Edited by
Karl Esser

THE MYCOTA

A Comprehensive Treatise on Fungi
as Experimental Systems for Basic and Applied Research

Human Fungal Pathogens

XII

Second Edition

Oliver Kurzai
Volume Editor

Springer

The Mycota

Edited by
K. Esser

The Mycota

I *Growth, Differentiation and Sexuality*
1st edition ed. by J.G.H. Wessels and F. Meinhardt
2nd edition ed. by U. Kües and R. Fischer

II *Genetics and Biotechnology*
1st and 2nd edition ed. by U. Kück

III *Biochemistry and Molecular Biology*
1st and 2nd edition ed. by R. Brambl and G. Marzluf

IV *Environmental and Microbial Relationships*
1st edition ed. by D. Wicklow and B. Söderström
2nd edition ed. by C.P. Kubicek and I.S. Druzhinina

V *Plant Relationships*
1st edition ed. by G. Carroll and P. Tudzynski
2nd edition ed. by H.B. Deising

VI *Human and Animal Relationships*
1st edition ed. by D.H. Howard and J.D. Miller
2nd edition ed. by A.A. Brakhage and P.F. Zipfel

VII *Systematics and Evolution*
1st edition ed. by D.J. McLaughlin, E.G. McLaughlin, and P.A. Lemke†
2nd edition ed. by D. McLaughlin and J.W. Spatafora

VIII *Biology of the Fungal Cell*
1st and 2nd edition ed. by R.J. Howard and N.A.R. Gow

IX *Fungal Associations*
1st and 2nd edition ed. by B. Hock

X *Industrial Applications*
1st edition ed. by H.D. Osiewacz
2nd edition ed. by M. Hofrichter

XI *Agricultural Applications*
1st and 2nd edition ed. by F. Kempken

XII *Human Fungal Pathogens*
1st edition ed. by J.E. Domer and G.S. Kobayashi
2nd edition ed. by O. Kurzai

XIII *Fungal Genomics*
1st edition ed. by A.J.P. Brown
2nd edition ed. by M. Nowrousian

XIV *Evolution of Fungi and Fungal-Like Organisms*
Ed. by S. Pöggeler and J. Wöstemeyer

XV *Physiology and Genetics: Selected Basic and Applied Aspects*
Ed. by T. Anke and D. Weber

The Mycota

A Comprehensive Treatise
on Fungi as Experimental Systems
for Basic and Applied Research

Edited by K. Esser

XII *Human Fungal Pathogens*
2nd Edition

Volume Editor:
O. Kurzai

Springer

Series Editor

Professor Dr. Dr. h.c. mult. Karl Esser
Allgemeine Botanik
Ruhr-Universität
44780 Bochum, Germany

Tel.: +49 (234)32-22211
Fax.: +49 (234)32-14211
e-mail: Karl.Esser@rub.de

Volume Editor

Professor Dr. Oliver Kurzai
Septomics Research Center
Friedrich-Schiller-Universität and Leibniz-Institut für
Naturstoff-Forschung und Infektionsbiologie - Hans-Knöll-Institut 07745
Albert-Einstein-Str. 10
Jena, Germany

Tel.: +49 (3641) 532 1347
Fax: +49 (3641) 532 0816
e-mail: oliver.kurzai@hki-jena.de

ISBN 978-3-642-39431-7 ISBN 978-3-642-39432-4 (eBook)
DOI 10.1007/978-3-642-39432-4
Springer Heidelberg New York Dordrecht London

Library of Congress Control Number: 2013955572

Karl Esser
(born 1924) is retired Professor of General Botany and Director of the Botanical Garden at the Ruhr-Universität Bochum (Germany). His scientific work focused on basic research in classical and molecular genetics in relation to practical application. His studies were carried out mostly on fungi. Together with his collaborators he was the first to detect plasmids in higher fungi. This has led to the integration of fungal genetics in biotechnology. His scientific work was distinguished by many national and international honors, especially three honorary doctoral degrees.

Oliver Kurzai
(born 1975) is an M.D. specialized in microbiology, virology and epidemiology of infection. He is full professor at the Septomics Research Center – a cross-faculty institution of the Friedrich-Schiller-University and the Leibniz-Institute for Natural Product Research and Infection Biology (Hans-Knöll-Institute), in Jena, Germany. His major research fields are infection biology and innate immune recognition of pathogenic fungi. He has been awarded several prizes and is elected Secretary of the German-speaking Mycological Society. In 2012, he was appointed as Editor-in-Chief of *Medical Mycology Case Reports* by the *International Society for Human and Animal Mycology (ISHAM).*

Series Preface

Mycology, the study of fungi, originated as a sub discipline of botany and was a descriptive discipline, largely neglected as an experimental science until the early years of this century. A seminal paper by Blakeslee in 1904 provided evidence for self incompatibility, termed "heterothallism", and stimulated interest in studies related to the control of sexual reproduction in fungi by mating-type specificities. Soon to follow was the demonstration that sexually reproducing fungi exhibit Mendelian inheritance and that it was possible to conduct formal genetic analysis with fungi. The names Burgeff, Kniep and Lindegren are all associated with this early period of fungal genetics research.

These studies and the discovery of penicillin by Fleming, who shared a Nobel Prize in 1945, provided further impetus for experimental research with fungi. Thus began a period of interest in mutation induction and analysis of mutants for biochemical traits. Such fundamental research, conducted largely with *Neurospora crassa*, led to the one gene: one enzyme hypothesis and to a second Nobel Prize for fungal research awarded to Beadle and Tatum in 1958. Fundamental research in biochemical genetics was extended to other fungi, especially to *Saccharomyces cerevisiae*, and by the mid-1960s fungal systems were much favored for studies in eukaryotic molecular biology and were soon able to compete with bacterial systems in the molecular arena.

The experimental achievements in research on the genetics and molecular biology of fungi have benefited more generally studies in the related fields of fungal biochemistry, plant pathology, medical mycology, and systematics. Today, there is much interest in the genetic manipulation of fungi for applied research. This current interest in biotechnical genetics has been augmented by the development of DNA-mediated transformation systems in fungi and by an understanding of gene expression and regulation at the molecular level. Applied research initiatives involving fungi extend broadly to areas of interest not only to industry but to agricultural and environmental sciences as well.

It is this burgeoning interest in fungi as experimental systems for applied as well as basic research that has prompted publication of this series of books under the title The Mycota. This title knowingly relegates fungi into a separate realm, distinct from that of either plants, animals, or protozoa. For consistency throughout this Series of Volumes the names adopted for major groups of fungi (representative genera in parentheses) areas follows:

Pseudomycota

Division:	Oomycota (Achlya, Phytophthora, Pythium)
Division:	Hyphochytriomycota

Eumycota

Division:	Chytridiomycota (Allomyces)
Division:	Zygomycota (Mucor, Phycomyces, Blakeslea)
Division:	Dikaryomycota
Subdivision:	Ascomycotina
Class:	Saccharomycetes (Saccharomyces, Schizosaccharomyces)
Class:	Ascomycetes (Neurospora, Podospora, Aspergillus)
Subdivision:	Basidiomycotina
Class:	Heterobasidiomycetes (Ustilago, Tremella)
Class:	Homobasidiomycetes (Schizophyllum, Coprinus)

We have made the decision to exclude from The Mycota the slime molds which, although they have traditional and strong ties to mycology, truly represent nonfungal forms insofar as they ingest nutrients by phagocytosis, lack a cell wall during the assimilative phase, and clearly show affinities with certain protozoan taxa.

The Series throughout will address three basic questions: what are the fungi, what do they do, and what is their relevance to human affairs? Such a focused and comprehensive treatment of the fungi is long overdue in the opinion of the editors.

A volume devoted to systematics would ordinarily have been the first to appear in this Series. However, the scope of such a volume, coupled with the need to give serious and sustained consideration to any reclassification of major fungal groups, has delayed early publication. We wish, however, to provide a preamble on the nature of fungi, to acquaint readers who are unfamiliar with fungi with certain characteristics that are representative of these organisms and which make them attractive subjects for experimentation.

The fungi represent a heterogeneous assemblage of eukaryotic microorganisms. Fungal metabolism is characteristically heterotrophic or assimilative for organic carbon and some nonelemental source of nitrogen. Fungal cells characteristically imbibe or absorb, rather than ingest, nutrients and they have rigid cell walls. The vast majority of fungi are haploid organisms reproducing either sexually or asexually through spores. The spore forms and details on their method of production have been used to delineate most fungal taxa. Although there is a multitude of spore forms, fungal spores are basically only of two types: (i) asexual spores are formed following mitosis (mitospores) and culminate vegetative growth, and (ii) sexual spores are formed following meiosis (meiospores) and are borne in or upon specialized generative structures, the latter frequently clustered in a fruit body. The vegetative forms of fungi are either unicellular, yeasts are an example, or hyphal; the latter may be branched to form an extensive mycelium.

Regardless of these details, it is the accessibility of spores, especially the direct recovery of meiospores coupled with extended vegetative haploidy, that have made fungi especially attractive as objects for experimental research.

The ability of fungi, especially the saprobic fungi, to absorb and grow on rather simple and defined substrates and to convert these substances, not only into essential metabolites but into important secondary metabolites, is also noteworthy.

The metabolic capacities of fungi have attracted much interest in natural products chemistry and in the production of antibiotics and other bioactive compounds. Fungi, especially yeasts, are important in fermentation processes. Other fungi are important in the production of enzymes, citric acid and other organic compounds as well as in the fermentation of foods.

Fungi have invaded every conceivable ecological niche. Saprobic forms abound, especially in the decay of organic debris. Pathogenic forms exist with both plant and animal hosts. Fungi even grow on other fungi. They are found in aquatic as well as soil environments, and their spores may pollute the air. Some are edible; others are poisonous. Many are variously associated with plants as copartners in the formation of lichens and mycorrhizae, as symbiotic endophytes or as overt pathogens. Association with animal systems varies; examples include the predaceous fungi that trap nematodes, the microfungi that grow in the anaerobic environment of the rumen, the many insect associated fungi and the medically important pathogens afflicting humans. Yes, fungi are ubiquitous and important.There are many fungi, conservative estimates are in the order of 100,000 species, and there are many ways to study them, from descriptive accounts of organisms found in nature to laboratory experimentation at the cellular and molecular level. All such studies expand our knowledge of fungi and of fungal processes and improve our ability to utilize and to control fungi for the benefit of humankind.

We have invited leading research specialists in the field of mycology to contribute to this Series. We are especially indebted and grateful for the initiative and leadership shown by the Volume Editors in selecting topics and assembling the experts. We have all been a bit ambitious in producing these Volumes on a timely basis and there in lies the possibility of mistakes and oversights in this first edition. We encourage the readership to draw our attention to any error, omission or inconsistency in this Series in order that improvements can be made in any subsequent edition.

Finally, we wish to acknowledge the willingness of Springer-Verlag to host this project, which is envisioned to require more than 5 years of effort and the publication of at least nine Volumes.

Bochum, Germany — Karl Esser
Auburn, AL, USA — Paul A. Lemke
April 1994 — *Series Editors*

Addendum to the Series Preface

During the Fourth International Mycological Congress in Regensburg (1989) while relaxing in a beer garden with Paul Lemke (USA), Dr. Czeschlik (Springer-Verlag) discussed with us the possibility to publish a series about Fungi. We both were at first somewhat reserved, but after a comprehensive discussion this idea looked promising. We decided to name this new series *The Mycota*.

Then Paul Lemke and I created a program involving seven volumes covering a wide area of Mycology. The first volume was presented in 1994 at the Fifth International Mycological Congress in Vancover (Canada). The other volumes followed step by step. After the early death of Paul Lemke (1995) I proceeded alone as Series Editor. However for Vols. X-XII I received support by Joan Bennett.

Since evidently the series was well accepted by the scientific community and since the broad area of Fungi was not completely covered, it was decided to proceed with eight more volumes. In addition, second editions of eleven volumes were published and two more are in preparation.

I would like to thank Springer-Verlag, represented by Andrea Schlitzberger for her support and cooperation.

Bochum, Germany
August 2013

Karl Esser

Volume Preface to the Second Edition

Books are old-fashioned. Books are never up-to-date, expensive and impractical. And books don't pay off for those who spend time and effort writing them. All these thoughts are among the first reactions evoked when contacting friends and colleagues to let them know you have accepted an offer to edit a book. Many of these reactions are – to say the worst – entirely true. So why take the effort? A very personal reason is that it was a book (and in fact a very old-fashioned, entirely not up-to-date book) that started my fascination for fungal pathogens when I was a young medical student starting to work on his M.D. thesis in 1997. This book was the 2nd edition of Frank Odds' outstanding compilation *Candida and Candidosis* published in 1988. For me it turned out to be a treasure of information and knowledge that guided me in exploring contemporary literature and helped in understanding a larger context. The reminiscence of this book made me happy and eager to try and put together an equally useful book when Karl Esser asked me to take over editorial responsibility for vol. XII of *The Mycota*, devoted to human fungal pathogens. Whereas the old edition of this volume had been a collection of nuggets from mycological research, I decided that the new edition should be an overview on basic principles in medical mycology, introducing the concepts of fungal virulence for selected and major fungal pathogens, emphasizing the importance of the host and especially the hosts' immune system for the development of fungal infection, and also addressing clinical issues like diagnosis and therapy of fungal infections. Putting together such a broad overview would not have been possible without the help of many colleagues and friends from all over the world. All of them are outstanding experts in their areas of expertise, who have guided and advanced medical mycology in recent years. Several have teamed up to join ideas and thoughts in their chapters. I want to thank all of them for their time and effort and their enthusiasm in contributing to this project despite many other obligations. In addition, my thanks go to Katrin Haupt and Silke Pfohl from my office for helping me to put all the chapters together.

The new volume is divided into four parts or sections. In the first part, we focus on fungal pathogens and introduce concepts of virulence for two model pathogenic fungi: *Aspergillus fumigatus*, an environmental species causing severe exogenous infections in immunocompromised patients, and *Candida albicans*, a colonizer of the human body that can disseminate from its natural habitat to cause invasive candidiasis. In addition, the tools of systems biology – a new approach to understanding complex networks of interaction in mycological research – are introduced. The second part is dedicated to the host, with a focus on innate and adaptive mechanisms of antifungal immunity. Basic concepts in

innate and adaptive antifungal immunity that have seen a remarkable and still-ongoing growth of knowledge in recent years are summarized. The major human pathogen *Cryptococcus neoformans* is introduced in the context of its interaction with macrophages, which is impressively multi-faceted and highly interesting. In addition, we learn about mucosal immunity in *Candida* infections and the pathogen–host interplay in mycoses caused by endemic fungal species. These chapters are complemented by a survey on modern tools for imaging fungus–host interactions, a field that has paved new ways to understanding infection biology. The third part focuses on a clinical point of view on fungal infections and introduces diagnostic principles, major patient collectives at risk and two other major fungal pathogens – *Pneumocystis jirovecii* and the *Mucoraceae*. Finally, in the last part, current approaches to treatment of fungal infection and pharmacokinetics of antifungal drugs are summarized.

I do hope that this overview on medical mycology, which integrates basic concepts with highly innovative up-to-date research, will stimulate the reader and help to interest newcomers while at the same time surprising insiders. If this works, then perhaps someday someone will do with a copy of this book what I did back then with the copy of Frank Odds' book, which is still in my bookshelf: save it from being sorted out by the library. This would make me happy.

Oliver Kurzai
Volume Editor

Volume Preface to the First Edition

Paralleling the increasing prominence of fungi as agents of disease has been the application of molecular genetic research on them. These studies have permitted sophisticated analyses of the ecological and environmental role fungi play in nature, the manner in which they cause disease, their interactions with the immune system, and their responses to antifungal agents. For this volume, we sought to recruit authors who were doing cutting-edge research with medically important fungi in these four broad areas of interest. Several of the studies summarized within the present volume contain extensions of earlier data presented in a previous volume of *The Mycota* (Vol. VI, D.H. Howard and J.D. Miller,Volume Editors). The emphasis in Volume VI, however, was on biochemical interactions of fungi with their hosts, both human and animal.

Much of the research summarized here places the study of pathogenic fungi squarely in the main stream of molecular biology. The basic and precise approaches provided by molecular biological studies allow one to draw conclusions at the genetic level that were not possible even a decade ago. While molecular methods are critically needed for a basic understanding of these fungi and the diseases they cause, there remain areas within the study of medically important fungi where classical and descriptive methods continue to be applied to great advantage in order to answer various basic questions. These include: (1) advances in diagnostic methods for invasive *Candida and Aspergillus* infection, (2) studies on the role melanin plays in pathogenesis, (3) all studies on the recently reclassified unique organism *Pneumocystis*, and (4) studies on the role of complement in disease. From the standpoint of opportunistic infections, species of *Candida and Aspergillus* are clearly the most important. Thus, two chapters are devoted to different aspects of infections caused by species of *Candida* and several chapters review various aspects of infections caused by species of *Aspergillus*.

In selecting topics for inclusion in Volume XII of *The Mycota*, we sought to feature research on medically important fungi currently being conducted by scientists at various international laboratories as well. To this end, outstanding mycologists from Brazil, France, Germany, Italy, and Great Britain were recruited as authors. The editors also recognize that the Mycota Series is devoted to all organisms in the Kingdom Fungi and that medically important fungi occupy only a small, although significant, niche. We have tried to provide the reader with a sampling of the sophisticated research that is currently being conducted on

medically important fungi and to further stimulate interest in applying molecular methods to the study of all fungi of interest in the realm of human and animal mycotic diseases.

Boone, NO, USA
St. Louis, MO,USA
July 2003

Judith E. Domer
George S. Kobayashi
Volume Editors

Contents

List of Contributors

Franco Aversa
Department of Clinical and Experimental Medicine, University of Parma, via Gramsci 14, Parma 43126, Italy

Elaine Bignell
Institute of Inflammation and Repair, University of Manchester, 46 grafton street, Manchester, M13 9NT, UK

Gordon D. Brown
Aberdeen Fungal Group and Section of Infection and Immunity, University of Aberdeen, Ashgrove Road West, Aberdeen AB25 2ZD, UK

Agostinho Carvalho
Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Polo Unico Sant'Andrea delle Fratte, Perugia 06132, Italy

Oliver A. Cornely
Department I of Internal Medicine, Hospital of the University of Cologne, Kerpener Strasse 62, Köln 50937, Germany

Cristina Cunha
Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Polo Unico Sant'Andrea delle Fratte, Perugia 06132, Italy

Robert J. Evans
Institute of Microbiology & Infection and the School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

Fedja Farowski
Clinic I for Internal Medizin, Clinical Infectiology, University Hospital Cologne, Cologne 50924, Germany

Neil A. R. Gow
School of Medical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK

Matthias Gunzer
Institute for Experimental Immunology and Imaging, University Duisburg-Essen, University Hospital, Hufelandstrasse 55, Essen 45147, Germany

Sarah E. Hardison
Aberdeen Fungal Group and Section of Infection and Immunity, University of Aberdeen, Ashgrove Road West, Aberdeen AB25 2ZD, UK

Mike Hasenberg
Institute for Experimental Immunology and Imaging, University Duisburg-Essen, University Hospital, Hufelandstrasse 55, Essen 45147, Germany

Philippe M. Hauser
Institute of Microbiology, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland

Bernhard Hube
Department of Microbial Pathogenicity Mechanisms, Leibniz Institute for Natural Product Research and Infection Biology – Hans Knöll Institute Jena (HKI), Friedrich Schiller University, Center for Sepsis Control and Care, Beutenbergstrasse 11a, Jena 07745, Germany

Daniela Kienzl
University Clinic for Radiodiagnostics, Medical University of Vienna, Währinger Gürtel 18-20, Vienna A-1090, Austria

Edda Klipp
Institute of Biology, Theoretical Biophysics, Humboldt-Universität zu Berlin, Invalidenstrasse 42, Berlin 10115, Germany

Joseph A. Kovacs
Critical Care Medicine Department, NIH Clinical Center, National Institutes of Health, Bethesda MD, USA

Sven Krappmann
Microbiology Institute – Clinical Microbiology, Immunology and Hygiene, University Hospital of Erlangen and Friedrich-Alexander-Universität Erlangen-Nürnberg, Wasserturmstrasse 3/5, Erlangen 91054, Germany

Karl Kuchler
Christian Doppler Laboratory Infection Biology, Max F. Perutz Laboratories, Medical University of Vienna, Vienna 1030, Austria

Oliver Kurzai
Septomics Research Center, Friedrich-Schiller-Universität and Leibniz-Institut für Naturstoff-Forschung und Infektionsbiologie – Hans-Knöll-Institut, Jena, Albert-Einstein-Strasse 10, Jena 07745, Germany

Robin C. May
Institute of Microbiology & Infection and the School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

Wouter Meersseman
Department of General Internal Medicine, Gasthuisberg University Hospital, Herestraat 49, Leuven 3000, Belgium

Caroline B. Moore
Mycology Reference Centre, Manchester Academic Health Science Centre, University Hospital of South Manchester, Manchester, UK; and University of Manchester, Manchester, UK

David L. Moyes
Department of Oral Immunology, Clinical and Diagnostic Sciences, King's College London Dental Institute, King's College London, London SE1 9RT, UK

Julian R. Naglik
Department of Oral Immunology, Clinical and Diagnostic Sciences, King's College London Dental Institute, King's College London, London SE1 9RT, UK

Chad A. Rappleye
Department of Microbiology, Department of Microbial Infection and Immunity, Ohio State University, Columbus, OH 43210, USA

Malcolm D. Richardson
Mycology Reference Centre, Manchester Academic Health Science Centre, University Hospital of South Manchester, Manchester, UK; and University of Manchester, Manchester, UK

Luigina Romani
Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Polo Unico Sant'Andrea delle Fratte, Perugia 06132, Italy

Martin Schaller
Department of Dermatology, Eberhard Karls University Tübingen, Tübingen 72076, Germany

Lanay Tierney
Christian Doppler Laboratory Infection Biology, Max F. Perutz Laboratories, Medical University of Vienna, Vienna 1030, Austria

Katarzyna Tyc
Institute of Biology, Theoretical Biophysics, Humboldt-Universität zu Berlin, Invalidenstrasse 42, Berlin 10115, Germany

Maria J. G. T. Vehreschild
Department I of Internal Medicine, Hospital of the University of Cologne, Kerpener Strasse 62, Köln 50937, Germany

Kerstin Wahlers
Department I of Internal Medicine, Hospital of the University of Cologne, Kerpener Strasse 62, Köln 50937, Germany

Günther Weindl
Institute of Pharmacy (Pharmacology and Toxicology), Freie Universität Berlin, Berlin 14195, Germany

Amy Whittington
School of Medical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK

Birgit Willinger
Division of Clinical Microbiology, Department of Laboratory Medicine, Medical University of Vienna, Währinger Gürtel 18-20/5P, Vienna A-1090, Austria

Basics of Fungal Virulence

1 From Commensal to Pathogen: *Candida albicans*

Amy Whittington[1], Neil A.R. Gow[1], Bernhard Hube[2]

CONTENTS

I. The Commensal School: Commensal and Pathogenic Attributes

Microbial infections are caused by bacteria, parasites or fungi that have distinct properties necessary for colonisation, survival and replication on or within their hosts, thereby causing damage and disease. They may have been acquired from the environment, from other hosts or from the host's own microbiota. However, not all hosts are equally susceptible to microbial infections and even closely related fungal species can have very different ecologies and relationships with their hosts. **Thus, although virulence is due to microbial attributes, these attributes are only expressed in a susceptible host** (Casadevall and Pirofski 2007). This susceptibility can be defined by the host species, certain variants of a host species, the immune status of a host or the normal microbial flora of the host.

Most human pathogenic fungi, for example *Aspergillus fumigatus*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Penicillium marneffei* and *Coccidioides immitis*, are environmental fungi, which normally live outside the human body and can cause exogenous infections. These fungi must have gained their pathogenic potential in certain environmental niches that resemble aspects of the human body. In these "environmental virulence schools" (Casadevall 2008), fungi must have been adapted to host-like environments during evolution and thus have acquired virulence attributes.

However, not all fungal pathogens of man come from the environment (or other non-human hosts). **In fact, the overwhelming proportion of infections caused by fungi are caused by organisms that are normally associated with human hosts.** These include *Candida*, *Malassezia* and *Pneumocystis* species and many dermatophytes (e.g. *Trichophyton rubrum*). With analogy to the environmental virulence schools, the virulence potential of these endogenous fungi can be explained by training in the "commensal virulence school" (Hube 2009). In the commensal environment, these fungi are constantly challenged by the host and other microbial habitants of the host and have evolved commensal factors, which can turn

[1]School of Medical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen, AB25 2ZD, UK; e-mail: n.gow@abdn.ac.uk
[2]Department of Microbial Pathogenicity Mechanisms, Leibniz Institute for Natural Product Research and Infection Biology – Hans Knöll Institute Jena (HKI), Friedrich Schiller University, Center for Sepsis Control and Care, Beutenbergstrasse 11a, 07745 Jena, Germany

Human Fungal Pathogens, 2nd Edition
The Mycota XII
O. Kurzai (Ed.)

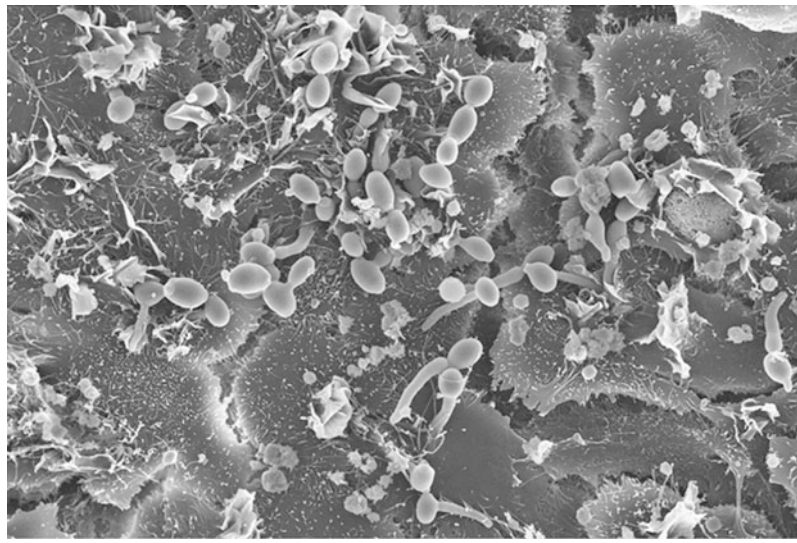


Fig. 1.1. Scanning electron micrograph of *C. albicans* growing on oral epithelial cell layer, in yeast, hyphal and pseudohyphal morphologies. Hyphae invade via active penetration and induce endocytosis, indicated by membrane ruffling (Zakikhany K, Holland G, Özel M, Hube B, with permission)

into virulence attributes once the conditions favour infection. In this chapter, we will describe some of the key commensal and virulence attributes of the most common pathogenic yeast, *C. albicans*.

II. Commensal Growth

During commensal growth on mucosal surfaces, *C. albicans* has to get hold of the mucus or adhere directly to epithelial cells (Fig. 1.1). Therefore, the fungus must express adhesion factors. Although yeast cells of *C. albicans* express adhesive proteins on their surface (Zupancic and Cormack 2007), some of the most dominant adhesins are known to be associated specifically with hyphal morphology in vitro. However, it is not clear whether yeast cells or hyphae or both are characteristic of commensal colonisation. Because the expression of hyphal-associated genes has been detected during asymptomatic carriage, it is possible that commensal cells include hyphae (Mochon et al. 2010; Naglik et al. 2006, 2003). However, it has also been shown that genes normally hyphal-associated can also be expressed by yeast cells under certain conditions (Andaluz et al. 2006; Fradin et al. 2005; Rosenbach et al. 2010; Sosinska et al. 2008; White et al. 2007). This includes the in vivo expression of certain hyphal-associated genes by *C. albicans* yeast cells colonising the murine gastrointestinal tract (after removal of the bacterial flora), a niche where the dominant morphology has been shown to be yeast cells (Rosenbach et al. 2010; White et al. 2007).

In the commensal phase, *C. albicans* co-exists and interacts with bacteria of the microflora (Piispanen and Hogan 2012). These probiotic bacteria generally contribute to a balanced and protective immunity and can produce molecules such as fatty acids, thereby directly inhibiting fungal growth, blocking epithelial binding sites and competing for nutrients. Although nutrients are relatively rich in the gut, they are quickly absorbed by the microbial flora and epithelial cells, forcing colonising *C. albicans* cells to **use nutrient acquisition mechanisms efficiently and to be metabolically flexible**. Changing conditions (e.g. pH, osmolarity) in different host niches probably require stress adaptation mechanisms to be deployed for growth to be permissible. It also seems feasible that commensal microbes, such as *C. albicans*, have developed immune evasion strategies to avoid recognition because the trigger of immune responses or inflammation would cause additional stress. Nevertheless, occasionally invading hyphae may cause mild local

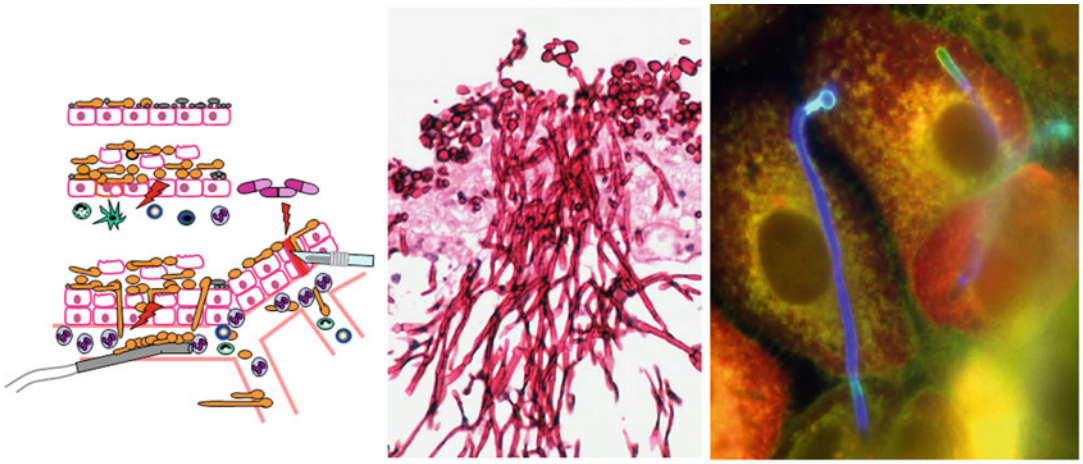


Fig. 1.2. *Left*: Cartoon of the various states of *C. albicans* from commensalism on the epithelial surface to invasion and pathogenicity due to insult via immunosuppression, injury and introduction as a biofilm on foreign bodies. *Middle*: Invasive properties of hyphae of *C. albicans* on model epithelium from chick chorioallantoic membrane (Gow NAR, with permission). *Right*: *C. albicans* inter-epithelial invasion of hyphae through one oral epithelial cell and invasion into an adjacent cell. *Dark blue* parts of hyphae are intracellular and *bright blue* parts are extracellular (Almeida R, Hube B, with permission)

damage, thereby attracting phagocytes. These transient confrontations with the immune system may select for fungal attributes that enable fungal cells to cope with situations beyond the commensal environment. This may include the refinement of transcriptional programs that prepare invading cells for challenges associated with the transition from commensalism to pathogenicity – a phenomenon that can be described as "predictive adaptation". One of these programs is the transition from yeast to hyphal growth and the expression of hyphal-associated genes. These genes encode adhesins, invasins, iron acquisition factors, hydrolases, damaging factors and enzymes that neutralise reactive oxygen species.

In the invasive phase, fungal hyphae, not yeast cells, invade into and through epithelial cell layers, damage their membranes and exploit their cellular content as nutrients. At this stage, fungal cells are recognised, the immune system is activated and armed, and phagocytes (monocytes, macrophages, dendritic cells, neutrophils) are attracted, establishing a local battlefield. If the fungus succeeds and invades into deeper tissue or gains access to blood vessels via damaged barriers, fungal cells may enter the blood stream and escape through the endothelial layers to colonise the major organs, causing life-threatening systemic infections (Fig. 1.2). Attributes that enable the fungus to invade, cause damage, counteract the immune system, cope with changing stresses, exploit host molecules as nutrients, and colonise non-commensal niches are true pathogenicity attributes and are described in this chapter.

A. Nutrient Acquisition and Metabolic Flexibility

During the transition from commensal growth to pathogenicity, and during dissemination and colonisation of the different host organs and niches, the available local nutrients change dramatically. The fact that *C. albicans* can proliferate in all these niches indicates a high degree of metabolic flexibility and suggests that several host sources can be exploited as nutrients. During systemic infections, the fungus disseminates via the bloodstream, which is relatively rich in glucose (6–8 mM), the preferred carbon source of *C. albicans*. However, circulating phagocytes, in particular neutrophils and to a lesser extent monocytes, efficiently phagocytose *C. albicans*. Within the phagosome, the fungus is exposed to a nutrient-starved environment and **switches from glycolysis to gluconeogenesis and activates**

the glyoxylate cycle. In fact, key enzymes of the glyoxylate cycle, such as isocitrate lyase and citrate synthase seem to be required for full virulence of *C. albicans* (Fradin et al. 2005; Lorenz and Fink 2002; Miramón et al. 2012). Single-cell profiling and global transcriptional profiling (Barelle et al. 2006; Thewes et al. 2007; Zakikhany et al. 2007) of hyphae invading epithelial tissues or organs showed that genes encoding for enzymes of both the glycolysis and glyoxylate cycles are activated, possibly reflecting different subpopulation of cells. Secreted lipases (Lips) and phospholipases B (PLBs) of *C. albicans* can degrade host lipids and phospholipids to provide non-glucose carbon sources. Secreted aspartic proteases (Saps) are able to hydrolyse host proteins, thereby releasing peptides and amino acids that can serve as a source of carbon and nitrogen. Indeed, *C. albicans* can use all natural amino acids as nitrogen sources (our unpublished data).

Interestingly, growth on carbon sources available within the host other than glucose, such as lactate or amino acids, can render *C. albicans* more resistant to environmental stresses and can increase its virulence potential in vivo (Ene et al. 2012). A combination of carbon sources, or alternatives to glucose and fructose such as pyruvate, sorbitol, oleic acids and galactose, increase resistance to osmotic stress. With the exception of oleic acid, this corresponds to an increase in kidney burden in systemically infected mice (Ene et al. 2012). However, the impact of carbon source on virulence depends on the site of infection in mouse models, e.g. cells grown on oleic acid produce greater vaginal infection and low systemic infection, although systemic infection is enhanced by growth on lactate and amino acids (Ene et al. 2012). Additionally, unlike the non-pathogenic *Saccharomyces cerevisiae*, *C. albicans* is able to assimilate lactate and oleic acid in the presence of glucose (Sandai et al. 2012), allowing *C. albicans* to swiftly adapt to an environment in which nutrient availability can change as quickly as a phagocyte can engulf a cell. This metabolic flexibility and the consequent protective benefits under stress give *C. albicans* an advantage within various host environments, under either commensal or pathogenic conditions.

B. Micronutrients

Iron is an essential element for both the host and *C. albicans*. However, in the host, iron is almost completely associated with host proteins, which prevents not only iron-dependent production of toxic free radicals, but also microbial growth ("nutritional immunity"). Like other commensals and many other pathogens, ***C. albicans* has developed several strategies for iron acquisition within the host** (Almeida et al. 2009). Although *C. albicans* cannot produce siderophores, the fungus can **exploit iron from siderophores produced by other microbes**, an iron acquisition strategy that may be particularly useful in the commensal stage. Siderophores and other iron complexes are taken up by the Sit1/Arn1 transporter (Heymann et al. 2002; Hu et al. 2002).

In addition to uptake of siderophores, *C. albicans* can **exploit iron from iron-containing host proteins.** For example, haemoglobin can be used as an iron source during the pathogenic stage. Haemoglobin iron utilisation may occur predominantly via hyphae, which can bind to erythrocytes (Moors et al. 1992). Following the release of haemoglobin by an unknown mechanism, specific receptors on the surface of *C. albicans* such as *RBT5*, *RBT51*, *WAP1/CSA1*, *CSA2* and *PGA7* mediate uptake (Weissman and Kornitzer 2004). This process also seems to be hyphal-associated because the expression of some haemoglobin receptors is co-regulated with hyphal formation (Braun et al. 2000). Once haemoglobin is internalised, it is hydrolysed or denatured and haem is released (Weissman et al. 2008). A haem oxygenase (Santos et al. 2003), encoded by *HXM1*, degrades haem intracellularly (Pendrak et al. 2004). Another host molecule that can serve as an iron source for *C. albicans*, at least in vitro, is transferrin. Although the transferrin receptor remains unknown, it was shown that binding is necessary for iron utilisation from transferrin (Knight et al. 2005). Furthermore, *C. albicans* is able to bind the main host iron storage protein ferritin on the surface of hyphae via the hyphal-associated adhesin and invasin Als (see below) (Almeida et al. 2008). Following binding, the iron content of ferritin must be released by a mechanism that probably includes acidification.

In fact, *C. albicans* is only able to use ferritin as an iron source under conditions that permit acid production, suggesting that iron acquisition from ferritin by *C. albicans* is pH-mediated (Almeida et al. 2008).

To utilise iron from transferrin, from ferritin or from the environment, *C. albicans* uses the reductive pathway, which consists of several ferric reductases, multicopper oxidase(s) and iron permeases. The extracellular pH greatly influences the availability of iron because neutral and alkaline pH favours the oxidisation from the soluble Fe^{2+} to the non-soluble Fe^{3+} ion. The *RIM101* pathway of *C. albicans* is required for an appropriate transcriptional response to the environmental pH by coordinating the upregulation of genes involved in the reductive pathway, for example by upregulation of ferric reductases and the ferritin receptor Als3 under alkaline conditions (Baek et al. 2008; Liang et al. 2009; Nobile et al. 2008).

In addition to iron, further **trace metals such as zinc, manganese and copper are also essential for growth and survival of *C. albicans*** in the commensal and pathogenic phases, suggesting that nutritional immunity is a much more extensive phenomenon than iron sequestration. Yet, the mechanisms of acquisition of metals other than iron are largely unknown. A novel mechanism for zinc acquisition by *C. albicans* was recently described (Citiulo et al. 2012) by which the fungus secretes the zinc-binding pH-regulated antigen 1 (Pra1). This binds extracellular zinc and re-associates with a Pra1 receptor and zinc transporter (Zrt1) on the cell surface. Because this system resembles iron uptake via siderophores, Pra1 was named a "zincophore".

III. Stress Response

Whether in the pathogenic phase or commensal phase of interaction with the host, *C. albicans* faces a number of environmental and immune-derived stresses. Each niche with which the fungus is associated presents unique environmental factors, from the varying pH of the gastrointestinal and urogenital tracts (Brown et al. 2012) to the osmotic pressures of the oral mucosa and kidneys. A multifaceted and robust stress response is crucial for commensal adaptation, but also for virulence (Brown et al. 2012).

Many environmental and stress signals in fungi are sensed via mitogen-activated protein kinase (MAPK) pathways. As with numerous classical signalling pathways, particularly MAPK pathways, signals are propagated as a series of phosphorylation events, each kinase phosphorylating the next in turn until transcription factors are activated. Three such MAPK pathways are activated in *C. albicans*, with each pathway distinguished by a specific kinase, Mkc1, Hog1 or Cek1 (Monge et al. 2006).

The Hog1 MAPK pathway is considered a core response and is activated after many types of stresses (Monge et al. 2006), including oxidative and osmotic stresses. Hog1 also plays a role in morphogenesis, cell wall formation and the response to thermal stress (Monge et al. 2006). However, Hog1 phosphorylation occurs at different times post stress in response to osmotic, oxidative and nitrosative stresses, even responding to osmotic stress from sodium chloride and sorbitol at distinct time points (Kaloriti et al 2012). Activation of Hog1 in response to osmotic stress leads to accumulation of glycerol within the cell, which counters the loss of water due to chemical gradients (Monge et al. 2006). Also responding to oxidative and osmotic stress, the Mkc1 pathway is primarily noted for cell wall salvage and biogenesis as well as for maintaining cellular integrity (Monge et al. 2006; Munro et al. 2007), whereas the Cek1 pathway mediates filamentation and mating, and possibly responds to quorum sensing molecules (Mayer et al. 2012; Monge et al. 2006). Cells without Cek1 are unable to form hyphae in low-nitrogen media, although this can be overcome by the presence of serum (Monge et al. 2006). As would be anticipated, the ability to respond to the host battery of stresses is crucial for the success of *C. albicans* as a pathogen because deletion of the major stress response components Mkc1, Hog1 or Cek1 results in attenuated virulence in the mouse model (Alonso-Monge et al. 1999; Csank et al. 1998; Diez-Orejas et al. 1997).

