New Insights in Medical Mycology

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Preface

Although the means of diagnosing and treating fungal infections have improved greatly over the last decade, fungi still represent a serious threat to the health of immunocompromised and immunodeficient patients. In addition to the more commonly encountered fungi, recent years have also seen the emergence of life-threatening infections due to fungi that had previously been seen only rarely in clinical practice. Many of these fungi are difficult to detect and to treat and their emergence as serious agents of disease among specific patient cohorts presents new challenges to the delivery of safe and effective antifungal therapy.

Recent developments in antifungal drug development have led to the welcome introduction of the Echinocandins and various azole derivatives into routine clinical use. Diagnosis of fungal infections has been improved with the utilization of PCR and immunoassay techniques. Despite these advances in diagnosis and therapy, fungi remain serious threats to the health of susceptible patients and we must strive to fully understand the fungi responsible for these infections and their interactions with the host's immune system if improved means of dealing with these infections are to be developed in the future.

The aim of this book is to give an in-depth assessment of our current understanding of the Biology of the main fungal pathogens and how they interact with the host. Each chapter focuses on a specific fungal pathogen or group of pathogens and examines their biology and the factors that allow the fungus colonize and disseminate within the host. In addition each chapter gives an indication of the challenges that remain to be tackled over the next 5–10 years in increasing our understanding of fungal pathogenicity. Each chapter is written by internationally recognized experts and this has ensured that the book is as comprehensive and authorative a text as is possible to assemble.

Chapter 1 gives a detailed description of the immune response of humans to pathogenic fungi and illustrates how an in-depth understanding of the host immune response can be utilized to better challenge fungal infection. Chapter 2 describes various *in vivo* models used to study the virulence of fungal pathogens. Chapter 3 presents an examination of the possibility of using 'alternative' animal models and demonstrates how the structural and functional similarities between the innate immune response of mammals and the insect immune response can be utilized to allow insect be used in place of mammals for the routine screening of fungal

mutants. Chapter 4 describes recent developments in the antifungal therapy and highlights the possibility of vaccines being used to prevent fungal infections.

Chapter 5 is the first chapter that deals with a specific pathogen and in this case the pathogen is *Candida albicans*, which has been the subject of much molecular examination in recent years to elucidate its virulence factors and this chapter also describes the current state of our knowledge and highlights the challenges that remain. Chapter 6 describes the biology of *Cryptococcus neoformans* and examines how this fungus interacts with the immune response. The Zygomycetes have emerged in recent years as serious etiological agents of disease in immunocompromised patients. In Chapter 7 the epidemiology, diagnosis, and treatment of zygomycosis is described. Recent developments in our understanding of the pathogenicity of *Aspergillus fumigatus* are described in Chapter 8, and Chapter 9 describes the factors affecting the virulence of *Penicillium marneffei*, which is a serious cause of disease in Southeast Asia among AIDS patients. Dermatophytic infections are one of the most widely encountered of all fungal infections and Chapter 10 describes how dermatophytes interact with the immune system to colonize areas of the body (skin, hair) that would normally be considered extremely hostile. Chapter 11 describes the biology of *Paracoccidioides brasiliensis*, which is the agent of the human systemic disease paracoccidioidomycosis that affects individuals in endemic areas extending from Argentina to Central America. Finally, Chapter 12 describes the occurrence and biology of *Fusarium* spp. and *Scedosporium* spp., which have recently emerged as important fungal pathogens causing significant morbidity and mortality especially in immunocompromised patients.

As well as providing a comprehensive assessment of our current understanding of the biology and pathogenicity of the principal fungal pathogens, each chapter provides an indication of the main challenges that remain to be tackled over the next 5–10 years in our efforts to improve patient recovery. It is the hope of all the contributors that this book will facilitate increased research into the interaction of pathogenic fungi with the immune response and allow the development of new and improved means of diagnosing and treating fungal infections.

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Chapter 1
Immunity to fungi

Luigina Romani

Abbreviations CMC – chronic mucocutaneous candidiasis; CMI – cell-mediated immunity; CR – complement receptor; CR3 – complement receptor 3; DC – dendritic cell; FcR – Fc receptor; IDO – indoleamine 2,3-dioxygenase; IL – interleukin; MBL – mannose-binding lectin; MR – mannose receptor; MyD88 – *Drosophila* myeloid differentiation primary response gene 88; PRR – pattern recognition receptor; TGF-β – transforming growth factor-β; Th, helper T cell; TLR – Toll-like receptor; Treg cell – regulatory T cell

Introduction

The kingdom of fungi consists of a number of species that are associated with a wide spectrum of diseases in humans and animals, ranging from allergy and autoimmunity to life-threatening infections. Most fungi (such as *Histoplasma capsulatum, Paracoccidioides brasiliensis, Coccidioides immitis, Blastomyces dermatitidis, Cryptococcus neoformans, Aspergillus fumigatus*, and *Pneumocystis jirovecii*) are ubiquitous in the environment. Some, including *Candida albicans*, establish lifelong commensalism on human body surfaces. Not surprisingly, therefore, human beings are constantly exposed to fungi, primarily through inhalation or traumatic implantation of fungal elements. The most common of the human diseases caused by fungi are the opportunistic fungal infections that occur in patients with defective immunity.

The switch of emphasis from morbidity to mortality has made the study of fungi a research priority. Because fungal pathogens are eukaryotes and therefore share many of their biological processes with humans, most antifungal drugs are associated with severe toxicity. No standardized vaccines exist for preventing any of the fungal infections of humans, a situation attributed both to the complexity of the pathogens involved and their sophisticated strategies for surviving in the host and evading immune responses (Dan & Levitz, 2006). However, provided that immunotherapy be tailored to specific immunocompromised states (Segal et al., 2006), the proper manipulation of the immune system is the challenge for future strategies that will prevent or treat fungal infections in susceptible patients.

Although not unique among infectious agents, fungi possess complex and unusual relationships with the vertebrate immune system, partly due to some

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prominent features. Among these, their ability to exist in different forms and to reversibly switch from one to the other in infection (Nemecek et al., 2006). Examples are the dimorphic fungi (*H. capsulatum, P. brasiliensis, C. immitis*, and *B. dermatitidis*) which transform from saprobic filamentous molds to unicellular yeasts in the host, the filamentous fungi (such as *Aspergillus* spp.) that, inhaled as unicellular conidia, may transform into a multicellular mycelium, and some species of *Candida*, capable of growing in different forms such as yeasts, blastospores, pseudohyphae, and hyphae. This implicates the existence of a multitude of recognition and effector mechanisms to oppose fungal infectivity at the different body sites. For commensals, two prominent features are also important, the highly effective strategies of immune evasion they must have evolved to survive in the host environment and the prolonged antigenic stimulation of the host that can have profound immunoregulatory consequences. Thus, in the context of the antagonistic relationships that characterize the host–pathogen interactions, the strategies used by the host to limit fungal infectivity are necessarily disparate (Romani, 2004b) and, in retaliation, fungi have developed their own elaborate tactics to evade or overcome these defenses (Romani, 2001; Huffnagle & Deepe, 2003; Shoham & Levitz, 2005; Hohl et al., 2006). This may have resulted in an expanded repertoire of cross-regulatory and overlapping antifungal host responses that makes it extremely difficult to define the relative contribution of individual components of the immune system in antifungal defense.

Within the limitations imposed by these considerations, this chapter is an advanced attempt to analyze the role of innate and adaptive immunity in resistance to pathogenic fungi. Through the involvement of different pattern recognition receptors, cells of the innate immune system not only discriminate between the different forms of fungi, but also contribute to discrimination between self and pathogens at the level of the adaptive T helper (Th) immunity (Romani, 2004b and references therein). Thus, the traditional dichotomy between the functions of innate and adaptive immunity in response to fungi has been recently challenged by the concept of an integrated immune response to fungi (Romani, 2004b and references therein).

