animals, the relationship between the primary/secondary immunosuppression and susceptibility to fungal infections is not fully explained yet. However, it is known that in cases of Severe Combined Immunodeficiency syndrome (SCID) occurring in mice, Arabian horses and dogs of

some breeds (Basset Hounds, Jack Russell Terriers and Cardigan Welsh Corgi) increased susceptibility to various infections, including fungal were observed, due to inability to form the proper immune response (Perryman 2004). In our experiments, we have observed significantly higher yeast colonization of diabetic dogs and cats comparing to group of healthy animals.

Although the mammalian endothermy and mechanisms of immunity are competent and sufficient in most cases, we have created new possibilities for fungi to invade and to inhabit our organisms. Soon we'll be forced to find the new ways to support our natural barriers and innate mechanisms of defense.

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