Within the host, further environmental stressors are also produced by the immune system, specifically phagocytic cells capable of producing reactive oxygen species (ROS) and reactive nitrogen species (RNS). In its role as a pathogen, *C. albicans* copes with both of these stresses with a series of **detoxifying enzymes**. The oxidative stress response to ROS such as hydroxyl radicals and peroxide includes synthesis of antioxidants, detoxifying enzymes such as catalase (Cta1) and superoxide dismutases (Sod1-5), and repair of damaged proteins (Brown et al. 2012; Hwang et al. 2002; Martchenko et al. 2004; Wysong

et al. 1998). *C. albicans* cells phagocytosed by neutrophils are exposed to RNS, which may be detoxified by flavohaemoglobin enzyme Yhb1 (Hromatka et al. 2005; Tillmann et al. 2011; Miramón et al. 2012). Loss or deletion of some of the enzymes that detoxify ROS and RNS impacts virulence in animal models (Hromatka et al. 2005; Hwang et al. 2002; Martchenko et al. 2004; Tillmann et al. 2011; Wysong et al. 1998).

Although much work has been done on responses to osmotic, oxidative and nitrosative stresses singly, within the host – and particularly when in contact with host phagocytic cells – it is usual for several stresses to confront *C. albicans* simultaneously. It has been found that a combination of stresses similar to those encountered in vivo by *C. albicans* can efficiently kill the fungus, despite intact stress response pathways (Kaloriti et al. 2012). The activation of the individual stress response and salvage pathways under combinatorial stress has not yet been described.

C. albicans shows a great deal of flexibility when confronted with the broad range of pH found in host tissues. Under conditions of acidic pH and in the presence of exogenous amino acids, *C. albicans* is able to rapidly raise extracellular pH through export of ammonia, autoinducing filamentation (Vylkova et al. 2011). Alkaline pH is a known inducer of filamentation in *C. albicans*, and this is dependent upon Rim101. Cells deprived of Rim101 are unable to form hyphae in alkaline pH (Davis et al. 2000). Both the ability to filament in alkaline pH and the alkalinisation of extracellular spaces provide the fungus with the means to escape from phagolysosomes.

Heat shock proteins (Hsp) prevent protein unfolding and aggregation by acting as chaperones, and the heat shock response is a conserved response to high temperature and oxidative stress (Lindquist 1992). Currently, six major and six small heat shock proteins have been identified in *C. albicans*, with functions in thermal tolerance, virulence, drug resistance and biofilm formation (Mayer et al. 2013). **Hsp90 facilitates thermal tolerance** via the Hog1, Cek1 and Mkc1 pathways (Leach et al. 2012) and regulates temperature-dependent morphological changes (Shapiro and Cowen 2010). The small heat shock protein **Hsp21 mediates both stress adaptation and virulence** via phosphorylation of Cek1 (Mayer et al. 2012). Heat stress also results in accumulation of trehalose, the function of which has not been fully elucidated (Brown et al. 2012).

IV. Structure and Function of the Cell Wall in the Host

The cell wall of *C. albicans* is composed almost exclusively of molecules that are absent from the human body and is therefore a key target for immune recognition (Netea et al. 2008; Romani 2011). The cell wall is also essential for viability and one sixth of the total genome is invested in its biosynthesis and maintenance. The cell wall is highly dynamic and the changing nature of the cell surface presents challenges for the detection and surveillance of the fungus. This is important in terms of commensalism and pathogenicity because **it is the molecules of the cell wall that activate immune recognition**. Over-activation of pro-inflammatory immune responses can be a major cause of tissue damage.

The wall exists in two readily distinguished layers: an **outer layer** that is enriched with highly glycosylated glycoproteins, many of which are expressed in a morphology-dependent fashion. Many proteins are found predominantly in the yeast or hyphal growth phase and hyphae-specific cell wall proteins play roles as adhesins and invasins and in modulating the immune responses (Liu and Filler 2011; Luo et al. 2010). There is less mannan and substantially more chitin in the hyphal wall. Cell wall mannan comprises short O-linked linear α-1,2 mannan; highly branched *N*-mannan comprises an α-1,6 mannan backbone and side chains composed of α-1,2 mannose and α-1,3 mannose residues, with β-1,2 mannose attached to the acid-stable and phosphodiester acid-labile side chains. Both *N*-mannan and *O*-mannan may also contain phosphomannosylated side chains. Mannan chemistry defines many of the *Candida* serotypes, further underlying the heterogeneity that exists in mannan structure (Suzuki 2002). *C. albicans O*-mannans consist of 1–5 mannose sugars that are almost

exclusively α-1,2 linked. The phosphomannan is exclusively β-1,2 mannose, which is a linkage that is not present in many other yeast species.

The **inner cell wall** represents the skeletal layer and is composed predominantly of two robust polysaccharides, β-1,3 glucan and lesser amounts of chitin, that confer strength and cell shape (Fig. 1.1). In budding yeast cells, a scar is left on the mother cell after separation of the bud and, at this site, components that constitute the inner layers of the cell wall, such as chitin and β-1,3 glucan, may become exposed at the surface (Gantner et al. 2005).

Therefore, although the basic components of the cell wall of *C. albicans* are similar in its yeast and filamentous forms, the signature of proteins and amounts of individual pathogen-associated molecular patterns (PAMPs) presented to immune cells **differs substantially in these different cell types**. In addition, the carbon source for growth (see above) and the growth conditions result in substantial remodelling of the cell wall, even if the morphology of the cell is not affected (Gow et al. 2002).

V. Morphology

The three major tissue-associated morphologies of *C. albicans* are the spheroid **yeast cell** and the broadly filamentous **hyphae** and **pseudohyphae** (Fig. 1.1) (Sudbery et al. 2004). Although the strict function of each morphological form within the host is up for debate, yeast cells are presumed to disseminate more readily through the host whereas hyphae exhibit more invasive tendencies (Gow et al. 2002; Sudbery et al. 2004). However, it must be kept in mind that yeast cells are the invasive form in other fungal pathogens – including all those dimorphic fungi that have an environmental hyphal phase. Less well characterised is the impact of the pseudohyphal form on the ability of *C. albicans* to cause disease or establish a commensal state within the host. Certainly, all three forms (yeast, pseudohyphal and hyphal) are found in clinical specimens as well as in animal models of infection.

Although it is clear that the majority of clinically relevant *Candida* species predominate in the yeast form (Thompson et al. 2011), the specific ability of *C. albicans* to form both yeast and hyphal cells in vivo seems to be an important attribute in the repertoire of virulence factors in this organism. The capacity to undergo reversible yeast–hyphae morphogenesis is linked to the virulence of *C. albicans* (Gow et al. 2002; Lo et al. 1997; Sudbery 2011). A wide range of studies have shown that mutants that are affected in the mechanism that regulates the yeast–hyphae and hyphae–yeast transition are less virulent in animal models than wild-type *C. albicans* strains (Lo et al. 1997; Saville et al. 2003). The dependence on the ability to switch to the hyphal form for virulence is evident in every commonly used animal model of *C. albicans* infection, both vertebrate and invertebrate (Brothers et al. 2011; Davis et al. 2011; Lo et al. 1997; Pukkila-Worley et al. 2009).

To date, numerous genes involved in the morphological switch from yeast to hyphae have been characterised and studied in detail, though very few are noted for their contribution to commensalism. The expression and activity of transcription factors Efg1 and Efh1, in particular, are considered modulators of the commensal state within the gut (Pierce and Kumamoto 2012; Pierce et al. 2013; White et al. 2007). It was found that when colonising the gut of mice, *C. albicans* remained primarily in the yeast form (White et al. 2007), and that the absence of Efg1 promoted high early colonisation in healthy and athymic mice (Pierce and Kumamoto 2012). It may therefore be inferred that *C. albicans* with low Efg1 levels are the most likely commensal form, even in immunocompromised hosts, unless and until an environmental shift promotes more pathogenic phenotypes.

Specimens from humans and experimental animals show that *C. albicans* **hyphae dominate at the primary site of infiltration** of epithelial cell layers and tissues, whereas yeast cells are generally found either on the epithelial cell surface or emerging from penetrating hyphae that are infiltrating tissue (Fig. 1.2, middle) (Ray and Payne 1988; Scherwitz 1982). Furthermore, it is proposed that there is a threshold for the amount of *C. albicans* that is tolerated. Therefore, the host immune defences have to keep the fungal burden below the threshold and have to distinguish a non-pathogenic *C. albicans* from an invasive and potentially life-threatening *C. albicans* in order to keep homeostasis (Moyes et al. 2010).

As detailed below, much recent progress has been made on the pattern recognition of *C. albicans*. However, much less is known regarding the differential recognition between the two main morphogenetic forms of *C. albicans*, yeasts and hyphae. Because this fungus reversibly transits between these forms during the progression of an infection, yeast–hyphal morphogenesis is likely to have a major effect on spatial and temporal aspects of the immune response.

VI. Adhesion, Invasion and Damage: Interaction with Epithelial Cells

Attachment of *C. albicans* to its host is a property that is required during both the commensal and the pathogenic phase. Adhesion is mediated by physical surface attributes such as hydrophobicity and specialised surface proteins, the **adhesins**. These proteins mediate adhesion to host cells, other *Candida* cells, other microbes or abiotic surfaces (e.g. catheters). The large number and variety of adhesins enables *C. albicans* to colonise several host niches. Adhesins are often glycosylphosphatidylinositol (GPI)-proteins or GPI-anchored proteins, which are exposed at the cell surface (Zordan and Cormack 2012). One of the best-studied group of *C. albicans* adhesins are the agglutinin-like sequence (Als) proteins, which form a family consisting of eight GPI-linked glycoproteins (Als1–Als7 and Als9) (Hoyer 2001). Each Als protein has three characteristic domains: a conserved N-terminal domain, a central domain with short tandemly repeated copies and variable length, and a C-terminal domain that varies in both sequence and length.

The hyphae-associated adhesin Als3 seems to play a key role for epithelial adhesion (Wächtler et al. 2012, 2011a) and the *ALS3* gene is expressed in vitro and in vivo during infection of oral epithelial cells and during vaginal infection (Cheng et al. 2005; Naglik et al. 2011; Wächtler et al. 2011a; Zakikhany et al. 2007). Another GPI-linked adhesin of *C. albicans* expressed during filamentation is Hwp1 (Staab et al. 1999). Hwp1 contributes to adherence to epithelial cells and is required for full virulence in vivo (Sundstrom et al. 2002a, b). Further GPI-linked adhesins are Eap1, Iff4 and Ecm33, the putative β-glucanase Mp65, the β-1,3 glucanosyl transferase Phr1 and the integrin-like surface protein Int1 (Naglik et al. 2011; Zhu and Filler 2010).

Following adhesion, *C. albicans* hyphae are able to invade epithelial cells via two different routes: induced **endocytosis** and **active penetration** (Dalle et al. 2010; Wächtler et al. 2011a; Zakikhany et al. 2007; Zhu and Filler 2010). Induced endocytosis is a host-driven process triggered by hyphae-associated invasins, in particular the adhesin and ferritin binding Als3, and Ssa1, a cell-surface-associated heat shock protein (Phan et al. 2005; Sun et al. 2010). These hyphae-associated invasins bind to epithelial ligands (e.g. E-cadherin) and thereby cause endocytosis of hyphae into the host cell. These processes have been described for oral and vaginal epithelial cells and endothelial cells, but not for intestinal cells (Caco-2 cell line). Even killed hyphae are endocytosed; therefore, induced endocytosis does not require fungal activities.

Active penetration directly into host cells or between host cells (Fig. 1.2, right) is an entirely fungal-driven process that requires viable *C. albicans* hyphae, fungal turgor, normal vacuole formation, cell wall integrity, hyphae extension and other physical forces (Dalle et al. 2010; Wächtler et al. 2011a). Hyphal growth of *C. albicans* along host surfaces is guided via contact sensing (thigmotropism) (Brand and Gow 2009). In addition, fungal growth and morphology is influenced by quorum sensing mechanisms within populations of *C. albicans* (Singh and Del Poeta 2011).

Although invasion is mostly considered a pathogenic process, it is possible that even commensal *C. albicans* hyphae moderately invade epithelial cells, thereby stabilising their attachment to proliferating epithelia. In fact, moderate invasion of viable hyphae and invasion of killed hyphae via induced endocytosis does not cause damage, cytokine production and attraction of neutrophils (Jacobsen et al. 2012; Wächtler et al. 2011b; Zhu and Filler 2010; our unpublished observation). However, locally, commensal invasion may cause counter-activities of the host and elimination of fungal hyphae that penetrate into deeper layers. Such continuous interactions may select for fungal immune evasion attributes (the commensal school), but may also prime the host for specific antifungal activities.

Immunological data obtained from sera of healthy individuals indicate that such continuous interactions between *C. albicans* and the host are likely to be common (Hube 2004; Mochon et al. 2010).

Although occasional, moderate invasion does not cause damage and may occur in the commensal phase, the cause of host **damage is a key characteristic of pathogenesis and disease**. Damage caused by *C. albicans* occurs when hyphae invade through epithelial cells and deep into epithelial layers (interepithelial invasion). It is still unknown which fungal factors cause host damage, but secretion of hydrolases, hyphae-associated damaging factors and hyphal extension (Wächtler et al. 2011a; Zhu and Filler 2010) seem to contribute to damage of host tissue. However, tissue damage and disease occurs not only by direct fungal activities, but may also be the result of an overreaction of the immune system, e.g. massive infiltration of neutrophils, which release toxic agents and damage epithelial cells, a hallmark of vaginal infections (Casadevall and Pirofski 2003; Yano et al. 2010). Furthermore, an inappropriate and unbalanced systemic response during invasive candidiasis may cause life-threatening sepsis. Therefore, immune responses to invading hyphae may not only be beneficial, but may also be a part of the disease process.

VII. Interaction with Phagocytes

Candida cells are phagocytosed by various cell types of the innate immune system, including macrophages, neutrophils and dendritic cells, as well as inducing their own phagocytosis by cells of the endothelium, as discussed above. **Phagocytosis is known to be critically dependent on *C. albicans* cell wall composition, on yeast–hyphae morphogenesis and on the immune cell type and state of activation** (Gow et al. 2002; McKenzie et al. 2010). For example, the glycosylation status of the *C. albicans* cell wall influences the rate of macrophage and neutrophil phagocytosis. Mutants deficient in cell wall phosphomannan (*mnn4*Δ, *pmr1*Δ and *mnt3*Δ/*mnt5*Δ) are taken up by macrophages more slowly, whereas some O-linked and N-linked mannan mutants (including *mns1*Δ and *mnt1*Δ/*mnt2* Δ) are taken up more rapidly (McKenzie et al. 2010). Cell wall N-linked mannan has been shown to be important for uptake into neutrophils (Sheth et al. 2011). Experiments in which phagocytes were present with alternative target yeast cells demonstrated that genus, species and morphology of the *Candida* cells were all-important factors in determining the efficiency of phagocytosis (Keppler-Ross et al. 2010). For example, *C. glabrata* and *S. cerevisiae* were more efficiently taken up by J774 macrophage cells than *C. albicans*, and *C. albicans* yeast cells were more efficiently phagocytosed than hyphae (Keppler-Ross et al. 2010). Macrophages also engulfed viable and UV-killed *C. albicans* more efficiently than glycosylation and morphology-locked mutants. Recent work also showed that phagocytosis efficiency was affected by hyphal length and the orientation and contact of the hyphae relative to the macrophages (Lewis et al. 2012). As first reported in *C. neoformans*, *C. albicans* can also promote its own non-lytic expulsion (vomitosis) from within the phagosome of macrophages (Bain et al. 2012).

Multiple groups have shown that *C. albicans* hypha formation within the phagolysosome of macrophages can lead to rupture and killing of the phagocyte (Lo et al. 1997; McKenzie et al. 2010). Mutants that are locked in the yeast form do not cause macrophage disruption. In the zebrafish model of infection it has been shown using in vivo imaging techniques that macrophages are better able to kill germ tube competent strains of *C. albicans*, but that the macrophages were ruptured by hyphae extension when the phagocytes were cultured and exposed to the fungus in vitro (Brothers et al. 2011). Therefore, there is evidence that the cell wall and morphogenesis are important for the interactions with phagocytes of the innate immune system, although the nature of these interactions may differ according to the conditions of the experimental set-up.

VIII. Host Recognition of the Cell Wall

The immune response to *Candida* starts by recognition of the cell wall – but this is a moving target. The wall properties and composition depends on

the available carbon source (Ene et al. 2012), hypoxia and morphogenesis. **The initial interactions with the wall trigger a series of events that ultimately lead to phagocytosis, signalling immune cell recruitment and modulation of subsequent specific responses** delivered by T cells and B cells through their interaction with antigen-presenting cells (especially dendritic cells; DC) (Hoebe et al. 2004).

Innate recognition of *C. albicans* is achieved by the pattern recognition receptors (PRRs), which bind to defined chemical moieties called PAMPs on the cell surface and in the cytoplasm and endosomes of lymphocytes. The PRR classes include Toll-like receptors (TLR) and C-type lectin receptors (LRs), which are mainly membrane-bound receptors whereas NOD-like receptors (NLRs) and RiG-I-receptors are intracellular. TLRs (TLR2, TLR4, TLR6 and TLR9) and LRs (e.g., macrophage mannose receptor, dectin-1, dectin-2, mincle and DC-SIGN) contribute to the recognition of fungal pathogens. These receptors have an extracellular pathogen-recognition domain and intracellular signalling domains that ultimately lead to activation of transcription factors that induce the expression of cytokines and other molecules required for the innate immune response. Some PRRs, such as dectin-1 and the MR are also phagocytic receptors that are required for the uptake of the pathogen and delivery into the phagosome (see also chapter "Receptor–Ligand Interactions in Fungal Infections").

The PAMP repertoire of a fungus represents its immunological signature and is recognised by the above PRRs. PAMPs are predominantly cell wall components and all of the major cell wall components of the *Candida* cell wall play some role in immune recognition. Recognition leads to phagocytosis, signalling, secretion of cytokines and chemokines, and ultimately the initiation of the adaptive immune response through the activation of various T cell subsets. Some cell wall PAMPs are exposed on the surface and immediately engage with PRRs, while others, such as the β-glucans and chitin, are initially masked by the outer mannan layers and presumably only influence the immune response as they become exposed as the cell wall is degraded or altered as a consequence of antibiotic exposure (Gow et al. 2011, 2007).

All of the cell wall components are relevant to the recognition and immune response to *C. albicans*. The outer cell wall mannan-rich layer represents the PAMPs for a wide range of PRRs and results in the initial inflammatory response. O-linked mannans are recognised by TLR4 whereas the N-linked mannan binds a range of C-type lectins including the mannose receptor, mincle, dectin-2 and DC-SIGN. β-1,2 mannan in the acid-stable and acid-labile *N*-mannan and in phospholipomannan is recognised by galectin-3 and TLR2 (Gow et al. 2011; Netea et al. 2008; Romani 2011; van de Veerdonk et al. 2008). The mannan acts as a shield that initially prevents underlying β-1,3 glucan from engaging with dectin-1 (Wheeler and Fink 2006), although β-1,3 glucan is naturally exposed at bud scars of yeast cells and can be revealed when the fungus is exposed to cell wall disrupting agents such as echinocandin antibiotics. β-1,6 Glucan, which is a linker molecule that connects the cell wall proteins to the glucan–chitin skeleton, acts as an opsonic receptor promoting phagocytosis by neutrophils (Rubin-Bejerano et al. 2007). Chitin dampens the proinflammatory response (Mora-Montes et al. 2011) and low molecular weight chitin engages with the internal NOD2 and TLR9 PRRs, leading to interleukin (IL)-10 secretion (Wagener and Gow, personal communication). Chitin is likely to be the last of the cell wall PAMPs to be exposed, and this may only occur after extensive cell wall damage and loss of fungal viability. The anti-inflammatory action of chitin may therefore help to mitigate inflammatory-mediated damage to the host due to the other pro-inflammatory molecules in the cell wall. Thus, β-1,3 glucan and chitin have opposite pro- and anti-inflammatory roles.

IX. Discrimination of Yeast and Hyphal Cells

The host defence mechanism of epithelial cells is triggered by certain danger signals that include an increase in yeast cell numbers on epithelial surfaces and the presence of tissue-infiltrating hyphae. Epithelial cells were shown recently to be able to discriminate between the yeast cells, which can regarded as non-harmful commensals, and invading hyphae (Moyes et al. 2010). The induction of a **first phase of c-Jun and c-Fos MAPK activation** was followed by a **second, hyphae-specific phase of c-Fos and MKP1 activation** through p38 and ERK1/ERK2 kinase pathways. These phases are required for induction of cytokine secretion by epithelial cells. Upregulation of TLR4 expression in *C. albicans*-infected human oral epithelium was reported to be correlated with protection

against *C. albicans* tissue invasion (Weindl et al. 2007), but TLR4 was not implicated in the activation of these MAPK pathways. TLR2, dectin-1 or mannose receptor were again not required for this biphasic activation of epithelial cells. Addition of neutrophils to the *Candida*-infected tissues of an oral epithelium model upregulated epithelial TLR4 expression, and cathelicidin (LL-37) secretion, which correlated with tissue protection (Weindl et al. 2011). It is still not known what repertoires of PRRs are required for recognition of hyphae and pseudohyphae during tissue invasion by *C. albicans*.

It is clear that the morphogenetic plasticity of *C. albicans*, comprising growth as yeast cells, pseudohyphae and hyphae, presents a moving target with respect to immune surveillance and recognition. Several studies have shown that the PAMPs present in these cell types induce substantially different immune responses. For example, unlike hyphae, yeast cells do not induce IL-12 production in DCs, but do induce IL-4 secretion (D' Ostiani et al. 2000). Hyphae do not induce the Th1- cytokine interferon-γ (IFN-γ), perhaps due to the lack of recognition by TLR4 (Van der Graaf et al. 2005).

It is not yet known what differences in the cell wall PAMPs account for such differences in cytokine stimulation by myeloid cells. Hyphae also do not produce bud scars and so β-glucan exposure is less on hyphae than on yeast cells (Gantner et al. 2005). The mannan fibrils of hyphae are shorter and less abundant than in yeast cells, but the permeability of the mannan layer of hyphae is less than in yeast cells (Cheng et al. 2011). It has also been suggested that differences in the mannan biochemical structure in yeast and hyphal cells accounts for differential recognition of mannans from hyphae and yeasts by dectin-2 (Bi et al. 2010). The precise nature of such changes in mannan is not yet clear (Kruppa et al. 2011; Shibata et al. 2007).

Mucosal defence is **critically dependent on Th17 function** and it now seems clear that Th17 activation responds differentially to colonising yeast cells and invading hyphae (Cheng et al. 2011). The Th17 response requires cytokines that include IL-23, IL-1, IL-6 and TGFβ (Acosta-Rodriguez et al. 2007a, b; van de Veerdonk et al. 2010). No difference in IL-23 and IL-6 production was found in macrophages exposed to yeasts and hyphae, and only hyphae induced IL-1β production by macrophages (Joly et al. 2009), which is required for induction of the Th17 responses mediated by the NLRP3 inflammasome (Cheng et al. 2011).

Monomorphic yeast-locked *C. albicans* mutants did not activate the NLRP3 inflammasome or the secretion of IL-1β by macrophages (Joly et al. 2009). This response also required the dectin-1 pathway for the induction of pro-IL-1β production, which requires cleavage into IL-1β (Van de Veerdonk et al. 2009) by the caspase-1 protease. This suggests that the germination of *C. albicans* yeasts into hyphae triggers activation of the NLRP3 inflammasome in macrophages, leading in turn to the activation of caspase-1. The transcription of pro-IL-1β depends on the activation of PRRs such as dectin-1, TLR2 and MR. However, when epithelia are colonised without the presence of invading hyphae, caspase-1 is not activated and therefore pro-IL-1β is not processed into its active form IL-1β. When macrophages engage *C. albicans* hyphae, caspase-1 is activated and bioactive IL-1β is produced, leading to the production of cytokines IL-17 and IL-22 and the induction of a protective Th17 response and neutrophil recruitment to that site (Ouyang et al. 2008). IL-22 also results in defensin production from epithelial cells (Eyerich et al. 2011), which contributes to the killing of invading hyphae. It is also possible that ATP that is released from damaged epithelial cells (Piccini et al. 2008) helps induce Th17 cell differentiation (Atarashi et al. 2008). Therefore, the production of hyphae acts as an alarm signal that activates a mucosal Th17 response that ultimately helps prevent tissue invasion and retains *C. albicans* in the commensal state.

X. Conclusions

C. albicans does not cause disease in most people most of the time, suggesting that it is adapted for life as a commensal. The evolutionary forces that have sculpted the genome of this fungus may have resulted in a gene set that enables the fungus to be retained in a commensal state on the mucosa when the immune system is vigorous and intact, and simultaneously to flourish and cause superficial and systemic disease under

conditions when immune surveillance is compromised. The roles of specific genes in this fungus are therefore context-dependent, meaning that the interpretation of knockout mutants of potential commensal or virulence-associated genes is complex. Nonetheless, it is clear that *C. albicans* is characterised by its metabolic and nutritional versatility and its ability to induce a wide variety of immune responses. This flexible phenotype is presumed to account for its prevalence, wide distribution and complex immune pathology, and makes it a remarkable and fascinating model system for studies of host–pathogen interactions.

Acknowledgements NG and AW were supported by the Wellcome Trust (080088, 086827, 075470 & 097377) and the European Union ALLFUN (FP7/2007 2013, HEALTH-2010-260338). BH is supported by the Federal Ministry of Education and Research (BMBF: ERA Net PathoGenoMics CandiCol 0315901B), the Center for Sepsis Control and Care (CSCC – BMBF 01EO1002), the Deutsche Forschungsgemeinschaft (DFG Hu 528/15, 16 and 17), the Jena School for Microbial Communication (JSMC), and the International Leibniz Research School for Microbial and Biomolecular Interactions (ILRS).

References

Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F (2007a) Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. Nat Immunol 8:942–949

Acosta-Rodriguez EV, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia A, Sallusto F, Napolitani G (2007b) Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. Nat Immunol 8:639–646

Almeida RS, Brunke S, Albrecht A, Thewes S, Laue M, Edwards JE, Filler SG, Hube B (2008) The hyphal-associated adhesin and invasin Als3 of *Candida albicans* mediates iron acquisition from host ferritin. PLoS Pathog 4:e1000217

Almeida RS, Wilson D, Hube B (2009) *Candida albicans* iron acquisition within the host. FEMS Yeast Res 9: 1000–1012

Alonso-Monge R, Navarro-García F, Molero G, Diez-Orejas R, Gustin M, Pla J, Snchez M, Nombela C (1999) Role of the mitogen-activated protein kinase Hog1p in morphogenesis and virulence of *Candida albicans*. J Bacteriol 181:3058–3068

Andaluz E, Ciudad T, Gómez-Raja J, Calderone R, Larriba G (2006) Rad52 depletion in *Candida albicans* triggers both the DNA-damage checkpoint and filamentation accompanied by but independent of expression of hypha-specific genes. Mol Microbiol 59:1452–1472

Atarashi K, Nishimura J, Shima T, Umesaki Y, Yamamoto M, Onoue M, Yagita H, Ishii N, Evans R, Honda K, Takeda K (2008) ATP drives lamina propria T(H)17 cell differentiation. Nature 455:808–812

Baek Y-U, Li M, Davis DA (2008) *Candida albicans* ferric reductases are differentially regulated in response to distinct forms of iron limitation by the Rim101 and CBF transcription factors. Eukaryot Cell 7:1168–1179

Bain JM, Lewis LE, Okai B, Quinn J, Gow NAR, Erwig L-P (2012) Non-lytic expulsion/exocytosis of *Candida albicans* from macrophages. Fungal Genet Biol 49: 677–678

Barelle CJ, Priest CL, Maccallum DM, Gow NAR, Odds FC, Brown AJP (2006) Niche-specific regulation of central metabolic pathways in a fungal pathogen. Cell Microbiol 8:961–971

Bi L, Gojestani S, Wu W, Hsu Y-MS, Zhu J, Ariizumi K, Lin X (2010) CARD9 mediates dectin-2-induced IkappaBalpha kinase ubiquitination leading to activation of NF-kappaB in response to stimulation by the hyphal form of *Candida albicans*. J Biol Chem 285:25969–25977

Brand A, Gow NAR (2009) Mechanisms of hypha orientation of fungi. Curr Opin Microbiol 12: 350–357

Braun BR, Head WS, Wang MX, Johnson AD (2000) Identification and characterization of TUP1-regulated genes in *Candida albicans*. Genetics 156: 31–44

Brothers KM, Newman ZR, Wheeler RT (2011) Live imaging of disseminated candidiasis in zebrafish reveals role of phagocyte oxidase in limiting filamentous growth. Eukaryot Cell 10:932–944

Brown A, Haynes K, Gow N, Quinn J (2012) Stress responses in *Candida*. In: Calderone R, Clancy C (eds) Candida and candidiasis. ASM, Washington, DC, pp 225–242

Casadevall A (2008) Evolution of intracellular pathogens. Annu Rev Microbiol 62:19–33

Casadevall A, Pirofski L (2003) The damage-response framework of microbial pathogenesis. Nat Rev Microbiol 1:17–24

Casadevall A, Pirofski L (2007) Accidental virulence, cryptic pathogenesis, martians, lost hosts, and the pathogenicity of environmental microbes. Eukaryot Cell 6:2169–2174

Cheng G, Wozniak K, Wallig MA, Fidel PL Jr, Trupin SR, Hoyer LL (2005) Comparison between *Candida albicans* agglutinin-like sequence gene expression

patterns in human clinical specimens and models of vaginal candidiasis. Infect Immun 73:1656–1663

Cheng S-C, Van de Veerdonk FL, Lenardon M, Stoffels M, Plantinga T, Smeekens S, Rizzetto L, Mukaremera L, Preechasuth K, Cavalieri D, Kanneganti TD, Van der Meer JWM, Kullberg BJ, Joosten LAB, Gow NAR, Netea MG (2011) The dectin-1/inflammasome pathway is responsible for the induction of protective T-helper 17 responses that discriminate between yeasts and hyphae of *Candida albicans*. J Leukoc Biol 90:357–366

Citiulo F, Jacobsen ID, Miramón P, Schild L, Brunke S, Zipfel P, Brock M, Hube B, Wilson D (2012) *Candida albicans* scavenges host zinc via Pra1 during endothelial invasion. PLoS Pathog 8:e1002777

Csank C, Schröppel K, Leberer E, Harcus D, Mohamed O, Meloche S, Thomas DY, Whiteway M (1998) Roles of the *Candida albicans* mitogen-activated protein kinase homolog, Cek1p, in hyphal development and systemic candidiasis. Infect Immun 66: 2713–2721

D' Ostiani CF, Del Sero G, Bacci A, Montagnoli C, Spreca A, Mencacci A, Ricciardi-Castagnoli P, Romani L (2000) Dendritic cells discriminate between yeasts and hyphae of the fungus *Candida albicans*. Implications for initiation of T helper cell immunity in vitro and in vivo. J Exp Med 191: 1661–1674

Dalle F, Wächtler B, L'Ollivier C, Holland G, Bannert N, Wilson D, Labruère C, Bonnin A, Hube B (2010) Cellular interactions of *Candida albicans* with human oral epithelial cells and enterocytes. Cell Microbiol 12:248–271

Davis D, Wilson RB, Mitchell AP (2000) RIM101-dependent and -independent pathways govern pH responses in *Candida albicans*. Mol Cell Biol 20:971–978

Davis MM, Alvarez FJ, Ryman K, Holm ÅA, Ljungdahl PO, Engström Y (2011) Wild-type *Drosophila melanogaster* as a model host to analyze nitrogen source dependent virulence of *Candida albicans*. PLoS One 6:e27434

Diez-Orejas R, Molero G, Navarro-García F, Pla J, Nombela C, Sanchez-Pérez M (1997) Reduced virulence of *Candida albicans* MKC1 mutants: a role for mitogen-activated protein kinase in pathogenesis. Infect Immun 65:833–837

Ene IV, Adya AK, Wehmeier S, Brand AC, MacCallum DM, Gow NAR, Brown AJP (2012) Host carbon sources modulate cell wall architecture, drug resistance and virulence in a fungal pathogen. Cell Microbiol 14: 1319–1335

Eyerich S, Wagener J, Wenzel V, Scarponi C, Pennino D, Albanesi C, Schaller M, Behrendt H, Ring J, Schmidt-Weber CB, Cavani A, Mempel M, Traidl-Hoffmann C, Eyerich K (2011) IL-22 and TNF-α represent a key cytokine combination for epidermal integrity during infection with *Candida albicans*. Eur J Immunol 41:1894–1901

Fradin C, De Groot P, MacCallum D, Schaller M, Klis F, Odds FC, Hube B (2005) Granulocytes govern the transcriptional response, morphology and proliferation of *Candida albicans* in human blood. Mol Microbiol 56:397–415

Gantner BN, Simmons RM, Underhill DM (2005) Dectin-1 mediates macrophage recognition of *Candida albicans* yeast but not filaments. EMBO J 24:1277–1286

Gow NAR, Brown AJP, Odds FC (2002) Fungal morphogenesis and host invasion. Curr Opin Microbiol 5:366–371

Gow NAR, Netea MG, Munro CA, Ferwerda G, Bates S, Mora-Montes HM, Walker L, Jansen T, Jacobs L, Tsoni V, Brown GD, Odds FC, Van der Meer JWM, Brown AJP, Kullberg BJ (2007) Immune recognition of *Candida albicans* beta-glucan by dectin-1. J Infect Dis 196:1565–1571

Gow NAR, Van de Veerdonk FL, Brown AJP, Netea MG (2011) *Candida albicans* morphogenesis and host defence: discriminating invasion from colonization. Nat Rev Microbiol 10:112–122

Heymann P, Gerads M, Schaller M, Dromer F, Winkelmann G, Ernst JF (2002) The siderophore iron transporter of *Candida albicans* (Sit1p/Arn1p) mediates uptake of ferrichrome-type siderophores and is required for epithelial invasion. Infect Immun 70:5246–5255

Hoebe K, Janssen E, Beutler B (2004) The interface between innate and adaptive immunity. Nat Immunol 5: 971–974

Hoyer LL (2001) The ALS gene family of *Candida albicans*. Trends Microbiol 9:176–180

Hromatka BS, Noble SM, Johnson AD (2005) Transcriptional response of *Candida albicans* to nitric oxide and the role of the YHB1 gene in nitrosative stress and virulence. Mol Biol Cell 16:4814–4826

Hu C-J, Bai C, Zheng X-D, Wang Y-M, Wang Y (2002) Characterization and functional analysis of the siderophore-iron transporter CaArn1p in *Candida albicans*. J Biol Chem 277:30598–30605

Hube B (2004) From commensal to pathogen: stage- and tissue-specific gene expression of *Candida albicans*. Curr Opin Microbiol 7:336–341

Hube B (2009) Fungal adaptation to the host environment. Curr Opin Microbiol 12:347–349

Hwang C-S, Rhie G, Oh J-H, Huh W-K, Yim H-S, Kang S-O (2002) Copper- and zinc-containing superoxide dismutase (Cu/ZnSOD) is required for the protection of *Candida albicans* against oxidative stresses and the expression of its full virulence. Microbiology 148:3705–3713 (Reading, Engl.)

Jacobsen ID, Wilson D, Wächtler B, Brunke S, Naglik JR, Hube B (2012) *Candida albicans* dimorphism as a therapeutic target. Expert Rev Anti Infect Ther 10: 85–93

Joly S, Ma N, Sadler JJ, Soll DR, Cassel SL, Sutterwala FS (2009) Cutting edge: *Candida albicans* hyphae formation triggers activation of the Nlrp3 inflammasome. J Immunol 183:3578–3581

Kaloriti D, Tillmann A, Cook E, Jacobsen M, You T, Lenardon M, Ames L, Barahona M, Chandrasekaran K, Coghill G, Goodman D, Gow NAR, Grebogi C, Ho H-L, Ingram P, McDonagh A, De Moura APS, Pang W, Puttnam M, Radmaneshfar E, Romano MC, Silk D, Stark J, Stumpf M, Thiel M, Thorne T, Usher J, Yin Z, Haynes K, Brown AJP (2012) Combinatorial stresses kill pathogenic *Candida* species. Med Mycol 50:699–709

Keppler-Ross S, Douglas L, Konopka JB, Dean N (2010) Recognition of yeast by murine macrophages requires mannan but not glucan. Eukaryot Cell 9: 1776–1787

Knight SAB, Vilaire G, Lesuisse E, Dancis A (2005) Iron acquisition from transferrin by *Candida albicans* depends on the reductive pathway. Infect Immun 73:5482–5492

Kruppa M, Greene RR, Noss I, Lowman DW, Williams DL (2011) *C. albicans* increases cell wall mannoprotein, but not mannan, in response to blood, serum and cultivation at physiological temperature. Glycobiology 21:1173–1180

Leach MD, Budge S, Walker L, Munro C, Cowen LE, Brown AJP (2012) Hsp90 orchestrates transcriptional regulation by Hsf1 and cell wall remodelling by MAPK signalling during thermal adaptation in a pathogenic yeast. PLoS Pathog 8:e1003069

Lewis LE, Bain JM, Lowes C, Gillespie C, Rudkin FM, Gow NAR, Erwig L-P (2012) Stage specific assessment of *Candida albicans* phagocytosis by macrophages identifies cell wall composition and morphogenesis as key determinants. PLoS Pathog 8:e1002578

Liang Y, Gui L, Wei D-S, Zheng W, Xing L-J, Li M-C (2009) *Candida albicans* ferric reductase FRP1 is regulated by direct interaction with Rim101p transcription factor. FEMS Yeast Res 9:270–277

Lindquist S (1992) Heat-shock proteins and stress tolerance in microorganisms. Curr Opin Genet Dev 2: 748–755

Liu Y, Filler SG (2011) *Candida albicans* Als3, a multifunctional adhesin and invasin. Eukaryot Cell 10: 168–173

Lo HJ, Köhler JR, DiDomenico B, Loebenberg D, Cacciapuoti A, Fink GR (1997) Nonfilamentous *C. albicans* mutants are avirulent. Cell 90:939–949

Lorenz MC, Fink GR (2002) Life and death in a macrophage: role of the glyoxylate cycle in virulence. Eukaryot Cell 1:657–662

Luo G, Ibrahim AS, Spellberg B, Nobile CJ, Mitchell AP, Fu Y (2010) *Candida albicans* Hyr1p confers resistance to neutrophil killing and is a potential vaccine target. J Infect Dis 201:1718–1728

Martchenko M, Alarco A-M, Harcus D, Whiteway M (2004) Superoxide dismutases in *Candida albicans*: transcriptional regulation and functional characterization of the hyphal-induced SOD5 gene. Mol Biol Cell 15:456–467

Mayer FL, Wilson D, Jacobsen ID, Miramón P, Slesiona S, Bohovych IM, Brown AJP, Hube B (2012) Small but crucial: the novel small heat shock protein Hsp21 mediates stress adaptation and virulence in *Candida albicans*. PLoS One 7:e38584

Mayer FL, Wilson D, Hube B (2013) *Candida albicans* pathogenicity mechanisms. Virulence 4:119–128

McKenzie CGJ, Koser U, Lewis LE, Bain JM, Mora-Montes HM, Barker RN, Gow NAR, Erwig LP (2010) Contribution of *Candida albicans* cell wall components to recognition by and escape from murine macrophages. Infect Immun 78:1650–1658

Miramón P, Dunker C, Windecker H, Bohovych IM, Brown AJP, Kurzai O, Hube B (2012) Cellular responses of *Candida albicans* to phagocytosis and the extracellular activities of neutrophils are critical to counteract carbohydrate starvation, oxidative and nitrosative stress. PLoS One 7:e52850

Mochon AB, Jin Y, Ye J, Kayala MA, Wingard JR, Clancy CJ, Nguyen MH, Felgner P, Baldi P, Liu H (2010) Serological profiling of a *Candida albicans* protein microarray reveals permanent host-pathogen interplay and stage-specific responses during candidemia. PLoS Pathog 6:e1000827

Monge RA, Romn E, Nombela C, Pla J (2006) The MAP kinase signal transduction network in *Candida albicans*. Microbiology 152:905–912 (Reading, Engl.)