The Immune Response to Fungi: From Microbe Sensing to Host Defensing

Most pathogenic fungi need a stable host–parasite interaction characterized by an immune response strong enough to allow host survival without elimination of the pathogen, thereby establishing commensalisms and latency. Therefore, the balance of pro-inflammatory and anti-inflammatory signaling is a prerequisite for successful host–fungus interaction. In light of these considerations, the responsibilities for virulence is shared by the host and the fungus at the pathogen–host interface, regardless the mode of its generation and maintenance. Studies with *C. albicans* have provided a paradigm that incorporates contributions from both the fungus and the host to explain the theme of the origin and maintenance of virulence for pathogens and commensals (Romani, 2004a). Through a high degree of

flexibility, the model accommodates the concept of virulence as an important component of fungus fitness *in vivo* within the plasticity of immune responses orchestrated by dendritic cells (DC). Conceptually, this implies that the qualitative development of adaptive response to a fungus may not primarily depend on the nature of the fungal form being presented but rather on the type of cell signaling initiated by the ligand–receptor interaction in DC. Therefore, the functional plasticity of DC at the pathogen–host interface may offer new interpretative clues to fungal virulence.

The host defense mechanisms against fungi are numerous, and range from protective mechanisms that appeared early in the evolution of multicellular organisms (referred to, collectively, as 'innate immunity') to sophisticated adaptive mechanisms, which are specifically induced during infection and disease ('adaptive immunity'). The innate mechanisms appeared early in the evolution of multicellular organisms and act early after the infection. Innate defense strategies are designed to detect broad and conserved patterns which differ between pathogenic organisms and their multicellular hosts. Most of the innate mechanisms are inducible upon infection and their activation requires specific recognition of invariant evolutionarily conserved molecular structures shared by large groups of pathogens by a set of pattern recognition receptors (PRR), including Toll-like receptors (TLR) (Akira & Takeda, 2004). In vertebrates, however, if the infectious organism can breach these early lines of defense an adaptive immune response will ensue, with generation of antigen-specific T helper (Th) effector and B cells that specifically target the pathogen and memory cells that prevent subsequent infection with the same microorganism. The two systems are intimately linked and controlled by sets of molecules and receptors that act to generate a highly coordinated and unitary process for protection against fungal pathogens. The dichotomous Th cell model has proven to be a useful construct that sheds light on the general principle that diverse effector functions are required for eradication of different fungal infections (Romani, 1999). The paradigm has greatly contributed to a better understanding of the host immune response to fungi from a regulatory perspective and has been helpful to accommodate clinical findings in a conceptual framework amenable to strategies of immune-interventions.

The Innate Immunity: The Art of Microbe Sensing and Shaping of Specific Immunity

The innate immune system distinguishes self from nonself and activates adaptive immune mechanisms by provision of specific signals. The constitutive mechanisms of defense are present at sites of continuous interaction with fungi and include the barrier function of body surfaces and the mucosal epithelial surfaces of the respiratory, gastrointestinal, and genitourinary tracts. Microbial antagonism (lactobacilli and bifidobacteria have shown efficacy in the biotherapy of candidiasis,

i.e. probiotics), defensins, and collectins comprise the major constitutive mechanisms of fungal immunity (Romani, 2004a).

Antigen-independent recognition of fungi by the innate immune system leads to the immediate mobilization of immune effector and regulatory mechanisms that provide the host with three crucial survival advantages: (i) rapid initiation of the immune response and creation of the inflammatory and co-stimulatory environment for antigen recognition; (ii) establishment of a first line of defense, which holds the pathogen in check during the maturation of the adaptive immune response; and (iii) steering of the adaptive immune response towards the cellular or humoral elements that are most appropriate for protection against the specific pathogen. Therefore, in order to achieve optimal activation of antigen-specific adaptive immunity, it is first necessary to activate the pathogen-detection mechanisms of the innate immune response.

The bulwark of the mammalian innate antifungal defense system is built upon effector mechanisms mediated by cells, cellular receptors, and a number of humoral factors (Romani, 2004a; Mansour & Levitz, 2002). The professional phagocytes, consisting of polymorphonuclear leukocytes (neutrophils), mononuclear leukocytes (monocytes and macrophages) and DC play an essential role. The antifungal effector functions of phagocytes include fungicidal and growth-inhibiting mechanisms, as well as processes to resist fungal infectivity, including inhibitory effects on dimorphism and promotion of phenotypic switching. The optimal restriction of fungal growth occurs via a combination of oxidative and complementary nonoxidative mechanisms, the latter consisting of intracellular or extracellular release of effector molecules, defensins, neutrophil cationic peptides, and iron sequestration. Enzymes such as the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and inducible nitric oxide synthase initiate the oxidative pathways known as the respiratory burst. Myeloperoxidase, a lysosomal hemoprotein found in azurophilic granules of neutrophils and monocytes, but not macrophages, is also a mediator in the oxygen-dependent killing of fungi. Patients with inherited X-linked chronic granulomatous disease (CGD), resulting from a deficiency in formation of activated oxygen radicals due to an NADPH-oxidase deficiency, have increased susceptibility to aspergillosis (Segal et al., 2000). However, transplantation of bone marrow cells transfected with the NADPH-oxidase gene has been shown to restore fungicidal activity of CGD patients (Barese et al., 2004). Myeloperoxidase deficiency predisposes to pulmonary candidiasis and aspergillosis, although it has not been shown to play an isolated role in fungal host defense in the absence of the NADPH oxidase.

The fact that both quantitative and qualitative defects of neutrophils are associated with an undue susceptibility to major disseminated fungal infections points to the important role that neutrophils play in the protective immunity to fungal diseases (Romani, 2004a; Fradin et al., 2006). Their functions may well go beyond microbicidal activity, and also include an immunoregulatory role in adaptive immunity (Romani et al., 1996). Myeloid suppressor cells are responsible for the immunosuppression observed in pathologies as dissimilar as tumor growth, immunosuppression, overwhelming infections, graft-versus-host disease, and pregnancy.

The reciprocal relationship of neutrophils and T lymphocytes further implies that the immune resistance to fungi is a highly coordinated and unitary process.

Macrophages are a heterogeneous population of tissue resident cells possessing the machinery for antigen presentation; however, their main contribution to antifungal defense is through phagocytosis and killing of fungi and immunomodulation (Vazquez-Torres & Balish, 1997; Cortez et al., 2006). Not surprisingly, therefore, fungi have exploited a variety of mechanisms or putative virulence factors to evade phagocytosis, escape destruction and survive inside macrophages (Woods, 2003; Alvarez & Casadevall, 2006). Macrophages serve as a protected environment in which the dimorphic fungi multiply and disseminate from the lung to other organs. *H. capsulatum* is a teaching example of a successful intracellular pathogen of mammalian macrophages.

Humoral factors contribute to and enhance the innate defense mechanisms. Mannose-binding protein or lectins (MBL), collectins, complement (a group of proteins activated in cascading fashion) and antibodies promote binding (opsonization) of the fungal organism and represent a recognition mechanism carried out by a variety of receptors and PRR that have a hierarchical organization. The collectin pentraxin 3 has shown a nonredundant role in antifungal resistance to *A. fumigatus* by promoting conidial recognition and phagocytosis, as well as activation of effector phagocytes (Garlanda et al., 2002). The specific biological activities of the complement system and antibodies, which contribute to host resistance are multifaceted and interdependent (Romani & Kaufmann, 1998 and references therein). For example, antibodies greatly contribute to the activation of the complement system by fungi and complement is essential for antibody-mediated protection. Each receptor on phagocytes not only mediates distinct downstream intracellular events related to clearance, but it also participates in complex and disparate functions related to immunomodulation and activation of immunity, depending on cell type. The receptors below are teaching examples. Engagement of CR3 (also known as CD11b/CD18) is one most efficient means of engulfing opsonized fungi, but it also has the remarkable characteristic of a broad recognition capacity of diverse fungal ligands. The multiplicity of binding sites and the existence of different activation states enable CR3 to engage in disparate (positive and negative) effector activities against fungi. Thus, because signaling through CR3 may not lead to phagocyte activation without the concomitant engagement of receptors for the Fc portion of immunoglobulins (FcR), this may contribute to intracellular fungal parasitism. It is of interest, therefore, that *H. capsulatum* uses this receptor for entry into macrophages, where it survives, and not into DC, where it is rapidly degraded. Likewise, *Candida* exploits entry through CR3 to survive inside DC. In contrast, ligation of FcR is usually sufficient to trigger phagocytosis, a vigorous oxidative burst, and the generation of pro-inflammatory signals. Ultimately, recognition of antibody-opsonized particles represents a high-level threat.

The absence of an association between deficits in antibodies and susceptibility to fungal infections and the presence of specific antibodies in patients with progressive fungal infections have been the main arguments against a protective role of antibodies in fungal infections. Recent advances have demonstrated that

both protective and nonprotective antibodies against fungi can be demonstrated, the relative composition and proportion of which may vary greatly in infections (Cassone et al., 2005). As a matter of fact, antibodies to HSP90 are associated with recovery from *C. albicans* infections, protection against disseminated disease in patients with AIDS, and synergize with antifungal chemotherapy (Pachl et al., 2006). Complement, antibodies and collectins not only fulfill the requirement of a first line of defense against fungi, but have also an impact on the inflammatory and adaptive immune responses, through several mechanisms, including regulation of cytokine secretion by and costimulatory molecule expression on phagocytes. The local release of these effector molecules regulates cell trafficking in various types of leukocytes, thus initiating an inflammatory response, activates phagocytic cells to a microbicidal state, and directs Th/Treg-cell development (Romani, 2004b).

Sensing Fungi: The TLR and Non-TLR Recognition System

TLR, which are broadly distributed on cells of the immune system, are arguably the best-studied immune sensors of invading pathogens, and the signaling pathways that are triggered by pathogen detection initiate innate immunity and help to strengthen adaptive immunity. TLR belong to the TIR (Toll/interleukin-1 (IL-1) receptor) superfamily, which is divided into two main subgroups: the IL-1 receptors and the TLR. All members of this superfamily signal in a similar manner owing to the presence of a conserved TIR domain in the cytosolic region, which activates common signaling pathways, most notably those leading to the activation of the transcription factor nuclear factor-κB (NF-κB) and stress-activated protein kinases that activate the transcription of the inflammatory and adaptive immune responses. The common signal pathways utilized by IL-1R and TLR involve the recruitment of several adapter proteins, including MyD88 (*Drosophila* myeloid differentiation primary response gene 88), that activates, in turn, a series of kinases that are crucially involved in innate immunity (Akira, 2003). Evidence suggests that individual members of the TLR family or other PRR interact with each other and cumulative effects of these interactions instruct the nature and outcome of the immune response to the provoking pathogen (Mukhopadhyay et al., 2004). TLR activation is a double-edged sword. It is essential for provoking the innate response and enhancing adaptive immunity against pathogens. However, members of the TLR family are also involved in the pathogenesis of autoimmune, chronic inflammatory inflammatory disorders, such as asthma, rheumatoid arthritis, and infectious diseases. Thus, by hyperinduction of pro-inflammatory cytokines, by facilitating tissue damage or by impaired protective immunity, TLR might also promote the pathogenesis of infections.

A number of cell wall components of fungi may act as TLR activators (Levitz, 2004; Netea et al., 2006). The different impact of TLR on the occurrence of the innate and adaptive Th immune response to fungi is consistent with the ability of each individual TLR to activate specialized antifungal effector functions on

phagocytes and DC. Although not directly affecting phagocytosis, TLR influence specific antifungal programs of phagocytes, such as the respiratory burst, degranulation, and production of chemokines and cytokines (Bellocchio et al., 2004a, b). As the quantity and specificity of delivery of toxic neutrophil products ultimately determine the relative efficiency of fungicidal activity versus inflammatory cytotoxicity to host cells (Bellocchio et al., 2004b), this implicates that TLR may contribute to protection and immunopathology against fungi. Although the simultaneous engagement of multiple TLR, as well as TLR cooperativity *in vivo*, makes it difficult to gauge the relative contribution of each single fungal morphotype in TLR activation and functioning, the emerging picture calls for: (i) the essential requirement for the MyD88-dependent pathway in the innate and Th1-mediated resistance to fungi (Bellocchio et al., 2004a; Biondo et al., 2005; Rivera et al., 2006); (ii) the crucial involvement, although not essential, of the TLR4/MyD88 pathway in recognition of and resistance to *A. fumigatus* (Netea et al., 2003; Bellocchio et al., 2004a); (iii) the beneficial effect of TLR9 stimulation on immune-mediated resistance to fungal pneumonia (Bozza et al., 2002; Edwards et al., 2004); (iv) the dependency of Treg induction on selected TLR (Netea et al., 2004); (v) the exploitation of TLR as a mechanism to divert and subvert host immune responses (Netea et al., 2004), and (vi) the association of selected TLR polymorphisms with susceptibility to fungal infections (Kesh et al., 2005).

C-type lectin receptors (i.e. Dectin-1 and 2, DC-SIGN, and the galectin family) are major mammalian PRR for several fungal components and are the prototype of innate non-TLR signaling pathway for innate antifungal sensing (Brown, 2006). The finding that N-linked mannosyl residues on fungal cells are bound by the MR, and O-linked mannosyl residues are bound by TLR4 (Netea et al., 2006) provide mechanistic insights into the cooperative signaling between TLR and non-TLR for full innate immune cell activation (Steele et al., 2005; Gross et al., 2006; Sato et al., 2006; Taylor et al., 2006; Saijo et al., 2006; Gersuk et al., 2006).

Tuning the Adaptive Immune Responses: The Instructive Role of DC

As DC are equipped with several TLR, they are the main connectors of the innate and adaptive immune systems. DC are bone marrow-derived cells of both lymphoid and myeloid stem cell origins that populate all lymphoid organs, as well as nearly all nonlymphoid tissues and organs. The dual activation/tolerization function of DC is mediated by their capacity to change the context of antigen presentation and to communicate to T cells the nature of the antigens they are presenting. This process exemplifies the importance of TLR not only in direct early immune responses, but also in activation of adaptive immunity. The DC system consists of a network of different subpopulations (Romani & Puccetti, 2006a). The ability of a given DC subset to respond with flexible activating programs to the different stimuli, as well as the ability of different subsets to convert

into each others confers unexpected plasticity to the DC system. DC are uniquely adept at decoding the fungus-associated information and translating it in qualitatively different adaptive T-cell immune responses (Romani & Puccetti, 2006a). PRR (such as CR, FcR, C-type lectins (such as DC-SIGN and dectin-1), MR, and TLR determine the functional plasticity of DC in response to fungi and contribute to the discriminative recognition of the different fungal morphotypes. DC (both human and murine) are now known to recognize and internalize a number of fungi, including *A. fumigatus, C. albicans, C. neoformans, H. capsulatum, Malassezia furfur*, and *Saccharomyces cerevisiae* and fungi and fungal products may affect DC functioning as well (Romani & Puccetti, 2006a; Buentke & Scheynius, 2003). DC are also known to cross-present exogenous fungal antigens through uptake of apoptotic macrophage-associated fungal antigens (Lin et al., 2005). Profiling gene expression on DC by microarray technologies has revealed that both shared response and a pathogen-specific gene expression program were induced upon the exposure to bacteria, viruses and fungi. Additional studies with *S. cerevisiae* have shown that recombinant yeast could represent an effective vaccine for the generation of broad-based cellular immune responses. It seems, therefore, that DC are uniquely able at decoding the fungus-associated information at the host–fungus interface. *Candida* and *Aspergillus* proved to be useful pathogen models to dissect events occurring at the fungus–DC interface. Murine and human DC internalize *Candida* yeasts, *Aspergillus* conidia and hyphae of both. The uptake of the different fungal elements occurred through different receptors and forms of phagocytosis. Transmission electronic microscopy indicated that internalization of yeasts and conidia occurred predominantly by coiling phagocytosis, characterized by the presence of overlapping bilateral pseudopods, which led to a pseudopodal stack before transforming into a phagosome wall. In contrast, entry of hyphae occurred by a more conventional zipper-type phagocytosis, characterized by the presence of symmetrical pseudopods which strictly followed the contour of the hyphae before fusion. Recognition and internalization of unopsonized yeasts and conidia occurred through the engagement of MR of different sugar specificity, DC-SIGN, dectin-1, and partly, CR3 (Claudia et al., 2002; Mansour et al., 2006). In contrast, entry of hyphae occurred by a more conventional, zipper-type phagocytosis and involved the cooperative action of FcγR II and III and CR3. Phagocytosis does not require TLR/MyD88. Consistent with the findings that signals from protein kinase C (PKC) and/or protein tyrosine kinases are required for phagocytosis in a variety of systems, the PKC inhibitor staurosporine was required for CR- and FcγR-mediated phagocytosis, while FcγR- and, to a lesser extent, MR-mediated phagocytosis required signaling through protein tyrosine kinases (Claudia et al., 2002). The results are consistent with the view that fungi have exploited common pathways for entry into DC, which may include a lectin-like pathway for unicellular forms and opsono-dependent pathways for filamentous forms.