Moors MA, Stull TL, Blank KJ, Buckley HR, Mosser DM (1992) A role for complement receptor-like molecules in iron acquisition by *Candida albicans*. J Exp Med 175:1643–1651

Mora-Montes HM, Netea MG, Ferwerda G, Lenardon MD, Brown GD, Mistry AR, Kullberg BJ, O'Callaghan CA, Sheth CC, Odds FC, Brown AJP, Munro CA, Gow NAR (2011) Recognition and blocking of innate immunity cells by *Candida albicans* chitin. Infect Immun 79:1961–1970

Moyes DL, Runglall M, Murciano C, Shen C, Nayar D, Thavaraj S, Kohli A, Islam A, Mora-Montes H, Challacombe SJ, Naglik JR (2010) A biphasic innate immune MAPK response discriminates between the yeast and hyphal forms of *Candida albicans* in epithelial cells. Cell Host Microbe 8:225–235

Munro CA, Selvaggini S, De Bruijn I, Walker L, Lenardon MD, Gerssen B, Milne S, Brown AJP, Gow NAR (2007) The PKC, HOG and Ca2+ signalling pathways co-ordinately regulate chitin synthesis in *Candida albicans*. Mol Microbiol 63:1399–1413

Naglik JR, Rodgers CA, Shirlaw PJ, Dobbie JL, Fernandes-Naglik LL, Greenspan D, Agabian N, Challacombe SJ (2003) Differential expression of

Candida albicans secreted aspartyl proteinase and phospholipase B genes in humans correlates with active oral and vaginal infections. J Infect Dis 188: 469–479

Naglik JR, Fostira F, Ruprai J, Staab JF, Challacombe SJ, Sundstrom P (2006) *Candida albicans* HWP1 gene expression and host antibody responses in colonization and disease. J Med Microbiol 55:1323–1327

Naglik JR, Moyes DL, Wächtler B, Hube B (2011) *Candida albicans* interactions with epithelial cells and mucosal immunity. Microbes Infect 13:963–976

Netea MG, Brown GD, Kullberg BJ, Gow NAR (2008) An integrated model of the recognition of *Candida albicans* by the innate immune system. Nat Rev Microbiol 6:67–78

Nobile CJ, Solis N, Myers CL, Fay AJ, Deneault J-S, Nantel A, Mitchell AP, Filler SG (2008) *Candida albicans* transcription factor Rim101 mediates pathogenic interactions through cell wall functions. Cell Microbiol 10:2180–2196

Ouyang W, Kolls JK, Zheng Y (2008) The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity 28:454–467

Pendrak ML, Chao MP, Yan SS, Roberts DD (2004) Heme oxygenase in *Candida albicans* is regulated by hemoglobin and is necessary for metabolism of exogenous heme and hemoglobin to alpha-biliverdin. J Biol Chem 279:3426–3433

Phan QT, Fratti RA, Prasadarao NV, Edwards JE Jr, Filler SG (2005) N-cadherin mediates endocytosis of *Candida albicans* by endothelial cells. J Biol Chem 280:10455–10461

Piccini A, Carta S, Tassi S, Lasiglié D, Fossati G, Rubartelli A (2008) ATP is released by monocytes stimulated with pathogen-sensing receptor ligands and induces IL-1beta and IL-18 secretion in an autocrine way. Proc Natl Acad Sci USA 105:8067–8072

Pierce JV, Kumamoto CA (2012) Variation in *Candida albicans* EFG1 expression enables host-dependent changes in colonizing fungal populations. MBio 3: e00117–00112

Pierce JV, Dignard D, Whiteway M, Kumamoto CA (2013) Normal adaptation of *Candida albicans* to the murine GI tract requires Efg1p-dependent regulation of metabolic and host defense genes. Eukaryot Cell 12 (1):37–49

Piispanen AE, Hogan DA (2012) *Candida* spp. in microbial populations and communities: molecular interactions and biological importance. In: Calderone RA, Clancy CJ (eds) Candida and candidiasis. ASM, Washington, DC, pp 331–342

Pukkila-Worley R, Peleg AY, Tampakakis E, Mylonakis E (2009) *Candida albicans* hyphal formation and virulence assessed using a *Caenorhabditis elegans* infection model. Eukaryot Cell 8:1750–1758

Ray TL, Payne CD (1988) Scanning electron microscopy of epidermal adherence and cavitation in murine candidiasis: a role for *Candida* acid proteinase. Infect Immun 56:1942–1949

Romani L (2011) Immunity to fungal infections. Nat Rev Immunol 11:275–288

Rosenbach A, Dignard D, Pierce JV, Whiteway M, Kumamoto CA (2010) Adaptations of *Candida albicans* for growth in the mammalian intestinal tract. Eukaryot Cell 9:1075–1086

Rubin-Bejerano I, Abeijon C, Magnelli P, Grisafi P, Fink GR (2007) Phagocytosis by human neutrophils is stimulated by a unique fungal cell wall component. Cell Host Microbe 2:55–67

Sandai D, Yin Z, Selway L, Stead D, Walker J, Leach MD, Bohovych I, Ene IV, Kastora S, Budge S, Munro CA, Odds FC, Gow NAR, Brown AJP (2012) The evolutionary rewiring of ubiquitination targets has reprogrammed the regulation of carbon assimilation in the pathogenic yeast *Candida albicans*. MBio 3:e00495–12

Santos R, Buisson N, Knight S, Dancis A, Camadro J-M, Lesuisse E (2003) Haemin uptake and use as an iron source by *Candida albicans*: role of CaHMX1-encoded haem oxygenase. Microbiology 149: 579–588 (Reading, Engl.)

Saville SP, Lazzell AL, Monteagudo C, Lopez-Ribot JL (2003) Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of *Candida albicans* during infection. Eukaryot Cell 2:1053–1060

Scherwitz C (1982) Ultrastructure of human cutaneous candidosis. J Invest Dermatol 78:200–205

Shapiro RS, Cowen LE (2010) Coupling temperature sensing and development. Virulence 1:45–48

Sheth CC, Hall R, Lewis L, Brown AJP, Odds FC, Erwig LP, Gow NAR (2011) Glycosylation status of the *C. albicans* cell wall affects the efficiency of neutrophil phagocytosis and killing but not cytokine signaling. Med Mycol 49:513–524

Shibata N, Suzuki A, Kobayashi H, Okawa Y (2007) Chemical structure of the cell-wall mannan of *Candida albicans* serotype A and its difference in yeast and hyphal forms. Biochem J 404:365–372

Singh A, Del Poeta M (2011) Lipid signalling in pathogenic fungi. Cell Microbiol 13:177–185

Sosinska GJ, De Groot PWJ, Teixeira de Mattos MJ, Dekker HL, De Koster CG, Hellingwerf KJ, Klis FM (2008) Hypoxic conditions and iron restriction affect the cell-wall proteome of *Candida albicans* grown under vagina-simulative conditions. Microbiology 154:510–520 (Reading, Engl.)

Staab JF, Bradway SD, Fidel PL, Sundstrom P (1999) Adhesive and mammalian transglutaminase substrate properties of *Candida albicans* Hwp1. Science 283:1535–1538

Sudbery PE (2011) Growth of *Candida albicans* hyphae. Nat Rev Microbiol 9:737–748

Sudbery P, Gow N, Berman J (2004) The distinct morphogenic states of *Candida albicans*. Trends Microbiol 12:317–324

Sun JN, Solis NV, Phan QT, Bajwa JS, Kashleva H, Thompson A, Liu Y, Dongari-Bagtzoglou A,

Edgerton M, Filler SG (2010) Host cell invasion and virulence mediated by *Candida albicans* Ssa1. PLoS Pathog 6:e1001181

Sundstrom P, Balish E, Allen CM (2002a) Essential role of the *Candida albicans* transglutaminase substrate, hyphal wall protein 1, in lethal oroesophageal candidiasis in immunodeficient mice. J Infect Dis 185:521–530

Sundstrom P, Cutler JE, Staab JF (2002b) Reevaluation of the role of HWP1 in systemic candidiasis by use of *Candida albicans* strains with selectable marker URA3 targeted to the ENO1 locus. Infect Immun 70:3281–3283

Suzuki S (2002) Serological differences among the pathogenic *Candida* spp. In: Calderone RA, Clancy C (eds) Candida and candidiasis. ASM, Washington, DC, pp 29–36

Thewes S, Kretschmar M, Park H, Schaller M, Filler SG, Hube B (2007) In vivo and ex vivo comparative transcriptional profiling of invasive and non-invasive *Candida albicans* isolates identifies genes associated with tissue invasion. Mol Microbiol 63:1606–1628

Thompson DS, Carlisle PL, Kadosh D (2011) Coevolution of morphology and virulence in *Candida* species. Eukaryot Cell 10:1173–1182

Tillmann A, Gow NAR, Brown AJP (2011) Nitric oxide and nitrosative stress tolerance in yeast. Biochem Soc Trans 39:219–223

Van de Veerdonk FL, Kullberg BJ, Van der Meer JWM, Gow NAR, Netea MG (2008) Host-microbe interactions: innate pattern recognition of fungal pathogens. Curr Opin Microbiol 11:305–312

Van de Veerdonk FL, Joosten LAB, Devesa I, Mora-Montes HM, Kanneganti T-D, Dinarello CA, Van der Meer JWM, Gow NAR, Kullberg BJ, Netea MG (2009) Bypassing pathogen-induced inflammasome activation for the regulation of interleukin-1beta production by the fungal pathogen *Candida albicans*. J Infect Dis 199:1087–1096

Van de Veerdonk FL, Marijnissen RJ, Marijnissen R, Joosten LAB, Kullberg BJ, Drenth JPH, Netea MG, Van der Meer JWM (2010) Milder clinical hyper-immunoglobulin E syndrome phenotype is associated with partial interleukin-17 deficiency. Clin Exp Immunol 159:57–64

Van der Graaf CAA, Netea MG, Verschueren I, Van der Meer JWM, Kullberg BJ (2005) Differential cytokine production and Toll-like receptor signaling pathways by *Candida albicans* blastoconidia and hyphae. Infect Immun 73:7458–7464

Vylkova S, Carman AJ, Danhof HA, Collette JR, Zhou H, Lorenz MC (2011) The fungal pathogen *Candida albicans* autoinduces hyphal morphogenesis by raising extracellular pH. MBio 2:e00055–00011

Wächtler B, Wilson D, Haedicke K, Dalle F, Hube B (2011a) From attachment to damage: defined genes of *Candida albicans* mediate adhesion, invasion and damage during interaction with oral epithelial cells. PLoS One 6:e17046

Wächtler B, Wilson D, Hube B (2011b) *Candida albicans* adhesion to and invasion and damage of vaginal epithelial cells: stage-specific inhibition by clotrimazole and bifonazole. Antimicrob Agents Chemother 55:4436–4439

Wächtler B, Citiulo F, Jablonowski N, Förster S, Dalle F, Schaller M, Wilson D, Hube B (2012) *Candida albicans*-epithelial interactions: dissecting the roles of active penetration, induced endocytosis and host factors on the infection process. PLoS One 7:e36952

Weindl G, Naglik JR, Kaesler S, Biedermann T, Hube B, Korting HC, Schaller M (2007) Human epithelial cells establish direct antifungal defense through TLR4-mediated signaling. J Clin Invest 117: 3664–3672

Weindl G, Wagener J, Schaller M (2011) Interaction of the mucosal barrier with accessory immune cells during fungal infection. Int J Med Microbiol 301: 431–435

Weissman Z, Kornitzer D (2004) A family of *Candida* cell surface haem-binding proteins involved in haemin and haemoglobin-iron utilization. Mol Microbiol 53:1209–1220

Weissman Z, Shemer R, Conibear E, Kornitzer D (2008) An endocytic mechanism for haemoglobin-iron acquisition in *Candida albicans*. Mol Microbiol 69:201–217

Wheeler RT, Fink GR (2006) A drug-sensitive genetic network masks fungi from the immune system. PLoS Pathog 2:e35

White SJ, Rosenbach A, Lephart P, Nguyen D, Benjamin A, Tzipori S, Whiteway M, Mecsas J, Kumamoto CA (2007) Self-regulation of *Candida albicans* population size during GI colonization. PLoS Pathog 3:e184

Wysong DR, Christin L, Sugar AM, Robbins PW, Diamond RD (1998) Cloning and sequencing of a *Candida albicans* catalase gene and effects of disruption of this gene. Infect Immun 66:1953–1961

Yano J, Lilly E, Barousse M, Fidel PL Jr (2010) Epithelial cell-derived S100 calcium-binding proteins as key mediators in the hallmark acute neutrophil response during *Candida* vaginitis. Infect Immun 78:5126–5137

Zakikhany K, Naglik JR, Schmidt-Westhausen A, Holland G, Schaller M, Hube B (2007) In vivo transcript profiling of *Candida albicans* identifies a gene essential for interepithelial dissemination. Cell Microbiol 9:2938–2954

Zhu W, Filler SG (2010) Interactions of *Candida albicans* with epithelial cells. Cell Microbiol 12:273–282

Zordan R, Cormack B (2012) Adhesins on opportunistic fungal pathogens. In: Calderone R, Clancy C (eds) Candida and candidiasis. ASM, Washington, DC, pp 243–259

Zupancic M, Cormack B (2007) *Candida* cell wall proteins at the host-pathogen interface. In: d' Enfert C, Hube B (eds) *Candida*: comparative and functional genomics. Caister Academic, Norfolk

2 *Aspergillus fumigatus*: Saprotroph to Pathogen

Elaine Bignell[1]

CONTENTS

[1]Institute of Inflammation and Repair, University of Manchester, Manchester, M13 9NT, UK; e-mail: elaine.bignell@manchester.ac.uk

I. Introduction

Most moulds propagate and disseminate via release of airborne spores, but none surpass the capacity of *Aspergillus fumigatus* to cause human disease. ***A. fumigatus* is a filamentous fungus that is capable of saprophytic growth in the natural environment and parasitic growth in susceptible human and animal hosts.** How does the transition from saprophytic soil-dweller to life-threatening pathogen come about? As the quest for *A. fumigatus* virulence factors continues apace, our understanding of the pathogenic qualities expressed by this "accidental pathogen" has reached a new, higher order appreciation of the host–pathogen interaction and how this fragile interrelationship might be tipped towards or against initiation or resolution of disease.

A. fumigatus has the ability, more than any other airborne fungal pathogen, to radically alter the trade-off between parasitic growth rate and host response in favour of fungal dominance. In this chapter, we will examine the pivotal processes occurring after spore (or conidia) inhalation and incorporate them into a three-step cause-and-effect framework, comprising a comprehensive overview of the host and pathogen factors that drive disease.

The genus *Aspergillus* includes several hundred species of filamentous, ascomycetous mould fungi, which disseminate via spore release (Bennet 2010; Baker and Bennet 2008). Ubiquitous on Earth, and occurring with a worldwide distribution, it is likely that all of these species contribute to recycling of planetary carbon and nitrogen via degradation of organic matter. Several species

Human Fungal Pathogens, 2nd Edition
The Mycota XII
O. Kurzai (Ed.)

have even greater significance to mankind, serving as conventional workhorses of food production or genetic study, or agents of devastating disease in plants and animals (Bennet 2010; Baker and Bennet 2008). *A. fumigatus* exerts, via a range of infectious and inflammatory diseases, a huge toll upon public health (Brown et al. 2012) and is the most significant mould pathogen of human beings (Latge 1999). Efforts to define the molecular basis of *A. fumigatus* pathogenicity have been ongoing for several decades and are continuing in the post-genomic era. Multiple excellent reviews have recently catalogued studies addressing *A. fumigatus* attributes required for pathogenicity and the host factors predisposing to disease (Dagenais and Keller 2009; Hohl and Feldmesser 2007; Askew 2008; Abad et al. 2010). These works, combined with an ever-increasing understanding of immune responses to infectious microbes, have revealed the intricacy of the *A. fumigatus* host–pathogen interaction and motivated the scientific community to reach a consensus on the huge significance of host factors in driving disease (Romani 2011). Although it is clear that this pathogen can utilise efficiently the nutrients provided by the mammalian niche and will do so in the absence of an adequate immune challenge, it is also clear that *A. fumigatus* draws upon multiple physiological traits, operative at distinct stages of the host–pathogen interaction, to exploit the weakened host and accomplish longevity in the host environment. Recent research has identified highly significant events that occur during the establishment of disease and contribute to the outcome of the host–pathogen interaction at the level of the whole animal.

II. *Aspergillus fumigatus*: A Three-Step Transition from Saprotroph to Pathogen

There are many host and pathogen factors that can drive initiation and establishment of human *Aspergillus*-related disease. To fully understand the pathogenic potential of *A. fumigatus* one must integrate a plethora of experimental and clinical data to compile a working model of the host–pathogen interaction. Currently, it remains difficult to assign relative measures of significance to many of these factors, and a more quantitative understanding will be required if immunomodulatory interventions are to be effectively implemented. Progress towards this aim will also permit a more standardised approach to the study of the host–pathogen interaction and place our understanding of it within a context having relevance to all types of *Aspergillus*-related disease.

To construct a fully panoramic view of the pathogenic status of *A. fumigatus* the progression from saprotroph to pathogen is here considered as a series of **three temporally distinct stages of the host–pathogen interaction: (1) primary encounter, (2) persistence and (3) permanence** (Fig. 2.1). Crucially, for the transition from saprotroph to pathogen to be realised all three stages must be completed thereby leading to a permanent alteration of host homeostasis. Within this framework, the activities of both host and pathogen can be readily incorporated as causes of disease (Fig. 2.1) and events that impact the outcome of infection are readily identifiable. In this chapter, the extent to which each of these activities impacts upon disease will be assessed within the context of the available experimental evidence.

III. Primary Encounter

A. Spore Size, Abundance and Human Exposure

A. fumigatus spores are a common component of the airborne microflora and are usually more abundant in outdoor, rather than indoor, air samples (Torpy et al. 2012). Despite seasonal variations in spore abundance, the estimated number of viable ***A. fumigatus*** **colony forming units per square metre (CFU/m^2) rarely exceeds 100**, so *A. fumigatus* spores are certainly not the most abundant of those we normally breathe. *Aspergillus* spp. are, however, the fungi most often cultured from the lungs of chronically ill humans (Lass-Florl et al. 1999) and since *Cladosporium* spores greatly outnumber those of *A. fumigatus* in outdoor air (O'Gorman and Fuller 2008) the spectrum of disease-causing fungi is not reflected by the results of air surveys. Clearly therefore, some mould spores enjoy a positive advantage in the host.

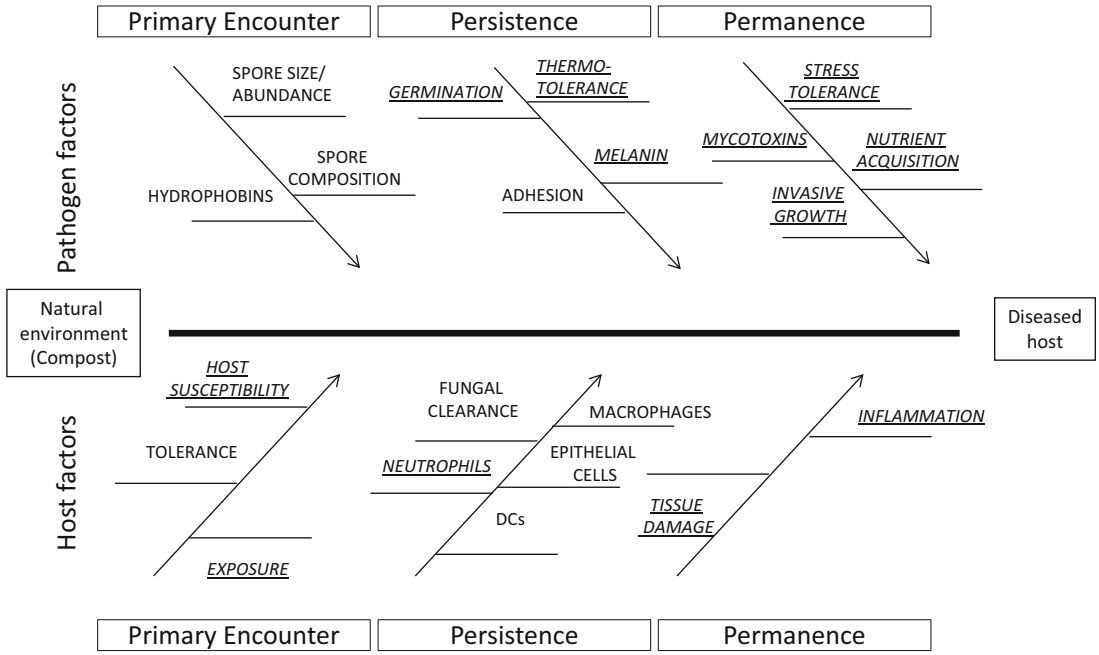


Fig. 2.1. Saprotroph to pathogen: a three-step cause-and-effect framework. Diseases caused by *A. fumigatus* arise as a result of both pathogen and host activities, and the transition from saprotroph to pathogen can be considered as occurring in three stages: primary encounter, persistence and permanence. A quantitative understanding of the contributions made by each factor is currently lacking; however, factors that can alter the outcome of the host–pathogen interaction, at the level of the whole animal host, are indicated in *italicised, underlined text*

***A. fumigatus* sporulates abundantly, producing green pigmented, uninucleate, mitotic spores of approximately 2 µm diameter extruded as chains from aerial hyphae positioned high above the vegetative mycelial mass** (Fig. 2.2a). This organisation of newly generated spores ensures efficient dispersal of spores in air currents, wherein *A. fumigatus* spores remain buoyant and viable for long periods of time. Spore dispersal is influenced by air mass movement, turbulence and thermal convection, with physical characteristics such as size, shape, density and surface texture exacting comparatively minor effects. Terminal velocity (or fall speed) is the dominant physical property, and actinomycete spores (approximating 2 µm diameter) are estimated to fall very slowly at approximately 40 µm/s (Lacey and West 2007). **According to the scheme proposed by** Lacey and West (2007)**, their extremely small size promotes the deepest possible penetration of the respiratory tree** (Fig. 2.2b).

Stored products, including hay, straw, grain, wood chips and composts, represent sources of *A. fumigatus* spores that can be aerosolised by human activities. Incredibly, the immediate vicinity of combine harvesters has been found to contain 200 million fungal spores per cubic metre, a concentration capable of eliciting an immediate hypersensitivity reaction (O'Gorman 2011). The potential for exposure to airborne *A. fumigatus* spores is heightened in the immediate vicinity of refuse dumps and landfill sites, and associated hazards to respiratory health have been formally assessed by Swan et al. (2013). In a recent review of the risk posed by *A. fumigatus* spores O'Gorman (2011) cites guidelines issued by the UK Environment Agency in 2009 that state that total emissions of bacterial and fungal spores from compost facilities not exceeding 10^3 CFU/m^3 are within "acceptable levels". Potential environmental reservoirs governing everyday exposures include dust, bedding, water supplies, air handling systems, fresh and dried flowers, the soil of ornamental plants, certain foods including tea, biscuits, fruits and spices, particularly pepper, and the smoking of tobacco and marijuana. Activities such as grass cutting, digging, potting plants, building works and household cleaning also aerosolise *A. fumigatus* conidia (O'Gorman 2011).

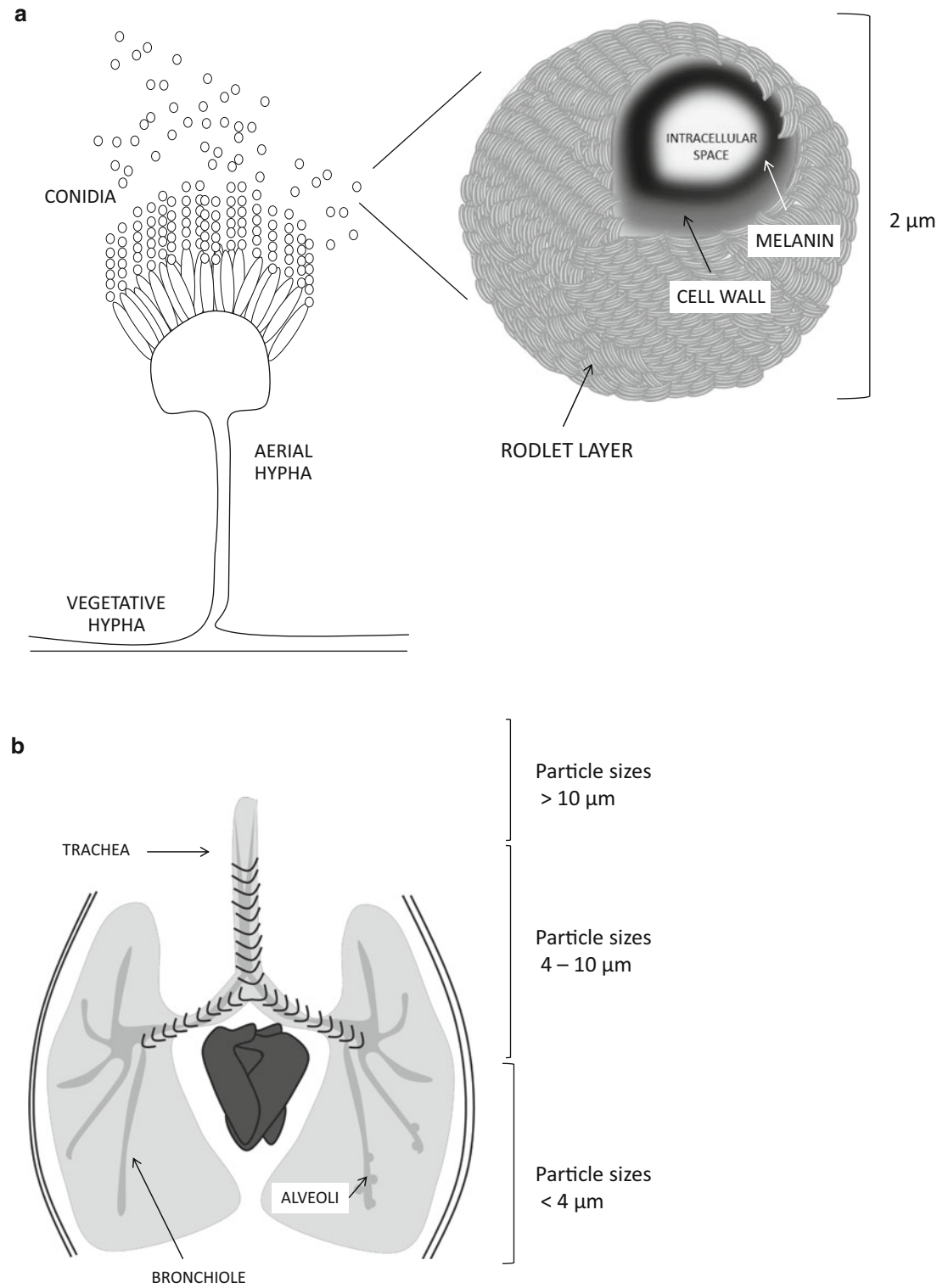


Fig. 2.2. Organisation of *A. fumigatus* colonies, conidial structure and airway penetration by particle size. (**a**) *A. fumigatus* is a prolific spore producer, generating thousands of uninucleate mitotic spores (conidia) per aerial hypha. Mitotic spores, approximately 2 µm in size, are extruded from aerial hyphae and become airborne. (**b**) *A. fumigatus* spores are small enough to achieve deepest possible penetration of the human airway

Although initiation of hypersensitivity reactions can be elicited by massive exposures to *A. fumigatus* spores, infections of immunocompromised hosts are probably initiated by single or repeated exposures to far fewer fungal spores. The true relationship between *Aspergillus* concentration in the air and the probability of invasive aspergillosis is not quantitatively known, and the circumstances surrounding initiation of infection pose important unanswered questions. **It is not certain whether prior carriage of, or repeated exposure to, *A. fumigatus* spores primes the host for subsequent infection.** Some have surmised that large inocula of fungal spores may overwhelm an otherwise adequately functioning immune system (Hope et al. 2005) whereas others have sought correlations between fungal colonisation and subsequent invasive aspergilloses (IA) (Einsele et al. 1998).

Leleu and co-workers (Leleu et al. 2013) used a combination of Bayesian modelling and in vivo experiments to determine the relationship between spore exposure and occurrence of IA. This study determined that in immunosuppressed (neutropenic) mice, exposed by aerosol to *A. fumigatus* spores, the median exposure dose required to achieve fatal infection of 50% of mice is $1.8–3.2 \times 10^4$ *A. fumigatus* spores. Animals exposed to lower doses were unlikely to contract infections causing death within 6 days of spore exposure. The authors acknowledge that a single exposure to $\sim 10^4$ spores is unlikely to occur in humans, where a cumulative dose over time is more likely. However, for patients with prolonged neutropenia or undergoing other immunosuppressive therapies, a significant cumulative infective exposure could be reached within a few hours or days. This study established, for the first time, a direct correlation between aerosol spore burdens and the probability of mammalian infection; however, the relevance of these data to human disease remains speculative because of the marked differences in the exposure conditions and in the respiratory system of mice and humans.

B. Physiological Traits That Favour Pathogenicity

The primary route of infection with *Aspergillus* is via the inhalation of airborne conidia, which are deposited in the bronchioles or alveolar spaces (Fig. 2.2b). **The average size of *A. fumigatus* conidia (2–3 μm) is ideal for infiltrating deep into the alveoli, whereas larger particles**, including the conidia of human pathogens such as *A. flavus* and *A. niger*, could be removed more easily by mucociliary clearance of the upper respiratory tract. Evaluation of parameters influencing the germination of *Aspergillus* conidia revealed significant differences in germination between differing pathogenic species, whereby ***A. fumigatus* germinates much more efficiently and in a shorter time frame than *Aspergillus flavus* or *Aspergillus niger*** (Araujo and Rodrigues 2004). Following 12 h of incubation in a nutritive medium, the average germination rate of *A. niger* strains was about 36.5%. A similar rate was obtained more quickly in *A. flavus* (8 h) and *A. fumigatus* (5.5 h). At ambient temperatures of up to 30°C, germination rates were similar for all three species; however, at higher temperatures germination of *A. flavus* decreased by 45%, and in the case of *A. niger* no germination was possible for 24 h. Thus, among pathogenic Aspergilli, *A. fumigatus* appears better equipped for initiating growth in the mammalian host.

The importance of thermotolerance for colonisation and infection of mammals is supported by the attenuation of virulence in a mutant lacking the *cgrA* gene product, which is involved in ribosome biogenesis. Δ*cgrA* mutants grow normally at 25°C and are fully virulent in a *Drosophila* insect model (25°C) of infection, but grow slower than the wild-type progenitor isolate at 37°C and are attenuated for virulence in mice (Bhabhra et al. 2004).

C. Host Susceptibility: How Does Immunity Fail the Host?

The potency of *A. fumigatus* as a pathogen and source of antigens is evidenced by the spectrum of diseases it causes in humans (Fig. 2.3). For example, in severely immunocompromised hosts

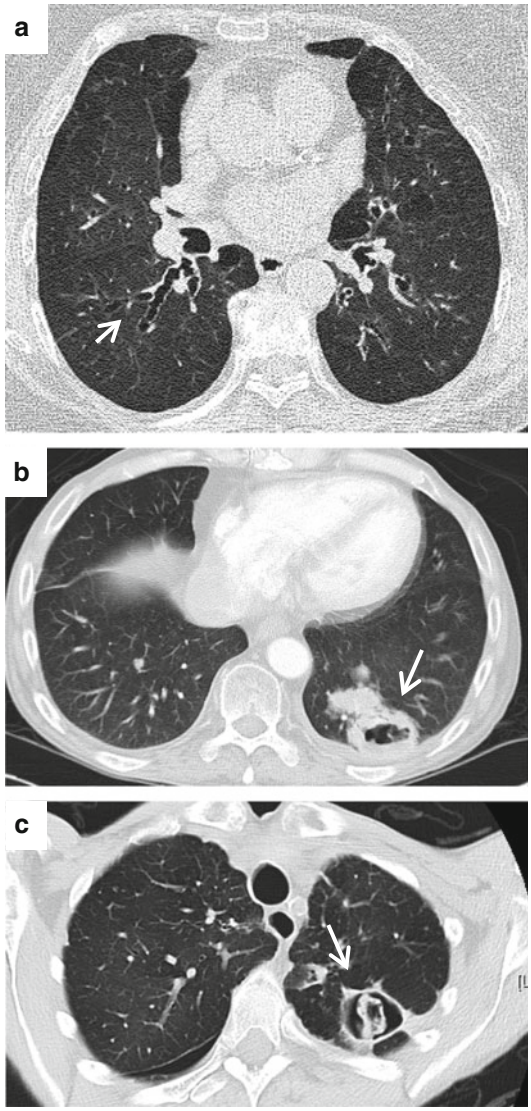


Fig. 2.3. Outcomes of *A. fumigatus* spore inhalation in humans. (**a**) Allergic bronchopulmonary aspergillosis (ABPA). Patient has longstanding ABPA with central bronchiectasis (*white arrow*). Total IgE is 500 times higher than normal at 5,000 versus 100 IU/ml. Patient has undergone itraconazole therapy for 7 years with stability and no exacerbations (**b**) Invasive pulmonary aspergillosis (IPA). Heart transplant recipient with a recent episode of rejection requiring pulse corticosteroids in high dose, presenting with chest complaints and headache. A cavitary lesion is apparent (*white arrow*), and patient also had multiple abscesses in the brain (**c**) Chronic pulmonary aspergillosis (CPA). A 65-year-old patient with rheumatoid arthritis and rheumatoid lung disease who gradually deteriorated, with weight loss and severe breathlessness over severalmonths. A cavity is evident (*white arrow*) that shows early signs of aspergilloma (fungus ball)

inhaled spores remain largely unchallenged by innate immune defences and tissue-invasive disease predominates. However, in atopic individuals an over-exuberant response to spore and/or hyphal antigens can prompt life-threatening allergy or chronic pulmonary inflammation (Zmeili and Soubani 2007). Thus, the problem posed by *A. fumigatus* extends beyond the mere ability to proliferate within the host environment. The disparate outcomes resulting from spore inhalation in immunologically distinct host settings demonstrate the crucial contribution made by the host environment to progression of disease. In humans, **Aspergillosis can be broadly grouped into three major categories: invasive aspergillosis (IA; with or without angioinvasion), chronic pulmonary aspergillosis (CPA; including aspergilloma) and allergic bronchopulmonary aspergillosis** (ABPA; Fig. 2.3).

D. Invasive Aspergillosis

In an authoritative review of the clinical presentations, risk factors and diagnosis of invasive aspergillosis, Hope and Denning (Hope et al. 2005) described an entire spectrum of distinct IA syndromes having differing pathophysiological profiles. For simplicity we here consider IA to include aspergillosis of both the angioinvasive and non-angioinvasive types, among which invasive pulmonary aspergillosis (IPA) is the commonest manifestation. Zmeili and Soubani (2007) defined seven major risk factors for IA: (i) prolonged neutropenia or neutrophil dysfunction (e.g., as seen in chronic granulomatous disease, CGD), (ii) organ or stem cell transplantation (highest risk is with lung and haematopoietic stem cell transplantation, HSCT), (iii) prolonged (>3 weeks) and high-dose corticosteroid therapy, (iv) haematological malignancy (risk is higher with leukaemia), (v) chemotherapy and (vi) advanced AIDS.

In humans, profound neutropenia (<100 neutrophils/μL) lasting more than 10–15 days is the major risk factor for invasive mould infections (Portugal et al. 2009) and this provides the major basis for assuming that neutrophils provide the most important defence to the human host. Hope and Denning (Hope et al. 2005) distinguish

between **angioinvasive** and **non-angioinvasive aspergilloses** on the basis of both clinical presentation, and predisposing risk factors whereby angioinvasive disease occurs exclusively in the neutropenic setting and is characterised by vascular invasion by hyphal elements, coagulative necrosis and haemorrhagic infarction. In contrast, non-angioinvasive disease lacks any evidence of vascular invasion but is characterised by any, all or some of: pyogranulomatous inflammatory infiltrate, inflammatory necrosis or cavitation and occurs in multiple non-neutropenic clinical settings, most significantly following corticosteroid therapy, non-neutropenic HSCT, graft-versus-host disease (GVHD), HIV/AIDS, CGD and solid organ transplantation. Murine models approximating the progression of angioinvasive, non-angioinvasive, CGD bone marrow and solid organ transplantation have provided a useful proxy for the study of the host–pathogen interaction as in all instances the major features of disease progression can be replicated (Clemons and Stevens 2005; Lewis and Wiederhold 2005; Balloy et al. 2005; Pollock et al. 1995). The major drawback of murine experimentation is the large infectious inocula that are routinely employed to achieve measurable readouts of disease progression. Future advances in bioimaging and mathematical modelling will permit the detailed study of diseases resulting from lower inocula.

E. Chronic Pulmonary Aspergillosis

Chronic forms of pulmonary aspergillosis are distinguishable from IA by the timescale of disease progression (Hope et al. 2005). In contrast to IPA, CPAs run a slowly progressive course over weeks to months and include several syndromes, variously characterised by slowly progressive cavitary lung disease, chronic respiratory symptoms and the presence of precipitating antibodies to *Aspergillus* spp. Hope and Denning (Hope et al. 2005) noted that chronic necrotising pulmonary aspergillosis (CPNA) may present with direct invasion of pulmonary parenchyma by hyphal elements but for the most part no clear evidence of parenchymal invasion can be secured, despite progressive tissue damage. Prior structural lung disease appears to be a crucial factor and defects in systemic immunity may also be important. The precise mechanism of new cavity formation remains unclear, and where cavities are present they may or may not contain fungus balls. The presence, or not, of an aspergilloma provides the basis upon which the distinction between aspergilloma (fungus ball) and CPA (cavitating disease) is made. *Aspergillus* tracheobronchitis is a further variant of CPA, occurring frequently in solid organ transplantations that incorporate calcineurin inhibition regimens. Tracheobronchitis occurs higher in the respiratory tree and involves profuse, but superficial inflammation with a mucus exudate. The depth of infection ranges from no, to extensive, involvement of the bronchial wall (Herbst et al. 2013; Zmeili and Soubani 2007).

F. Allergic Bronchopulmonary Aspergillosis

Allergic bronchopulmonary aspergillosis (ABPA) is an immunological disorder caused by hypersensitivity to *Aspergillus fumigatus* and occurs predominantly **in people with asthma or cystic fibrosis** (Patterson and Strek 2010; Zmeili and Soubani 2007). Sensitisation to Aspergillus prompts activation of T helper 2 lymphocytes, which play a key role in recruiting eosinophils and other inflammatory mediators. Clinical presentations therefore include granulomatous inflammation consisting of histiocytes, lymphocytes and eosinophils. Local inflammation results in mucus production, airway hyperreactivity and, ultimately, bronchiectasis in which fungal hyphae may be seen, but without evidence of tissue invasion. Early diagnosis and treatment is likely to prevent disease progression, parenchymal damage and loss of lung function. To this end, ABPA is defined by a combination of clinical and laboratory criteria that include asthma, serum eosinophilia, elevated total IgE, pulmonary opacity, bronchiectasis and sensitisation to *A. fumigatus* antigens by skin testing. Treatment with corticosteroids can be effective but may be required indefinitely. The incidence of ABPA in patients with asthma is approximately 2%, and 1–15% of cystic fibrosis sufferers develop ABPA (Sorci et al. 2011; Agarwal 2009; Eaton et al. 2000).

G. Aspergillosis in Animals

Aspergillosis is relatively uncommon in other mammals, but dogs, horses, cows and dolphins are susceptible and **birds are particularly susceptible** to infection. Avian aspergillosis affects both immunocompetent and immunosuppressed birds, in particular turkeys, penguins, raptors and waterfowl. Immunosuppression caused by forced production or physical exertion can predispose to infection. As in humans, *A. fumigatus* is the major pathogen (Tell 2005).

H. Spore Composition

A. fumigatus spores are the particles with highest relevance to the primary encounter between host and pathogen. Dependent upon the immunosuppressive regimen adopted, germination of *A. fumigatus* spores in murine airways can take as little as 6–8 h (severely neutropenic hosts) or more than 24 h (corticosteroid-treated hosts) (Balloy et al. 2005). Spores can therefore enter into extended interactions with host cells prior to germination or elimination. This is a key consideration in the study of the Aspergillus host–pathogen interaction because (a) the antigenic identity of spores, swollen conidia and hyphae differs considerably and (b) in contrast to *Candida albicans*, which conditionally undergoes filamentation in response to relevant stimuli, ***A. fumigatus* is obliged to undergo the morphogenetic transition from uninucleate single cells to multinucleate hyphae during vegetative growth.** During IA, this transition might be rapid or slow, but it will always occur.

Concordant with a role for adhesion to host cells during initiation of mammalian infection, histological analysis of murine lung tissue at early time-points of experimental murine aspergillosis reveals the apparent adherence of spores to alveolar and bronchial epithelial cells. The molecular basis for such adhesion has been probed via in vitro experimentation, revealing that spores of *A. fumigatus* specifically bind to extracellular matrix (ECM) components of A549 epithelial cells. They also bind specifically to fibrinogen, fibronectin, laminin, type I collagen, and type IV collagen. Pre-incubation of spores with an Arg-Gly-Asp tripeptide inhibits binding to fibronectin and type I collagen by 50% (Tronchin et al. 1997; Bouchara et al. 1997; Penalver et al. 1996; Gil et al. 1996). Both carbohydrate and protein molecules on the conidial surface are involved in binding to host proteins (Sheppard 2011).

The outermost cell-wall layer of the *A. fumigatus* conidium is adorned with a mesh of interwoven rodlet proteins (Fig. 2.2a), including an immunoprotective rodlet protein RodA, which confers conidial hydrophobicity. A rodlet-lacking *A. fumigatus* mutant, *ΔrodA*, (Thau et al. 1994) is defective in adherence to collagen and albumin, but retains the capacity to bind host cells (both in vivo and in vitro) and is fully virulent. In a modified murine model of pulmonary aspergillosis, using milder immunosuppressive drug regimens, Shibuya and colleagues (Shibuya et al. 1999) studied the inflammatory response to infections with *A. fumigatus* wild-type and *ΔrodA* conidia. In comparison to mice infected with the wild-type isolate, pulmonary lesions induced by the rodletless mutant were limited and inflammatory responses were weak. The mechanistic basis of this pathogenic phenotype was proposed as being due to RodA-mediated depletion of neutrophils and macrophages during the early stages of infection. However, although this hypothesis has not formally been disproved in whole animal studies, it is now clear that the **RodA hydrophobin acts by exerting an immunoprotective barrier to recognition by host cells** (Aimanianda et al. 2009).

Early observations of the outermost conidial surfaces, conducted via electron microscopy, determined that spore swelling and germination lead to alterations in surface characteristics and adherence of *A. fumigatus* conidia. Furthermore, the cell wall was noted to undergo reorganisation during swelling and germination (Tronchin et al. 1995). **The ultrastructural appearance of the conidial cell wall differs significantly from that of hyphal cells** (Bernard and Latge 2001) **whereby the conidial cell wall is composed of a dense pigmented outer layer (containing melanin) and a translucent inner layer** (Fig. 2.2a). Wasylnka and colleagues (Wasylnka et al. 2001) showed that *A. fumigatus* conidia bind significantly better than those of other *Aspergillus*

species to intact lung cell basal lamina and, seeking the mechanism of this binding, found that neither desialylation nor complete deglycosylation of fibronectin decreased the binding of *A. fumigatus* conidia. This suggested that fibronectin binding was not oligosaccharide-mediated, a finding that was further supported by binding of *A. fumigatus* spores to a nonglycosylated 40 kDa fragment of fibronectin containing the glycosaminoglycan (GAG)-binding domain. Because binding could be inhibited by negatively charged carbohydrates, the authors proposed a novel mechanism of conidial attachment whereby adherence to fibronectin (and other basal lamina proteins) might be mediated via negatively charged carbohydrates on the conidial surface. In another study, the same authors demonstrated that surface-localised sialic acids, at an estimated density of 7×10^5 sialic acid residues per conidium, mediated adhesion of conidia to the positively charged polymer poly(L-lysine) (Wasylnka and Moore 2000). Thus, sialic acids on the conidial wall may be involved in adhesion to fibronectin as such binding is strongly inhibited in the presence of a sialylated glycoprotein. Sialic acids are terminal components of many glycoproteins and glycolipids, contributing to the structural properties of these molecules and regulating cellular and molecular interactions (Kelm and Schauer 1997). Sialic acid residues can act as, or to mask, recognition sites such as subterminal carbohydrate structures or proteins. Although the role of such residues in mediating the binding of pathogens to host cells has been documented over many years, the identity of the *A. fumigatus* sialic acid-presenting moiety is unknown.

Levdansky and colleagues (Sharon et al. 2011) performed a molecular genetic analysis of conidial cell wall integrity, initially focusing upon the cell wall-associated protein CspA, originally identified as one of four putatively GPI-anchored cell wall proteins whose gene sequences contained different numbers of DNA repeats in different patient isolates. The predicted *cpaA* translation product includes a long serine-threonine-proline-rich N-terminal region, followed by a variably (18–47 repeats) large six-amino-acid serine-proline [P-G-Q-P-S-(A/V)]-rich tandem repeat region having significant homology to the repeat domains found in mammalian type XXI collagen. A *myc*-tagged *cspA*-encoded protein was detectable in hydrofluoric acid extracts of conidial cell walls, suggesting covalent linkage of CspA to cell wall glucan via a GPI anchor. Further, discrepancy between predicted and actual sizes of the protein suggests heavy glycosylation. Immunofluorescence microscopy revealed a hyphal-specific expression profile (6–12 h post-germination). However, western blotting revealed an abundance of the protein in dormant and swollen conidia. Taken together, these observations suggest that CspA is located below the cell wall surface and becomes unmasked as germination proceeds. In support of this theory, sonication of conidia, intended to remove the outer hydrophobin layer, significantly heightened surface exposure of CspA. In combination with deletions of genes encoding the GPI-anchored cell wall proteins *ecm33* or *gel2*, *cpsA* deletion additively affects conidal adhesion to culture-derived ECM, and leads to morphological abnormality of the conidial cell wall, which exhibits defective layering of melanin-rich and carbohydrate-rich domains, and high levels of exposed chitin, mannose and glucan. Although these structural reorganisations of the cell wall could be correlated with increased internalisation of the double mutants by human monocyte-derived macrophages, and heightened susceptibility to hyphal damage mediated by polymorphonuclear monocytes, virulence was unaltered in neutropenic mice.

I. Immunological Tolerance of Inhaled *Aspergillus fumigatus* Spores

Hyperinflammatory responses to inhaled spores occur in patients and mice suffering from CGD (Song et al. 2011; Patterson and Strek 2010) or ABPA (Patterson and Strek 2010). Clearly then, *A. fumigatus* spores are potently capable of evoking immune responses in the mammalian host and, given the frequency with which *A. fumigatus* spores enter the human pulmonary tract, mammalian immunity has evolved to cope with this omnipotent challenge. In 2009, Aimanianda and co-workers (Aimanianda et al. 2009), in attempting to dissect the reasons for the immunologically inert nature of the dormant conidia, made some findings that could explain this phenomenon. Analysis of a hydrofluoric acid extract derived from *A. fumigatus* conidia identified three protein species, the molecular weights of which (32, 16 and 14.5 kDa) corresponded, respectively, to the dimeric form of the native RodA protein, native RodA and partially degraded or processed RodA. Crucially, RodA was undetectable in fungal

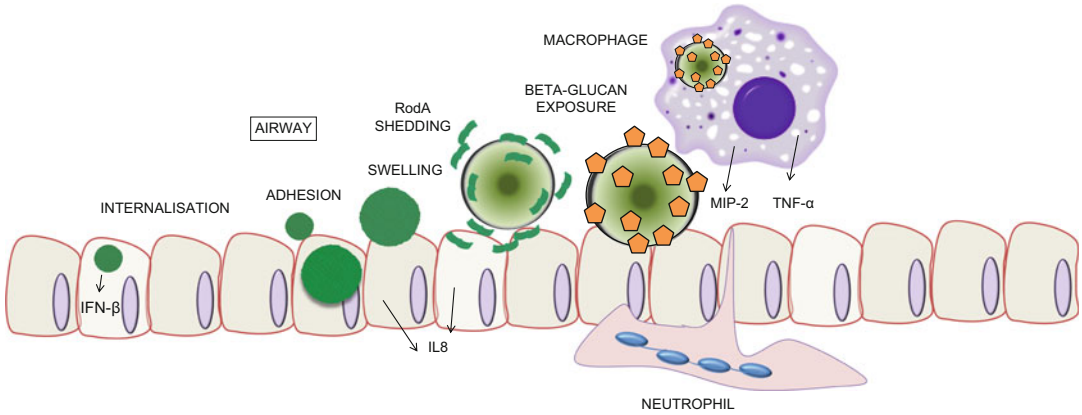


Fig. 2.4. First encounter, immune evasion and fungal persistence in the host. *A. fumigatus* spores, both resting and swollen, can prompt inflammatory responses in epithelial cells (ECs). ECs internalise both resting and swollen spores, eliciting differential responses to each morphotype. *A. fumigatus* spores are adorned with a layer of proteinaceous rodlets, which are shed upon swelling to reveal cell-wall-associated polysaccharides, some of which act as pathogen-associated molecular patterns. *Orange pentagons* represent β-glucan moieties

culture supernatant following conidial germination, indicating that the RodA rodlet layer, covalently bound to the cell wall of dormant conidia (Figs. 2.2 and 2.4), was shed upon spore germination. **RodA extracted directly from *A. fumigatus* condia was found to be immunologically inert.** This is in contrast to germinating conidia, which prompt, in dendritic cells, significant expression of co-stimulatory molecules (CD80, CD86, CD40 and CD83) and the antigen-presenting molecule human leukocyte antigen DR (HLA-DR), and secretion of pro- and inflammatory and anti-inflammatory cytokines. The removal of RodA, either chemically (using hydrofluoric acid), genetically (*ΔrodA* mutant) or biologically (germination) resulted in immune activation. Thus, the hydrophobic rodlet layer on the *A. fumigatus* conidial cell surface acts as an "immunological silencer". A similar effect was documented in murine alveolar macrophages where inflammatory cytokines, chemokines and reactive oxygen intermediates (produced in response to hydrofluoric acid-treated conidia, *ΔrodA* dormant conidia and germinated conidia) were undetectable in macrophages exposed to dormant conidia or RodA protein. The authors concluded that the surface rodlet layer of the conidial cell wall makes *A. fumigatus* conidia inert to both innate and adaptive immunity. Evidence first documented by Hohl et al. (2005) revealed a further layer of immunomodulatory control, manifested by the gradual exposure of cell surface β-glucan during swelling and germination of *A. fumigatus* spores (see subsequent discussions).

IV. Persistence

A. Spore Germination

In environments conducive to germination, *A. fumigatus* spores become metabolically active and increase in size, eventually germinating to produce tip-extending elongated cells called hyphae, a hallmark characteristic of filamentous fungi. In most fungi, conidial germination is governed by moisture, oxygen and nutrients and, when dormancy is broken, *A. fumigatus* conidia begin nuclear division and morphological development (Momany and Taylor 2000). This morphological transition multiply impacts upon *A. fumigatus* pathogenicity (Fig. 2.4), from shedding of the RodA hydrophobic layer (Aimanianda et al. 2009) to gradual exposure of pathogen-associated molecular pattern molecules (PAMPs) and activation of immune responses (Hohl et al. 2005) and invasion of the host epithelium (Kamai et al. 2009).

Recognising that morphological landmarks coincide with specific events in the *S. cerevisiae*

cell cycle, Momany and Taylor (2000) sought similar landmarks in the transition of *A. fumigatus* and *Aspergillus nidulans* from conidia to hyphae. To score *A. fumigatus* cells for mitotic and morphological events, Hoechst 33258 and calcofluor white, respectively, were used to stain fungal cells. Following inoculation into rich medium, the first nuclear division in *A. fumigatus* occurs after a lag of just over 4 h, with subsequent nuclear divisions every 45 min. This represents a significantly faster rate of nuclear duplication than that observed in *A. nidulans*, where nuclei divide once per hour. Initially spherical, conidia of both *A. fumigatus* and *A. nidulans* assume a pear-type shape that indicates that an axis of polarity has been established. This event often (22%) happened prior to the first nuclear division in *A. fumigatus* cells, which also differs significantly from the less pathogenic *A. nidulans* where only 12% were polarised after the first mitosis. A further important difference between the morphogenetic transitions of these two species included a reduction in second germ tube emergence in *A. fumigatus* (19% versus 98%) after the fifth mitosis. The authors proposed that by directing growth primarily in one direction, the lower percentage of second germ tubes allows *A. fumigatus* to scavenge nutrients more efficiently in the host.

Dague and colleagues (2008) used real-time atomic force microscopy with a temperature-controlled 37°C stage to probe the structural and Physiochemical dynamics of single *A. fumigatus* conidia during germination. By capturing images of the same cell after 20, 60 and 120 min, significant swelling could be quantified. Ultrastructural alterations were imaged via high-resolution images of the spore surface (Fig. 2.2a). At early germination times, the spore surface was observed to be covered with an array of 10 nm diameter rodlets. Notably, dramatic changes in cell surface structure were observed after 2 h of germination, corresponding to the swelling of the conidium. At this point the rodlet layer was found to alter into a layer of amorphous material, thereby buttressing the hypothesis that significant remodelling of the cell surface, including shedding of RodA (Aimanianda et al. 2009), represents a significant restructuring event during spore germination.

From a molecular perspective, the regulatory mechanisms that govern conidial germination in *A. fumigatus* have not been fully described, and much is extrapolated from studies in the model ascomycete *A. nidulans*. However, the number of gene products that have been demonstrated as important for conidial germination is rapidly growing, and a plethora of functional categorisations have been implicated, from calcineurin-mediated signalling (da Silva Ferreira et al. 2007; Steinbach et al. 2006) to MAPK signalling (Liebmann et al. 2004; Xue et al. 2004; Zhao et al. 2006) and glucose metabolism (Fleck and Brock 2010). An integrated and hierarchical understanding of the process will gradually emerge once functional genomics approaches reveal the entire cohort of gene products involved.

Fortwendel et al. (2006) recently established the mechanism by which a membrane-associated Ras GTPase switch, RasA, governs morphological transitions in *A. fumigatus*. Ras-GTPases regulate a multiplicity of cellular processes in a highly precise spatial and temporal manner, commanding control over differential pathway activation, which is governed in part by post-translational modification and consequent modulation of subcellular localisation. Deletion of RasA in *A. fumigatus* causes delayed germination and subsequent formation of wide, blunted hyphae that continually switch polarised growth axes.

A functional RasA-GFP translational fusion was used to interrogate the correlation between RasA localisation and morphogenesis. RasA was found to localise at the cell periphery of emerging germ tubes and the outer edge of hyphae, consistent with a plasma membrane association of the protein during polarised hyphal growth. Incubation of fungal cultures with a nonreversible inhibitor of protein palmitoylation inhibited hyphal growth in a dose-dependent manner and prompted aberrant localisation of GFP-RasA to internal foci. Taken together, these observations suggested that palmitoylation might be important for proper RasA localisation and polarised hyphal growth. The targeted double mutation ($RasA^{C206/207S}$) of a conserved RasA palmitoylation motif prevented full complementation of the RasA hyphal growth blockade and resulted in a dramatic reduction of GFP signal at the plasma membrane. $GFP\text{-}RasA^{C206/207S}$ was mislocalised from the hyphal periphery and septa to the cytosol and internal patches, presumably to compartments of the endomembrane system. Both a RasA null mutant and the $GFP\text{-}RasA^{C206/207S}$ mutant displayed low germination rates and formed stunted, highly branched germlings

during early phases of hyphal growth and displayed heightened sensitivity to cell wall damaging agents and aberrant deposition of β-glucan. To determine the contribution of plasma membrane localisation of RasA to *A. fumigatus* virulence, the *ΔrasA* and GFP-RasA$^{C206/207S}$ mutants were compared to the wild-type strain in a murine model of invasive aspergillosis. Virulence of both the *ΔrasA* and GFP-RasA$^{C206/207S}$ mutants was significantly attenuated ($P < 0.0001$), with infected mice displaying 25% and 20% mortality at 15 days postinoculation, respectively. Appropriate RasA localisation to the plasma membrane is therefore essential for full virulence in *A. fumigatus.*

Several groups have applied functional genomics techniques to the study of the conidial to hyphal transition in *A. fumigatus*. Lamarre and colleagues (2008) adopted a transcriptomic approach to analyse the exit of *A. fumigatus* spores from dormancy, identifying a **shift from fermentative to respiratory metabolism and immediate protein synthesis upon breaking dormancy**. In the first 30 minutes of incubation in YPD medium at 37°C, modulation of expression of 787 genes (of a possible 3,000) was observed in freshly harvested conidia. Downregulated genes encoded functions involved in fermentative metabolism and oxidoreductase activity whereas upregulated genes indicated a heightened emphasis upon RNA and phosphorus metabolism, amino acid and protein biosynthesis and protein complex assembly. Concordant with the predicted timing of mitosis in germinating spores, the authors found no evidence of upregulation of DNA processing/replication or cell cycle regulation. Additionally, expression of multiple genes involved in amino acid biosynthesis were upregulated during the first 30 minutes of the study, perhaps reflecting the rapid depletion of a pre-existing pool of free amino acids. This trend was supported by observed upregulation of genes in other pathways leading to amino acid biosynthesis, including glutamate dehydrogenase and glutamate synthase, NADPH isocitrate and succinate dehydrogenase. Downregulation of 28 mitochondrial genes, along with genes encoding alcohol dehydrogenases, lactate dehydrogenase, pyruvate decarboxylase and phosphoenol pyruvate synthase suggested a shift from a fermentative metabolism when the conidium is in a dormant stage to a respirative metabolism as soon as the germination process has started. Combining microarray and quantitative proteomic data, Cagas et al. (2011) were also able to identify a trend towards protein biosynthesis in time-series analyses of germinating conidia, during 0–16 hours of growth. A further quantitative proteomic analysis performed by Suh et al. (2012) identified an abundance of small, lineage-specific proteins amongst the conidial proteome. Teutschbein and colleagues (2010) applied two-dimensional polyacrylamide gel electrophoresis to establish a reference map of conidial proteins, identifying 449 different proteins, 57 of which were more abundant in conidia relative to mycelia. These included enzymes involved in detoxification of reactive oxygen intermediates, pigment biosynthesis and conidial rodlet layer formation. In agreement with the transcriptional analyses of Lamarre et al. (2008), pyruvate decarboxylase and alcohol dehydrogenase were detectable in dormant conidia, further supporting the notion that alcoholic fermentation plays a role during dormancy or early germination.

These studies set the scene for analysing differences between germination undergone in vitro and germination occurring during mammalian infection. Although the technologies to analyse the infecting fungal proteome thus far elude us, the transcriptional data from murine infections are already under analysis as part of our research programme.

B. Thermotolerance

The ability to thrive at 37°C is characteristic of all human pathogens and distinguishes *A. fumigatus* from most other environmental moulds. Relative to other *Aspergillus* spp., *A. fumigatus* has a natural propensity for thermotolerance, and in being capable of growth at temperatures that approach the upper limit for all eukaryotes, approximates an extremophilic mode of growth in the human host (Bhabhra and Askew 2005; Bhabhra et al. 2004). An intuitive hypothesis is that mutants that fail to grow at 37°C will be attenuated for virulence. This holds true in at least once case as *A. fumigatus* mutants lacking the *cgrA* gene product are attenuated for virulence. CgrA is required for the synthesis of ribosomes during conidial germination and

has a distinct nucleolar localisation in *A. fumigatus*.

Mutants deficient in trehalose biosynthesis also demonstrate thermosensitive phenotypes in *A. fumigatus* (Al-Bader et al. 2010). Contingent with a role in stress tolerance, the trehalose content of *A. fumigatus* hyphae becomes elevated in response to heat shock and two genes, *tpsA* and *tpsB*, whose expression can be correlated with heightened trehalose content, serve partially redundant roles in trehalose accumulation during development and heat shock. A Δ*tpsAB* double mutant is devoid of trehalose and exhibits delayed (by 2–3 h) germination at 37°C and is nonviable at 50°C. However, virulence of the double mutant was unabated in a murine model of aspergillosis. Trehalose is regarded as an important source of energy during fungal development and acts to heighten stress tolerance by preventing the aggregation of denatured proteins. Being absent from mammalian cells it is of obvious therapeutic interest. It is intriguing that, despite the prevailing belief that rapidity of spore germination at 37°C provides a competitive advantage for *A. fumigatus* in the face of the host environment (Araujo and Rodrigues 2004), a mutant such as the Δ*tpsAB* double mutant does not suffer any deficit in the diseased mammalian host. A plausible explanation put forward for this involves aberrancy of cell wall biosynthesis in the double mutant, which might exacerbate the immune response sufficiently to worsen disease outcome.

C. Adhesion

Adhesion is postulated, but not yet formally proven, to be crucial for *A. fumigatus* virulence; however, significant correlations between the capacity to adhere to host proteins in vitro and the ability to cause mammalian disease have been documented (Sheppard 2011). The polysaccharide **galactosaminogalactan** (GAG), discovered in both cell wall extracts and culture media (Fontaine et al. 2000), is a polymer of galactopyranose linked to *N*-acetylgalactosamine. GAG is implicated as an important component of the *A. fumigatus* extracellular matrix, as evidenced by analyses of human biopsy material (Muller et al. 2011). **In independent studies seeking genes that govern biofilm formation and adherence Gravelat et al. (2010) identified a regulatory gene, *medA*, that acts to modulate GAG synthesis.** Transcriptomic analyses identified the genetic locus that directs GAG biosynthesis as a cluster of genes on chromosome 3. MedA was found to mediate biofilm formation and adherence to several substrates including fibronectin and pulmonary epithelial cells, and a *ΔmedA* mutant was attenuated for virulence in a murine model of disease. GAG-deficient strains also exhibited a reduction in their ability to injure and stimulate pulmonary epithelial cells in vitro.

D. Host-Mediated Fungal Clearance and Immune Evasion by *Aspergillus fumigatus*

In the immune-competent host, the respiratory epithelium and alveolar macrophages present the first host interface encountered by the fungal pathogen (Fig. 2.4). Alveolar macrophages are potent inactivators of *A. fumigatus* spores in vitro and, following spore recognition and internalisation, they efficiently kill ungerminated spores.

In human and murine **pulmonary macrophages, acidification of *A. fumigatus* phagolysosomes and phagocyte oxidase-derived reactive oxygen intermediates (ROIs) are requisite for killing of *A. fumigatus* spores** (Ibrahim-Granet et al. 2003; Philippe et al. 2003). **Obligatory morphological transitions undertaken by the invading pathogen ensure that invasive, but not dormant, fungal elements act as immunostimulants for macrophages**, a phenomenon that is governed by the stage-specific cell surface exposure of cell wall β-glucans (Fig. 2.4).

Macrophages secrete tumour necrosis factor-α (TNF-α) and macrophage inflammatory protein-2 (MIP-2) in response to metabolically active, but not heat-killed spores (Hohl et al. 2005). Swollen spores, live or dead, recruit macrophages and neutrophils to the murine lung following intratracheal infection (Hohl et al. 2005).

Unlike macrophages, neutrophils kill both conidia and hyphae, the latter probably being simply too large to be phagocytosed prior to

killing. Human (McCormick et al. 2010) and murine (Bonnett et al. 2006) neutrophils inhibit germination of *A. fumigatus* conidia, and delayed polymorphonuclear neutrophil (PMN) recruitment leads to conidial germination in murine lungs (Bonnett et al. 2006). Phagocytic uptake of conidia by human neutrophils is crucial for the observed inhibition of germination (McCormick et al. 2010). Neutrophil extracellular traps (NETs) limit hyphal growth in vitro but have minimal, if any, direct capacity for killing hyphae (McCormick et al. 2010); however, the soluble pattern recognition receptor (PRR) PTX3, which is nonredundant in whole animals for defence against *A. fumigatus* infection, is localised in specific PMN granules and is secreted in response to exposure to *A. fumigatus* spores (Jaillon et al. 2007). Neutrophils release ROIs onto *A. fumigatus* hyphae in a NADPH oxidase-dependent manner, requiring the class IA phosphatidylinositol 3-kinases PI3Kβ and PI3Kδ, which have also been found to regulate the spreading of neutrophils over the hyphal surface. β2-Integrins play a major role in the activation of the NADPH oxidase in response to hyphae, whereas dectin-1 has only a minor, redundant role. Neutrophil recruitment in immunocompetent mice requires CXCR2, neutralisation of which leads to marked impairment of neutrophil influx and severe IA with nearly 100% mortality. Similarly, CXCR2-deficient animals challenged with intrapulmonary conidia suffer impaired recruitment of neutrophils to the lungs, permitting conidial germination. Pulmonary dendritic cells phagocytose conidia and hyphae via distinct receptors and orchestrate differential cytokine responses to each fungal morphology to instruct local and peripheral Th1-type inflammatory responses (Bozza et al. 2002, 2003). Release of cytokines by fungus-stimulated dendritic cell (DC) subsets recruits and activates other immune cells, thereby boosting innate responses and helping to initiate adaptive immunity. Pulmonary DCs transport *Aspergillus* conidia or hyphae to the draining lymph nodes and spleens and mount differential responses to the two distinct morphological forms of the fungus. Whereas TNF-α is produced in response to either form, IL-12p70 is produced upon exposure to conidia, but not to hyphae, and IL-4 and IL-10 production is prompted by phagocytosis of hyphae, but not conidia.

Plasmacytoid DCs (pDCs), comprising 0.2–0.8% of the total peripheral blood mononuclear cells (PBMCs) link innate to adaptive immunity by secreting cytokines such as IFN-α and TNF-α and by differentiating into mature pDCs with upregulated major histocompatibility complexes (MHC) and co-stimulatory molecules capable of priming naive T cells. Pulmonary DCs are required for effective antifungal defences in vivo, as mice depleted of pDCs are hypersusceptible to invasive aspergillosis (Ramirez-Ortiz et al. 2011).

Natural killer (NK) cells are directly cytotoxic to *A. fumigatus* spores and hyphae and germinated *A. fumigatus* morphologies are highly immunogenic, able to induce a Th1-like response and capable of upregulating cytokines such as IFN-γ and TNF-α. NK cells do not mediate anti-*Aspergillus* cytotoxicity through degranulation of their cytotoxic proteins (perforin, granzymes, granulysine), but via IFN-γ, which directly damages *A. fumigatus*, attributing new properties to both human NK cells and IFN-γ and suggesting them as possible therapeutic tools against IA.

In addition to the RodA hydrophobin, which prevents inhaled fungal spores from immediately initiating inflammatory responses in the host (Aimanianda et al. 2009), a further layer of immunomodulatory control, documented simultaneously by Hohl et al. (2005) and Steele et al. (2005), was revealed. This phenomenon is manifested by the gradual exposure of cell surface β-glucan during swelling and germination of *A. fumigatus* spores (Fig. 2.4). In contrast to dormant spores, **germinating conidia induce neutrophil recruitment to the airways and TNF-α/MIP-2 secretion by alveolar macrophages, with fungal β-glucans acting as the inflammatory trigger via their stage-specific exposure on the surface of germinating conidia.** Dectin-1, the innate immune receptor that mediates this immune response is recruited in vivo to alveolar macrophage phagosomes that have internalised conidia with exposed β-glucans.

E. Interactions of Spores with Epithelial Cells: Reservoirs for Fungal Persistence?

Airway epithelial cells (ECs) are central participants in innate and adaptive immune responses as well as in mucosal inflammation and allergy. At present, the contribution of epithelial cell activities to defence against *A. fumigatus* is unknown, but this is a highly active area of current research (Osherov 2012) and present theories implicate both immunomodulatory and directly antimicrobial roles of epithelial cells in anti-*Aspergillus* defence (Osherov 2012). Additionally, not conversely. **The ability of ECs to internalise and kill *A. fumigatus* spores has prompted the suggestion that epithelial cells might provide a reservoir in which dormant *A. fumigatus* spores escape clearance by innate immune cells.**

Using electron microscopy, Paris et al. (1997) were the first to show that epithelial cells (of rabbit tracheal, rat alveolar and human umbilical cord origins) internalise *A. fumigatus* conidia. These observations were replicated in several subsequent studies (Wasylnka and Moore 2002, 2003). Germinating, but not resting, conidia were found to induce production of interleukin (IL)-8 (Balloy et al. 2008) in human bronchial epithelial cells (HBECs). IL-8 production was found to be governed by phosphatidylinositol 3-kinase, p38 MAPK and ERK1/2 but was independent of the TLR-MyD88 pathway. However, integrity of the MyD88 pathway was found to be required for *A. fumigatus*-mediated NF-κB activation, suggesting that two independent signalling pathways become activated in respiratory epithelial cells by *A. fumigatus*.

Resting conidia are also recognised by, and stimulate, differentiated HBECs in an internalisation-dependent manner, resulting in the activation of the interferon (IFN)-β signalling pathway (Beisswenger et al. 2012). Conida-derived double-stranded RNA was found to be responsible for IFN-β and IP-10 expression when it was transfected into HBECs, but not when RNA was added directly to the culture medium. This supports the theory that internalisation and degradation of *A. fumigatus* spores leads to RNA release and IFN-β signalling. The observation that these responses to *A. fumigatus* challenge require internalisation of spores was substantiated by inhibition of endocytosis using cytochalasin D, which prompted the dose-dependent reduction of IFN-β and IP-10 expression.

In the epithelial cell line A549, and primary respiratory epithelial cells, internalised conidia are directed into the endosomal–lysosomal compartment where they are acidified and some are killed (Wasylnka and Moore 2003). Internalisation of conidia by type II pneumocytes such as A549 cells may be important in the development of aspergillosis in vivo because sequestration by these cells could allow conidia to evade the immune response of the host.

F. Melanin

A. fumigatus produces at least two types of melanin, pyomelanin and dihydroxynaphthalene (DHN)-melanin (Heinekamp et al. 2012), the latter being a crucial contributor to fungal resilience in the host environment. Detailed molecular genetic studies, (reviewed by Heinekamp et al. 2012), employing a variety of mutants in DHN biosynthesis have documented the **contribution of DHN-melanin to intracellular trafficking of *A. fumigatus* in phagocytic cells and also to mammalian virulence.**

The phagocytosis and intracellular degradation of conidia by phagocytic cells contributes to fungal and proinflammatory responses, thus making a dual contribution to essential host defences. Following phagocytosis, the intracellular fusion of conidia-containing phagosomes with lysosomes is a crucial step in microbial killing because the resulting phagolysosome provides an acidic environment conducive to antimicrobial activities, including degrading enzymes. **Conidia of the *A. fumigatus pksP* mutant, which lack DHN-melanin, produce smooth white conidia and have a much higher propensity than wild-type spores to localise**

in phagolysosomes (Thywissen et al. 2011). DHN-melanin probably provides a further morphotype-specific example of the host-modulating potential of *A. fumigatus* particles because resting or fixed conidia showed stronger inhibition of phagolysosome acidification than swollen conidia.

V. Permanence

A. Fungal Stress Tolerance and Nutrient Acquisition

To identify fungal attributes preferentially employed during adaptation to the host niche, McDonagh et al. (2008) compared the transcriptomes of developmentally matched *A. fumigatus* isolates following laboratory culture or initiation of infection in the neutropenic murine lung. This analysis unveiled a remarkable level of co-ordinated gene expression during mammalian pathogenesis, including hallmarks of growth in alkaline, iron-limited and nutrient-limiting environments. A minimum of 11 siderophore biosynthesis/transport genes were identified as having heightened relevance during growth in the murine lung, as well as 13 amino acid permease genes and the general amino acid permease, Gap1. The functional categories of ergosterol biosynthesis, heme biosynthesis and aerobic respiration were significant among genes underrepresented during infection, relative to laboratory culture, as well as multiple functional categories representing ribosome biogenesis and assembly, and protein biosynthesis and processing. This may reflect the poor nutritional value of murine lung relative to YPD medium. A further intriguing aspect of the host adapting transcriptome was the co-ordinated expression of multiple groups of physically clustered genes, including genes known to direct biosynthesis of siderophores, and two known secondary metabolites, pseurotin and gliotoxin. These insights have been borne out by a plethora of subsequent studies addressing the role of stress tolerance and nutrient acquisition, a selection of which are discussed below.

B. Iron

Iron is an essential nutrient, the acquisition of which is exquisitely regulated in *A. fumigatus* to guard against the toxic effects of an excess of this element [recently reviewed by Haas (2012)]. This requires the co-ordinated activities of two transcription factors, SreA and HapX. When iron is in sufficient supply, **SreA represses iron uptake** to exclude toxic effects. When iron is limiting for growth, **HapX acts to repress iron-consuming pathways** such as heme biosynthesis. With respect to mammalian virulence, iron acquisition is a greater concern for the infecting fungus because **deficiency in HapX, but not in SreA, attenuates virulence of *A. fumigatus* in a murine model of aspergillosis** (Schrettl et al. 2008, 2010). In a series of eloquent molecular genetic analyses, Haas and colleagues have, to date, identified and characterised 24 genes involved in iron homeostasis in *A. fumigatus* and/or *A. nidulans*. In 10 out of 19 described *A. fumigatus* genes, knockout mutants proved to be attenuated for murine virulence. When it comes to iron acquisition, *A. fumigatus* differs somewhat from *A. nidulans* in possessing an additional capacity for high-affinity iron uptake by reductive iron assimilation (RIA); however, both organisms produce low molecular weight chaperones called siderophores to assist with high-affinity iron uptake. **The first committed step in the biosynthesis of siderophores, and in *A. fumigatus* for RIA, is the hydroxylation of ornithine catalysed by the ornithine monooxygenase SidA.** Subsequently, the pathways for biosynthesis of extra and intracellular siderophores split but, crucially, **in the absence of sidA both RIA and siderophore biosynthesis are abolished.** This has catastrophic consequences for *A. fumigatus* survival in the host, and a SidA null mutant is completely attenuated for virulence in neutropenic mice (Schrettl et al. 2004).

C. Hypoxia

Hypoxia ranks high amongst abiotic stimuli and varies greatly between the natural and

other environments colonised by *A. fumigatus*. Variations in ambient oxygen concentrations from 21% (atmospheric) through 14% (alveolar) and 4% (human tissues) to 1.5% (compost) are predicted as being well within the repertoire of manageable stresses imposed upon the activities of this ubiquitous organism. **In *A. fumigatus*, the sterol-regulatory element binding protein (SREBP) transcription factor governs growth in hypoxic conditions and mammalian virulence** (Willger et al. 2008) but is not required for oxidative stress resistance and resistance to macrophage killing. Seeking to verify the direct involvement of hypoxic microenvironments upon observed attenuation of SREB functionality, Grahl et al. (2011) used 1H-NMR metabolomics to forage for ethanol production in the lungs of mice infected with *A. fumigatus*, thus confirming, via a surrogate marker of fungal anaerobic respiration, that oxygen-depleted microenvironments occur during murine infection. The additional use of a chemical hypoxia reporter, pimonidazole hydrochloride, confirmed via histopathological investigations that hypoxia could be observed during *A. fumigatus* infection of neutropenic, X-CGD and corticosteroid-treated mice. Relative to mice infected with a wild-type isolate, animals infected with an alcohol dehydrogenase-deficient mutant suffered heightened inflammation and a reduction in fungal burden. In conclusion, therefore, environmental conditions encountered by *A. fumigatus* in the mammalian host probably alter fungal metabolism in a manner conducive to effecting local modulation of the host immune response.

D. Invasive Growth and Tissue Damage

It has long been assumed that fungal proteases support the degradation of host lung tissue during invasive aspergillosis. However, due to the assumed redundancy of multiple such functions in *A. fumigatus*, no mutant lacking a single protease-encoding gene has ever demonstrated attenuation of virulence in murine aspergillosis. The assimilation of proteinaceous substrates is an essential facet of the fungal lifestyle and multiple studies here, and elsewhere (Lamarre et al. 2008; Latge 1999; Abad et al. 2010; Dagenais and Keller 2009; Krappmann et al. 2004; Oliver et al. 2012; McDonagh et al. 2008) documented have identified a **clear requirement for amino acid acquisition/biosynthesis during germinative growth in vitro and during murine virulence**. Two research groups (Sharon et al. 2009; Krappmann et al. 2004) simultaneously assessed the importance of extracellular proteases during in vitro growth and virulence by assessing the impact of PrtT (a transcriptional regulator that acts to modulate the expression of secreted proteases) on utilisation of alternative nitrogen sources and in murine models of invasive aspergillosis. The Δ*prtT* strain proved unable to grow on bovine serum albumin as the sole nitrogen source, and in a number of other assays for proteinaceous activity the mutant fared less well than the wild-type progenitor. Concordant with the phenotypic assays, transcriptomic analyses identified that the mutant was defective in expressing six extracellular proteases; however, both groups, using three independent approaches to virulence analysis, concluded that the mutant is fully virulent. Although it is tempting to conclude that the ability to degrade extracellular protein is dispensable for virulence, Sharon et al. (2009) duly noted that still other secreted proteases might be crucial during *A. fumigatus* lung infection. Indeed, of the proteases upregulated in the transcriptome study of McDonagh et al. (2008), an abundance of transcripts for the secreted proteases MEP, Dpp4, Dpp5, AFUA_6G10250 and AFUA_3G07850 was uncovered. Of these, only MEP and Dpp4 exhibited reduced transcription in the *ΔprtT* mutant.

Study of PrtT gathered further relevance when the effects of the gene deletion on infection of epithelial cells was investigated. Previous studies had shown that *A. fumigatus* proteases disrupt A549 cell actin fibres and focal adhesions, leading to cell detachment and death. In a further study, culture filtrates derived from the *ΔprtT* mutant were shown to cause significantly less A549 cell detachment and cell death (Sharon et al. 2011). Comparing the A549 transcriptional and cell signalling responses activated in the presence of *A. fumigatus* wild-type and *ΔprtT* conidia and culture

filtrates, differential phosphorylation, and therefore activation, of JNK and ERK1/2 was observed in response to challenge with the two isolates. Inhibition of JNK or ERK1/2 kinase activity substantially decreased CF-induced cell damage, including cell peeling, actin-cytoskeleton damage, reduced metabolic activity and necrotic death. Because deletion of PrtT results primarily in the loss of secreted protease activity, it is likely that protease activity may be responsible for the subsequent MAPK activation in the treated A549 cells. Inhibition of this effect might provide a route to protecting the host against fungus-mediated damage and resultant inflammation.

E. Mycotoxins

Like other filamentous fungi, *A. fumigatus* produces multiple small molecule compounds biosynthesised by secondary metabolism. **The demonstration that a wide domain regulator of secondary metabolism gene expression, LaeA, is required for fungal virulence affirms the potential importance of secondary metabolite "cocktails" produced in the lung of the infected host.** Furthermore, a LaeA regulatory signature, characterised by transcriptomic analysis of a LaeA null mutant (Perrin et al. 2007) was found to be significantly similar to that derived from analyses of fungal infection, performed by McDonagh et al. (2008), and indicates that the selective expression of a subset of secondary metabolite loci might facilitate initiation of mammalian infection. In the post-genomic era, interest in secondary metabolism has become refuelled as bioinformatic scrutiny of the genome sequence has revealed interspecies variation in secondary metabolism genes, offering a plausible explanation for differential virulence amongst *Aspergillus* spp. (Khaldi et al. 2010; Nierman et al. 2005; Machida et al. 2005; Galagan et al. 2005).

At the time of its discovery, the DHN-melanin gene cluster was the largest cluster of fungal biosynthetic genes to be reported. Gene deletion mutants lacking any of six open reading frames, including those encoding a polyketide synthase, scytalone dehydratase and HN reductase, lacked the typical blue-green spore pigmentation characteristic of *A. fumigatus* and regulation of gene expression was observed to be developmentally linked to sporulation (Tsai et al. 1999). As discussed earlier in this chapter, conidial pigment biosynthesis in *A. fumigatus* appears to be an important virulence factor in the establishment of infection.

The right subtelomere of *A. fumigatus* chromosome 3 contains two predicted (Perrin et al. 2007) secondary metabolism gene clusters, Afu3g15200-Afu3g15340 and Afu3g14560-Afu3g14760, whose biosynthetic products are unknown. The former of the two gene clusters contains a polyketide synthase encoding the gene designated *pes3*, which has recently been shown to affect murine virulence and insect virulence (O'Hanlon et al. 2011). A Pes3 null mutant was found to be increased for fungal burden in corticosteroid-treated mice at 5 days post-infection, a phenotype that was accompanied by a more rapid germination of spores within murine lung tissues and a reduction in proinflammatory cytokine release, relative to wild type, from macrophages exposed to Δ*pes3* mutants in vitro. Subtelomeres 4 (left and right arms), 5 (left) and 7 (left) collectively accommodate five predicted (Perrin et al. 2007) secondary metabolism gene clusters, the biosynthetic products of which are currently unknown.

Subtelomere 8 (left) is exceptional because it encodes genes for predominantly secondary metabolism ($n=61$), including multiple polyketide synthase-encoding genes (Afu8g00370 and Afu8g00890), a hybrid polyketide synthase/NRPS-encoding gene (Afu8g00540) and a further NRPS (Afu8g00170). Sheppard and colleagues noted the presence of genes in this region that are known to be required for sterigmatocystin/aflatoxin biosynthesis, as well as genes required for ergot alkaloid synthesis (Sheppard et al. 2005). Intriguingly, this combination of secondary metabolism genes is not found in other *Aspergillus* species. Previous gene deletion studies by Turner and colleagues have defined the biosynthetic products of some subclusters within this supercluster on chromosome 8, including the pseurotin A gene cluster (Maiya et al. 2007) and the fumitremorgin gene cluster (Maiya et al. 2006). Work in my laboratory is currently addressing the role of such metabolites in murine virulence. Indications to date are that loss of at least one of the metabolites

whose biosynthesis is directed by genes resident in the chromosome 8 supercluster can impact virulence at whole animal level (Bignell, unpublished observations). However, our recent analyses indicate only a partial requirement for these metabolites during infection, as strain-dependent variability is observed when similar mutants are constructed in different genetic backgrounds. Thus, further characterisation is required before firm conclusions on the role of the metabolites encoded by genes in this region can be conclusively ascertained. A further crucial point is that the combinatorial activity of *A. fumigatus* secondary metabolites is a possibility thus far unexplored. It is highly feasible that certain secondary metabolites act in concert to disable residual host immune defences. The construction of mutants lacking multiple biosynthetic properties is therefore required to address this important question.