The engagement of distinct receptors by distinct fungal morphotypes translates into downstream signaling events, ultimately regulating cytokine production and costimulation, an event greatly influenced by fungal opsonins, such as MBL, C3,

and/or antibodies (Romani et al., 2004; Romani et al., 2002). Entry through MR and dectin-1 resulted in the production of pro-inflammatory cytokines, including IL-12, upregulation of costimulatory molecules and histocompatibility Class II antigens. IL-12 production by DC required the MyD88 pathway with the implication of distinct TLR. These events were all suppressed upon entry through CR3. In contrast, coligation of CR3 with FcγR, as in the phagocytosis of hyphae, resulted in the production of IL-4/IL-10 and upregulation of costimulatory molecules and histocompatibility Class II antigens. The production of IL-10 was largely MyD88-independent. Therefore, TLR collaborate with other innate immune receptors in the activation of DC against fungi through MyD88-dependent and MyD88-independent pathways (Romani & Puccetti, 2006a).

A remarkable and important feature of DC is their capacity to produce IL-10 in response to fungi. These IL-10-producing DC activate $CD4^+$ $CD25^+$ Treg cells that are essential components of antifungal resistance (see below). Thus, by subverting the morphotype-specific program of activation of DC, opsonins, antibodies, and other environmental factors may qualitatively affect DC functioning and Th/Treg selection *in vivo*, ultimately impacting on fungal virulence. In this scenario, the qualitative development of the Th cell response to a fungus may not primarily depend on the nature of the fungal form being phagocytosed and presented. Rather, the nature of the cell response is strongly affected by the type of cell signaling initiated by the ligand–receptor interaction in DC. For *Candida*, the paradigm would predict that dimorphism per se can no longer be considered as the single most important factor in determining commensalism versus infection, nor can specific forms of the fungus be regarded as absolutely indicative of saprophytism or infection at a given site. The selective exploitation of receptor-mediated entry of fungi into DC could explain the full range of host immune–parasite relationships, including saprophytism and infection. Importantly, as both fungal morphotypes, but particularly hyphae, activate gut DC for the local induction of Treg cells and because the morphogenesis of *C. albicans* is activated *in vivo* by a wide range of signals, it appears that the discriminative response towards Treg cell function is of potential teleological meaning. It could indeed allow for fungal persistence in the absence of the pathological consequences of an exaggerated immunity and possible autoimmunity, a condition which represents the very essence of fungal commensalism. Therefore, in addition to the induction of phase-specific products enhancing fungal survival within the host, transition to the hyphal phase of the fungus could implicate the induction of immunoregulatory events that will benefit the host.

Fungus-pulsed DC translated fungus-associated information to Th1, Th2, and Treg cells, *in vitro* and *in vivo* (Romani & Puccetti, 2006a). *In vivo*, the balance among the different DC subsets determined whether protective or nonprotective antifungal cell-mediated immune responses developed. Fungus-pulsed DC activated different $CD4^+$ Th cells upon adoptive transfer in a murine model of allogeneic bone marrow transplantation (Bozza et al., 2005; Romani et al., 2006). The ability of fungus-pulsed DC to prime for Th1 and Th2 cell activation upon adoptive transfer *in vivo* correlated with the occurrence of resistance and susceptibility to the infections. Recent data have shown that the infusion of fungus-pulsed

DC of the different subsets accelerated the recovery of peripheral antifungal Th1 immunity and increased resistance to fungal infections in a murine model of allogeneic bone marrow transplantation (Romani et al., 2006). However, only the co-infusion of DC of both subsets resulted in: (i) induction of T reg cells capable of a fine control over the inflammatory pathology; (ii) tolerization toward alloantigens; and (iii) diversion from alloantigen-specific to antigen-specific Tcell responses in the presence of donor T lymphocytes. Thus, the adoptive transfer of DC may restore antifungal immunocompetence in hematopoietic transplantation by contributing to the educational program of T cells through the combined action of activating and tolerizing DC. These results, along with the finding that fungus- pulsed DC could reverse T-cells anergy of patients with fungal diseases (Romani & Puccetti, 2006a), may suggest the utility of DC for fungal vaccines and vaccination (Bozza et al., 2004; Lam et al., 2005).

The Adaptive Immunity: Th1, Th2, and Th17 Cells

Serological and skin reactivity surveys indicate that fungal infections are common, but clinical disease is rare, consistent with the development of acquired immunity. Underlying acquired immunity to *C. albicans*, such as the expression of a positive delayed type hypersensitivity, is demonstrable in adult immunocompetent individuals, and is presumed to prevent mucosal colonization from progression to symptomatic infection (Puccetti et al., 1995). Lymphocytes from healthy subjects show strong proliferative responses after stimulation with fungal antigens and produce a number of different cytokines (Romani, 2004b). For many fungal pathogens, the effective tissue response to invasion is granulomatous inflammation, a hallmark of cell-mediated immunity (CMI). There is extensive plasticity in the T-cell response to fungi (Romani & Puccetti, 2006a). The heterogeneity of the $CD4^+$ and $CD8^+$ T cell repertoire may account for the multiplicity and redundancy of effector mechanisms through which T lymphocytes participate in the control of fungal infections. The flexible program of T lymphocytes also implicates the production of a number of mediators, including cytokines. Due to their action on circulating leukocytes, the cytokines produced by fungus-specific T cells are instrumental in mobilizing and activating antifungal effectors, thus providing prompt and effective control of infectivity once the fungus has established itself in tissues or spread to internal organs. Therefore, host resistance to fungi appears to be dependent upon the induction of cellular immunity, mediated by T lymphocytes, cytokines, and a number of effector phagocytes (Romani, 2004b).

The clinical circumstances in which fungal infections occur definitely suggest an association with impaired CMI. AIDS and severe hematological malignancies are examples of acquired defects in T-cell function that predispose to severe fungal infections. Interestingly, however, defective CMI may also be a consequence of fungal virulence (Fischer et al., 1978; Yauch et al., 2006). Furthermore, the occurrence of severe disseminated infections by filamentous fungi in non-granulocytopenic

patients, as well as in concomitance with the onset of graft-versus-host disease in bone marrow transplant recipients are compelling evidence of the pathogenic role of T-cell dysreactivity in infection. In endemic mycosis, the severity of the disease correlates with the degree of impairment of CMI, associated with elevated levels of antibodies (Romani, 2004b).

Generation of a dominant Th1 response driven by IL-12 is essentially required for the expression of protective immunity to fungi. Through the production of the signature cytokine IFN-γ and help for opsonizing antibodies, the activation of Th1 cells is instrumental in the optimal activation of phagocytes at sites of infection. Therefore, the failure to deliver activating signals to effector phagocytes may predispose patients to overwhelming infections, limit the therapeutic efficacy of antifungals and antibodies, and favor persistency and/or commensalism. Immunological studies in patients with polar forms of paracoccidioidomycosis demonstrate an association between Th1-biased reactivity and the asymptomatic and mild forms of the infection, as opposed to the positive correlation of Th2 responses with the severity of the disease. Not surprisingly, therefore, patients with disseminated infection show defective production of IFN-γ and DTH anergy, associated with elevated levels of type 2 cytokines (IL-4 and IL-5), IgE, IgG4 and IgA, and eosinophilia, which is a marker of poor prognosis in endemic mycoses (Romani & Kaufmann, 1998 and references therein). In patients with defective IL-12/IFN-γ pathway, such as those with hyperimmunoglobulinemia E syndrome, fungal infections, and allergy are both observed (Romani, 2004b). Deficient IFN-γ receptor-mediated signaling occurs in neonates and may predispose to fungal infections. IL-4 is one major discriminative factor of susceptibility and resistance in most fungal infections. The most important mechanism underlying the inhibitory activity of IL-4 in infections relies on its ability to act as the most potent proximal signal for commitment to Th2 reactivity that dampens protective Th1 responses and favors fungal allergy. In atopic subjects, the suppressed DTH response to fungi is associated with elevated levels of antifungal IgE, IgA, and IgG. However, susceptibility to fungal infections may not always be associated with an overt production of IL-4. For instance, although an association between chronic disseminated candidiasis and genetic variants of IL-4 has been recently described (Romani, 2004b), IL-4 or IL-5 are not always increased in patients with chronic mucocutaneous candidiasis (CMC), despite a defective type 1 cytokine production (Lilic et al., 2003).