The chemical structures of a large number of metabolites have been well characterised, e.g. gliotoxin, which impacts virulence of *A. fumigatus* in corticosteroid, but not neutropenic mice (Sugui et al. 2007; Cramer et al. 2006; Bok et al. 2006; Kupfahl et al. 2006; Spikes et al. 2008) **Thus far, gliotoxin, which has multiple toxic and inhibitory effects upon host cells is the only toxic secondary metabolite to have been isolated from the sera of rodents and patients suffering from invasive aspergillosis.**

Until recently, few data on the identity of other toxic metabolites have been forthcoming and no attempt had been made to evaluate the toxicity of these metabolites on the respiratory tract. To address this, Gauthier and co-workers (Gauthier et al. 2012) extracted metabolites from the spores of *A. fumigatus* and evaluated their cytotoxic activity towards A549 epithelial cells using an MTT assay. The composition of each fraction regarding other secondary metabolites produced by *Aspergillus fumigatus* was determined by LC-MS analysis. The most toxic fractions were those containing all of the metabolite compounds. Sequential analyses of toxic fractions, by a deductive process, identified toxicity in several fractions that could be attributed to trypacidin and other fractions containing trypacidin precursor compounds. Of all fractions tested, viability of A549 cells exposed to spore extracts ranged from 92.5% (vehicle) to 0.2%. The use of cell lysis assays demonstrated that 50 μm trypacidin reduced cell viability by nearly 100% and triggered 85% cell lysis whereas the other metabolites showed only slight or no toxicity at this concentration. The question remains whether trypacidin could be toxic to epithelial and other cells in vivo.

F. Inflammation

Our advancing understanding of host–fungus interactions has revealed that fungal elements pose a potent antigenic stimulus (Romani 2011). For *A. fumigatus*, resting and germinating conidia and hyphae are recognised by host PRRs on epithelial cells and, in various cell types, induce the production of cytokines and chemokines such as IL-6, TNF-α, IL-8 and IFN-β. Importantly, amelioration of this inflammatory response can be achieved via corticosteroid administration, providing support for the importance of epithelial cells in directing host responses and resolution of infection. Macrophages activate at least two receptor-mediated signalling pathways in response to *A. fumigatus*. Proinflammatory cytokine production following challenge of peritoneal macrophages from TLR2 and TLR4 knockout mice revealed that TLR4 plays an important role in TNF-α, IL-1α and IL1-β production in response to conidia but that the stimulus for such production during spore-to-hyphal conversions is lost. TLR2, on the other hand, is required for responses of both conidia and hyphae (Gersuk et al. 2006; Netea et al. 2003).

Dectin-1, an innate immune receptor that recognises fungal β-glucans, associates with inhaled *A. fumigatus* conidia in a β-glucan-dependent manner (Hohl et al. 2005) and the production of TNF-α, IL-1α, IL-1β, IL-6, CXCL2/MIP-2, CCL3/ MIP-1α, granulocyte-colony stimulating factor (G-CSF), and granulocyte monocyte-CSF (GM-CSF), in response to live *A. fumigatus* is dectin-1 dependent (Steele et al. 2005). This complex inflammatory milieu requires precise regulation in order to ensure protection against host damage. Most likely, for the *A. fumigatus* host–pathogen interaction, deregulation of immune responses contribute as much to host damage as do pathogen-associated pathogenicity factors.

In seeking a unifying framework within which to study diseases caused by *A. fumigatus*, this working model can be applied to all types of disease caused by this mould. Allergic bronchopulmonary aspergillosis (ABPA) is a good case in point; in this disease host activities are probably more important than pathogen activities in driving host damage since allergic inflammation leads to infiltration of inflammatory cells and injury and detachment of the epithelium. ABPA occurs predominantly in asthmatic or cystic fibrosis (CF) patients and up to 15% of CF patients mount an allergic response, which is associated with exaggerated Th2 responses to the organism (Patterson and Strek 2010; Eaton et al. 2000). In a study assessing ABPA in CF patients, the binding and internalisation of *A. fumigatus* spores by epithelial cells was monitored in normal and mutated epithelial cells derived from both mice and cell lines (Chaudhary et al. 2012). Cell lines and murine tracheal epithelial cells (MTECS) that harbour defects in the cystic fibrosis transmembrane conductance regulator (CFTR) were found to bind, internalise and kill conidia less well than control cells. Histological analysis of murine infections (wild-type and CFTR null mice) clearly showed invasion of the lung parenchyma at 24 h in the absence of the CFTR channel. Proinflammatory mediators were measured after MTEC exposure to inactivated conidia and hyphal cells. Compared to wild-type MTEC, unstimulated CFTR null cells demonstrated an overall decreased production of MIP-2, IL-6 and IP-10. These analyses demonstrated a defective uptake of *A. fumigatus* conidia as well as aberrant inflammatory and apoptotic responses to different forms of *A. fumigatus*, thus contributing to altered pulmonary inflammation in the setting of CF, at least in part associated with ineffective epithelial cell clearance of conidia and subsequent aberrant inflammatory responses to germinated *A. fumigatus* morphotypes.

G. Perspectives

A unified model of the *A. fumigatus* host–pathogen interaction is required to understand the basis of diseases caused by this pathogen. The outcome of disease can be equally impacted by host and pathogen activities, and this must be accommodated into the design of novel therapeutic strategies. Although the administration of harsh antifungal agents is the mainstay of the current antifungal arsenal, it is likely that moderation of the host immune response might be equally effective and potentially less damaging to the host. A quantitative understanding of the host–pathogen interaction and the impact of antifungal drugs upon host homeostasis will be a necessary prerequisite to confidently apply the next generation of interventative strategies.

References

Abad A, Fernandez-Molina JV, Bikandi J, Ramirez A, Margareto J, Sendino J, Hernando FL, Ponton J, Garaizar J, Rementeria A (2010) What makes *Aspergillus fumigatus* a successful pathogen? Genes and molecules involved in invasive aspergillosis. Rev Iberoam Micol 27:155–182

Agarwal R (2009) Allergic bronchopulmonary aspergillosis. Chest 135:805–826

Aimanianda V, Bayry J, Bozza S, Kniemeyer O, Perruccio K, Elluru SR, Clavaud C, Paris S, Brakhage AA, Kaveri SV, Romani L, Latge JP (2009) Surface hydrophobin prevents immune recognition of airborne fungal spores. Nature 460:1117–1121

Al-Bader N, Vanier G, Liu H, Gravelat FN, Urb M, Hoareau CM, Campoli P, Chabot J, Filler SG, Sheppard DC (2010) Role of trehalose biosynthesis in *Aspergillus fumigatus* development, stress response, and virulence. Infect Immun 78:3007–3018

Araujo R, Rodrigues AG (2004) Variability of germinative potential among pathogenic species of *Aspergillus*. J Clin Microbiol 42:4335–4337

Askew DS (2008) *Aspergillus fumigatus*: virulence genes in a street-smart mold. Curr Opin Microbiol 11:331–337

Baker SE, Bennet JW (2008) An overview of the genus *Aspergillus*. In: Goldman GH, Osmani SA (eds) The Aspergilli: genomics, medical aspects and research methods. Taylor & Francis, Boca Raton, pp 3–13

Balloy V, Huerre M, Latge JP, Chignard M (2005) Differences in patterns of infection and inflammation for corticosteroid treatment and chemotherapy in experimental invasive pulmonary aspergillosis. Infect Immun 73:494–503

Balloy V, Sallenave JM, Wu Y, Touqui L, Latge JP, Si-Tahar M, Chignard M (2008) *Aspergillus fumigatus*-induced interleukin-8 synthesis by respiratory epithelial cells is controlled by the phosphatidylinositol 3-kinase, p38 MAPK, and ERK1/2 pathways and not by the toll-like receptor-MyD88 pathway. J Biol Chem 283:30513–30521

Beisswenger C, Hess C, Bals R (2012) *Aspergillus fumigatus* conidia induce interferon-beta signalling in respiratory epithelial cells. Eur Respir J 39:411–418

Bennet JW (2010) An overview of the genus *Aspergillus*. In: Masayuki M, Katsuya G (eds) *Aspergillus:* molecular biology and genomics. Caister Academic, Norfolk, pp 1–18

Bernard M, Latge JP (2001) *Aspergillus fumigatus* cell wall: composition and biosynthesis. Med Mycol 39 (Suppl 1):9–17

Bhabhra R, Askew DS (2005) Thermotolerance and virulence of *Aspergillus fumigatus*: role of the fungal nucleolus. Med Mycol 43(Suppl 1):S87–S93

Bhabhra R, Miley MD, Mylonakis E, Boettner D, Fortwendel J, Panepinto JC, Postow M, Rhodes JC, Askew DS (2004) Disruption of the *Aspergillus fumigatus* gene encoding nucleolar protein CgrA impairs thermotolerant growth and reduces virulence. Infect Immun 72:4731–4740

Bok JW, Chung D, Balajee SA, Marr KA, Andes D, Nielsen KF, Frisvad JC, Kirby KA, Keller NP (2006) GliZ, a transcriptional regulator of gliotoxin biosynthesis, contributes to *Aspergillus fumigatus* virulence. Infect Immun 74:6761–6768

Bonnett CR, Cornish EJ, Harmsen AG, Burritt JB (2006) Early neutrophil recruitment and aggregation in the murine lung inhibit germination of *Aspergillus fumigatus* conidia. Infect Immun 74:6528–6539

Bouchara JP, Sanchez M, Chevailler A, Marot-Leblond A, Lissitzky JC, Tronchin G, Chabasse D (1997) Sialic acid-dependent recognition of laminin and fibrinogen by *Aspergillus fumigatus* conidia. Infect Immun 65:2717–2724

Bozza S, Gaziano R, Spreca A, Bacci A, Montagnoli C, Di FP, Romani L (2002) Dendritic cells transport conidia and hyphae of *Aspergillus fumigatus* from the airways to the draining lymph nodes and initiate disparate Th responses to the fungus. J Immunol 168:1362–1371

Bozza S, Perruccio K, Montagnoli C, Gaziano R, Bellocchio S, Burchielli E, Nkwanyuo G, Pitzurra L, Velardi A, Romani L (2003) A dendritic cell vaccine against invasive aspergillosis in allogeneic hematopoietic transplantation. Blood 102:3807–3814

Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, White TC (2012) Hidden killers: human fungal infections. Sci Transl Med 4:165rv13

Cagas SE, Jain MR, Li H, Perlin DS (2011) The proteomic signature of *Aspergillus fumigatus* during early development. Mol Cell Proteomics 10:M111

Chaudhary N, Datta K, Askin FB, Staab JF, Marr KA (2012) CFTR regulates epithelial cell response to *Aspergillus* and resultant pulmonary inflammation. Am J Respir Crit Care Med 185(3):301–310

Clemons KV, Stevens DA (2005) The contribution of animal models of aspergillosis to understanding pathogenesis, therapy and virulence. Med Mycol 43(Suppl 1):S101–S110

Cramer RA Jr, Gamcsik MP, Brooking RM, Najvar LK, Kirkpatrick WR, Patterson TF, Balibar CJ, Graybill JR, Perfect JR, Abraham SN, Steinbach WJ (2006) Disruption of a nonribosomal peptide synthetase in *Aspergillus fumigatus* eliminates gliotoxin production. Eukaryot Cell 5:972–980

da Silva Ferreira ME, Heinekamp T, Hartl A, Brakhage AA, Semighini CP, Harris SD, Savoldi M, de Gouvea PF, de Souza Goldman MH, Goldman GH (2007) Functional characterization of the *Aspergillus fumigatus* calcineurin. Fungal Genet Biol 44:219–230

Dagenais TR, Keller NP (2009) Pathogenesis of *Aspergillus fumigatus* in invasive aspergillosis. Clin Microbiol Rev 22:447–465

Dague E, Alsteens D, Latge JP, Dufrene YF (2008) High-resolution cell surface dynamics of germinating *Aspergillus fumigatus* conidia. Biophys J 94:656–660

Eaton T, Garrett J, Milne D, Frankel A, Wells AU (2000) Allergic bronchopulmonary aspergillosis in the asthma clinic. A prospective evaluation of CT in the diagnostic algorithm. Chest 118:66–72

Einsele H, Quabeck K, Muller KD, Hebart H, Rothenhofer I, Loffler J, Schaefer UW (1998) Prediction of invasive pulmonary aspergillosis from colonisation of lower respiratory tract before marrow transplantation. Lancet 352:1443

Fleck CB, Brock M (2010) *Aspergillus fumigatus* catalytic glucokinase and hexokinase: expression analysis and importance for germination, growth, and conidiation. Eukaryot Cell 9:1120–1135

Fontaine T, Simenel C, Dubreucq G, Adam O, Delepierre M, Lemoine J, Vorgias CE, Diaquin M, Latge JP (2000) Molecular organization of the alkali-insoluble fraction of *Aspergillus fumigatus* cell wall. J Biol Chem 275:41528

Fortwendel JR, Juvvadi PR, Rogg LE, Asfaw YG, Burns KA, Randell SH, Steinbach WJ (2006) Plasma membrane localization is required for RasA-mediated polarized morphogenesis and virulence of *Aspergillus fumigatus*. Eukaryot Cell 11(8):966–977. doi:10.1128/EC.00091-12 (Epub 4 May 2012)

Galagan JE, Calvo SE, Cuomo C, Ma LJ, Wortman JR, Batzoglou S, Lee SI, Basturkmen M, Spevak CC, Clutterbuck J, Kapitonov V, Jurka J, Scazzocchio C, Farman M, Butler J, Purcell S, Harris S, Braus GH, Draht O, Busch S, D'Enfert C, Bouchier C, Goldman GH, Bell-Pedersen D, Griffiths-Jones S, Doonan JH, Yu J, Vienken K, Pain A, Freitag M, Selker EU, Archer DB, Penalva MA, Oakley BR, Momany M, Tanaka T, Kumagai T, Asai K, Machida M, Nierman WC, Denning DW, Caddick M, Hynes M, Paoletti M, Fischer R, Miller B, Dyer P, Sachs MS, Osmani SA, Birren BW (2005) Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. Nature 438:1105–1115

Gauthier T, Wang X, Dos Sifuentes SJ, Fysikopoulos A, Tadrist S, Canlet C, Artigot MP, Loiseau N, Oswald IP, Puel O (2012) Trypacidin, a spore-borne toxin from *Aspergillus fumigatus*, is cytotoxic to lung cells. PLoS One 7:e29906

Gersuk GM, Underhill DM, Zhu L, Marr KA (2006) Dectin-1 and TLRs permit macrophages to distinguish between different *Aspergillus fumigatus* cellular states. J Immunol 176:3717–3724

Gil ML, Penalver MC, Lopez-Ribot JL, O'Connor JE, Martinez JP (1996) Binding of extracellular matrix proteins to *Aspergillus fumigatus* conidia. Infect Immun 64:5239–5247

Grahl N, Puttikamonkul S, Macdonald JM, Gamcsik MP, Ngo LY et al (2011) In vivo hypoxia and a

fungal alcohol dehydrogenase influence the pathogenesis of invasive pulmonary aspergillosis. PLoS Pathog 7(7):e1002145

Gravelat FN, Ejzykowicz DE, Chiang LY, Chabot JC, Urb M, Macdonald KD, Al-Bader N, Filler SG, Sheppard DC (2010) *Aspergillus fumigatus* MedA governs adherence, host cell interactions and virulence. Cell Microbiol 12:473–488

Haas H (2012) Iron – a key nexus in the virulence of *Aspergillus fumigatus*. Front Microbiol 3:28

Heinekamp T, Thywissen A, Macheleidt J, Keller S, Valiante V, Brakhage AA (2012) *Aspergillus fumigatus* melanins: interference with the host endocytosis pathway and impact on virulence. Front Microbiol 3:440

Herbst S, Shah A, Carby M, Kikkeri N, Dorling A, Bignell E, Shaunak S, Armstrong-James D (2013) A new and clinically relevant model of solid-organ transplant aspergillosis. Dis Model Mech 6(3):643–651

Hohl TM, Feldmesser M (2007) *Aspergillus fumigatus*: principles of pathogenesis and host defense. Eukaryot Cell 6:1953–1963

Hohl TM, Van Epps HL, Rivera A, Morgan LA, Chen PL, Feldmesser M, Pamer EG (2005) *Aspergillus fumigatus* triggers inflammatory responses by stage-specific beta-glucan display. PLoS Pathog 1:e30

Hope WW, Walsh TJ, Denning DW (2005) The invasive and saprophytic syndromes due to *Aspergillus* spp. Med Mycol 43(Suppl 1):S207–S238

Ibrahim-Granet O, Philippe B, Boleti H, Boisvieux-Ulrich E, Grenet D, Stern M, Latge JP (2003) Phagocytosis and intracellular fate of *Aspergillus fumigatus* conidia in alveolar macrophages. Infect Immun 71:891–903

Jaillon S, Peri G, Delneste Y, Fremaux I, Doni A, Moalli F, Garlanda C, Romani L, Gascan H, Bellocchio S, Bozza S, Cassatella MA, Jeannin P, Mantovani A (2007) The humoral pattern recognition receptor PTX3 is stored in neutrophil granules and localizes in extracellular traps. J Exp Med 204:793–804

Kamai Y, Lossinsky AS, Liu H, Sheppard DC, Filler SG (2009) Polarized response of endothelial cells to invasion by *Aspergillus fumigatus*. Cell Microbiol 11:170–182

Kelm S, Schauer R (1997) Sialic acids in molecular and cellular interactions. Int Rev Cytol 175:137–240

Khaldi N, Seifuddin FT, Turner G, Haft D, Nierman WC, Wolfe KH, Fedorova ND (2010) SMURF: genomic mapping of fungal secondary metabolite clusters. Fungal Genet Biol 47:736–741

Krappmann S, Bignell EM, Reichard U, Rogers T, Haynes K, Braus GH (2004) The *Aspergillus fumigatus* transcriptional activator CpcA contributes significantly to the virulence of this fungal pathogen. Mol Microbiol 52:785–799

Kupfahl C, Heinekamp T, Geginat G, Ruppert T, Hartl A, Hof H, Brakhage AA (2006) Deletion of the gliP gene of *Aspergillus fumigatus* results in loss of gliotoxin production but has no effect on virulence of the fungus in a low-dose mouse infection model. Mol Microbiol 62:292–302

Lacey ME, West JS (2007) The aerobiology pathway. In: The air spora: a manual for catching and identifying airborne biological particles. Springer, Berlin, p 156

Lamarre C, Sokol S, Debeaupuis JP, Henry C, Lacroix C, Glaser P, Coppee JY, Francois JM, Latge JP (2008) Transcriptomic analysis of the exit from dormancy of *Aspergillus fumigatus* conidia. BMC Genomics 9:417

Lass-Florl C, Salzer GM, Schmid T, Rabl W, Ulmer H, Dierichi MP (1999) Pulmonary *Aspergillus* colonization in humans and its impact on management of critically ill patients. Br J Haematol 104:745–747

Latge JP (1999) *Aspergillus fumigatus* and aspergillosis. Clin Microbiol Rev 12:310–350

Leleu C, Menotti J, Meneceur P, Choukri F, Sulahian A, Garin Y-J, Denis J-B, Derouin F (2013) Bayesian development of a dose-response model for *Aspergillus fumigatus* and invasive aspergillosis. Risk Anal 33(4):1–13. doi:10.1111/risa.12007

Lewis RE, Wiederhold NP (2005) Murine model of invasive aspergillosis. Methods Mol Med 118:129–142

Liebmann B, Muller M, Braun A, Brakhage AA (2004) The cyclic AMP-dependent protein kinase a network regulates development and virulence in *Aspergillus fumigatus*. Infect Immun 72:5193–5203

Machida M, Asai K, Sano M, Tanaka T, Kumagai T, Terai G, Kusumoto K, Arima T, Akita O, Kashiwagi Y, Abe K, Gomi K, Horiuchi H, Kitamoto K, Kobayashi T, Takeuchi M, Denning DW, Galagan JE, Nierman WC, Yu J, Archer DB, Bennett JW, Bhatnagar D, Cleveland TE, Fedorova ND, Gotoh O, Horikawa H, Hosoyama A, Ichinomiya M, Igarashi R, Iwashita K, Juvvadi PR, Kato M, Kato Y, Kin T, Kokubun A, Maeda H, Maeyama N, Maruyama J, Nagasaki H, Nakajima T, Oda K, Okada K, Paulsen I, Sakamoto K, Sawano T, Takahashi M, Takase K, Terabayashi Y, Wortman JR, Yamada O, Yamagata Y, Anazawa H, Hata Y, Koide Y, Komori T, Koyama Y, Minetoki T, Suharnan S, Tanaka A, Isono K, Kuhara S, Ogasawara N, Kikuchi H (2005) Genome sequencing and analysis of *Aspergillus oryzae*. Nature 438:1157–1161

Maiya S, Grundmann A, Li SM, Turner G (2006) The fumitremorgin gene cluster of *Aspergillus fumigatus*: identification of a gene encoding brevianamide F synthetase. Chembiochem 7:1062–1069

Maiya S, Grundmann A, Li X, Li SM, Turner G (2007) Identification of a hybrid PKS/NRPS required for pseurotin A biosynthesis in the human pathogen *Aspergillus fumigatus*. Chembiochem 8:1736–1743

McCormick A, Heesemann L, Wagener J, Marcos V, Hartl D, Loeffler J, Heesemann J, Ebel F (2010) NETs formed by human neutrophils inhibit growth of the pathogenic mold *Aspergillus fumigatus*. Microbes Infect 12:928–936

McDonagh A, Fedorova ND, Crabtree J, Yu Y, Kim S, Chen D, Loss O, Cairns T, Goldman G, Armstrong-James D, Haynes K, Haas H, Schrettl M, May G, Nierman WC, Bignell E (2008) Sub-telomere directed gene expression during initiation of invasive aspergillosis. PLoS Pathog 4:e1000154

Momany M, Taylor I (2000) Landmarks in the early duplication cycles of *Aspergillus fumigatus* and *Aspergillus nidulans*: polarity, germ tube emergence and septation. Microbiology 146(Pt 12):3279–3284

Muller FM, Seidler M, Beauvais A (2011) *Aspergillus fumigatus* biofilms in the clinical setting. Med Mycol 49(Suppl 1):S96–S100

Netea MG, Warris A, Van der Meer JW, Fenton MJ, Verver-Janssen TJ, Jacobs LE, Andresen T, Verweij PE, Kullberg BJ (2003) *Aspergillus fumigatus* evades immune recognition during germination through loss of toll-like receptor-4-mediated signal transduction. J Infect Dis 188:320–326

Nierman WC, Pain A, Anderson MJ, Wortman JR, Kim HS, Arroyo J, Berriman M, Abe K, Archer DB, Bermejo C, Bennett J, Bowyer P, Chen D, Collins M, Coulsen R, Davies R, Dyer PS, Farman M, Fedorova N, Fedorova N, Feldblyum TV, Fischer R, Fosker N, Fraser A, Garcia JL, Garcia MJ, Goble A, Goldman GH, Gomi K, Griffith-Jones S, Gwilliam R, Haas B, Haas H, Harris D, Horiuchi H, Huang J, Humphray S, Jimenez J, Keller N, Khouri H, Kitamoto K, Kobayashi T, Konzack S, Kulkarni R, Kumagai T, Lafon A, Latge JP, Li W, Lord A, Lu C, Majoros WH, May GS, Miller BL, Mohamoud Y, Molina M, Monod M, Mouyna I, Mulligan S, Murphy L, O'Neil S, Paulsen I, Penalva MA, Pertea M, Price C, Pritchard BL, Quail MA, Rabbinowitsch E, Rawlins N, Rajandream MA, Reichard U, Renauld H, Robson GD, de Rodriguez CS, Rodriguez-Pena JM, Ronning CM, Rutter S, Salzberg SL, Sanchez M, Sanchez-Ferrero JC, Saunders D, Seeger K, Squares R, Squares S, Takeuchi M, Tekaia F, Turner G, Vazquez de Aldana CR, Weidman J, White O, Woodward J, Yu JH, Fraser C, Galagan JE, Asai K, Machida M, Hall N, Barrell B, Denning DW (2005) Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. Nature 438:1151–1156

O'Gorman C (2011) Airborne *Aspergillus fumigatus* conidia: a risk factor for aspergillosis. Fungal Biol Rev 25(3):151–157

O'Gorman C, Fuller HT (2008) Prevalence of culturable airborne spores of selected allergenic and pathogenic fungi in outdoor air. Atmos Environ 42 (18):4355–4368

O'Hanlon KA, Cairns T, Stack D, Schrettl M, Bignell EM, Kavanagh K, Miggin SM, O'Keeffe G, Larsen TO, Doyle S (2011) Targeted disruption of nonribosomal peptide synthetase pes3 augments the virulence of *Aspergillus fumigatus*. Infect Immun 79:3978–3992

Oliver JD, Kaye SJ, Tuckwell D, Johns AE, Macdonald DA, Livermore J, Warn PA, Birch M, Bromley MJ (2012) The *Aspergillus fumigatus* dihydroxyacid dehydratase Ilv3A/IlvC is required for full virulence. PLoS One 7:e43559

Osherov N (2012) Interaction of the pathogenic mold *Aspergillus fumigatus* with lung epithelial cells. Front Microbiol 3:346

Paris S, Boisvieux-Ulrich E, Crestani B, Houcine O, Taramelli D, Lombardi L, Latge JP (1997) Internalization of *Aspergillus fumigatus* conidia by epithelial and endothelial cells. Infect Immun 65:1510–1514

Patterson K, Strek ME (2010) Allergic bronchopulmonary aspergillosis. Proc Am Thorac Soc 7:237–244

Penalver MC, O'Connor JE, Martinez JP, Gil ML (1996) Binding of human fibronectin to *Aspergillus fumigatus* conidia. Infect Immun 64:1146–1153

Perrin RM, Fedorova ND, Bok JW, Cramer RA, Wortman JR, Kim HS, Nierman WC, Keller NP (2007) Transcriptional regulation of chemical diversity in *Aspergillus fumigatus* by LaeA. PLoS Pathog 3:e50

Philippe B, Ibrahim-Granet O, Prevost MC, Gougerot-Pocidalo MA, Sanchez PM, Van der Meeren A, Latge JP (2003) Killing of *Aspergillus fumigatus* by alveolar macrophages is mediated by reactive oxidant intermediates. Infect Immun 71:3034–3042

Pollock JD, Williams DA, Gifford MA, Li LL, Du X, Fisherman J, Orkin SH, Doerschuk CM, Dinauer MC (1995) Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production. Nat Genet 9:202–209

Portugal RD, Garnica M, Nucci M (2009) Index to predict invasive mold infection in high-risk neutropenic patients based on the area over the neutrophil curve. J Clin Oncol 27:3849–3854

Ramirez-Ortiz ZG, Lee CK, Wang JP, Boon L, Specht CA, Levitz SM (2011) A nonredundant role for plasmacytoid dendritic cells in host defense against the human fungal pathogen *Aspergillus fumigatus*. Cell Host Microbe 9:415–424

Romani L (2011) Immunity to fungal infections. Nat Rev Immunol 11:275–288

Schrettl M, Bignell E, Kragl C, Joechl C, Rogers T, Arst HN Jr, Haynes K, Haas H (2004) Siderophore biosynthesis but not reductive iron assimilation is essential for *Aspergillus fumigatus* virulence. J Exp Med 200:1213–1219

Schrettl M, Kim HS, Eisendle M, Kragl C, Nierman WC, Heinekamp T, Werner ER, Jacobsen I, Illmer P, Yi H, Brakhage AA, Haas H (2008) SreA-mediated iron regulation in *Aspergillus fumigatus*. Mol Microbiol 70:27–43

Schrettl M, Beckmann N, Varga J, Heinekamp T, Jacobsen ID, Jochl C, Moussa TA, Wang S, Gsaller F, Blatzer M, Werner ER, Niermann WC, Brakhage AA, Haas H (2010) HapX-mediated adaption to iron starvation is crucial for virulence of *Aspergillus fumigatus*. PLoS Pathog 6:e1001124

Sharon H, Hagag S, Osherov N (2009) Transcription factor PrtT controls expression of multiple secreted proteases in the human pathogenic mold *Aspergillus fumigatus*. Infect Immun 77:4051–4060

Sharon H, Amar D, Levdansky E, Mircus G, Shadkchan Y, Shamir R, Osherov N (2011) PrtT-regulated proteins secreted by *Aspergillus fumigatus* activate MAPK signaling in exposed A549 lung cells leading to necrotic cell death. PLoS One 6:e17509

Sheppard DC (2011) Molecular mechanism of *Aspergillus fumigatus* adherence to host constituents. Curr Opin Microbiol 14:375–379

Sheppard DC, Doedt T, Chiang LY, Kim HS, Chen D, Nierman WC, Filler SG (2005) The *Aspergillus fumigatus* StuA protein governs the up-regulation of a discrete transcriptional program during the acquisition of developmental competence. Mol Biol Cell 16:5866–5879

Shibuya K, Takaoka M, Uchida K, Wakayama M, Yamaguchi H, Takahashi K, Paris S, Latge JP, Naoe S (1999) Histopathology of experimental invasive pulmonary aspergillosis in rats: pathological comparison of pulmonary lesions induced by specific virulent factor deficient mutants. Microb Pathog 27:123–131

Song E, Jaishankar GB, Saleh H, Jithpratuck W, Sahni R, Krishnaswamy G (2011) Chronic granulomatous disease: a review of the infectious and inflammatory complications. Clin Mol Allergy 9:10

Sorci G, Giovannini G, Riuzzi F, Bonifazi P, Zelante T, Zagarella S, Bistoni F, Donato R, Romani L (2011) The danger signal S100B integrates pathogen- and danger-sensing pathways to restrain inflammation. PLoS Pathog 7:e1001315

Spikes S, Xu R, Nguyen CK, Chamilos G, Kontoyiannis DP, Jacobson RH, Ejzykowicz DE, Chiang LY, Filler SG, May GS (2008) Gliotoxin production in *Aspergillus fumigatus* contributes to host-specific differences in virulence. J Infect Dis 197:479–486

Steele C, Rapaka RR, Metz A, Pop SM, Williams DL, Gordon S, Kolls JK, Brown GD (2005) The beta-glucan receptor dectin-1 recognizes specific morphologies of *Aspergillus fumigatus*. PLoS Pathog 1:e42

Steinbach WJ, Cramer RA Jr, Perfect BZ, Asfaw YG, Sauer TC, Najvar LK, Kirkpatrick WR, Patterson TF, Benjamin DK Jr, Heitman J, Perfect JR (2006) Calcineurin controls growth, morphology, and pathogenicity in *Aspergillus fumigatus*. Eukaryot Cell 5:1091–1103

Sugui JA, Pardo J, Chang YC, Zarember KA, Nardone G, Galvez EM, Mullbacher A, Gallin JI, Simon MM, Kwon-Chung KJ (2007) Gliotoxin is a virulence factor of *Aspergillus fumigatus*: gliP deletion attenuates virulence in mice immunosuppressed with hydrocortisone. Eukaryot Cell 6:1562–1569

Suh MJ, Fedorova ND, Cagas SE, Hastings S, Fleischmann RD, Peterson SN, Perlin DS, Nierman WC, Pieper R, Momany M (2012) Development stage-specific proteomic profiling uncovers small, lineage specific proteins most abundant in the *Aspergillus fumigatus* conidial proteome. Proteome Sci 10:30

Swan J, Gilbert E, Kelsey A, Crook B (2013) Occupational and environmental exposure to bioaerosols from composts and potential health effects - a critical review of published data. HSE Books, Sudbury

Tell LA (2005) Aspergillosis in mammals and birds: impact on veterinary medicine. Med Mycol 43 (Suppl 1):S71–S73

Teutschbein J, Albrecht D, Potsch M, Guthke R, Aimanianda V, Clavaud C, Latge JP, Brakhage AA, Kniemeyer O (2010) Proteome profiling and functional classification of intracellular proteins from conidia of the human-pathogenic mold *Aspergillus fumigatus*. J Proteome Res 9:3427–3442

Thau N, Monod M, Crestani B, Rolland C, Tronchin G, Latge JP, Paris S (1994) Rodletless mutants of *Aspergillus fumigatus*. Infect Immun 62:4380–4388

Thywissen A, Heinekamp T, Dahse HM, Schmaler-Ripcke J, Nietzsche S, Zipfel PF, Brakhage AA (2011) Conidial dihydroxynaphthalene melanin of the human pathogenic fungus *Aspergillus fumigatus* interferes with the host endocytosis pathway. Front Microbiol 2:96

Torpy FR, Irga PJ, Brennan J, Burchett MD (2012) Do indoor plants contribute to the aeromycota in city buildings? Aerobiologia (in press) doi: 10.1007/s10453-012-9282-y

Tronchin G, Bouchara JP, Ferron M, Larcher G, Chabasse D (1995) Cell surface properties of *Aspergillus fumigatus* conidia: correlation between adherence, agglutination, and rearrangements of the cell wall. Can J Microbiol 41:714–721

Tronchin G, Esnault K, Renier G, Filmon R, Chabasse D, Bouchara JP (1997) Expression and identification of a laminin-binding protein in *Aspergillus fumigatus* conidia. Infect Immun 65:9–15

Tsai HF, Wheeler MH, Chang YC, Kwon-Chung KJ (1999) A developmentally regulated gene cluster involved in conidial pigment biosynthesis in *Aspergillus fumigatus*. J Bacteriol 181:6469–6477

Wasylnka JA, Moore MM (2000) Adhesion of *Aspergillus* species to extracellular matrix proteins: evidence for involvement of negatively charged carbohydrates on the conidial surface. Infect Immun 68:3377–3384

Wasylnka JA, Moore MM (2002) Uptake of *Aspergillus fumigatus* conidia by phagocytic and nonphagocytic cells in vitro: quantitation using strains expressing green fluorescent protein. Infect Immun 70:3156–3163

Wasylnka JA, Moore MM (2003) *Aspergillus fumigatus* conidia survive and germinate in acidic organelles of A549 epithelial cells. J Cell Sci 116:1579–1587

Wasylnka JA, Simmer MI, Moore MM (2001) Differences in sialic acid density in pathogenic and nonpathogenic *Aspergillus* species. Microbiology 147:869–877

Willger SD, Puttikamonkul S, Kim KH, Burritt JB, Grahl N, Metzler LJ, Barbuch R, Bard M, Lawrence CB, Cramer RA Jr (2008) A sterol-regulatory element binding protein is required for cell polarity, hypoxia adaptation, azole drug resistance, and virulence in *Aspergillus fumigatus*. PLoS Pathog 4: e1000200

Xue T, Nguyen CK, Romans A, May GS (2004) A mitogen-activated protein kinase that senses nitrogen regulates conidial germination and growth in *Aspergillus fumigatus*. Eukaryot Cell 3:557–560

Zhao W, Panepinto JC, Fortwendel JR, Fox L, Oliver BG, Askew DS, Rhodes JC (2006) Deletion of the regulatory subunit of protein kinase A in *Aspergillus fumigatus* alters morphology, sensitivity to oxidative damage, and virulence. Infect Immun 74:4865–4874

Zmeili OS, Soubani AO (2007) Pulmonary aspergillosis: a clinical update. QJM 100:317–334

3 Systems Biology Approaches to Understanding and Predicting Fungal Virulence

Lanay Tierney[1], Katarzyna Tyc[2], Edda Klipp[2], Karl Kuchler[1]

CONTENTS

Lanay Tierney and Katarzyna Tyc contributed equally.
[1]Christian Doppler Laboratory Infection Biology, Max F. Perutz Laboratories, Medical University of Vienna, 1030 Vienna, Austria; e-mail: karl.kuchler@meduniwien.ac.at
[2]Institute of Biology, Theoretical Biophysics, Humboldt-Universität zu Berlin, Invalidenstrasse 42, 10115 Berlin, Germany; e-mail: edda.klipp@biologie.hu-berlin.de

I. Introduction: Fungi as Model Organisms for SysBio Studies

Systems Biology (SysBio) is an overarching term used to describe a collection of methods that attempt to understand biological systems in a quantitative fashion, primarily using experimental data to formulate mathematical models that enable the prediction of the future behavior of a system or emergent properties in response to environmental stimuli. A **"system"** in this context can be defined as any group of interacting biological components that either directly or indirectly perform a specific and measurable function; it can include a small or large number of components. For example, a protein complex, a regulatory pathway, an organ, a cell or the entire organism could all be systems in different experimental contexts. This field is a recent addition to the scientific community. Although its exact origins are difficult to pinpoint, many ascribe its beginnings to the late 1960s when the first attempts to investigate metabolic pathways in bacterial cells were performed (von Bertalanffy 1969). The formulation of the metabolic control theory (MCT) by the groups of Kacser and Burns (1973) and Heinrich and Rapoport (1974) marks the birth of SysBio. MCT mathematically describes metabolic flux as an inherent system property, and that the flux control through different reactions is shared within the system, influencing one another.

"Scientific fields, like species, arise by descent with modification" (Kirschner 2005), and tellingly, to date, a consensus definition of what SysBio concretely means is intensely debated. Nevertheless, SysBio has some key

Human Fungal Pathogens, 2nd Edition
The Mycota XII
O. Kurzai (Ed.)

characteristics that differentiate it from classical reductionist-driven biological studies. Historically, scientific experiments have taken a reductionist approach, investigating a single gene under a single condition. However, biological systems are dynamic and nonlinear in nature, making the reductionist approach often unsuitable for investigating the behavior of a gene of interest. **A primary goal of SysBio is to understand the wiring and connectivity in biological networks that define genotype–phenotype relations** (Kohl et al. 2010). A "systems approach," sometimes referred to as the "desk–bench–desk loop" (Mustacchi et al. 2006), combines computational and experimental tools to formulate a model based on prior knowledge of the system (i.e., from published literature, databases, and genome-wide data sets). This model is then tested under laboratory conditions and analyzed for biological insights that would have been otherwise difficult to gain without an interdisciplinary approach (Fig. 3.1). The recent introduction of whole-genome sequencing and genome-wide experimental platforms such as RNA sequencing (RNA-seq), metabolomics, microarray, and mass spectrometry (MS), among several others, form the core "omics-based" methodologies for data generation that drive SysBio studies.

The integration of data from these various technologies into SysBio models has remained a formidable challenge. Hence, SysBio approaches have been classified as "top-down," "bottom-up," or "middle-out" (Bray 2003; O'Malley and Dupre 2005; Bruggeman and Westerhoff 2007; Petranovic and Nielsen 2008). A **top-down** approach aims at extracting principles from experimental data representing molecular properties of the studied system. A top-down approach focuses on the comparison of genome-wide data sets, such as transcriptome and proteome, to formulate a focused and testable biological hypothesis. A **bottom-up** approach uses the knowledge of molecular properties of the system components to predict the behavior of the system as a whole. In short, the bottom-up approach connects smaller entities to predict, identify, and simulate the behavior of a bigger system. A bottom-up approach usually starts with prior knowledge of a specific gene, and a model is then generated based on these data to investigate the system as a whole. A **middle-out** approach describes a process of starting at the level for which the best information for the process of interest is available, and then combining higher and lower levels of structural and functional information, essentially breaking out of a more strict top-down and bottom-up loop in order to validate the hypothesis at the current state of biological understanding (Brenner et al. 2001). Regardless of the approach used, SysBio strategies differentiate themselves from more classical biological methods by consciously taking into consideration different levels and dynamics of biological data (DNA, RNA, protein) simultaneously.

Fungal SysBio was ushered in with the completion of the *Saccharomyces cerevisiae* genome sequence, the first completely sequenced eukaryotic genome (Goffeau et al. 1996). Since then, the genomes of many of the most common fungal pathogens including, but not limited to, *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans*, have become available (Loftus et al. 2005; Nierman et al. 2005; van het Hoog et al. 2007). Community-wide initiatives such as the Fungal Genome Initiative (Cuomo and Birren 2010) and the 1,000 Fungal Genomes Project (http://1000.fungalgenomes.org/) have been useful tools for studying the evolution of fungal virulence. The discovery of key genes positively and negatively regulated during the infection process, and understanding the function of their products, will drive the design of new strategies to combat fungal pathogens.