Recent studies have suggested a greater diversification of the $CD4^+$ T cell effector repertoire than that encompassed by the Th1/Th2 paradigm (Dong, 2006). Th17 cells are now thought to be a separate lineage of effector Th cells contributing to immune pathogenesis previously attributed to the Th1 lineage. Although the developmental pathways leading to Th17 differentiation *in vitro* are still unclear, IL-23 is a critical cytokine for the generation and maintenance of this lineage. IL-12 and IL-23 are members of a small family of pro-inflammatory heterodimeric cytokines that share a common p40 subunit linked to the IL-12p35 chain or the IL-23p19 chain. IL-23 functions through a receptor complex composed of the IL-12Rβ1 subunit and a unique component, the IL-23R chain. Both cytokines induce IFN-γ expression in $CD4^+$ T cells, though only IL-23 facilitates a T-helper state marked by production of the pro-inflammatory cytokine, IL-17. Despite these similarities,

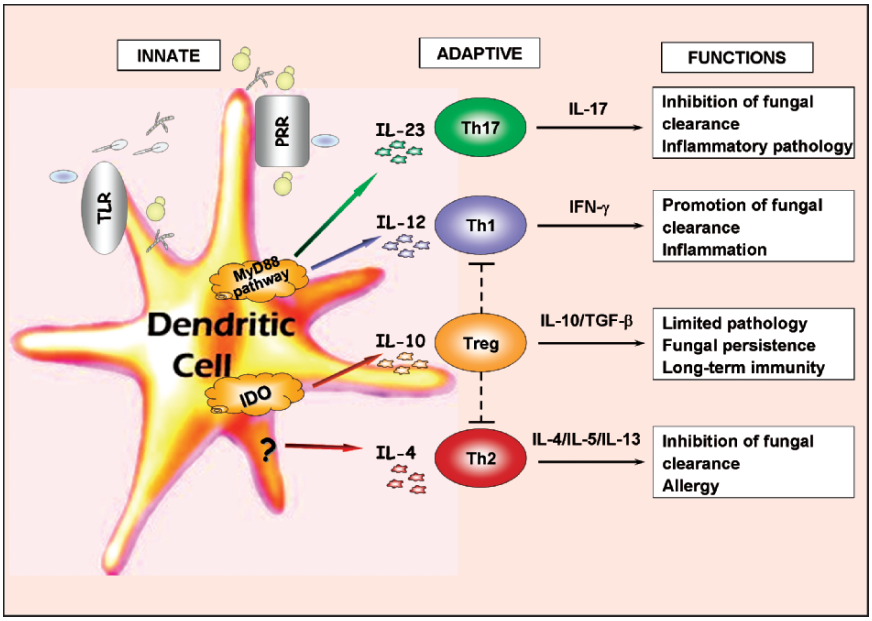

Figure 1.1 Pathways of innate and adaptive antifungal immunity: the role of dendritic cells, tryptophan catabolism, and Th subsets. The majority of fungi are detected and destroyed within hours by innate defense mechanisms. These mechanisms act immediately and are followed some hours later by an early induced response, which must be activated by infection but does not generate lasting protective immunity. These early phases help to keep infection under control. In vertebrates, however, if the infectious organism can breach these early lines of defense an adaptive immune response will ensue, with generation of antigen-specific T helper (Th) effectors and regulatory T (Treg) cells that specifically target the pathogen and induce memory cells that prevent subsequent infection with the same microorganism. Dendritic cells (DC) sample fungi at the site of colonization/infection, transport them to the draining lymph nodes and activate disparate Th/Treg cells in a morphotype- and tissue-dependent fashion. The activity of DC involves the pattern recognition receptors (PRR), including Toll-like receptors (TLR) and the enzyme indoleamine 2,3-dioxygenase (IDO)-dependent metabolic pathways leading to T cell activation and regulation. As the different Th cell subsets release a distinct panel of cytokines, capable of delivering, activating, and deactivating feedback signals to effector phagocytes, the activation of the appropriate Th subset is instrumental in the generation of a successful immune response to fungi. Counter-regulatory Treg cells may serve to dampen the excessive inflammatory reactions and to contribute to the development of memory antifungal immunity. Solid and broken lines refer to positive and negative signals, respectively

there is increasing evidence that IL-12 and IL-23 drive divergent immunological pathways (Trinchieri et al., 2003). Th cells primed for IL-17 production appear to have important roles in autoimmune diseases (Harrington et al., 2006). Moreover, although less clear, the production of high levels of IL-23/IL-17, more than IL-12/IFN-γ, better correlates with disease severity and immunopathology in diverse infections (Hunter, 2005). These studies suggest that IL-12 and IL-23 have distinct roles in promoting antimicrobial immune responses and diseases *in vivo*. Recent evidence indicated that the IL-23/IL-17 developmental pathway may act as a negative regulator of the Th1-mediated immune resistance to

fungi and played an inflammatory role previously attributed to uncontrolled Th1 cell responses. Both inflammation and infection were exacerbated by a heightened Th17 response against *C. albicans* and *A. fumigatus*. Both IL-23 and IL-17 subverted the inflammatory program of neutrophils and promoted fungal virulence, which impacted severely on tissue inflammatory pathology associated with infection (author's unpublished observations). Our data support a model in which IL-23-driven inflammation promotes infection and impairs antifungal immune resistance (Figure 1.1). Thus, modulation of the inflammatory response represents a potential strategy to stimulate protective immune responses to fungi.

Dampening Inflammation and Allergy to Fungi: A Job for Treg Cells

The inflammatory response to fungi may serve to limit infection but may also contribute to pathogenicity, as documented by the occurrence of severe fungal infections in patients with immunoreconstitution disease (Cheng et al., 2000). These patients may experience intractable fungal infections despite recovery from neutropenia and the occurrence of adaptive immune responses. The above considerations imply that immunoregulation may be essential in fine-tuning inflammation and adaptive Th reactivity to fungi and fungal diseases. This imposes a new job upon the immune system. In addition to efficient control of pathogens, tight regulatory mechanisms are required in order to balance protective immunity and immunopathology. To limit the pathologic consequences of an excessive inflammatory cell-mediated reaction, the immune system resorts to a number of protective mechanisms. $CD4^+$ T cells making immunoregulatory cytokines such as IL-10, transforming growth factor (TGF)-β and IL-4 have long been known and discussed in terms of immune deviation or class regulation. Recently, Treg cells, capable of fine-tuning protective antimicrobial immunity in order to minimize harmful immune pathology, have become an integral component of the immune response (Montagnoli et al., 2002; Montagnoli et al., 2006; Romani & Puccetti, 2006b; Hori et al., 2002; Cavassani et al., 2006; McKinley et al., 2006). The decision of how to respond will still be primarily determined by interactions between pathogens and cells of the innate immune system, but the actions of Treg cells will feed back into this dynamic equilibrium to regulate subsequent immune responses. Usually, Treg cells serve to restrain exuberant immune reactivity, which in many chronic infections benefits the host by limiting tissue damage. However, the natural Treg cell responses may handicap the efficacy of protective immunity. Conceptually, similar to their effect on immunity against pathogens, Treg cells can also impede effective immunosurveillance of tumors. Nowadays, aberrant numbers and/or functions of Treg cells are incorporated within the view of counter-regulatory elements affecting the self versus nonself discrimination and influencing the outcome of infection, autoimmunity, transplantation, cancer, and even allergy.

A number of clinical observations suggest an inverse relationship between IFN-γ and IL-10 production in patients with fungal infections. High levels of IL-10, negatively affecting IFN-γ production, are detected in chronic candidal diseases, in the severe form of endemic mycoses and in neutropenic patients with aspergillosis (Romani, 2004b). Fungal polysaccharides are known to negatively modulate CMI through the production of IL-10, a finding suggesting that IL-10 production may be a consequence of infection (Romani & Puccetti, 2006b). However, tolerance to fungi can also be achieved through the induction of Treg cells capable of finely tuning antifungal Th reactivity. Naturally occurring Treg cells operating in the respiratory or the gastrointestinal mucosa accounted for the lack of pathology associated with fungal clearance in mice with fungal pneumonia or mucosal candidiasis (Montagnoli et al., 2002, 2006). Distinct Treg populations capable of mediating anti-inflammatory or tolerogenic effects are coordinately induced after exposure to *Aspergillus* conidia. Ultimately, the inherent resistance to *Aspergillus* diseases suggests the existence of regulatory mechanisms that provide the host with protection from infection and tolerance to allergy. It has been demonstrated that a division of labor occurs between functionally distinct Treg cells that are coordinately activated after exposure of mice to *Aspergillus* resting conidia. Early in infection, inflammation is controlled by the expansion, activation, and local recruitment of Treg cells suppressing neutrophils through the combined actions of IL-10 and cytotoxic T lymphocyte antigen-4 on the enzyme indoleamine 2,3-dioxygenase (IDO) (see below). Late in infection, and similarly in allergy, tolerogenic Treg cells which produce IL-10 and TGF-β inhibit Th2 cells and prevent allergy to the fungus.

It has long been known that the ability of *C. albicans* to establish an infection involves multiple components of the fungus, but its ability to persist in host tissue might involve primarily the immunosuppressive property of a major cell wall glycoprotein, mannan (Nelson et al., 1991). Although epitopes of mannan exist endowed with the ability to induce protective antibodies to the fungus, mannan and oligosaccharide fragments of it could be potent inhibitors of cell-mediated immunity and appear to reproduce the immune deficiency in patients with the mucocutaneous form of candidiasis (Fischer et al., 1978). CMC, although encompassing a variety of clinical entities, has also been associated with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (Peterson et al., 1998). Interestingly, in APECED, the mutated gene has been proposed to be involved in the ontogeny $CD25^+$ Treg cells (Sakaguchi et al., 1996). In CMC, both anergy and active lymphoproliferation and variable-delayed hypersensitivity to the fungus are indeed observed (Lilic, 2002). As already discussed, this has been associated with a defective type 1 cytokine production without obvious increase in type 2 cytokine production (namely IL-4 or IL-5). However, variable, either increased or not, levels of IL-10 have also been observed, a finding that may lead to the speculation that an inherent alteration in receptor-mediated signaling in response to fungal polysaccharide, may predispose patients with CMC to a dysfunctional induction of Treg, negatively affecting the capacity of the Th1-dependent clearance of the fungus and without the activation of Th2 cells.

Collectively, these observations suggest that the capacity of Treg cells to inhibit aspects of innate and adaptive immunity may be central to their regulatory activity in fungal infections. This may result in the generation of immune responses vigorous

enough to provide adequate host defense, without necessarily eliminating the pathogen (which could limit immune memory) or causing an unacceptable level of host damage. In the last two decades the immunopathogenesis of fungal infections and associated diseases was explained primarily in terms of Th1/Th2 balance. While the pathogenetic role of either subset may still hold true, the reciprocal regulation of both subsets is apparently outdated. It appears that a combination of different types of Treg cells controls the Th1, as well as the Th2 inflammatory responses (Figure 1.1).

The Central Role of the Tryptophan Metabolic Pathway in Tolerance and Immunity to Fungi

The inflammatory/anti-inflammatory state of DC is strictly controlled by the metabolic pathway involved in tryptophan catabolism and mediated by the enzyme IDO. IDO has a complex role in immunoregulation in infection, pregnancy, autoimmunity, transplantation, and neoplasia (Mellor & Munn, 2004). IDO-expressing DC are regarded as regulatory DC specialized to cause antigen-specific deletional tolerance or otherwise negatively regulating responding T cells. In experimental fungal infections IDO blockade greatly exacerbated infections, the associated inflammatory pathology and swept away resistance to reinfection, as a result of deregulated innate and adaptive immune responses caused by the impaired activation and functioning of suppressor $CD4^+$ $CD25^+$ Treg cells producing IL-10 (Bozza et al., 2005; Montagnoli et al., 2006). The results provide novel mechanistic insights into complex events that, occurring at the fungus–pathogen interface, relate to the dynamics of host adaptation by fungi. The production of IFN-γ may be squarely placed at this interface, where IDO activation likely exerts a fine control over inflammatory and adaptive antifungal responses.

The implication for IDO in immunoregulation in candidiasis may help to accommodate several, as yet unexplained findings. As *C. albicans* is a commensal of the human gastrointestinal and genitourinary tracts and IFN-γ is an important mediator of protective immunity to the fungus, the IFN-γ/IDO axis may accommodate fungal persistence in a host environment rich in IFN-γ. In its ability to downregulate antifungal Th1 response in the gastrointestinal tract, IDO behaves in a fashion similar to that described in mice with colitis where IDO expression correlates with the occurrence of local tolerogenic responses. Alternatively, the high levels of IL-10 production, such as those seen in patients with CMC, may be a consequence of IDO activation by the fungus, impairing antifungal Th1 immunity and thus favoring persistent infection (Romani & Puccetti, 2006b). In aspergillosis, the level of inflammation and IFN-γ in the early stage set the subsequent adaptive stage by conditioning the IDO-dependent tolerogenic program of DC and the subsequent activation and expansion of tolerogenic Treg cells preventing allergy to the fungus. Therefore, regulatory mechanisms operating in the control of inflammation and allergy to the fungus are different but interdependent as the level of the inflammatory response early in infection may impact on susceptibility to allergy, in conditions of

continuous exposure to the fungus. Early Treg cells, by affecting IFN-γ-production, indirectly exert a fine control over the induction of late tolerogenic Treg cells. Thus, a unifying mechanism linking natural Treg cells to tolerogenic respiratory Treg cells in response to the fungus is consistent with the revisited 'hygiene hypothesis' of allergy in infections, and may provide at the same time mechanistic explanations for the significance of the variable level of IFN-γ seen in allergic diseases and asthma and for the paradoxical worsening effect on allergy of Th1 cells. IDO has a unique and central role in this process as it may participate in the effector and inductive phases of anti-inflammatory and tolerogenic Treg cells.

Conclusions

The discovery of TLR, DC, and Treg cells have been major breakthroughs in the field of fungal immunology, which may offer new grounds for a better comprehension of the cells and immune pathways that are amenable to manipulation in patients with or at risk of fungal infections. A variety of cytokines, including chemokines and growth factors proved to be beneficial in experimental and human fungal infections (Mencacci et al., 2000; Kawakami, 2003). The Th1/Th2 balance itself can be the target of immunotherapy (Koguchi & Kawakami, 2002; Mencacci et al., 2000). It now appears that a combination of different types of Treg cells controls the Th1, as well as the Th2 inflammatory responses. Consequently, manipulation of Treg cells is thought of as a promising therapeutic approach devoid of risks associated with interference with homeostatic mechanisms of the immune system. Further understanding of the cooperation of various multiple innate immune receptors in fungal recognition potentially provides a basis for novel therapeutic strategies for immunomodulation, which will very likely contribute to successfully coping with the threat of severe fungal infections. Notwithstanding the redundancy and overlapping repertoire of antifungal effector mechanisms, the deliberate targeting of cells and pathways of antifungal CMI may represent a useful strategy in developing fungal vaccines capable of both sterilizing immunity and protecting against fungal reactivation.

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References

Akira, S. (2003). *Curr. Opin. Immunol*., 15:5–11.
Akira, S. & Takeda, K. (2004). *Nat. Rev. Immunol*., 4:499–511.
Alvarez, M. & Casadevall, A. (2006). *Curr. Biol*., 16:2161–2165.
Barese, C. N., Goebel, W. S., & Dinauer, M. C. (2004). *Expert Opin. Biol. Ther*., 4:1423–1434.