In this chapter, we provide a comprehensive overview of recent SysBio methods suitable for study of fungal virulence, including genome sequencing, -omics technologies, and bioinformatics tools, with an emphasis on computational and modeling-based approaches. We focus on the genera *Candida* and *Saccharomyces*; the latter stands out as a "workhorse" of fungal SysBio in which many of the methods described herein were originally established or tested (Mustacchi et al. 2006; Santamaria et al. 2011). These approaches are used to identify molecular wiring and dynamics in biological networks, with the goal of identifying their biological function and eventually identifying novel therapeutic options. We describe the

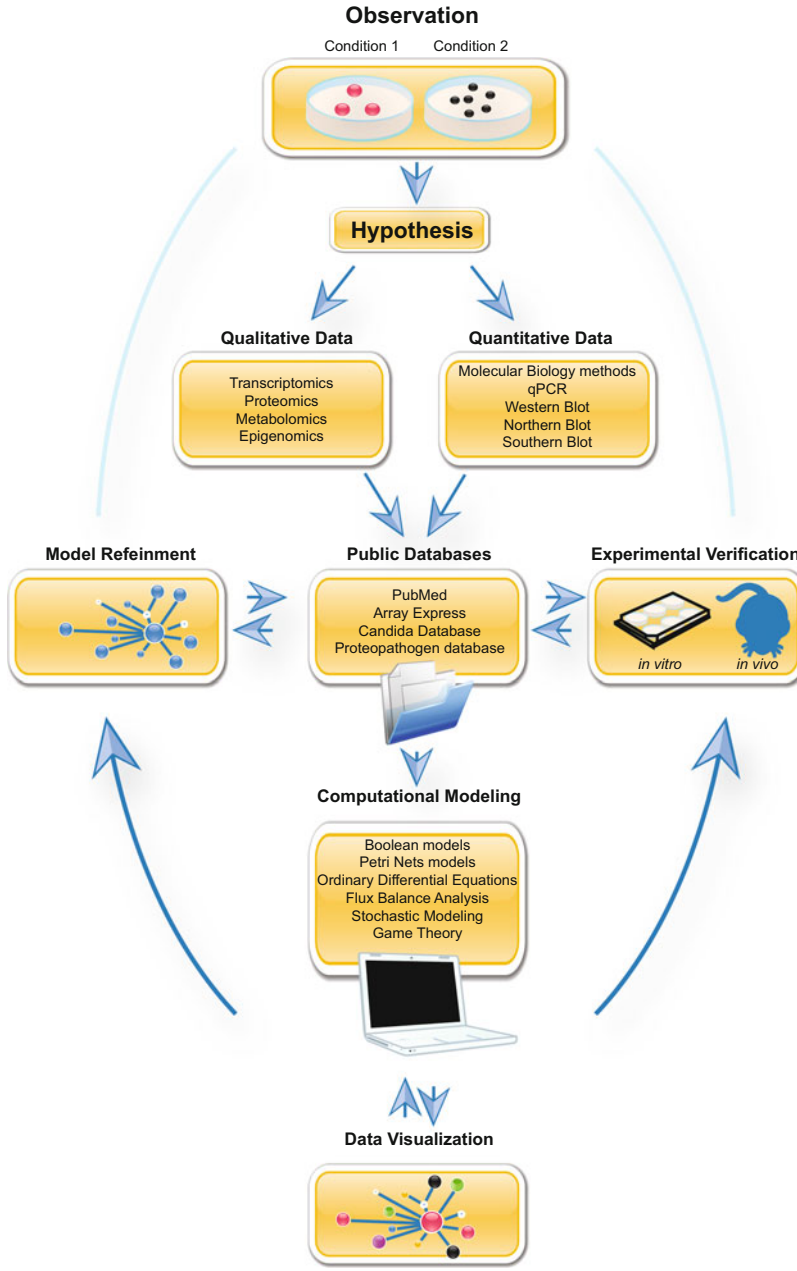


Fig. 3.1. The "bench–desk–bench" loop of SysBio. A typical SysBio workflow. Based on an observation, such as a difference in yeast colony size and phenotype under two different conditions, a hypothesis is formed based on the experimental results and further supported by prior knowledge in published literature. This hypothesis can be addressed using a number of qualitative and quantitative methods, the results of which are deposited in publically available databases. With these data, modeling approaches attempt to mimic, predict, and visualize data. Once modeled, experimental verification and refinement of the model, creates the bench–desk–bench loop, where iterative cycles of prediction and verification are undertaken until the model and experiment validation are representative of one another

applicability of each method to specific experimental questions using numerous case examples and critically discuss some of the current pitfalls in the analysis of SysBio data sets.

II. High-Throughput and –Omics-Based Methods for Studying Fungal Virulence

SysBio approaches, especially top-down analyses, incorporate genome-wide data sets such as comparative genomics, transcriptomics, and proteomics data. These approaches fit into the category of "–omics" or "–ome" studies, which attempt to analyze a genome-wide response to a specific condition. –Omics studies represent an important shift in the way biological data is both produced and interpreted, complementing traditional hypothesis-driven research (Weinstein 2001). In order to understand SysBio as a whole, it is important to understand the types of data sets that it utilizes to address a given question. Several important methods have established themselves in this field over the past decade and have been used extensively to investigate fungal virulence. For clarity, we have divided these methods into qualitative and quantitative approaches. We address some of the most popular methods used at different levels of biological understanding, including DNA, RNA, and protein, as well as epigenetic modifications and validation methods, and examine the key contributions they have made to the understanding of fungal virulence.

A. Genomics

The initial genomic sequencing of *S. cerevisiae* was a monumental international collaboration that included some 600 scientists worldwide (Goffeau et al. 1996).

This sequencing was performed using a series of hybrid plasmids, called "cosmids." Cosmids had the advantage that a much longer DNA sequence stretches could be incorporated than using normal plasmids, and at the same time longer DNA stretches could be sequenced to build up the genomic library. Sequencing polymerase chain reaction (PCR) fragments then filled the remaining gaps between sequence stretches of the assembled genomic library to complete the genome (Dujon 1993). The *Candida* Genome Sequencing project began directly after the *S. cerevisiae* sequencing in 1996, ending in 2004 with the *C. albicans* genome assembly known as Assembly 19 (Jones et al. 2004). This genome assembly was divided into 412 contigs (consensus stretches of DNA that are assembled to form the scaffold of the genome assembly) and sequenced with a shotgun-based sequencing strategy. In order to obtain a more complete view of the diploid sequence, Assembly 21 was created using a fosmid library, which is conceptually similar to a cosmid library, except that it is based instead on a bacterial F-plasmid and is more stable than a cosmid because of its low copy number (Hall 2004). These early sequencing projects took years because of the low throughput.

Today, genome and transcriptome sequencing has become routine, with ever-increasing stability, coverage (several fungal genomes can now be sequenced in a couple of days), and bioinformatics assembly tools publically available. As DNA sequencing technologies have become more efficient, there has been a surge in the number of sequenced genomes, with over 150 fungi sequenced so far (Marcet-Houben and Gabaldon 2009). These sequences facilitate functional and comparative genomics studies. **Functional genomics** aims to understand relationships between genotype and phenotype. **Comparative genomics** attempts to identify genes or genetic rearrangements between closely related species based on their DNA sequence; in the case of fungal pathogens, this often includes a highly virulent species compared to a significantly less or even avirulent species. This is done in order to identify genetic transitions that might explain the evolutionary divergence of pathogens or the identification of novel virulence factors.

Comparative genomics studies use two main techniques: comparative whole genome sequencing or hybridization-based microarrays. Comparative whole genome sequencing literally attempts to identify genetic elements present in one species and absent in another based on the genome sequence; this is done by overlapping the genome sequences and identifying outlier sequence stretches that do not match between them. Comparative genomic hybridization (CGH) arrays identify genome-wide variation in gene copy number. CGH experiments assume that the binding

ratio of the experimental sample to the control is proportional to the sequencing concentration in the samples. These methods provided significant insight into the evolution of pathogenicity for many fungal species. For example, early comparative studies of fungi identified a strong sequence homolog among 228 genes in *S. cerevisiae*, *Schizosaccharomyces pombe*, *Aspergillus niger*, *Magnaporthe grisea*, *C. albicans* and *Neurospora crassa* genomes for which no homology was found in the human or mouse genomes, representing potential targets for pan-fungal treatment (Braun et al. 2005).

Numerous studies have investigated the evolution of pathogenicity within a single fungal clade. For example, in the *Candida* clade, eight genomes were sequenced, including the *C. albicans* WO-1 strain (which is characterized for white-opaque switching and is associated with specificity to host tissues), along with the de novo sequencing of *C. tropicalis* and *C. parapsilosis, Lodderomyces elongisporus, C. guilliermondii* and *C. lusitaniae,* many of which are now classified as emerging fungal pathogens (Butler et al. 2009). These strains were compared to the previously sequenced genomes of *C. albicans* clinical isolate SC5314, the marine yeast *Debaryomyces hansenii*, and species from the *Saccharomyces* clade. Some 21 gene families emerged that were enriched in pathogenic species as compared to nonpathogenic fungi. A related study investigated the closest known relative of *C. albicans*, *C. dubliniensis*, which, despite its similarities, is significantly less virulent than *C. albicans*. Comparative sequence analysis has identified almost 200 species-specific genes in *C. albicans*, including the absence of the key *C. albicans* invasion gene *ALS3* in *C. dubliniensis*, and members of the aspartyl proteinase family *SAP4* and *SAP5* (Jackson et al. 2009). ALS3 is among the most important virulence factors in *C. albicans*. It is a cell surface protein that plays a major role in adhesion to host cells and in maintenance of infection (Hoyer 2001; Hoyer et al. 2008). Notably, numerous translocations were identified in *C. dubliniensis,* especially in the SAP family, which is known to play a role in *Candida* pathogenesis. Comparative genomics has even lent itself to the investigation of genetic variations at chromosome level, using a single *C. albicans* isolate that had been passaged multiple times in an in vivo model organism using CGH (Forche et al. 2009), showing the environmental impact on the host strain evolution. Together, these studies collectively demonstrate that even closely related species have significantly diverged at their genomic levels, suggesting mechanisms for the evolution of fungal virulence factors.

A number of resources for fungal genomics research have recently been made available. Large genome databases, including the Broad Institute (http://www.broad.mit.edu/annotation/fgi/), the Sanger Center (http://www.sanger.ac.uk/Projects/Fungi/), the Institute for Genomic Research (http://www.tigr.org/tdb/fungal/), and the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/genomes/FUNGI/funtab.html), are all publically available.

B. Transcriptomics

Looking at the DNA level, one can investigate how the information stored in the genetic code is translated into protein molecules; however, it does not provide information on the diverse molecules actually produced in the cell normally or in response to different environmental conditions. This takes place at the RNA level by looking at the complete set of RNA species produced in a given population of cells at a specific time. This field is referred to as **transcriptomics.**

There are two main methods to investigate transcriptional dynamics, also known as expression profiling. These include genome-wide **microarrays** and, more recently, **next-generation sequencing** (NGS) technologies, most notably, RNA-seq. Microarray experiments employ special microarray chips carrying printed copies of the entire genome and are used for assessing the relative differences in gene expression between a control sample and a treated sample. A microarray chip is, in principle, able to measure the relative changes in expression levels of all known genes simultaneously. Southern blotting was the inspiration for the microarray technology (Maskos and Southern 1992). Southern blotting involves the

hybridization of a DNA probe to a specific DNA fragment on a solid substrate. Microarrays use the same principle but cover a genome-wide scale rather than single genes. The chip itself is made up of probes, corresponding to short DNA stretches for all genes in the genome. Depending on the specific experimental question, there may be multiple probes for each gene of interest and the number of spotted probes is often well into the thousands. In a typical microarray experiment, RNA is collected from an organism of interest, transcribed into cDNA (the sample is also referred to as the "target"), and hybridized to the chip where the target forms hydrogen bonds with the probes. In order to determine the relative abundance of the transcripts, the chip is then scanned with the hybridized sample. In theory, if a gene is expressed in the organism, it will have hybridized to the probe on the chip. The abundance of a gene product is then measured by the detection of chemiluminescent-labeled targets. Based on the intensity of the target–probe hybridization, the relative abundance of the RNA produced by the organism in response to a condition can be measured. Because microarray technology is chip-based, its ability to detect a specific gene or transcript is limited to the original spotting on the chip itself. This is especially important to keep in mind for certain organisms, where only incomplete genomic sequences are available, leading to low quality annotations and unknown alternative spliced products, which would remain undetected if not already taken into account in the original design of the microarray.

The first genome-wide array was developed for *S. cerevisiae* in 1997 (Lashkari et al. 1997). Since then, microarrays for fungi have evolved into high-density tiling arrays (Sellam et al. 2010) and splicing-sensitive exon-junction arrays (Inada and Pleiss 2010), among many others. Microarray technology has been extensively used to investigate global changes in gene expression in response to changing environmental conditions and genetic knockouts. It has also been used in conjunction with immune cell and animal models at different infections stages in vitro and in vivo to investigate different infection stages. For example, the transcriptional response of both *S. cerevisiae* and *Candida glabrata* to antifungal agents and other chemical stress agents in vitro was profiled (Lelandais et al. 2008). To identify pathogen-specific responses on the side of *C. glabrata*, the authors compared the transcriptional profile of both species after treatment. Surprisingly, they found a high conservation among the regulated genes during infection, and a subpopulation of genes that were pathogen-specific. Further, in vitro infection experiments of human blood cells with *C. albicans* identified a number of differentially expressed genes, which may be important in the survival of *Candida* during bloodstream infections (Fradin et al. 2003). Transcriptional profiling has been used to identify effects of phagocytosis of *C. albicans* by immune cells, including neutrophils (Rubin-Bejerano et al. 2003) and macrophages (Lorenz et al. 2004). These studies identified the extent of the amino-acid-deficient environment within the phagosome, and characterized the dynamic starvation response of *Candida* over the time course of infection. The first dual transcriptional profiling using microarrays for a host and pathogen interaction was also performed with conidia of *A. fumigatus* during infection of human airway epithelial cells. This work confirmed the upregulation of inflammatory interleukin (IL)-6 and the immune response to conidia, as well as pathways whose activation had previously only been investigated from either the host or pathogen perspective alone (Oosthuizen et al. 2011).

Transcription profiling using **RNA-seq** is conceptually similar to microarray, insofar as the end result of the experiment is often a list of differently expressed genes. However, the sample is sequenced using a parallel sequencing approach referred to as next-generation sequencing (NGS) instead of using hybridization-based methods. Based on Sanger sequencing methods, high-throughput technology began with tag-based methods that were developed so that multiple sequencing reactions could be run in parallel. These included serial analysis of gene expression (SAGE) (Velculescu et al. 1995), cap analysis of gene expression (CAGE) (Kodzius et al. 2006) and massive parallel signature sequencing (MPSS) (Reinartz et al. 2002). In order to increase the scale of reactions taking place, a number of novel sequencing strategies and commercially available platforms have been developed. These included Roche/454, Illumina/Solexa, Life/APG Helicos BioSciences, and Pacific Biosciences. Each system has pros and cons, depending on the biological application (Metzker 2010).

In a typical RNA-seq experiment, cDNA is first fragmented; these templates are then attached to a substrate

(which will vary with the technology used) with the aid of adaptor sequences. The immobilization of the template samples gives the advantage of allowing billions of simultaneous sequencing reactions, differentiating itself from first generation sequencing technology in terms of capacity and cost (Metzker 2010). Templates can be sequenced either from one end (single-end sequencing) or both ends (paired-end sequencing). The resulting sequencing reads can vary in length, depending on the technology used, from less than 30 bp to over 300 bp (Wang et al. 2009; Metzker 2010). Reads are then mapped back to the reference genome to determine gene expression and, when compared to other samples, differential gene expression.

RNA-seq has rapidly gained in broad popularity over the past few years, especially because of its ability to sequence to a high depth and also because it detects low abundance transcripts, offering a more complete view of the transcriptional profile of an organism than microarrays.

The sequencing technology has significant advantages over microarray, especially for non-model organism species, **as the detection of expressed genes is not dependent on having a priori knowledge of the gene investigated.** Moreover, RNA-seq does not have intrinsic limitations to the dynamic range of detection (Royce et al. 2007). RNA-seq has been especially important in the detection of novel noncoding RNA species and small RNAs, as well as for de novo annotation (Wang et al. 2009). Under in vitro conditions, a de novo annotation of the *C. albicans* transcriptome under nine different environmental conditions was recently performed and was able to identify over 600 novel transcriptionally active regions and introns from a total of 177 million uniquely mapped reads (Bruno et al. 2010). Similarly, with *A. fumigatus*, RNA-seq was used to investigate planktonic and biofilm growth to identify differences in pathological and morphological characteristics in these two stages. Numerous biofilm-specific genes were identified as being regulated, representing targets for biofilm development.

Most recently, the first **dual-species RNA-seq** approach, sequencing RNA mixture comprising both host and fungal pathogen transcriptomes over a time course of infection, has been accomplished. Furthermore, this study predicted, using mathematical approaches, and experimentally verified novel host–pathogen regulatory networks implicated in the interaction. The use of a **combination of sequence analysis and network inference enabled this dual-systems approach** (Tierney et al. 2012). This study presents the first adaptation of network inference to model host–pathogen interactions, validating the use of network inference for the analysis of multiple species data sets.

1. Clustering Gene Expression Data Sets

The most common output of transcriptomics is a list of differentially expressed genes in one condition versus a control condition. Differential gene expression analysis begins with a testing for the statistical significance of the variation within the sample. Statistical approaches for determining differential expression have been extensively reviewed elsewhere (Cui and Churchill 2003), as have a number of freely available tools to aid in statistical analysis for both microarray (Steinhoff and Vingron 2006) and RNA-seq data sets (Sun and Zhu 2012). This method reduces a genome-wide comparison down to only those genes significantly affected under a specific condition. Convenient analysis pipelines, especially for RNA-seq data, have been recently created to help non-computational biologists in the analysis of sequencing from the raw data file to a list of differentially expressed genes (Oshlack et al. 2010; Garber et al. 2011). High-dimensionality data such as microarray or RNA-seq samples complicate data analysis due to the inequality of variables measured compared to the sample number. Because the list of differentially expressed genes can still be on an order of magnitude of several hundred genes, additional **methods to reduce complexity** are often necessary.

Partitioning expression data into subgroups of genes, called clusters, facilitates data visualization and interpretation underlying a biological process of interest. Depending on the approach used, the groupings can then be visualized by scatter plots, histograms, dendrograms, or heat maps. Genes are clustered into specific categories, which can be functional,

structural, temporal, or a combination of the above. A number of clustering approaches have been developed, including principle component analysis (PCA), hierarchical clustering, fuzzy clustering, biclustering, and mutual information analysis, each of which tackle different potential bias aspects of the data set (Eisen et al. 1998; Kerr et al. 2008).

PCA identifies data trends within samples, called principal components, such that very large data sets can be graphically represented using a smaller number of dimensions (Ringner 2008). This technique is especially useful for visually identifying batch effects or noise between samples, which may otherwise negatively affect downstream analysis.

Hierarchical clustering aims to create a hierarchy of gene groups, whereby relationships among genes are represented by a dendrogram. The shorter the length of dendrogram branches between objects, the more closely related the gene expression patterns are. These differences are assessed by pair-wise similarity functions. In this way, the method builds a hierarchy of gene groups by progressively merging clusters (Eisen et al. 1998). One of the major limitations of hierarchical clustering is that the decision-making for gene assignments is focused locally, without considering the data set as a whole, which can affect downstream interpretation (Tamayo et al. 1999).

Fuzzy clustering (also referred to as **soft clustering**) was developed to partially counteract the local bias of hierarchical clustering approaches. Fuzzy clustering allows for data elements to simultaneously belong to multiple groups with respect to a given criteria. Each data element has a "degree of belonging" to a cluster, instead of being assigned to an individual cluster and this degree represents how close the fit is in multiple clusters (Dembele and Kastner 2003; Fu and Medico 2007). This is in contrast to **hard clustering**, where data elements only are allowed to belong to one group.

Some of the newest clustering approaches have attempted to incorporate prior biological knowledge into the clustering algorithm. This has been attempted with a form of **biclustering** (Madeira and Oliveira 2004), a matrix-based clustering approach that includes both genes and conditions in the algorithm. One example algorithm, called cMonkey, was used to identify and cluster sequence motifs in *Helicobacter pylori*, *S. cerevisiae*, and *Escherichia coli* based on microarray data sets (Reiss et al. 2006). A similar clustering approach that incorporates prior knowledge, called **mutual information analysis**, has also been shown to identify transcriptional interactions with a high fidelity in mammalian cells (Margolin et al. 2006). Finally, a number of standardized tools and analysis techniques are already publically available (Table 3.1) to facilitate transcriptional data analysis. To date, they have been able to provide detailed views of changing transcriptional landscapes in response to different environmental conditions on a functional level, and have been highly beneficial for the identification and prediction of virulence factors in fungi.

C. Proteomics

The term "proteome" was coined in 1996 to describe the complete set of proteins that is synthesized by a cell (Wilkins et al. 1996). The proteome provides the highest level of functional information of a cell, revealing the end product of the transcription and downstream transcriptional processing. The use of proteomics data sets is also becoming a popular approach for studying proteins involved in virulence.

The major areas in proteomics research include identification of proteins and their posttranslational modifications as well as protein–protein interactions.

These areas are investigated using two main methods, traditional two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and, increasingly, mass spectrometry (MS). In **2D-PAGE**, protein samples are resolved by two intrinsic properties: first in one dimension on an SDS gel, and then in the second dimension, at a 90° rotation (O'Farrell 1975); These properties can include their isoelectric point, protein complex mass in the native state, and protein mass. The properties chosen will depend on the specific experiment. Proteins are then visualized by staining of gels, often using silver, Coomassie Blue or Ponceau S staining techniques. Once visible, spots can then be picked out by hand or more often using automated detection software based on

Table 3.1. OMICS resources

-Omics	Methods	Standards	Databases	Analysis resources and tools	Fungal-specific resources
Genomics	CGA, NGS	MIGS	**1000 Genomes** http://www.1000genomes.org	**ClustalW2** http://www.ebi.ac.uk/Tools/msa/clustalw2	***Candida* Database** http://www.candidagenome.org
			ENSEMBL http://www.ensembl.org	**UCSC Genome Browser** http://genome.ucsc.edu	***Aspergillus* Database** http://www.aspgd.org
			GOLD http://www.genomesonline.org	**IGV** http://www.broadinstitute.org/igv	***Saccharomyces* Database** http://www.yeastgenome.org
Transcriptomics	Microarray, RNA-seq	MIAME, MINSEQE	**Array Express** www.ebi.ac.uk/arrayexpress	**Galaxy** https://main.g2.bx.psu.edu	**YEASTRACT** http://www.yeastract.com
			GEO www.ncbi.nlm.nih.gov/geo	**Bioconductor** http://www.bioconductor.org	**Filamentous Fungal Gene Expression Database** http://bioinfo.townsend.yale.edu
Proteomics	2D-PAGE, MS	MIAPE	**PRIDE** http://www.ebi.ac.uk/pride	**InterProScan** http://www.ebi.ac.uk/Tools/pfa/iprscan	**Proteopathogen** http://proteopathogen.dacya.ucm.es/
			MassBank http://www.massbank.jp	**APEX** http://pfgrc.jcvi.org/index.php/bioinformatics/apex	**NetPhosYeast** http://www.cbs.dtu.dk/services/NetPhosYeast
Metabolomics	NMR, HPLC-MS	MIAMET, CIMR	**HMDB** http://www.hmdb.ca	**Arcadia** http://arcadiapathways.sourceforge.net	**YEASTNET** http://www.comp-sys-bio.org/yeastnet
			GOLM http://gmd.mpimp-golm.mpg.de	**MetaboAnalyst** http://www.metaboanalyst.ca	**FunSecKB** http://proteomics.ysu.edu/secretomes/fungi
Epigenomics	ChIP-chip, ChIP-seq	MIAME, MINSEQE	**Roadmap** http://www.roadmapepigenomics.org	**STAR Genome Browser** http://tabit.ucsd.edu/sdec	**ChromatinDB** http://www.bioinformatics2.wsu.edu/ChromatinDB
			ENDCODE http://genome.ucsc.edu/ENCODE	**RMAP** http://rulai.cshl.edu/rmap	**Nucleosome Acetylation and Methylation in Yeast** http://younglab.wi.mit.edu/nucleosome

The table includes a nonexhaustive list of -omics methods and resources with an emphasis on those available for fungi. Reporting standards are abbreviated as follows with their corresponding reference: *MIGS* minimum information about a genome sequence (Field et al. 2008); *MIAME* minimum information about a microarray experiment (Brazma et al. 2001); *MINSEQE* minimum information about a high-throughput sequencing experiment (http://www.mged.org/ minseqe/); *MIAPE* minimum information about a proteomics experiment (Taylor et al. 2007); *MIAMET* minimum information about a metabolomics experiment (Bino et al. 2004); and *CIMR* core information for metabolomics reporting (http://msi-workgroups.sourceforge.net/).

their location on the gel. The identified spots are then excised, proteolytically digested, and then subjected to MS analysis. Briefly, **MS measures the mass-to-charge ratio of charged particles such as peptides**, and this information can then be used to identify the composition of the peptide and the gene it is derived from. Experimentally, MS samples are first vaporized and then ionized using an electron beam. The produced ions are then detected by the mass analyzer, which sorts the ions by their masses, and then processed into mass spectra where the detector measures the quality and quantity of the ions present. Variations of MS, including liquid chromatography tandem MS (LC-MS/MS) (Yates et al. 1999) and gel-free proteomics techniques (2012; Stastna and Van Eyk 2012) are also widely used approaches. These facilitate the analysis of proteins that are not easily separated in 2D gels due to their high hydrophobicity or high molecular weight, as in the case of many integral membrane proteins (Aebersold and Mann 2003; de Godoy et al. 2008). MS/MS involves additional rounds of ionization; however, the reproducibility between technical replicates of a sample remains in the range of 35–60% overlap (Tabb et al. 2010). Unfortunately, absolute protein quantification remains out of reach at the moment (Peng et al. 2012). Major hurdles remain to improve the reproducibility and standardization of the MS-based methods (Kniemeyer et al. 2011). Nonetheless, since 1996, the percentage of protein-coding genes in *S. cerevisiae* for which some biological function has been identified has increased to over 80%, greater than for any other sequenced eukaryotic genome (Botstein and Fink 2011). Proteomics studies have been highly beneficial in achieving this.

Proteomics approaches have led to the identification of a number of fungal virulence factors. Using an in vitro approach, the proteome of *C. albicans* yeast-form cells in the exponential or stationary growth phase was investigated in response to nutrient limitation using 2D-PAGE. The authors aimed to identify metabolic response patterns in these two cell types that might confer a tolerance phenotype (Kusch et al. 2008) similar to that observed in *S. cerevisiae* in response to stress (Herman 2002). They observed that the stationary phase cells upregulated a number of proteins, including those involved in the defense against reactive oxygen species and heat stress, as compared to exponentially growing cells. The ability to undergo morphological transitions between yeast and hyphal cells is an important virulence trait of many but not all *Candida* spp. This is especially important as the cell wall itself is always subjected to recognition by the host cell surface and is thus exposed to immune recognition.

For example, a number of proteins are expressed in the yeast or hyphal stage only, suggesting a potential mechanism for secretion of cytosolic proteins, which may contribute its overall virulence in these different morphological states (Ebanks et al. 2006). These data further support the idea that the regulation of Hsp90, an essential chaperone protein that is activated in response to stress, is posttranscriptional in hyphal cells.

Additional variations of MS, such as matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS has been shown to be a useful tool in drug susceptibility screening of *C. albicans* to fluconazole (Marinach et al. 2009). A complete map of the yeast proteome using MS has most recently been completed, using a combination approach of high-throughput peptide synthesis in conjunction with MS for the *S. cerevisiae* proteome. This study provides insight into the evolution of yeast proteins and protein complexes (Picotti et al. 2013).

For *A. fumigatus,* the conidia mediates the initial contact with the immune system of the host and therefore is an interesting target for proteomics studies looking for fungal virulence factors and posttranscriptional responses upon host recognition. For example, comparison of the proteome profiles of *A. fumigatus* conidia and mycelial cells revealed some 50 conidia-specific proteins (Teutschbein et al. 2010). Interestingly, the data suggested that many proteins that are not needed during the resting stage are stored, perhaps for a rapid response to the activation of metabolic processes or in response to recognition by the immune system.

In vitro co-culture approaches with immune cells have also been influential in investigating differential protein expression in response to infection conditions. Using a time course of interaction between *C. albicans* and macrophages, a combination of proteomics and transcriptomics techniques highlighting specific pathways related to the virulence of *Candida* spp, including the regulation of apoptosis (Fernandez-Arenas et al. 2004; Fernandez-Arenas et al. 2007), was performed. The authors used a *C. albicans* strain of attenuated virulence, in which the kinase HOG1, important for the

oxidative stress response, was absent. They were able to identify several novel *C. albicans* antigens and further characterized the protective antibody response of mice against *C. albicans* infection. The use of proteomics methods, in general, has been useful in validating transcriptional data sets. However, they also revealed a number of discrepancies between the transcriptome and the proteome, which remains an active area of research in the validation of fungal virulence factors. Finally, **many resources have recently become available**, including the Proteopathogen Database for studying host–pathogen interactions (http://proteopathogen.dacya.ucm.es) with *C. albicans,* Compluyeast (http://compluyeast2dpage.dacya.ucm.es/cgi-bin/2d/2d.cgi), which catalogues 2D-PAGE data sets from *C. albicans*, *Mus musculus*, and *S. cerevisiae* for comparative proteomics.

E. Metabolomics

Metabolites are the products of metabolism or reaction intermediates and are usually small molecules serving a number of functions within the cell, including signaling and inhibition or stimulation of enzymes, among a number of other functions. As reaction intermediates, **metabolites provide the "missing-link" between DNA, RNA, and protein interactions within a cell**. One of the major themes of metabolomics is to investigate the influence of metabolites on cellular phenotypes. The metabolome is composed of intracellular metabolites and the exometabolome, also referred to as the secretome, which contains all small molecules secreted from a cell. It has been estimated that over 70% of metabolites participate in more than two biological reactions, and therefore represent interesting molecules for SysBio approaches (Nielsen 2003). Furthermore, from an evolutionary perspective, it is expected that a number of the filamentous fungi share their primary metabolism with their yeast ancestor *S. cerevisiae*, suggesting a broad applicability of the metabolomics research in the fungal research community. In fungal cells, there is an estimated number of more than 1,000 metabolites in the steady state (Smedsgaard and Nielsen 2005), some of which are extremely short-lived or of low abundance, making their quantification a formidable challenge.

A number of methods are in use to identify metabolite profiles in cells. The most common are nuclear magnetic resonance (NMR) spectroscopy, MS (see Sect. II.C) as well as metabolic labeling with radioactive isotopes (Niittylae et al. 2009; Zamboni and Sauer 2009). Another method for investigation of metabolomics is gas chromatography coupled to mass spectrometry (GC-MS). GC-MS utilizes GC with detection by MS. GC is used in analytical chemistry to separate and identify molecules based on their migration within a capillary system. The sample is vaporized and travels through the capillary using an inert carrier gas. The time it takes for each molecule to elute from the column will vary according to its molecular properties and therefore can be used to identify compounds. Combining this elution with MS gives a highly detailed description of the molecule. High performance liquid chromatography (HPLC) is often used in combination with MS (HPLC-MS). HPLC is a chromatographic purification technique using a high-pressured capillary tube system, allowing for the fine separation of molecules. These methods, among others, provide a comprehensive way to identify the structure of metabolites on a genome-wide scale.

The identification and function of metabolites is highly relevant for a better understanding of fungal virulence. Fungi, more so than other pathogenic species, are notoriously known for the diversity of metabolites produced in response to host immune defense, and are thus useful organisms for studying metabolic diversity (Jewett et al. 2006). Notably, about a dozen *A. fumigatus* **secondary metabolites** have been implicated in niche adaptation and virulence (Galagan et al. 2005). To date, significant progress has only been made in metabolic profiling of fungi such as *S. cerevisiae*. The first metabolic network reconstruction of *S. cerevisiae* used an extensive data-mining approach of previous literature in combination with mathematical techniques to identify approximately 600 metabolites (Forster et al. 2003). Shortly thereafter, GC-MS methods were able to verify the presence of approximately 100 of these metabolites under standard laboratory growth conditions (Villas-Boas et al. 2005). **Metabolic flux in over 30 *S. cerevisiae* mutants demonstrated robustness and inherent redundancies built into yeast metabolism** (Blank et al. 2005).

In *C. albicans,* LC-tandem MS was used to profile the regulation of the secretome under standard laboratory conditions (Sorgo et al. 2010) and in response to the antifungal agent fluconazole (Sorgo et al. 2011), identifying numerous immunogenic peptides as novel vaccine candidates for antifungal therapy. Recently, the metabolome of *A. fumigatus* was investigated using 1H-NMR metabolomics under infection conditions (Grahl et al. 2011). Using this technique, the authors detected ethanol in the lungs in a murine model of invasive pulmonary aspergillosis, suggesting a role for fungal alcohol dehydrogenase in pathogenesis (Grahl et al. 2011). 1H-NMR metabolomics also enabled the identification of pneumococcal or cryptococcal meningitis without prior sample culture, which if implemented in a clinical setting would speed up the time it takes for patients to be diagnosed (Himmelreich et al. 2009).

F. Epigenomics

Among the biologically relevant –omics approaches, the most recent addition, epigenomics, has entered center stage. **The epigenome describes the global epigenetic modifications that take place within a cell.** Epigenetic modifications take place on the DNA, histones, and chromatin in its various functional states. They use numerous posttranslational modifications, including, but not limited to, the addition of single or multiple methyl residues, ubiquitination, acetylation, phosphorylation, or adenylation just to name the most common modifications [for review see (Hnisz et al. 2011)]. Most importantly, many modifications are reversible, providing an additional and even heritable level of cellular regulation.

The most common methods for investigation of the epigenetic landscape are studies on the variation of chromatin states using chromatin immunoprecipitation (ChIP).

Combinations of ChIP with microarray technology, known as ChIP-Chip or ChIP-on-Chip, and a similar combination of ChIP with NGS technology, termed ChIP-seq, have been recently introduced. ChIP identifies transient in vivo protein–DNA complexes by crosslinking DNA and associated proteins within a cell lysate. The DNA is then fragmented either by sonication or nuclease digestion. The proteins of interest are then selected using an antibody, precipitated, purified, and the associated DNA is either sequenced or placed on a microarray, depending on the technology used.

ChIP-Chip has been used to investigate genome-wide changes in patterns of histone methylation in the fission yeast *S. pombe*. A complex composed of two proteins, Swm1 and Swm2, mediates demethylation of lysine 9 in histone H3 (H3K9) (Opel et al. 2007). Epigenetic regulation via this complex, in concert with additional histone deacetylases and chromatin remodelers, is a major factor in the transcriptional regulation of *S. pombe* (Opel et al. 2007). In *C. albicans*, Nobile and colleagues identified the transcriptional network for controlling biofilm formation using a combination of ChIP-Chip and in vivo animal models. The six identified core transcriptional regulators, regulating over 1,000 target genes, provide insight into biofilm formation during host infection (Nobile et al. 2012). In yeast, ChIP-Chip was used to investigate histone and gene deletion mutants during environmental stress, highlighting the importance of epigenetic regulation in this process (Weiner et al. 2012).

In *C. neoformans*, the size of the capsule increases under infection conditions and is a well-established virulence factor of the species. The direct targets of Ada2 in *C. neoformans* were recently investigated using ChIP-seq (Haynes et al. 2011). Ada2 is a member of the Spt-Ada-Gcn5 acetyltransferase (SAGA) complex, which regulates transcription by histone acetylation. The authors identified a relationship between the function of Ada2 and capsule size, linking this epigenetic modification and its targets to the overall virulence of the species (Haynes et al. 2011). Most recently, in *C. albicans*, a role of chromatin-modifying enzymes in the inhibition of the yeast-to-hyphal transition was discovered using a combined approach of ChIP-seq and RNA-seq. The authors identified a role for the histone deacetylase Set3/Hos2 complex (Set3C) as a transcriptional cofactor of metabolic and morphogenesis-related gene expression. They found that the acetylation status of

C. albicans chromatin influences transcription kinetics at target genes, showing that the epigenetic regulation supersedes a core transcriptional factor circuit involved in morphogenesis, a circuit that might be shared among other fungal pathogens (Hnisz et al. 2012).

G. Data Mining Approaches and Genome-Wide Fungal Resources

1. Databases

High-throughput molecular biology techniques have enormously increased the sheer volume of data generated and the need for proper data storage has never been higher (Kersey and Apweiler 2006). The value of this biological information is dependent on the ability of researchers to access and extract the information in a quick and reliable format, but also requires **high-level curation**.

Databases classify, organize, and systematize information. The maintenance of databases is essential in disseminating biological data to the community. The early development of two excellent databases for *S. cerevisiae,* the *Saccharomyces* Genome Database (www.yeastgenome.org/) and the Yeast Proteome Database (http://www.proteome.com/YPDhome.html) led to the rapid use of *S. cerevisiae* as a functional genomics tool and model organism (Botstein and Fink 2011). Despite their importance, maintaining high-quality, reliable databases is a constant struggle (Baker 2012), partly because of inherent high costs required for the curation of ever-changing biological data sets. Systems biologists extensively use databases for retrieval of prior knowledge, both qualitative and quantitative, on the biological question to increase the strength predictability, and identifiability models.

Major database initiatives include PubMed (http://www.ncbi.nlm.nih.gov/pubmed/), Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/), and Gene Ontology (GO) http://www.geneontology.org, all of which have established themselves as staple websites for researcher. Specialized databases are also becoming increasingly popular, such as the database of virulence factors in fungal pathogens (http://sysbio.unl.edu/DFVF/), which enable inclusion of more in-depth information about a specific topic that may not be sufficiently covered in larger databases.

An additional benefit to including scientific data into databases is the ability to standardize the reporting format, facilitating both the integration of distinct data sets from different laboratories and the development of analysis tools. Standardization has been greatly aided with the push for minimum reporting guidelines for biological and biomedical information (Taylor et al. 2008) (http://mibbi.sourceforge.net/). Reporting guidelines now exist for all major –omics methodologies (Table 3.1) and there has been a general push from the scientific community to adhere to and popularize these standards for biological information.

2. Strain Collections

Genome-wide profiling at the RNA and protein level has been greatly aided by **publically available strain libraries** in the form of loss-of-function (deletions) and gain-of-function (overexpression) collections. They have provided an efficient screening tool for scientists worldwide to investigate transcriptional and posttranscriptional changes in response to external stimuli, such as drug treatment and environmental variation, or exposure to host immune surveillance. Specifically for fungi, they have increased the throughput of virulence factor screening.

Of all fungi, *S. cerevisiae* has contributed the most number of strain collections. Starting with the yeast knockout (YKO) strain collection, this set methodologically deletes open reading frames (ORFs) by substituting the gene of interest with a selectable drug-resistance cassette, allowing for the systematic screening of the effects of gene loss (Winzeler et al. 1999; Giaever et al. 2002). More than 20,000 strains are currently available from the *Saccharomyces* Genome Deletion Project (http://www-sequence.stanford.edu/group/yeast_deletion_project/), including both homozygous and heterozygous diploid deletions, *MAT*α and *MAT***a** haploids, green fluorescent protein (GFP)-tagged (Huh et al. 2003), even essential temperature-sensitive collections (Li et al. 2004, 2011; Yan et al. 2008).

To investigate the posttranscriptional regulation of a gene and the number of protein complexes in the *S. cerevisiae* by proteomics, a Tandem Affinity Purification (TAP) collection was created in which each ORF is tagged with a high-affinity epitope expressing the protein at its native locus (Ghaemmaghami et al. 2003). To investigate protein–protein interactions with *S. cerevisiae,* a yeast two-hybrid collection was created, where hybrid proteins were derived from over 6,000 transformations and a Gal4 transcription–activation domain vector was inserted to create a hybrid protein for each ORF (Uetz et al. 2000). Data sets generated using this collection have also been made publically available (http://portal.curagen.com/). Several overexpression libraries for *S. cerevisiae* are also available, including a yeast GAL-GST library of over 5,000 strains, containing inducible overexpressed tagged ORFs from the GAL1 promoter, covering over 80% of the genome (Sopko et al. 2006). Additionally, the overexpression transformable plasmid based library of *S. cerevisiae* has been created, including over 13,000 entries with over 95% functional coverage of the genome (Jones et al. 2008). Approximately three-quarters of the *S. cerevisiae* proteome are also covered by the chromosomally C-terminal-tagged GFP fusion proteins strains (Huh et al. 2003). Using this library of 4,159 yeast–GFP clones, the localization of proteins in response to external stimuli can be easily visualized by live-cell fluorescence microscopy.

After the early success of strain collections in *S. cerevisiae*, more focused collections of pathogenic fungi have been created in order to specifically address fungal virulence. In *C. albicans*, a homozygous deletion library of approximately 670 homozygous deletion strains affecting 11% of the *C. albicans* genome was used to screen for virulence in a mouse model of infection, identifying 115 infectivity-attenuated mutants (Noble et al. 2010). A knockout collection of *C. albicans* transcriptional regulators includes over 100 strains, which were screened in 55 different growth conditions (Homann et al. 2009). Among the phenotypes identified, a number of them showed altered susceptibility towards antifungal treatment. These results also support the theory that there is a high redundancy among transcriptional regulatory circuitry, where a single knockout does not greatly affect the strain's overall virulence. In *C. neoformans,* a knockout collection of 1,201 genes was screened in an in vitro model of murine lung tissue for virulence phenotypes (Liu et al. 2008). Using these collections, a number of previously uncharacterized genes were identified as virulence factors, including those involved in growth at body temperature and in melanization, and those dependent and independent of capsule formation.