Bellocchio, S., Montagnoli, C., Bozza, S., Gaziano, R., Rossi, G., et al. (2004a). *J. Immunol*., 172:3059–3069.
Bellocchio, S., Moretti, S., Perruccio, K., Fallarino, F., Bozza, et al. (2004b). *J. Immunol*., 173:7406–7415.
Biondo, C., Midiri, A., Messina, L., Tomasello, F., Garufi, G., et al. (2005). *Eur. J. immunol*., 35:870–878.
Bozza, S., Fallarino, F., Pitzurra, L., Zelante, T., Montagnoli, C., et al. (2005). *J. Immunol*., 174:2910–2918.
Bozza, S., Gaziano, R., Lipford, G. B., Montagnoli, C., Bacci, A., et al. (2002). *Microbes Infect*., 4:1281–1290.
Bozza, S., Montagnoli, C., Gaziano, R., Rossi, G., & Nkwanyuo, G., (2004). *Vaccine*, 22:857–864.
Brown, G. D. (2006). *Nat. Rev. Immunol*., 6:33–43.
Buentke, E. & Scheynius, A. (2003). *APMIS*, 111:789–796.
Cassone, A., de Bernardis, F., & Torososantucci, A. (2005). *Curr. Mol. Med*., 5:377–382.
Cavassani, K. A., Campanelli, A. P., Moreira, A. P., Vancim, J. O., Vitali, L. H., et al. (2006). *J. Immunol*., 177:5811–5818.
Cheng, V. C., Yuen, K. Y., Chan, W. M., Wong, S. S., Ma, E. S., & Chan, R. M. (2000). *Clin. Infect. Dis*., 30:882–892.
Claudia, M., Bacci, A., Silvia, B., Gaziano, R., Spreca, A., & Romani, L. (2002). *Curr. Mol. Med*., 2:507–524.
Cortez, K. J., Lyman, C. A., Kottilil, S., Kim, H. S., Roilides, E., et al. (2006). *Infect. Immun*., 74:2353–2365.
Dan, J. M. & Levitz, S. M. (2006). *Drug Resist. Updat*., 9:105–110.
Dong, C. (2006). *Nat. Rev. Immunol*., 6:329–333.
Edwards, L., Williams, A. E., Krieg, A. M., Rae, A. J., Snelgrove, R. J., & Hussell, T. (2004). *Eur. J. Immunol*., 35:273–281.
Fischer, A., Ballet, J. J., & Griscelli, C. (1978). *J. Clin. Invest*., 62:1005–1013.
Fradin, C., Mavor, A. L., Weindl, G., Schaller, M., Hanke, K., et al. (2006). *Infect. Immun*.
Gersuk, G. M., Underhill, D. M., Zhu, L., & Marr, K. A. (2006). *J. Immunol*., 176:3717–3724.
Gross, O., Gewies, A., Finger, K., Schafer, M., & Sparwasser, T., (2006). *Nature*, 442:651–656.
Harrington, L. E., Mangan, P. R., & Weaver, C. T. (2006). *Curr. Opin. Immunol*., 18:349–356.
Hohl, T. M., Rivera, A., & Pamer, E. G. (2006). *Curr. Opin. Immunol*., 18:465–472.
Hori, S., Carvalho, T. L., & Demengeot, J. (2002). *Eur. J. Immunol*., 32:1282–1291.
Huffnagle, G. B. & Deepe, G. S. (2003). *Curr. Opin. Microbiol*., 6:344–350.
Kawakami, K. (2003). *J. Infect. Chemother*., 9:201–209.
Kesh, S., Mensah, N. Y., Peterlongo, P., Jaffe, D., Hsu, K. M., (2005). *Ann. N. Y. Acad. Sci*., 1062:95–1103.
Koguchi, Y. & Kawakami, K. (2002). *Int. Rev. Immunol*., 21;423–438.
Lam, J. S., Mansour, M. K., Specht, C. A., & Levitz, S. M. (2005). *J. Immunol*., 175:7496–7503.
Levitz, S. M. (2004). *Microbes Infect*., 6:1351–1355.
Lilic, D. (2002). *Curr. Opin. Infect. Dis*., 15:143–147.
Lilic, D., Gravenor, I., Robson, N., Lammas, D. A., Drysdale, P., et al. (2003). *Infect. Immun*., 71:5690–5699.
Lin, J. S., Yang, C. W., Wang, D. W., & Wu-Hsieh, B. A. (2005). *J. Immunol*., 174:6282–6291.
Mansour, M. K., Latz, E., & Levitz, S. M. (2006). *J. Immunol*., 176:3053–3061.
Mansour, M. K. & Levitz, S. M. (2002). *Curr. Opin. Microbiol*., 5:359–365.
McKinley, L., Logar, A., McAllister, F., Zheng, M., Steele, C., & Kolls, J. (2006). *J. Immunol*., 177:6215–6226.
Mellor, A. & Munn, D. H. (2004). *Nat. Rev. Immunol*., 4:762–774.
Mencacci, A., Cenci, E., Bacci, A., Montagnoli, C., Bistoni, F., & Romani, L. (2000). *Curr. Pharm. Biotechnol*., 1:235–251.
Montagnoli, C., Bacci, A., Bozza, S., Gaziano, R., Mosci, P., et al. (2002). *J. Immunol*., 169:6298–6308.

Montagnoli, C., Fallarino, F., Gaziano, R., Bozza, S., Bellocchio, S., et al. (2006). *J. Immunol*., 176:1712–1723.
Mukhopadhyay, S., Herre, J., Brown, G., & Gordon, S. (2004). *Immunology*, 112:521–530.
Nelson, R., Shibata, N., Podzorski, R., & Herron, M. (1991). *Clin. Microbiol. Rev*., 4:1–19.
Nemecek, J., Wuthrich, M., & Klein, B. (2006). *Science*, 312:583–588.
Netea, M., Ferwerda, G., van der Graaf, C., van der Meer, J., & Kullberg, B. (2006). *Curr. Pharm. Des*., 12:4195–4201.
Netea, M., van der Meer, J., & Kullberg, B. (2004). *Trends Microbiol*., 12:484–488.
Netea, M., Warris, A., van der Meer, J., Fenton, M., Verver-Janssen, T., et al. (2003). *J. Infect. Dis*., 188:320–326.
Pachl, J., Svoboda, P., Jacobs, F., Vandewoude, K., van der Hoven, B., et al. (2006). *Clin. Infect. Dis*., 42:1404–1413.
Peterson, P., Nagamine, K., Scott, H., Heino, M., Kudoh, J., et al. (1998). *Immunol. Today*, 19:384–386.
Puccetti, P., Romani, L., & Bistoni, F. (1995). *Trends Microbiol*., 3:237–240.
Rivera, A., Ro, G., van Epps, H., Simpson, T., Leiner, I., Sant'angelo, D., & Pamer, E. (2006). *Immunity*, 25:665–675.
Romani, L. (1999). *Curr. Opin. Microbiol*., 2:363–367.
Romani, L. (2001). Chapter overview of the fungal pathogens. In: S. H. *Immunology of Infectious Diseases*, Kaufmann, A. Sher and Ahmed R., Eds., ASM Press, Washington, DC, pp. 25–37.
Romani, L. (2004a). Chapter innate immunity to fungi: the art of speed and specificity. In: *Pathogenic Fungi. Host Interactions and Emerging Strategies for Control*, G. san-Blas, and R. A. Calderone Eds., Caister Academic Press, Norfolk, England, pp. 167–214.
Romani, L. (2004b). *Nat. Rev. Immunol*., 4:1–23.
Romani, L., Bistoni, F., Perruccio, K., Montagnoli, C., Gaziano, R., et al. (2006). *Blood*, 108:2265–2274.
Romani, L., Bistoni, F., & Puccetti, P. (2002). *Trends Microbiol*., 10:508–514.
Romani, L. & Kaufmann, S. (1998). *Res. Immunol*., 149:277–281.
Romani, L., Mencacci, A., Cenci, E., Puccetti, P., & Bistoni, F. (1996). *Res. Immunol*., 147:512–518.
Romani, L., Montagnoli, C., Bozza, S., Perruccio, K., Spreca, A., et al. (2004). *Int. Immunol*., 16:149–161.
Romani, L. & Puccetti, P. (2006a). Dendritic cells in immunity and vaccination against fungi. In: *Handbook of Dendritic Cells. Biology, Diseases and Therapies*, 2 vol. Lutz, M. and Steinkasserer A. Eds., Wiley-VCH Verlag Gmbh & Co., Weinham, Germany, pp. 915–930.
Romani, L. & Puccetti, P. (2006b). *Trends Microbiol*., 14:183–189.
Saijo, S., Fujikado, N., Furuta, T., Chung, S., Kotaki, H., et al. (2006). *Nat. Immunol*.
Sakaguchi, S., Toda, M., Asano, M., Itoh, M., Morse, S., & Sakaguchi, N. (1996). *J. Autoimmun*., 9:211–220.
Sato, K., Yang, X., Yudate, T., Chung, J., Wu, J., et al. (2006). *J. Biol. Chem*., 281:38854–38866.
Segal, B., Kwon-Chung, J., Walsh, T., Klein, B., Battiwalla, M., et al. (2006). *Clin. Infect. Dis*., 42:507–515.
Segal, B., Leto, T., Gallin, J., Malech, H., & Holland, S. (2000). *Medicine (Baltimore)*, 79:170–200.
Shoham, S. & Levitz, S. M. (2005). *Br. J. Haematol*., 129:569–582.
Steele, C., Rapaka, R., Metz, A., Pop, S., Williams, D., et al. (2005). *Plos Pathog*., 1:e42.
Taylor, P., Tsoni, S., Willment, J., Dennehy, K., Rosas, M., et al. (2006). *Nat. Immunol*., 6:33–43.
Trinchieri, G., Pflanz, S., & Kastelein, R. (2003). *Immunity*, 19:641–644.
Vazquez-Torres, A. & Balish, E. (1997). *Microbiol. Mol. Biol. Rev*., 61:170–192.
Woods, J. P. (2003) *Curr. Opin. Microbiol*., 6:327–331.
Yauch, L., Lam, J., & Levitz, S. (2006). *Plos Pathog*., 2:e120.