Smaller arrayed mutant collections of *Neurospora crassa* and *A. fumigatus*, among other pathogenic fungi, are available from the Fungal Genetics Stock Center (http://www.fgsc.net/) for screening. Finally, a single gene deletion collection comprising around 650 haploid *C. glabrata* genes will become available to the community shortly (Schwarzmüller, unpublished data).

III. Modeling Biological Phenomena

Most, if not all, biological processes follow a **dynamic, nonlinear pattern**. Nonetheless, a biological process can be approximated via a set of mathematical expressions to form a mathematical model. In this context, a "model" is referred to as a description of a biological process using mathematical expressions of quantitative data rather than a graphical representation. Although SysBio studies do not exclusively rely on either high or low throughput data sets, and lean towards a combination of both when possible, mathematical models have become increasingly useful ways of representing the information. These studies integrate computational approaches with experimental data to gain a more complete picture of how cells, tissues, and organs of species function and how the entire genetic information is wired and connected. Although current experimental techniques allow detailed measurements, it is impossible to gain full information about the system from discrete data sets without considering the topology and dynamics of the interacting

components. Depending on what is known a priori about the specific system under investigation, and what one wants or needs to learn about it, different modeling approaches are required. Identifying the proper modeling approach is a critical point, as not all methods will be appropriate for each experimental question. The pros and cons for each approach should be weighed for each biological question (Di Ventura et al. 2006; Karlebach and Shamir 2008). This needs a close interaction between mathematicians and experimentalists because very often it is impossible to generate the experimental data required for a particular model. Visual examples and a summary of the pros and cons for the use of Boolean models, ordinary differential equations (ODEs), and Petri Nets (PNs) are provided in Fig. 3.2.

In short, mathematical models represent a **simplified and abstract view** of the studied phenomena. They are extremely useful in understanding dynamic and multifaceted systems and their perturbations . These models often encompass different levels of biological understanding. Although it is important to know the consecutive action of the individual components of the system, an understanding of the time scale under which these interactions take place is essential for the whole picture.

Processes such as the ability of the cell to respond and adapt to a stimulus can be modeled using a set of ODEs. These processes are triggered by molecular interactions, which are not spontaneous and any environmental signal will result in a cellular response. A cellular response involves consecutive activation of a list of proteins that together establish a signaling pathway. These proteins pass the signal to the transcriptional machinery via a transcription factor (TF) that sometimes shuttles between cytoplasm and nucleus. TFs regulate gene expression and define the amplitude, magnitude, and duration of the cellular response. Such interactions can be graphically represented by "networks" that connect the interacting molecules. For example, gene regulatory networks (GRNs) are types of pathways that consist only of genes; in the network, gene A is connected with gene B if its product regulates the activity of gene B. A Boolean approach is often applied to study the topology of GRNs. Thus, based on the specific experimental question, different modeling approaches will be applicable. Here, we discuss in detail several computational approaches commonly applied to model experimental data sets, emphasizing how key attributes of fungal virulence have so far been investigated using modeling approaches.

A. Boolean Models

Boolean models are often used to infer GRNs from microarray data or other types of expression analysis (Hickman and Hodgman 2009). Boolean models were first introduced by Kauffman and colleagues (Glass and Kauffman 1973). In contrast to ODE models, a Boolean approach is a discrete type of modeling where time and states are represented by discrete values. Boolean models are suitable for studying biological problems that can be interpreted using a rather simple on/off behavior, such as gene transcription. For example, an algorithm called REVEAL (Liang et al. 1998) infers the network topology from expression data. A measure of element interactions is used to derive logic functions that define them. Boolean models are useful for studying the existence of steady states or whether a given network topology is robust (Li et al. 2004). Furthermore, the approach can be used when the precise network topology is uncertain and our primary goal is to understand the wiring of the interactions in the system. The topology of the system of interest must be known before implementing kinetic models. As an example, the Boolean approach was used to infer a *Drosophila* segment polarity GRN (Ay and Arnosti 2011).

A Boolean model is defined by n entities interconnected via k edges, forming a directed graph. Each model entity is in a state either "on" (1) or "off" (0). Using the example of gene transcription, each gene can be considered as either expressed, 1, or not, 0. In the **synchronous Boolean model**, the states of the model entities are simultaneously evaluated and updated at time $t+1$, according to the regulatory functions and variables states at time t. Such Boolean models are purely deterministic.

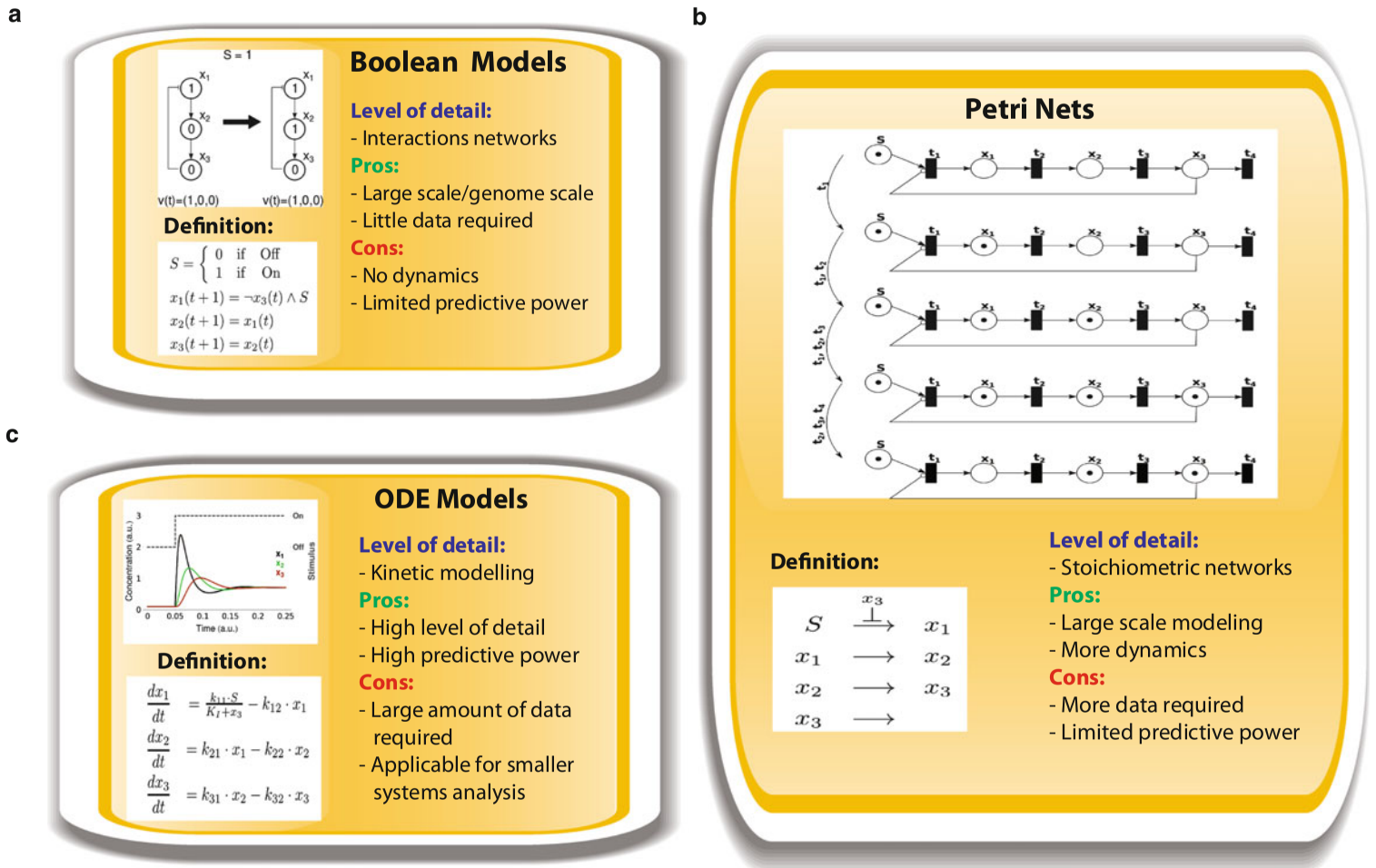


Fig. 3.2. Simulation of a negative feedback loop with different modeling techniques. (**a**) Boolean models incorporate only two values: 0 for the inactive state and 1 for the active state of a variable. They represents only an interaction network where no kinetic information can be included. (**b**) Petri Nets are an extension to the Boolean approach such that they include the stoichiometric information of the considered reaction network. The reaction can take place only if the right amount of variable is present. (**c**) ODE model. Parameters used: $x_1^0 = 0.1$, $x_2^0 = 0.1$, $x_3^0 = 0.1$, $S^{\text{off}} = 1$, $S^{\text{On}} = 50$, $k_{11} = 1$, $k_{12} = 99$, $K_I = 0.001$, $k_{21} = k_{22} = 50$, $k_{31} = k_{32} = 50$. Both the Boolean modeling approach and PNs indicate oscillatory behavior of the system, whereas the ODE model, which incorporates higher level of detail, suggests that a system adapts to the external stimuli by reaching a new steady state

Regulatory relations are described via logic functions, such as the Boolean operators "and," "or," "not." For *vi* being a vector representing a state of the model, we call a state space a set of all possible vectors *vi*. Thus, the state space has 2^n elements for *n* entities in the network, and *vi* are vectors of 0s and 1s. The elements of the state space are connected via arrows indicating the flow of model states.

In the **asynchronous Boolean model**, one node at a time is chosen and updated, and the evaluation of the next selected node state takes this change into account. If the order of choosing the nodes is fixed, then the model is called a **deterministic asynchronous Boolean model**; if the nodes are chosen at random, then the model is termed a **stochastic asynchronous Boolean model**. The state space typically contains single point attractors, which are fixed points (also called steady states) towards which the systems evolve into both synchronous and asynchronous Boolean models. However, the time needed to reach the fixed point can vary between synchronous and asynchronous modeling variants. Cyclic attractors, e.g., limit cycles, can be lost in the stochastic Boolean model (Wang et al. 2012). In either case, the identification of point and cyclic attractors of large-scale Boolean models is not a trivial task but there are algorithms that deal with this problem (Wang et al. 2012). Because stochastic processes influence any biological process, Boolean networks have been further developed to account for noise in the system and for making the approach suitable for the study of stochasticity and uncertainty. These include development of probabilistic Boolean networks (Shmulevich et al. 2002) or Boolean models where stochasticity is implemented by reversing a node's state at some probability rate, or by implementing stochasticity of a biological function that fails to be executed (stochasticity in function, SIF, models) (Garg et al. 2009).

Although a Boolean approach only allows for a very simplified representation of a biological system, it can be a powerful method for studying its underlying nature. For instance, a Boolean model can be used for systematic screening of possible networks that reproduce a pattern of interest (Giacomantonio and Goodhill 2010). Recently, Boolean modeling was used to study the interplay between gene expression, chromatin modifications, and DNA methylation, where the authors linked the epigenetic landscape with the probability state space (Flöttmann et al. 2012). This innovative application shows that there are other possible ways of analyzing Boolean models that are waiting to be explored.

B. Petri Nets

There is a growing interest in applying Petri Nets (PNs) to modeling and analysis of biological networks. **In principle, a PN is a directed bipartite graph, whose nodes are called either "places" or "transitions"**. Places indicate resources and they are indicated with circles; transitions are events or biochemical reactions, which are shown as boxes. Both types of nodes are connected by arrows. An arrow (called an "arc") from a place (input place) to a transition indicates that a compound is necessary for the reaction. Further arcs point from the reaction to its products (see Fig. 3.3).

Each place holds a nonnegative number of tokens, which indicate resources of a given substance in the system. The state of a system is represented by an allocation of tokens at a given time point, which is called marking (M). Initial marking takes place at the time point zero. A transition will fire if there is a sufficient number of tokens on the input places, and it is at least equal to the edge's weight. Once a transition fires, tokens are transferred into the respective output places and the number of tokens in the output places is again indicated by the weight of the arcs. In summary, formally a PN is a tuple $PN = (P, T, F, W, M_0)$, where P is a set of places, T is a set of transitions, F is a set of arcs, W is a map that assigns each arc with a specific weight, and M_0 is initial marking (Ackermann 2011). For the analysis of PNs, concepts like P-invariants and T-invariants are introduced. For instance, for a stoichiometric matrix N of a PN, a P-invariant, is any vector x where $x^T N = 0$ and it holds $\langle x, M \rangle = const.$ for any marking M

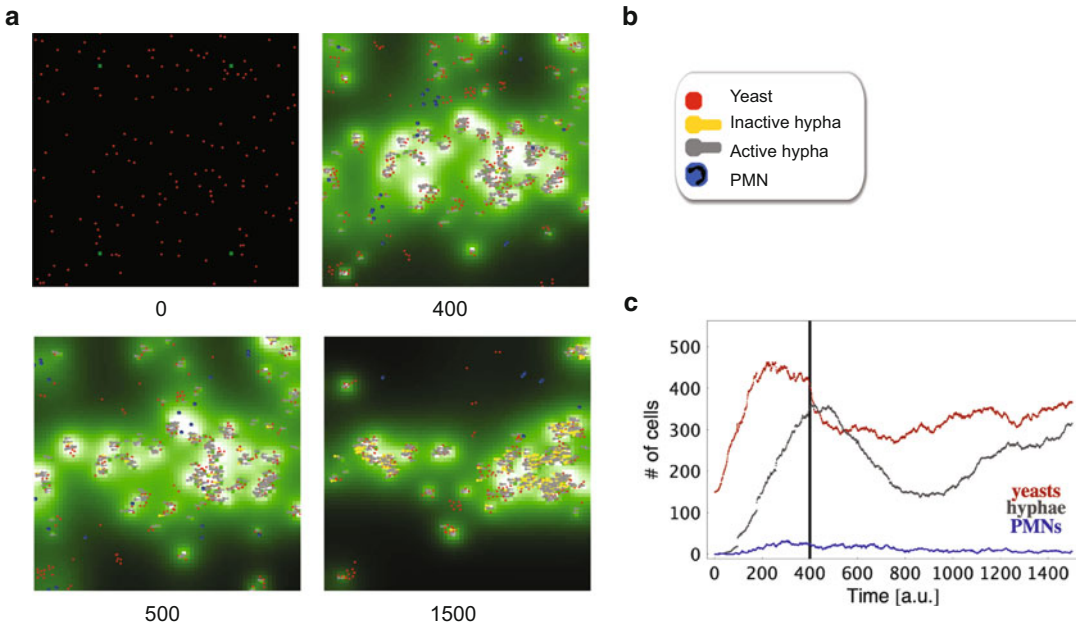


Fig. 3.3. Model simulation of the system treated with a low drug dose. Drug is applied at time $t=400$ (arbitrary units). Treatment does not clear the infection. After initial reduction, $t=500$, the fungal population recovers from the drug stress, $t=1{,}500$. (**a**) Model simulation output at different time points. (**b**) Agents considered in the model: yeast cells (*red*), inactive hyphal cells (*yellow*), active hyphal cells causing damage to the host (*gray*), polymorphonuclear neutrophils (*blue*). (**c**) Graphical representation of the model simulation over time; *y*-axis gives the total cell number

that appears during the simulation of PN. The scalar product $\langle x, M\rangle = const.$ represents the conservation relation, e.g., ATP + ADP = const. A T-invariant on the other side is any vector x where $Nx = 0$. These provide a decomposition of the network, and a transition that does not belong to any T-invariant can be removed from the PN. A set of T-invariants corresponds to the elementary flux modes operating in the system. The marking graph of a PN is a directed graph that represents the evolution of markings during PN simulation. This concept is similar to state space in Boolean models.

PN approaches are applicable for discrete modeling, or modeling of GRNs. For GRNs, like with Boolean models, one can consider both synchronous and asynchronous modes. PNs can also be applied to perform quantitative analysis and establish stochastic models. Fuzzy modeling has also been introduced based on PNs, and details on the application of the techniques and examples have been published (Ackermann 2011).

A stochastic version of a Petri Net (SPN) has been applied to the study of the cell cycle in budding yeast (Mura and Csikasz-Nagy 2008). The authors provide a fair comparison of the results from SPN with the results of the deterministic version of the corresponding ODE model, as proposed earlier (Novak 2002). Recently, a PN-based technique has been applied to integrate signaling, metabolic, and regulatory events participating in the *S. cerevisiae* HOG signaling pathway (Tomar et al. 2013).

C. Ordinary Differential Equation Models

Dynamics of biological processes are most often described via ODE models or partial differential equations (PDEs) when space is included. Optimally, ODE models are used for systems that can be considered "well-stirred" and that comprise large molecule numbers. When this condition is met, changes in molecule numbers can then be considered as

continuous (Di Ventura et al. 2006). ODE models can also be used to address questions that regard changes in cellular phenotypes (Le Novere et al. 2005; Karlebach and Shamir 2008). Each equation in an ODE model describes temporal changes of one variable such as molecule concentration, phosphorylation levels, cell mass, or volume. If we consider a system with n variables, characterized by k, and positively valued parameters p, then the equation is:

$$\frac{dx_i}{dt} = f_i\ (x_1, \ldots, x_n,\ p_1, \ldots, p_k,\ t) \qquad (3.1)$$

where t represents time. The equation describes dynamical changes of the variable x_i. A mathematical expression describing an increased production of a compound or its activation enters Eq. 3.1 with a positive sign; expressions for degradation or deactivation enter with a negative sign. Taken together, the equation evolves, having m reactions determined by the topology of our network of n state variables, which may or may not influence each other. Thus, we can also represent Eq. 3.1 in a matrix form:

$$\frac{dx}{dt} = \mathrm{Nv} \qquad (3.2)$$

where N is a ***n x m*** stoichiometric matrix and $\mathrm{v} = (v_1, \ldots, v_m)^{\mathrm{T}}$ is a vector that stores rates for all the reactions taking place in our system. For practical examples, refer to detailed earlier descriptions (Klipp et al. 2009). The solution of an ODE model is a time course simulation. Given the initial state in a deterministic model, all the future states can be computed. Simulations of the model reproducing experimental data are used for understanding the time course dynamics of the interacting molecules, generating testable predictions, and for design of new experiments. Understanding of the system through these simulations has proven useful for the study of fungal virulence (Chen et al. 2004; Klipp et al. 2005; Leach et al. 2012). Only using the analysis of the model simulations can reveal the purpose of integrating by the cell certain molecular circuits, e.g., by negative or positive feedback loops.

Another example is a molecular autoregulatory loop integrated by *C. albicans* adapting to heat stress (Leach et al. 2012). In this case, the authors developed a dynamic model of the heat stress response in *C. albicans* using a set of ODEs supported with experimental data. The model reveals several features of the system such as a memory for acquired thermotolerance. For example, when pretreated with a mild heat shock, the system becomes more resistant to a severe shock. Moreover, the simulations of the model indicate a transient molecular memory in the system that is mediated through phosphorylation of heat shock transcription factor Hsf1.

D. Flux-Balance Analysis

Flux-balance analysis (FBA) is a mathematical framework that is widely used for the analysis of the **flow of metabolites** throughout a metabolic network (Orth et al. 2010). This structural modeling approach solely requires knowledge of the stoichiometric matrix N of the biological network. This is generally a well-known property for metabolic networks and, hence, opens the way for genome-scale studies (Edwards and Palsson 2000; Price et al. 2003; Yus et al. 2009). The FBA approach aims to identify the optimal distribution of fluxes in the steady state, i.e., fluxes v that satisfy the following equation:

$$\frac{dx}{dt} = \mathrm{N}v \stackrel{!}{=} 0 \qquad (3.3)$$

FBA aims to find fluxes where a given objective function reaches extreme values, for example, fluxes leading to the maximal growth rate (Feist and Palsson 2010) or the minimal production of toxic metabolites. The problem takes the form of linear programming. Here, problem constraints are included on the basis of the experimental results in the steady state (for instance, thermodynamics, biomass produced, or energy availability). These data are required to reduce the degree of freedom in the solution space.

The major advantage of using FBA is that there is no need to know reaction kinetics and metabolite concentrations, because FBA addresses steady-state conditions. However, the approach is inherently deprived of quantitative information (e.g., enzyme concentrations). Because of this restriction, FBA cannot

be used to predict, for example, how specific levels of a certain enzyme must be changed in order to achieve a desired effect on flux. It is important to keep in mind that FBA does not help us to understand the dynamics of the system because it can only reveal steady state properties.

To enable the re-use of FBA models, they should be provided in the Systems Biology Markup Language (SBML) format, similar to the case of ODE models. FBA models can then be imported and solved using algorithms such as Matlab or COBRA Toolbox (http://systemsbiology.ucsd.edu/Downloads/Cobra_Toolbox) (Becker et al. 2007).

An extension of FBA, the regulated flux-balance analysis (rFBA) has been extensively reviewed (Karlebach and Shamir 2008) and aims at integrating the metabolic network with regulatory processes that are expressed by Boolean logic (Covert et al. 2001). Regulatory events can reflect situations when, for instance, certain regulatory proteins are not expressed and then appropriate fluxes will be shut down and thus set to zero.

E. Stochastic Modeling

ODE models, which are deterministic, are suitable for the analysis of dynamical behavior of the population on average. These, however, do not provide information about whether and how stochastic switches or noise impact the outcome of the biological process. For instance, the lysogenic and lytic cycle of **λ** phage (Arkin et al. 1998) could not be explained by deterministic modeling. Stochastic models describe **random processes that evolve and change over time**. It is convenient to use stochastic modeling in cases where one wants to investigate processes in which a molecule with small copy number affects key components of the model or if steady states are unstable. In general, simulating stochastic models is computationally more expensive. Moreover, to enable statistically significant conclusions, many simulation results have to be analyzed together. Stochastic simulations can be performed using tools such as COPASI (Hoops et al. 2006) or Cain (http://cain.sourceforge.net/). For a thorough introduction to stochastic modeling, we refer to recent work in yeast (Klipp et al. 2009). The ideal type of data for stochastic modeling are time-resolved measurements of single molecules, e.g., by microscopic measurements. In practice, experiments can rarely be repeated for the same single cells.

F. Monte Carlo Simulation

Monte Carlo (MC) simulation is a hit-or-miss sampling method. It is typically applied to find extremes of a function in a restricted region of possible parameter space. The MC sampling method can be viewed as randomly choosing parameters (x,y) and keeping the pair that gives, for example, the highest value of $f(x,y)$. This method, however, is not a systematic approach to approximate the optimal solution; simply put – each time the simulation is performed, we either hit or miss the solution.

For each run, parameter values are randomly changed as, for example, initial conditions or kinetic constants. Then, it is recorded whether such perturbations influence the final result of the model and, if so, how. The parameters to vary are those where a significant uncertainty is encountered. From the analysis of the created range of estimated final values of the model, one can estimate how likely it is that a certain outcome will occur. MC simulation usually evaluates the model from hundreds to tens of thousands of times to estimate the solution to the model.

The MC simulation method was applied to study the dependence of drug dosage treatments on host resistance against disseminated candidiasis (Hope et al. 2006). In another study, the MC method was applied to study anticancer drug target inhibition strategies on the epidermal growth factor signaling pathway. The authors investigated the influence of changes in kinetic parameters by comparing parallel simulation runs (Wierling et al. 2012). MC simulation was also proposed as a method for assessing the degree of completeness of GRNs, where information on gene interactions is often missing (Kuhn et al. 2009). Among other tools for performing MC simulations are PyBios (http://pybios.molgen.mpg.de/), MATLAB, and Statistics ToolBox or Simulink.

Finally, MC Markov Chain (MCMC) is an optimization method in high dimensional spaces. It is designed to randomly search the parameter space such that the optimal value can be approximated. The way that the parameter space is searched and tested for whether or not parameters are accepted is often defined by methods such as Gibbs sampling or Metropolis–Hastings algorithms (Hastings 1970). MCMC is applied to solve integrals in high dimensional spaces when the traditional numerical methods fail. A complete description of MCMC is beyond the scope of this chapter. For further details on the method, we refer readers elsewhere (Gamerman and Lopes 2006).

G. Agent-Based Models

Agent-based models (ABMs) are counterparts to ODE models, where simple rules for each agent's action can lead to complex dynamics in the population of interacting agents. **Agents are autonomous entities that can represent molecules, cells, and organisms.** ODE models are often applied to study the dynamical properties of regulatory pathways, leaving out information on spatial distribution of the molecules in the cell. This can be tackled using ABMs (Pogson et al. 2006, 2008). For example, ABM was applied to examine the pathogenesis of gut-derived sepsis using the example of *Pseudomonas aeruginosa* interaction with its host (Seal et al. 2011). In general, ABMs are suitable for study of systems where the spatial and temporal distribution of agents influences the systems dynamics, such as chemotaxis, while sensing a gradient of quorum molecules (Netotea et al. 2009; Fozard et al. 2012), inducing biofilm formation (Mitri et al. 2011), or pheromone concentration gradients during mating. ABM has been applied to study the functionality of the immune system (Folcik et al. 2011), granuloma formation (Segovia-Juarez et al. 2004) and for predicting the outcome of different immunotherapy strategies (Pappalardo et al. 2011).

All ABMs are systems of agents, whose actions and decisions are specified by the user. The agents are typically living in a 2-D world, which is a square divided into a grid of patches (or triangulated space). These patches, representing the environment, can influence an agent's actions, and agents can affect the attributes of the environment themselves as well. ABM is a discrete and stochastic modeling approach. At each step, an agent makes a decision according to its status at the time. Dynamics of biological systems are complex and although ABMs appear conceptually clear, the code of ABMs tend to have particularly long lines of code, numbering even into the thousands, which can make them difficult to handle.

ABMs have gained popularity, particularly in modeling the immune system, disease dynamics, and as a tool for elucidating the nature of host–pathogen interactions. Agent-based modeling is a modeling technique where each agent's action can be defined with simple rules. Furthermore, using ABM simulations, **it is possible to track dynamics of a single agent rather than the averaged behavior of a population.** Tools have already been developed for simulating immune dynamics whereby the user can specify the rules for an agent's interactions, including IMMSIM, SIMMUNE, SIS, and reactive animation [reviewed in (Bauer et al. 2009)]. Computational-oriented studies on host–pathogen interactions can be performed using CyCells, PathSim, MASyV (Bauer et al. 2009), or BSim (Gorochowski et al. 2012), which are freely available at http://bsim-bccs.sf.net.

ABMs have been implemented for the study of fungal pathogenesis using an additional ABM tool referred to as NetLogo (freely available at http://ccl.northwestern.edu/netlogo/docs/). NetLogo was used to study *C. albicans* interactions with its human host (Tyc and Klipp 2011). The authors explored the rules that determine the dynamics of a fungal population influenced by host phagocytic cells (Fig. 3.4). The model was then used to investigate the effects of potential drug treatments on fungal populations and their clearance. Another example is a study on *A. fumigatus* population clearance by neutrophils, where different rules defining neutrophil movement were examined, such as chemotaxis along a chemokine gradient, random walk, and communication between the phagocytes (Tokarski et al. 2012).

H. Game Theory

Humans have developed numerous strategies to protect themselves against invading patho-

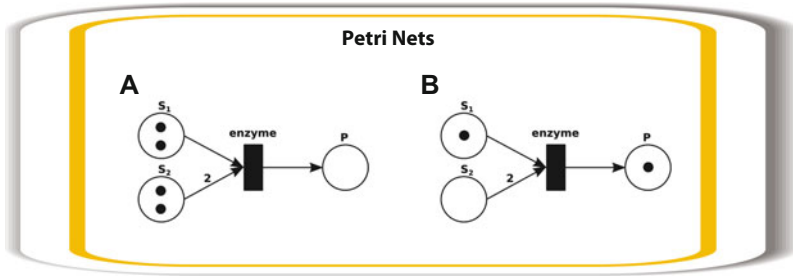


Fig. 3.4. Petri Nets. An example PN of the reaction $S_1 + 2S_2 \xrightarrow{enzyme} P$ is shown. Marking of the PN is presented (**a**) before the reaction takes place and (**b**) after enabling the reaction

gens, such as controlling pathogen growth and dissemination by clearing pathogens from the body through activation of the innate and adaptive immune system. Host–microbe interactions are therefore processes that can be viewed as shifting the balance between different populations of cells that will lead to either a healthy or diseased host state. Populations evolve and they establish equilibrium states in accordance with the equilibrium of the other populations, and this process repeats *ad infinitum* or until one partner is removed from the interaction, i.e., by death of the host or through pathogen clearance. Evolutionary game theory is an approach suitable for modeling dynamics of evolutionary processes that are assumed to modify the population fitness landscape. This can be assumed because normal optimization methods for population fitness or flux rates are not affected by interactions between individuals and the environment (Pfeiffer and Schuster 2005). In short, game theory (GT) is a framework suitable for modeling biological systems where **distinct strategies can be assigned to the individual partners.**

GT has been applied to the study of survival strategies of *C. albicans* in macrophages (Hummert et al. 2010) and to growth strategies adapted by individual cells when different carbon sources are given (Friesen et al. 2004). GT was also exploited to study distinct utilization of metabolic pathways by cell populations (Pfeiffer and Schuster 2005; Ruppin et al. 2010), to study cooperation during evolution (Nowak et al. 2004), and as an application for optimizing altruistic behavior in microbial populations (Schuster et al. 2010). GT was used in the analysis of cancer cells (Gatenby and Vincent 2003), for multiple knockout analysis in *S. cerevisiae* (Kaufman et al. 2004), and for the analysis of microarrays data via defining coalitional game sets (Albino et al. 2008; Moretti et al. 2007, 2008).

I. Model Parameters

Simulation of a dynamic model requires knowledge of the kinetic parameter values. These, however, are often unavailable or extremely difficult to obtain from experimental data. Thus, a process often termed parameter estimation or more precisely, regression (Jaqaman and Danuser 2006) has to be employed. Parameter estimation is a typical inverse problem (i.e., deducing from effects to their causes) and its objective function is the set of parameter values that best represent the data. There are many algorithms suitable for solving such optimization problems that minimize the distance between experimental data points and simulation results of the model, which focus both on local and global optimization methods (Moles et al. 2003; Baker et al. 2010). Although global optimization methods search for the solution to the problem by scanning the entire parameter space, they are computationally more expensive and time-consuming than local optimization methods. Comparing a global optimization method and a local one, the latter method is faster; however, it is limited to providing a suboptimal solution, which in some cases might be only a local optimum. Regardless of the method utilized, algorithms tend to minimize a sum of squared residuals (RSS) given by:

$$\min_{\mathrm{p}} \sum_{i=1}^{n} |y(t_i) - \mathrm{M}(x(t_\mathrm{i}), \mathrm{p})|^2 \qquad (3.4)$$

where $y(t_i)$ are experimental data points and $\mathrm{M}(x(t_\mathrm{i}), \mathrm{p})$ are the values of the simulated model; $x(t_i)$ is a corresponding model variable and p is a set of parameter values.

J. Sensitivity Analysis

Most mathematical models require parameters to be estimated from experimental data sets. The quality of the data fit, however, depends both on reproducibility of experimental data and model structure itself. We can study how the changes in model parameters influence its output – a technique termed **sensitivity analysis**. Parameters with marginal effects on a model output cannot be estimated from experimental data, nor will their numerical values significantly affect the quality of model predictions. Therefore, in order to estimate a set of parameters for a given model, one needs to **focus on generating experimental data for the components that are strongly influenced by these parameters**. Sensitivity analysis can be performed either locally or globally. Using local sensitivity analysis, we can analyze the effects of comparatively small perturbation changes in the parameter (p) on the model output (O), such as at steady state (SS), and can represent the effects using the following mathematical equation:

$$R_p^O = \frac{p}{O^{SS}} \frac{\partial O^{SS}}{\partial p} \qquad (3.5)$$

Local sensitivity analysis does not consider multiple parameter interdependencies. It also tends not to be robust, meaning that results will be partially affected by the parameters used in the model. By contrast, global techniques for performing sensitivity analysis search the entire parameter space, taking into account many parameters values rather than only one. Global techniques not only consider larger variability in parameter values, but they also provide a measure for parameter interactions (Frey and Patil 2002; Marino et al. 2008).

The matrix of coefficients obtained from sensitivity analysis can be analyzed to address the question of structural identification of our model. Specifically, whenever (i) each column contains a large absolute value (i.e., each parameter has a strong effect on at least one model variable), and (ii) columns are linearly independent, then the model is structurally identifiable (Jaqaman and Danuser 2006). In summary, sensitivity analysis can help the process of parameter estimation. Although a strategy for estimating parameters is very model-dependent, there are no golden standards and, hence, it will rely on the modeler's experience and the biological context.

K. Standards for Modeling

Standards in computational biology are necessary to ease the exchange of the results of research between scientific communities. SBML has been proposed for describing models of signaling pathways and GRNs (Hucka et al. 2003). SBML is used for formatting ODE models or a system of ordinary differential and algebraic equations (DAE) such that they can be re-used in other software tools. The need for standardization of models is evident from an ever-increasing number of software tools for model implementation. Lack of standards will inevitably lead to incompatibility of the models between different tools, and make their broad use impossible. **Minimum information requested in the annotation of biochemical models (MIRIAM) has been proposed** (Le Novere et al. 2005). Proper annotations of model components (using ontology names and unique database identifiers) allow for the comparison of different models but also enable model merging. Model annotation can be done either manually or using tools such as semanticSBML (http://www.semanticsbml.org, (Krause et al. 2010)).

IV. Conclusions and Future Perspectives

As experimental techniques improve and evolve, the dimensionality of the biological problems under investigation increases in parallel. Our knowledge about system properties increases exponentially with the amount of biological data. Initially, studies were focused on understanding the functionality of single genes and followed strictly reductionist schemes. The availability of high-throughput and genome-wide data sets (genomics, metabolomics, proteomics epigenomics, etc.) has dramatically extended the size and complexity of biological problems because we have remained almost paralyzed in our efforts to integrate data to gain a better understanding of systems. Hence, there is an emerging need to come up with creative and efficient solutions for integrating large-scale data into meaningful biological context.

The ever-increasing amount of genome-wide data sets has significantly aided our understanding of the nature of cellular responses and how these have evolved. It has helped us to identify novel network components so that it is possible to identify what makes, for example, one species more resistant to an antifungal drug compared to another.

Modeling techniques across the board have been useful in the visualization of both large- and small-scale data sets. For each technique, it is important to keep in mind the kind of information that is being included, and what the expected outcome of the system is. For instance, protein–protein interactions between molecules do not always correlate with a conserved GRN (Roguev et al. 2008). One should also interpret these models cautiously, especially when perturbing a mathematical model by including gene mutations or alternations in the system, since such alterations may also change physical interactions of the proteins and therefore may not fit the proposed model structure (Goh et al. 2007; Zhong et al. 2009). There are also benefits and limitations to modeling a single cell versus a cell population. It is imperative to weigh the pros and cons of each of these limitations when modeling biological data.

The generation and cataloguing of further data sets and models will become increasingly important in fostering our understanding of fungal virulence and for the prediction of alternative or even novel therapeutic strategies.

Acknowledgments Work on systems biology in the KK group has been supported by a grant from the Christian Doppler Research Society, and by grants of the Austrian Science Foundation (FWF-DACH Proj.: I-746-B11, FWF-P25333-B09), by the SysMo-MOSES project through the FFG, the WWTF, and by the IP-UNICELLSYS from the FP7 programme. Work on systems biology of fungal infection in the EK group has been supported by grants from the German Research Council (DFG: CRC618), the EU Marie-Curie Program (FinSysB, PITN-GA-2008-214004) and the EU FP7 programme (IP-UNICELLSYS and IP-SysteMtb). We would like to thank Sara Tierney for contributing to the artwork.