Chapter 2
In Vitro Models to Analyse Fungal Infection

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Introduction

According to the molecular Koch's postulates (Falkow, 1988), putative virulence traits can be identified in a pathogen because deletion of the gene encoding a virulence factor in an otherwise wild-type strain generates a mutant with reduced pathogenicity in a certain model of experimental infection.

Recent advances in molecular genetics have led to the generation of such altered strains in several clinically relevant fungi, including *Candida albicans, Cryptococcus neoformans, Aspergillus fumigatus*, and *Histoplasma capsulatum*. This has, in turn allowed the identification of several virulence genes involved in important physiological processes in the pathogen. These processes include, among others, the biogenesis of the cell wall, the acquisition of nutrients, the production of extracellular enzymes, and the tolerance to stress. In experimental infection models these mutants frequently display attenuated or abolished virulence. While this methodology provides global information on whether a gene is involved in virulence or not, it does not define the specific step(s) in the pathogenic process that is (are) impaired by the molecular lesion.

During the pathoenic cycle fungi interact with various types of host cells, which may lead to dissemination from the original entry site and deep-seated infection of inner organs (Figure 2.1).

This interaction is characterized by successive events at the cellular level. Fungi first attach and then enter the host cells, where they may persist or even proliferate before they leave and infect other host cells and tissues. Each of these steps may be crucial for the development of the disease, and virulence factors may contribute in each of these steps by different molecular mechanisms (Figure 2.2). *In vitro* models of infection provide a defined experimental set-up to characterize the host–pathogen interplay at a cellular level and allow us to ascertain more precisely the molecular lesion present in the mutant and the corresponding step of the pathogenic process specifically altered.

This chapter reviews the main *in vitro* models for epithelial, endothelial, and immune system cells. It outlines the available techniques to characterize and to quantify host cell–pathogen interactions following a structure as preset by the

K. Kavanagh (ed.), *New Insights in Medical Mycology.*

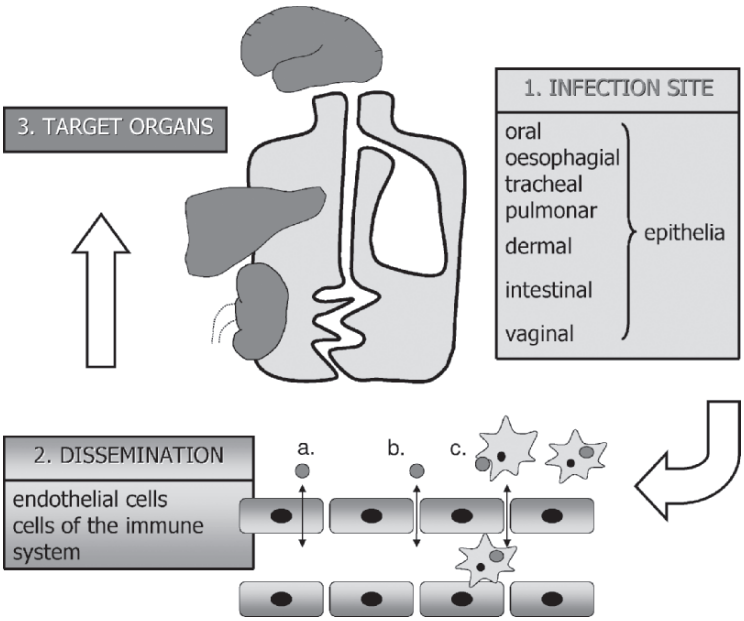

Figure 2.1 Stations of the pathogenic cycle. Fungi can enter the body interior through various epithelial sites which mark the boundary between inside and outside (1). The host's defence either limits the interaction to the epithelial surface or the fungus overcomes the barrier. The infection becomes invasive and no longer restricted to the primary infection site. A key feature initiating dissemination (2) is the interaction of the pathogen with host cells of the blood system – endothelial or immune cells. The fungus can get access to the bloodstream by transcellular (a), paracellular (b), or 'Trojan horse' (c) mechanisms by which it might also leave the vascular system to attack finally vital organs as the brain, the liver, or the kidneys (3)

pathogenic course itself (Figure 2.2). Examples on how the *in vitro* methodology has contributed to clarify the effective molecular mechanism in the single steps are included for the different fungal pathogens.

Host Cells in Conjunction – Epithelial and Endothelial Models

Invading pathogens are confronted with two structural barriers which are the epithelia and endothelia located at diverse sites of the body. They are conjunctions of cells that confer, by their assembly, organ-specific physiological characteristics and functions. Thus, *in vitro* models for the investigation of fungal interactions with epithelial and endothelial cells (EC) are basically layers of varying degrees of complexity that are attached to surfaces. They aim to reflect the physiological properties

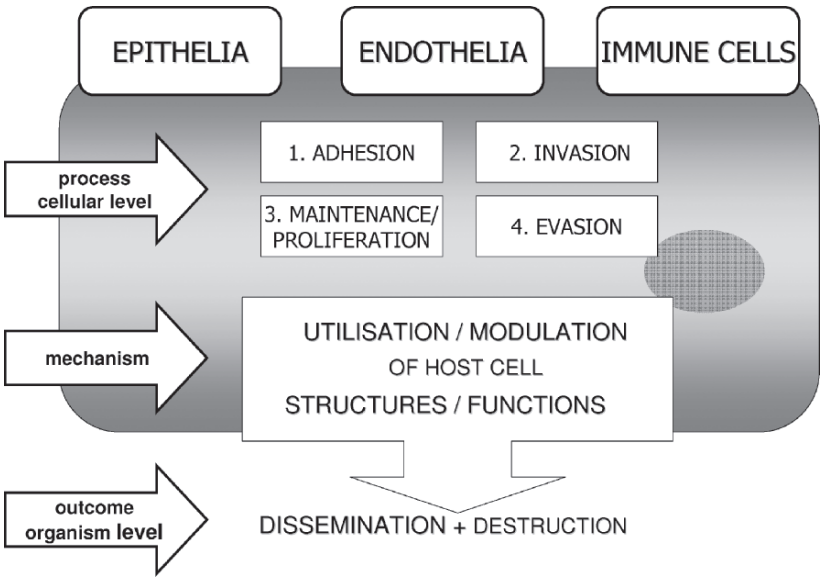

Figure 2.2 Host–pathogen interaction – players and processes. During the course of infection fungi interact with different host cells such as epithelial, endothelial, and immune system cells. This interaction is characterized by successive events at the cellular level. They include: adhesion (1), entrance (2), persistence or even proliferation inside (3), and finally exit from host cells (4). Pathogens contrive these steps by using and manipulating host cell structures and functions. Each of the processes can be crucial for the overall outcome of the interaction

of the corresponding natural organs and ongoing advances in tissue engineering have progressed towards models of an organizational level that represents an intermediate stage between single cell type culture and organ culture.

Apart from the fact that epithelial and EC form part of the host barriers they may play additional roles during the course of a fungal infection. In recent years it has been recognized that pathogens induce their own endocytosis upon interaction with these usually non-phagocytic cells. The possibility that epithelial and EC could serve as a reservoir for pathogens, which hide themselves intracellularly from the immune system is a matter of current research (for a recent review see Filler & Sheppard, 2006).

Epithelial Cell Models

Epithelia are formed by cells in close proximity to each other lying on a basal membrane. This structure is fed through the connective, highly vascularized tissue, which underlies the basal membrane. Epithelia cover every surface of the human