References

Ackermann J, Koch I (2011) Quantitative analysis. In: Koch I, Reisig W, Schreiber F (eds) Modeling in systems biology: the Petri Net approach. Computational biology, vol 16. Springer, Berlin, pp 153–178

Aebersold R, Mann M (2003) Mass spectrometry-based proteomics. Nature 422:198–207

Albino D, Scaruffi P, Moretti S, Coco S, Truini M, Di Cristofano C et al (2008) Identification of low intratumoral gene expression heterogeneity in neuroblastic tumors by genome-wide expression analysis and game theory. Cancer 113:1412–1422

Arkin A, Ross J, McAdams HH (1998) Stochastic kinetic analysis of developmental pathway bifurcation in phage lambda-infected *Escherichia coli* cells. Genetics 149:1633–1648

Ay A, Arnosti DN (2011) Mathematical modeling of gene expression: a guide for the perplexed biologist. Crit Rev Biochem Mol Biol 46:137–151

Baker M (2012) Databases fight funding cuts. Nature 489:19

Baker SM, Schallau K, Junker BH (2010) Comparison of different algorithms for simultaneous estimation of multiple parameters in kinetic metabolic models. J Integr Bioinform 7:3

Bauer AL, Beauchemin CA, Perelson AS (2009) Agent-based modeling of host-pathogen systems: the successes and challenges. Inform Sci 179:1379–1389

Becker SA, Feist AM, Mo ML, Hannum G, Palsson BO, Herrgard MJ (2007) Quantitative prediction of cellular metabolism with constraint-based models: the COBRA toolbox. Nat Protoc 2:727–738

Bino RJ, Hall RD, Fiehn O, Kopka J, Saito K, Draper J et al (2004) Potential of metabolomics as a functional genomics tool. Trends Plant Sci 9:418–425

Blank LM, Kuepfer L, Sauer U (2005) Large-scale 13C-flux analysis reveals mechanistic principles of metabolic network robustness to null mutations in yeast. Genome Biol 6:R49

Botstein D, Fink GR (2011) Yeast: an experimental organism for 21st century biology. Genetics 189:695–704

Braun BR, van Het Hoog M, d'Enfert C, Martchenko M, Dungan J, Kuo A et al (2005) A human-curated annotation of the *Candida albicans* genome. PLoS Genet 1:36–57

Bray D (2003) Molecular networks: the top-down view. Science 301:1864–1865

Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C et al (2001) Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nat Genet 29:365–371

Brenner S, Noble D, Sejnowski T, Fields RD, Laughlin S, Berridge M, Segel L, Prank K, Dolmetsch RE (2001) Understanding complex systems: top-down, bottom-up or middle-out? In: Bock G, Goode J (eds) Complexity in biological information processing, vol 239, Novartis foundation symposium. Wiley, Chichester, pp 150–159

Bruggeman FJ, Westerhoff HV (2007) The nature of systems biology. Trends Microbiol 15:45–50

Bruno VM, Wang Z, Marjani SL, Euskirchen GM, Martin J, Sherlock G et al (2010) Comprehensive annotation of the transcriptome of the human fungal pathogen *Candida albicans* using RNA-seq. Genome Res 20:1451–1458

Butler G, Rasmussen MD, Lin MF, Santos MA, Sakthikumar S, Munro CA et al (2009) Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. Nature 459:657–662

Chen KC, Calzone L, Csikasz-Nagy A, Cross FR, Novak B, Tyson JJ (2004) Integrative analysis of cell cycle control in budding yeast. Mol Biol Cell 15:3841–3862

Covert MW, Schilling CH, Palsson B (2001) Regulation of gene expression in flux balance models of metabolism. J Theor Biol 213:73–88

Cui X, Churchill GA (2003) Statistical tests for differential expression in cDNA microarray experiments. Genome Biol 4:210

Cuomo CA, Birren BW (2010) The fungal genome initiative and lessons learned from genome sequencing. Methods Enzymol 470:833–855

de Godoy LM, Olsen JV, Cox J, Nielsen ML, Hubner NC, Frohlich F et al (2008) Comprehensive mass-spectrometry-based proteome quantification of haploid versus diploid yeast. Nature 455:1251–1254

Dembele D, Kastner P (2003) Fuzzy C-means method for clustering microarray data. Bioinformatics 19:973–980

Di Ventura B, Lemerle C, Michalodimitrakis K, Serrano L (2006) From in vivo to in silico biology and back. Nature 443:527–533

Dujon B (1993) Mapping and sequencing the nuclear genome of the yeast *Saccharomyces cerevisiae*: strategies and results of the European enterprise. Cold Spring Harb Symp Quant Biol 58:357–366

Ebanks RO, Chisholm K, McKinnon S, Whiteway M, Pinto DM (2006) Proteomic analysis of *Candida albicans* yeast and hyphal cell wall and associated proteins. Proteomics 6:2147–2156

Edwards JS, Palsson BO (2000) The *Escherichia coli* MG1655 in silico metabolic genotype: its definition, characteristics, and capabilities. Proc Natl Acad Sci USA 97:5528–5533

Eisen MB, Spellman PT, Brown PO, Botstein D (1998) Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 95:14863–14868

Feist AM, Palsson BO (2010) The biomass objective function. Curr Opin Microbiol 13:344–349

Fernandez-Arenas E, Molero G, Nombela C, Diez-Orejas R, Gil C (2004) Contribution of the antibodies response induced by a low virulent *Candida albicans* strain in protection against systemic candidiasis. Proteomics 4:1204–1215

Fernandez-Arenas E, Cabezon V, Bermejo C, Arroyo J, Nombela C, Diez-Orejas R et al (2007) Integrated proteomics and genomics strategies bring new insight into *Candida albicans* response upon macrophage interaction. Mol Cell Proteomics 6:460–478

Field D, Garrity G, Gray T, Morrison N, Selengut J, Sterk P et al (2008) The minimum information about a genome sequence (MIGS) specification. Nat Biotechnol 26:541–547

Flöttmann M, Scharp T, Klipp E (2012) A stochastic model of epigenetic dynamics in somatic cell reprogramming. Front Physiol 3:216

Folcik VA, Broderick G, Mohan S, Block B, Ekbote C, Doolittle J et al (2011) Using an agent-based model to analyze the dynamic communication network of the immune response. Theor Biol Med Model 8:1

Forche A, Magee PT, Selmecki A, Berman J, May G (2009) Evolution in *Candida albicans* populations during a single passage through a mouse host. Genetics 182:799–811

Forster J, Famili I, Fu P, Palsson BO, Nielsen J (2003) Genome-scale reconstruction of the *Saccharomyces cerevisiae* metabolic network. Genome Res 13:244–253

Fozard JA, Lees M, King JR, Logan BS (2012) Inhibition of quorum sensing in a computational biofilm simulation. Bio Syst 109:105–114

Fradin C, Kretschmar M, Nichterlein T, Gaillardin C, d'Enfert C, Hube B (2003) Stage-specific gene expression of *Candida albicans* in human blood. Mol Microbiol 47:1523–1543

Frey HC, Patil SR (2002) Identification and review of sensitivity analysis methods. Risk Anal 22:553–578, Risk analysis: an official publication of the Society for Risk Analysis

Friesen ML, Saxer G, Travisano M, Doebeli M (2004) Experimental evidence for sympatric ecological

diversification due to frequency-dependent competition in *Escherichia coli*. Evolution; Int J Org Evol 58:245–260

Fu L, Medico E (2007) FLAME, a novel fuzzy clustering method for the analysis of DNA microarray data. BMC Bioinformatics 8:3

Galagan JE, Calvo SE, Cuomo C, Ma LJ, Wortman JR, Batzoglou S et al (2005) Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. Nature 438:1105–1115

Gamerman D, Lopes HF (2006) Markov chain Monte Carlo: stochastic simulation for Bayesian inference. Taylor & Francis, Boca Raton

Garber M, Grabherr MG, Guttman M, Trapnell C (2011) Computational methods for transcriptome annotation and quantification using RNA-seq. Nat Methods 8:469–477

Garg A, Mohanram K, Di Cara A, De Micheli G, Xenarios I (2009) Modeling stochasticity and robustness in gene regulatory networks. Bioinformatics 25: i101–i109

Gatenby RA, Vincent TL (2003) An evolutionary model of carcinogenesis. Cancer Res 63:6212–6220

Ghaemmaghami S, Huh WK, Bower K, Howson RW, Belle A, Dephoure N et al (2003) Global analysis of protein expression in yeast. Nature 425:737–741

Giacomantonio CE, Goodhill GJ (2010) A Boolean model of the gene regulatory network underlying mammalian cortical area development. PLoS Comput Biol 6

Giaever G, Chu AM, Ni L, Connelly C, Riles L, Veronneau S et al (2002) Functional profiling of the *Saccharomyces cerevisiae* genome. Nature 418:387–391

Glass L, Kauffman SA (1973) The logical analysis of continuous, non-linear biochemical control networks. J Theor Biol 39:103–129

Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H et al (1996) Life with 6000 genes. Science 274(546):63–67

Goh KI, Cusick ME, Valle D, Childs B, Vidal M, Barabasi AL (2007) The human disease network. Proc Natl Acad Sci USA 104:8685–8690

Gorochowski TE, Matyjaszkiewicz A, Todd T, Oak N, Kowalska K, Reid S et al (2012) BSim: an agent-based tool for modeling bacterial populations in systems and synthetic biology. PLoS One 7: e42790

Grahl N, Puttikamonkul S, Macdonald JM, Gamcsik MP, Ngo LY, Hohl TM et al (2011) In vivo hypoxia and a fungal alcohol dehydrogenase influence the pathogenesis of invasive pulmonary aspergillosis. PLoS Pathog 7:e1002145

Hall BG (2004) Predicting the evolution of antibiotic resistance genes. Nat Rev Microbiol 2:430–435

Hastings WK (1970) Monte Carlo sampling methods using Markov chains and their applications. Biometrika 57:97–109

Haynes BC, Skowyra ML, Spencer SJ, Gish SR, Williams M, Held EP et al (2011) Toward an integrated model of capsule regulation in *Cryptococcus neoformans*. PLoS Pathog 7:e1002411

Heinrich R, Rapoport TA (1974) A linear steady-state treatment of enzymatic chains. General properties, control and effector strength. Eur J Biochem/FEBS 42:89–95

Herman PK (2002) Stationary phase in yeast. Curr Opin Microbiol 5:602–607

Hickman GJ, Hodgman TC (2009) Inference of gene regulatory networks using Boolean-network inference methods. J Bioinform Comput Biol 7:1013–1029

Himmelreich U, Malik R, Kuhn T, Daniel HM, Somorjai RL, Dolenko B et al (2009) Rapid etiological classification of meningitis by NMR spectroscopy based on metabolite profiles and host response. PLoS One 4:e5328

Hnisz D, Tscherner M, Kuchler K (2011) Targeting chromatin in fungal pathogens as a novel therapeutic strategy: histone modification gets infectious. Epigenomics 3:129–132

Hnisz D, Bardet AF, Nobile CJ, Petryshyn A, Glaser W, Schock U et al (2012) A histone deacetylase adjusts transcription kinetics at coding sequences during *Candida albicans* morphogenesis. PLoS Genet 8: e1003118

Homann OR, Dea J, Noble SM, Johnson AD (2009) A phenotypic profile of the *Candida albicans* regulatory network. PLoS Genet 5:e1000783

Hoops S, Sahle S, Gauges R, Lee C, Pahle J, Simus N et al (2006) COPASI–a complex pathway simulator. Bioinformatics 22:3067–3074

Hope WW, Warn PA, Sharp A, Howard S, Kasai M, Louie A et al (2006) Derivation of an in vivo drug exposure breakpoint for flucytosine against *Candida albicans* and Impact of the MIC, growth rate, and resistance genotype on the antifungal effect. Antimicrob Agents Chemother 50:3680–3688

Hoyer LL (2001) The ALS gene family of *Candida albicans*. Trends Microbiol 9:176–180

Hoyer LL, Green CB, Oh SH, Zhao X (2008) Discovering the secrets of the *Candida albicans* agglutinin-like sequence (ALS) gene family–a sticky pursuit. Med Mycol 46:1–15

Hucka M, Finney A, Sauro HM, Bolouri H, Doyle JC, Kitano H et al (2003) The systems biology markup language (SBML): a medium for representation and exchange of biochemical network models. Bioinformatics 19:524–531

Huh WK, Falvo JV, Gerke LC, Carroll AS, Howson RW, Weissman JS et al (2003) Global analysis of protein localization in budding yeast. Nature 425: 686–691

Hummert S, Hummert C, Schroter A, Hube B, Schuster S (2010) Game theoretical modelling of survival strategies of *Candida albicans* inside macrophages. J Theor Biol 264:312–318

Inada M, Pleiss JA (2010) Genome-wide approaches to monitor pre-mRNA splicing. Methods Enzymol 470:51–75

Jackson AP, Gamble JA, Yeomans T, Moran GP, Saunders D, Harris D et al (2009) Comparative genomics of the fungal pathogens *Candida dubliniensis* and *Candida albicans*. Genome Res 19:2231–2244

Jaqaman K, Danuser G (2006) Linking data to models: data regression. Nat Rev Mol Cell Biol 7:813–819

Jewett MC, Hofmann G, Nielsen J (2006) Fungal metabolite analysis in genomics and phenomics. Curr Opin Biotechnol 17:191–197

Jones T, Federspiel NA, Chibana H, Dungan J, Kalman S, Magee BB et al (2004) The diploid genome sequence of *Candida albicans*. Proc Natl Acad Sci USA 101:7329–7334

Jones GM, Stalker J, Humphray S, West A, Cox T, Rogers J et al (2008) A systematic library for comprehensive overexpression screens in *Saccharomyces cerevisiae*. Nat Methods 5:239–241

Kacser H, Burns JA (1973) The control of flux. Symp Soc Exp Biol 27:65–104

Karlebach G, Shamir R (2008) Modelling and analysis of gene regulatory networks. Nat Rev Mol Cell Biol 9:770–780

Kaufman A, Kupiec M, Ruppin E (2004) Multi-knockout genetic network analysis: the Rad6 example. Proc IEEE Comput Syst Bioinform Conf 2004:332–340

Kerr G, Ruskin HJ, Crane M, Doolan P (2008) Techniques for clustering gene expression data. Comput Biol Med 38:283–293

Kersey P, Apweiler R (2006) Linking publication, gene and protein data. Nat Cell Biol 8:1183–1189

Kirschner MW (2005) The meaning of systems biology. Cell 121:503–504

Klipp E, Nordlander B, Kruger R, Gennemark P, Hohmann S (2005) Integrative model of the response of yeast to osmotic shock. Nat Biotechnol 23:975–982

Klipp E, Wierling WLC, Kowald A, Lehrach H, Herwig R (2009) Systems biology: a textbook. Wiley-Blackwell, Weinheim

Kniemeyer O, Schmidt AD, Vodisch M, Wartenberg D, Brakhage AA (2011) Identification of virulence determinants of the human pathogenic fungi *Aspergillus fumigatus* and *Candida albicans* by proteomics. Int J Med Microbiol 301:368–377

Kodzius R, Kojima M, Nishiyori H, Nakamura M, Fukuda S, Tagami M et al (2006) CAGE: cap analysis of gene expression. Nat Methods 3:211–222

Kohl P, Crampin EJ, Quinn TA, Noble D (2010) Systems biology: an approach. Clin Pharmacol Ther 88:25–33

Krause F, Uhlendorf J, Lubitz T, Schulz M, Klipp E, Liebermeister W (2010) Annotation and merging of SBML models with semanticSBML. Bioinformatics 26:421–422

Kuhn C, Wierling C, Kuhn A, Klipp E, Panopoulou G, Lehrach H et al (2009) Monte Carlo analysis of an ODE model of the sea urchin endomesoderm network. BMC Syst Biol 3:83

Kusch H, Engelmann S, Bode R, Albrecht D, Morschhauser J, Hecker M (2008) A proteomic view of *Candida albicans* yeast cell metabolism in exponential and stationary growth phases. Int J Med Microbiol 298:291–318

Lashkari DA, DeRisi JL, McCusker JH, Namath AF, Gentile C, Hwang SY et al (1997) Yeast microarrays for genome wide parallel genetic and gene expression analysis. Proc Natl Acad Sci USA 94:13057–13062

Le Novere N, Finney A, Hucka M, Bhalla US, Campagne F, Collado-Vides J et al (2005) Minimum information requested in the annotation of biochemical models (MIRIAM). Nat Biotechnol 23:1509–1515

Leach MD, Tyc KM, Brown AJ, Klipp E (2012) Modelling the regulation of thermal adaptation in *Candida albicans*, a major fungal pathogen of humans. PLoS One 7:e32467

Lelandais G, Tanty V, Geneix C, Etchebest C, Jacq C, Devaux F (2008) Genome adaptation to chemical stress: clues from comparative transcriptomics in *Saccharomyces cerevisiae* and *Candida glabrata*. Genome Biol 9:R164

Li F, Long T, Lu Y, Ouyang Q, Tang C (2004) The yeast cell-cycle network is robustly designed. Proc Natl Acad Sci USA 101:4781–4786

Li Z, Vizeacoumar FJ, Bahr S, Li J, Warringer J, Vizeacoumar FS et al (2011) Systematic exploration of essential yeast gene function with temperature-sensitive mutants. Nat Biotechnol 29:361–367

Liang S, Fuhrman S, Somogyi R (1998) Reveal, a general reverse engineering algorithm for inference of genetic network architectures. In: Pacific symposium on biocomputing. Pacific symposium on biocomputing, pp 18–29

Liu OW, Chun CD, Chow ED, Chen C, Madhani HD, Noble SM (2008) Systematic genetic analysis of virulence in the human fungal pathogen *Cryptococcus neoformans*. Cell 135:174–188

Loftus BJ, Fung E, Roncaglia P, Rowley D, Amedeo P, Bruno D et al (2005) The genome of the basidiomycetous yeast and human pathogen *Cryptococcus neoformans*. Science 307:1321–1324

Lorenz MC, Bender JA, Fink GR (2004) Transcriptional response of *Candida albicans* upon internalization by macrophages. Eukaryot Cell 3:1076–1087

Madeira SC, Oliveira AL (2004) Biclustering algorithms for biological data analysis: a survey. IEEE/ACM Trans Comput Biol Bioinf 1:24–45

Marcet-Houben M, Gabaldon T (2009) The tree versus the forest: the fungal tree of life and the topological diversity within the yeast phylome. PLoS One 4: e4357

Margolin AA, Wang K, Lim WK, Kustagi M, Nemenman I, Califano A (2006) Reverse engineering cellular networks. Nat Protoc 1:662–671

Marinach C, Alanio A, Palous M, Kwasek S, Fekkar A, Brossas JY et al (2009) MALDI-TOF MS-based drug susceptibility testing of pathogens: the

example of *Candida albicans* and fluconazole. Proteomics 9:4627–4631
Marino S, Hogue IB, Ray CJ, Kirschner DE (2008) A methodology for performing global uncertainty and sensitivity analysis in systems biology. J Theor Biol 254:178–196
Maskos U, Southern EM (1992) Oligonucleotide hybridizations on glass supports: a novel linker for oligonucleotide synthesis and hybridization properties of oligonucleotides synthesised in situ. Nucleic Acids Res 20:1679–1684
Metzker ML (2010) Sequencing technologies - the next generation. Nat Rev Genet 11:31–46
Mitri S, Xavier JB, Foster KR (2011) Social evolution in multispecies biofilms. Proc Natl Acad Sci USA 108 (Suppl 2):10839–10846
Moles CG, Mendes P, Banga JR (2003) Parameter estimation in biochemical pathways: a comparison of global optimization methods. Genome Res 13:2467–2474
Moretti S, Fioravante P, Bonassi S (2007) The class of microarray games and the relevance index for genes. TOP 15:256–280
Moretti S, van Leeuwen D, Gmuender H, Bonassi S, van Delft J, Kleinjans J et al (2008) Combining Shapley value and statistics to the analysis of gene expression data in children exposed to air pollution. BMC Bioinformatics 9:361
Mura I, Csikasz-Nagy A (2008) Stochastic Petri Net extension of a yeast cell cycle model. J Theor Biol 254:850–860
Mustacchi R, Hohmann S, Nielsen J (2006) Yeast systems biology to unravel the network of life. Yeast 23:227–238
Netotea S, Bertani I, Steindler L, Kerenyi A, Venturi V, Pongor S (2009) A simple model for the early events of quorum sensing in *Pseudomonas aeruginosa*: modeling bacterial swarming as the movement of an "activation zone". Biol Direct 4:6
Nielsen J (2003) It is all about metabolic fluxes. J Bacteriol 185:7031–7035
Nierman WC, Pain A, Anderson MJ, Wortman JR, Kim HS, Arroyo J et al (2005) Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. Nature 438:1151–1156
Niittylae T, Chaudhuri B, Sauer U, Frommer WB (2009) Comparison of quantitative metabolite imaging tools and carbon-13 techniques for fluxomics. Methods Mol Biol 553:355–372
Nobile CJ, Fox EP, Nett JE, Sorrells TR, Mitrovich QM, Hernday AD et al (2012) A recently evolved transcriptional network controls biofilm development in *Candida albicans*. Cell 148:126–138
Noble SM, French S, Kohn LA, Chen V, Johnson AD (2010) Systematic screens of a *Candida albicans* homozygous deletion library decouple morphogenetic switching and pathogenicity. Nat Genet 42:590–598
Novak B, Tyson J (2002) Cell cycle controls. Springer, Berlin, pp 261–284
Nowak MA, Sasaki A, Taylor C, Fudenberg D (2004) Emergence of cooperation and evolutionary stability in finite populations. Nature 428:646–650
O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250: 4007–4021
O'Malley MA, Dupre J (2005) Fundamental issues in systems biology. Bioessays 27:1270–1276, BioEssays: news and reviews in molecular, cellular and developmental biology
Oosthuizen JL, Gomez P, Ruan J, Hackett TL, Moore MM, Knight DA et al (2011) Dual organism transcriptomics of airway epithelial cells interacting with conidia of *Aspergillus fumigatus*. PLoS One 6:e20527
Opel M, Lando D, Bonilla C, Trewick SC, Boukaba A, Walfridsson J et al (2007) Genome-wide studies of histone demethylation catalysed by the fission yeast homologues of mammalian LSD1. PLoS One 2:e386
Orth JD, Thiele I, Palsson BO (2010) What is flux balance analysis? Nat Biotechnol 28:245–248
Oshlack A, Robinson MD, Young MD (2010) From RNA-seq reads to differential expression results. Genome Biol 11:220
Pappalardo F, Martinez Forero I, Pennisi M, Palazon A, Melero I, Motta S (2011) SimB16: modeling induced immune system response against B16-melanoma. PLoS One 6:e26523
Peng M, Taouatas N, Cappadona S, van Breukelen B, Mohammed S, Scholten A et al (2012) Protease bias in absolute protein quantitation. Nat Methods 9:524–525
Petranovic D, Nielsen J (2008) Can yeast systems biology contribute to the understanding of human disease? Trends Biotechnol 26:584–590
Pfeiffer T, Schuster S (2005) Game-theoretical approaches to studying the evolution of biochemical systems. Trends Biochem Sci 30:20–25
Picotti P, Clement-Ziza M, Lam H, Campbell DS, Schmidt A, Deutsch EW et al (2013) A complete mass-spectrometric map of the yeast proteome applied to quantitative trait analysis. Nature 494:266–270
Pogson M, Smallwood R, Qwarnstrom E, Holcombe M (2006) Formal agent-based modelling of intracellular chemical interactions. Bio Syst 85:37–45
Pogson M, Holcombe M, Smallwood R, Qwarnstrom E (2008) Introducing spatial information into predictive NF-kappaB modelling–an agent-based approach. PLoS One 3:e2367
Price ND, Papin JA, Schilling CH, Palsson BO (2003) Genome-scale microbial in silico models: the constraints-based approach. Trends Biotechnol 21:162–169
Reinartz J, Bruyns E, Lin JZ, Burcham T, Brenner S, Bowen B et al (2002) Massively parallel signature

sequencing (MPSS) as a tool for in-depth quantitative gene expression profiling in all organisms. Brief Funct Genomic Proteomic 1:95–104

Reiss DJ, Baliga NS, Bonneau R (2006) Integrated biclustering of heterogeneous genome-wide datasets for the inference of global regulatory networks. BMC Bioinformatics 7:280

Ringner M (2008) What is principal component analysis? Nat Biotechnol 26:303–304

Roguev A, Bandyopadhyay S, Zofall M, Zhang K, Fischer T, Collins SR et al (2008) Conservation and rewiring of functional modules revealed by an epistasis map in fission yeast. Science 322:405–410

Royce TE, Rozowsky JS, Gerstein MB (2007) Toward a universal microarray: prediction of gene expression through nearest-neighbor probe sequence identification. Nucleic Acids Res 35:e99

Rubin-Bejerano I, Fraser I, Grisafi P, Fink GR (2003) Phagocytosis by neutrophils induces an amino acid deprivation response in *Saccharomyces cerevisiae* and *Candida albicans*. Proc Natl Acad Sci USA 100:11007–11012

Ruppin E, Papin JA, de Figueiredo LF, Schuster S (2010) Metabolic reconstruction, constraint-based analysis and game theory to probe genome-scale metabolic networks. Curr Opin Biotechnol 21:502–510

Santamaria R, Rizzetto L, Bromley M, Zelante T, Lee W, Cavalieri D et al (2011) Systems biology of infectious diseases: a focus on fungal infections. Immunobiology 216:1212–1227

Schuster S, Kreft JU, Brenner N, Wessely F, Theissen G, Ruppin E et al (2010) Cooperation and cheating in microbial exoenzyme production–theoretical analysis for biotechnological applications. Biotechnol J 5:751–758

Schwarzmüller T, Ma B, Hiller E, Istel F, Tscherner M, Brunke S, Ames L, Firon A, Green B, Cabral V, Marcet-Houben M, Jacobsen ID, Quintin13 J, Seider K, Frohner I, Glaser W, Jungwirth H, d'Enfert C, Ferrandon D, Gabaldón T, Hube B, Rupp S, Cormack B, Haynes K, Kuchler K (in preparation) Systematic phenotyping of a genome-scale candida glabrata deletion collection reveals novel antifungal tolerance genes

Seal JB, Alverdy JC, Zaborina O, An G (2011) Agent-based dynamic knowledge representation of *Pseudomonas aeruginosa* virulence activation in the stressed gut: towards characterizing host-pathogen interactions in gut-derived sepsis. Theor Biol Med Model 8:33

Segovia-Juarez JL, Ganguli S, Kirschner D (2004) Identifying control mechanisms of granuloma formation during *M. tuberculosis* infection using an agent-based model. J Theor Biol 231:357–376

Sellam A, Hogues H, Askew C, Tebbji F, van Het Hoog M, Lavoie H et al (2010) Experimental annotation of the human pathogen *Candida albicans* coding and noncoding transcribed regions using high-resolution tiling arrays. Genome Biol 11:R71

Shmulevich I, Dougherty ER, Kim S, Zhang W (2002) Probabilistic Boolean networks: a rule-based uncertainty model for gene regulatory networks. Bioinformatics 18:261–274

Smedsgaard J, Nielsen J (2005) Metabolite profiling of fungi and yeast: from phenotype to metabolome by MS and informatics. J Exp Bot 56:273–286

Sopko R, Huang D, Preston N, Chua G, Papp B, Kafadar K et al (2006) Mapping pathways and phenotypes by systematic gene overexpression. Mol Cell 21:319–330

Sorgo AG, Heilmann CJ, Dekker HL, Brul S, de Koster CG, Klis FM (2010) Mass spectrometric analysis of the secretome of *Candida albicans*. Yeast 27:661–672

Sorgo AG, Heilmann CJ, Dekker HL, Bekker M, Brul S, de Koster CG et al (2011) Effects of fluconazole on the secretome, the wall proteome, and wall integrity of the clinical fungus *Candida albicans*. Eukaryot Cell 10:1071–1081

Stastna M, Van Eyk JE (2012a) Improved and emerging gel-free separation and detection methods for proteomics. Proteomics 12:2902–2903

Stastna M, Van Eyk JE (2012b) Analysis of protein isoforms: can we do it better? Proteomics 12:2937–2948

Steinhoff C, Vingron M (2006) Normalization and quantification of differential expression in gene expression microarrays. Brief Bioinform 7:166–177

Sun Z, Zhu Y (2012) Systematic comparison of RNA-seq normalization methods using measurement error models. Bioinformatics 28:2584–2591

Tabb DL, Vega-Montoto L, Rudnick PA, Variyath AM, Ham AJ, Bunk DM et al (2010) Repeatability and reproducibility in proteomic identifications by liquid chromatography-tandem mass spectrometry. J Proteome Res 9:761–776

Tamayo P, Slonim D, Mesirov J, Zhu Q, Kitareewan S, Dmitrovsky E et al (1999) Interpreting patterns of gene expression with self-organizing maps: methods and application to hematopoietic differentiation. Proc Natl Acad Sci USA 96:2907–2912

Taylor CF, Paton NW, Lilley KS, Binz PA, Julian RK Jr, Jones AR et al (2007) The minimum information about a proteomics experiment (MIAPE). Nat Biotechnol 25:887–893

Taylor CF, Field D, Sansone SA, Aerts J, Apweiler R, Ashburner M et al (2008) Promoting coherent minimum reporting guidelines for biological and biomedical investigations: the MIBBI project. Nat Biotechnol 26:889–896

Teutschbein J, Albrecht D, Potsch M, Guthke R, Aimanianda V, Clavaud C et al (2010) Proteome profiling and functional classification of intracellular proteins from conidia of the human-pathogenic mold *Aspergillus fumigatus*. J Proteome Res 9:3427–3442

Tierney L, Linde J, Muller S, Brunke S, Molina JC, Hube B et al (2012) An interspecies regulatory network inferred from simultaneous RNA-seq of *Candida albicans* invading innate immune cells. Front Microbiol 3:85

Tokarski C, Hummert S, Mech F, Figge MT, Germerodt S, Schroeter A et al (2012) Agent-based modeling approach of immune defense against spores of opportunistic human pathogenic fungi. Front Microbiol 3:129

Tomar N, Choudhury O, Chakrabarty A, De RK (2013) An integrated pathway system modeling of *Saccharomyces cerevisiae* HOG pathway: a Petri Net based approach. Mol Biol Rep 40(2):1103–1125

Tyc KM, Klipp E (2011) Modeling dissemination of pathogenic fungi within a host: a cartoon for the interactions of two complex systems. J Comput Sci Syst Biol S1:001

Uetz P, Giot L, Cagney G, Mansfield TA, Judson RS, Knight JR et al (2000) A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*. Nature 403:623–627

van het Hoog M, Rast TJ, Martchenko M, Grindle S, Dignard D, Hogues H et al (2007) Assembly of the *Candida albicans* genome into sixteen supercontigs aligned on the eight chromosomes. Genome Biol 8:R52

Velculescu VE, Zhang L, Vogelstein B, Kinzler KW (1995) Serial analysis of gene expression. Science 270:484–487

Villas-Boas SG, Moxley JF, Akesson M, Stephanopoulos G, Nielsen J (2005) High-throughput metabolic state analysis: the missing link in integrated functional genomics of yeasts. Biochem J 388:669–677

von Bertalanffy L (1969) In: General systems theory: foundations, development, applications. Revised (ed) Vol. pp. George Braziller, New York

Wang Z, Gerstein M, Snyder M (2009) RNA-seq: a revolutionary tool for transcriptomics. Nat Rev Genet 10:57–63

Wang RS, Saadatpour A, Albert R (2012) Boolean modeling in systems biology: an overview of methodology and applications. Phys Biol 9:055001

Weiner A, Chen HV, Liu CL, Rahat A, Klien A, Soares L et al (2012) Systematic dissection of roles for chromatin regulators in a yeast stress response. PLoS Biol 10:e1001369

Weinstein JN (2001) Searching for pharmacogenomic markers: the synergy between omic and hypothesis-driven research. Dis Markers 17:77–88

Wierling C, Kuhn A, Hache H, Daskalaki A, Maschke-Dutz E, Peycheva S et al (2012) Prediction in the face of uncertainty: a Monte Carlo-based approach for systems biology of cancer treatment. Mutat Res 746:163–170

Wilkins MR, Pasquali C, Appel RD, Ou K, Golaz O, Sanchez JC et al (1996) From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and amino acid analysis. Biotechnology 14:61–65

Winzeler EA, Shoemaker DD, Astromoff A, Liang H, Anderson K, Andre B et al (1999) Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science 285:901–906

Yan Z, Costanzo M, Heisler LE, Paw J, Kaper F, Andrews BJ et al (2008) Yeast barcoders: a chemogenomic application of a universal donor-strain collection carrying bar-code identifiers. Nat Methods 5:719–725

Yates JR 3rd, Carmack E, Hays L, Link AJ, Eng JK (1999) Automated protein identification using microcolumn liquid chromatography-tandem mass spectrometry. Methods Mol Biol 112:553–569

Yus E, Maier T, Michalodimitrakis K, van Noort V, Yamada T, Chen WH et al (2009) Impact of genome reduction on bacterial metabolism and its regulation. Science 326:1263–1268

Zamboni N, Sauer U (2009) Novel biological insights through metabolomics and 13C-flux analysis. Curr Opin Microbiol 12:553–558

Zhong Q, Simonis N, Li QR, Charloteaux B, Heuze F, Klitgord N et al (2009) Edgetic perturbation models of human inherited disorders. Mol Syst Biol 5:321

Immune Response to Fungal Infections

4 Receptor–Ligand Interactions in Fungal Infections

Sarah E. Hardison[1], Gordon D. Brown[1]

CONTENTS

I. Introduction

Pattern recognition receptors (PRRs) play a key role in the antifungal immune response by recognizing pathogen-associated molecular patterns (PAMPs) on the surface of fungi and initiating antimicrobial defenses. PRRs are found on myeloid and epithelial cells and provide the first line of immune defense by stimulating phagocytosis, respiratory burst, release of antimicrobial compounds, leukocyte recruitment, and importantly, release of inflammatory lipids and cytokines. PRR engagement drives adaptive responses through the release of cytokines that polarize Th1 and Th17-type effector T cells, which are crucial for the control of fungi. PRRs primarily recognize fungal cell wall carbohydrates (Fig. 4.1); these include the structural carbohydrate β-1,3-glucan and mannan modifications found on cell wall proteins. Chitin has demonstrable immunomodulatory properties, but no PRR has been identified that recognizes it (Reese et al. 2007). PRRs are selective for their individual ligands, but receptors will collaborate to elicit orchestrated responses to individual microbes. To date, known classes of PRR that recognize fungi and modulate cellular responses include the Toll-like (TLR), C-type lectin (CLR), Nod-like (NLR), scavenger and complement receptor families. Although TLRs are the best-described class of PRR with regards to other types of pathogens, CLRs are emerging as the major receptor for the recognition of fungi.

II. Pattern Recognition Receptors and Their Fungal Ligands

A. Toll-Like Receptors

The TLRs are the best-characterized family of PRR, and are part of a larger "interleukin-1/Toll-like receptor" superfamily of interleukin (IL) and Toll-like receptors. The members of this superfamily share a characteristic intracellular TIR (Toll-IL-1 receptor) domain, which functions as a protein–protein interaction motif for TIR-containing adaptors such as MyD88 and Trif. TLRs are single membrane-spanning

[1]Aberdeen Fungal Group and Section of Infection and Immunity, University of Aberdeen, Ashgrove Road West, Aberdeen AB25 2ZD, UK; e-mail: gordon.brown@abdn.ac.uk

Human Fungal Pathogens, 2nd Edition
The Mycota XII
O. Kurzai (Ed.)

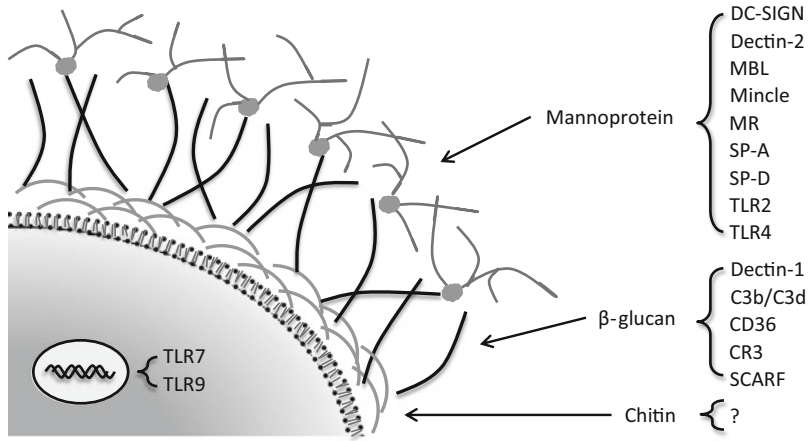


Fig. 4.1. Fungal PAMPs. The fungal cell wall is rich in carbohydrates such as α-mannan (mannosylated proteins), β-glucan, and chitin. The cell wall is a dynamic structure capable of considerable changes, particularly during morphological transition such as yeast to hyphae. Furthermore, the composition of the cell wall varies between different fungal species. Several CLRs have been identified that recognize these cell wall structures, including transmembrane and soluble CLRs. No receptors have been identified that recognize chitin; however, it has demonstrable immunomodulatory properties

noncatalytic receptors that bind microbial ligands and initiate signaling via MyD88 and Trif pathways. The extracellular ligand binding domain of TLRs has the characteristic horseshoe-like structure of the leucine-rich repeat (LRR) family of proteins. Upon ligand binding, TLRs homo- or heterodimerize with other TLRs or the adaptor protein MD2. TLR ligands include such diverse microbial products as bacterial lipopolysaccharide (LPS), lipoproteins, glycolipids, flagellin, double-stranded RNA, and unmethylated CpG DNA.

A number of TLRs have been implicated in fungal recognition, including TLR1, TLR2, TLR4, TLR6, TLR7, and TLR9 (Netea et al. 2008; Brown 2011; Wuthrich et al. 2012). Fungal ligands are not determined for every TLR; although we do know that **TLR2 and TLR4 recognize phospholipomannan and N- or O-linked glycans, respectively, and that TLR9 recognizes fungal DNA**. The individual roles of each TLR in their ability to initiate immune responses to fungi are not fully defined and often controversial. For example, TLR4 stimulates tumor necrosis factor-α (TNF-α) production and intracellular signaling during *Aspergillus fumigatus*, *Candida albicans*, and *Cryptococcus neoformans* infections, yet TLR4-driven cytokine production in response to *C. albicans* is highly variable between different strains (Netea et al. 2010), and this may be reflected by strain-dependent alterations in cell wall structure. Further examples include TLR2 recognition of *C. albicans,* whose activity in some mouse models is immunosuppressive, while in others it is protective (Netea et al. 2004; Villamon et al. 2004).

Mice lacking the TLR adaptor protein MyD88 are susceptible to infections with many fungal species, including *C. albicans*, *Paracoccidioides brasiliensis*, *A. fumigatus*, and *C. neoformans*, thus TLR signaling pathways are crucial for the control of fungal infection. Furthermore, even if the specific roles of TLRs in fungal infection are unclear, polymorphisms in specific TLRs have been linked to human susceptibility to fungal disease in immunosuppressed patients (Netea and van der Meer 2011). It is important to note, however, that immunocompetent individuals who lack MyD88 and other critical downstream signaling components are not predisposed (von Bernuth et al. 2008). Recent evidence demonstrates that TLRs and their signaling components can cooperate with other types of PRR, particularly CLRs, to initiate immune responses to fungi. The interaction of TLRs or MyD88 with CLRs is an integral

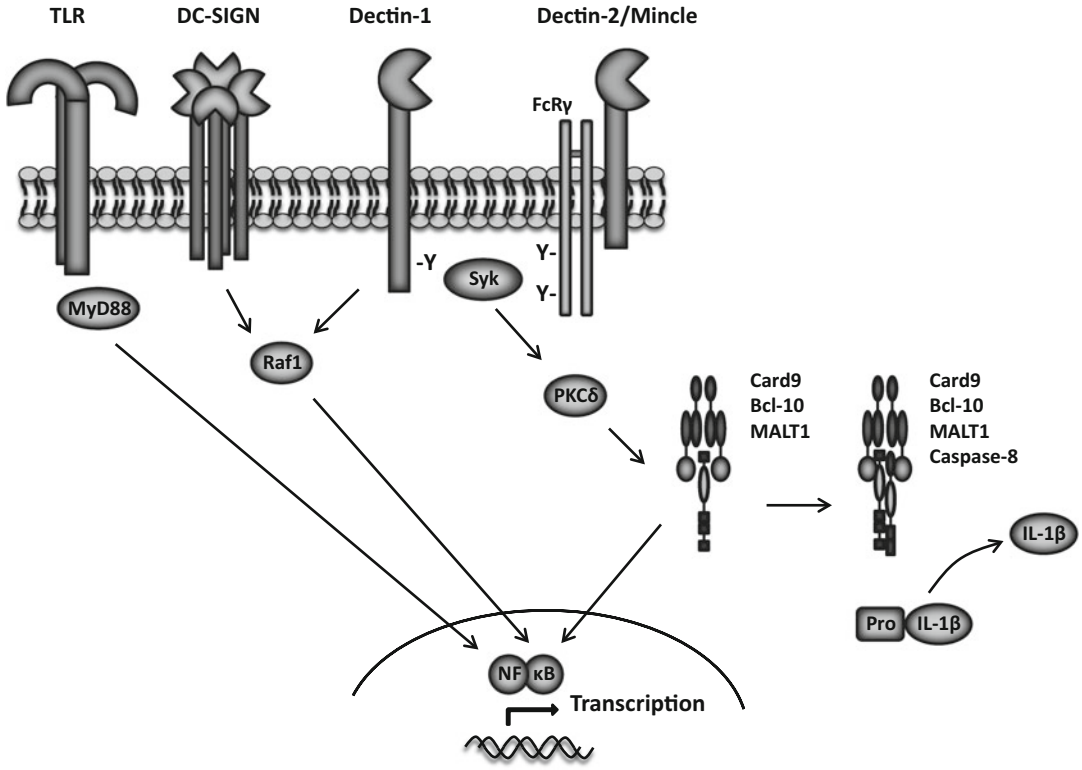


Fig. 4.2. PRR signal transduction pathways. Transmembrane PRRs involved in antifungal immunity and their intracellular signaling pathways. Dectin-1, Dectin-2, and Mincle induce intracellular signaling via immunoreceptor tyrosine (Y)-based activation motifs, which recruit and activate Syk kinase either directly or indirectly through the FcRγ adaptor chain. Syk signals through protein kinase C (PKC)δ to activate the Card9–Bcl-10–Malt1 complex, inducing gene transcription and the production of various inflammatory mediators. When caspase-8 is included in this complex, it can cleave pro-IL-1 β into is biologically active form. Dectin-1 additionally signals through MAPK and NFAT; DC-SIGN and Dectin-1 can signal via the Raf-1 kinase pathway, which modulates the activity of other signaling pathways and gene transcription. TLR primarily signal through MyD88, but can also utilize TRIF

component of antifungal immunity and will be discussed in more detail in later sections of this chapter.

B. C-Type Lectin Receptors

The CLR superfamily consists of soluble and transmembrane proteins defined by a characteristic carbohydrate recognition domain (CRD) or, more accurately, a C-type lectin-like domain (CTLD). The "C" denotes a historical requirement for calcium binding, although calcium binding is not required for all the CLRs known to date. Collectively, CLR bind nearly every fungal species that infects vertebrates, including *C. albicans*, *A. fumigatus*, *Pneumocystis carinii*, *Coccidioides immitis*, *P. brasiliensis*, *Histoplasma capsulatum*, *Malassezia* sp., *Trichophyton* sp., and *C. neoformans* (Taylor et al. 2005; Brown 2006; McGreal et al. 2006; Sato et al. 2006; Wuthrich et al. 2012). Many CLR are coupled either directly or indirectly to the Src family signal transduction kinase Syk, which activates MAPK, NFAT, and through the PKCδ-CARD9-Bcl10-MALT1 axis, nuclear factor-κB (NFκB) (Fig. 4.2) (Sato et al. 2006; LeibundGut-Landmann et al. 2007; Robinson et al. 2009). Genetic deletion of Syk or CARD9 in mice or humans abrogates protective immune responses to fungal particles and intact *C. albicans* yeasts, and these deficiencies are greater than deletions in any single CLR receptor (Rogers et al. 2005;

Gross et al. 2006; LeibundGut-Landmann et al. 2007). Some CLR activate noncanonical NFκB subunits through the Raf1 kinase (Gringhuis et al. 2007, 2009b), and the signaling pathways for other CLR are unknown at the time of writing this chapter. **CLR initiate immunity to fungi through stimulating phagocytosis, respiratory burst, and the release of cytokines that polarize adaptive responses toward protective Th1/Th17 immunity.** Here we will cover the transmembrane and soluble CLRs that are known to initiate immunity against fungi.

1. Dectin-1

Dendritic cell-associated C-type lectin receptor-1 (Dectin-1) was initially identified in a subtractive cloning screen as a dendritic cell-associated receptor with unknown function (Ariizumi et al. 2000b). Later, **zymosan screening of a macrophage cDNA expression library identified Dectin-1 as the first known pattern recognition receptor for β-glucan** (Brown and Gordon 2001). The gene encoding for Dectin-1, *CLEC7a*, is found within the natural killer cell (NKC) gene complex within a cluster of genes encoding for several PRRs that is now known as the Dectin-1 cluster (Huysamen and Brown 2009). Dectin-1 contains an immunoreceptor tyrosine-based activation motif (ITAM)-like domain (also called a Hemi-ITAM) in its cytoplasmic tail that mediates cellular activation through the phosphorylation of a key tyrosine residue that serves as a docking site for the signal transduction kinase Syk. Dectin-1 lacks calcium-binding motifs and thus does not require calcium for ligand recognition.

Dectin-1 selectively and specifically binds β-1,3-glucans, which are structural carbohydrates in the fungal cell wall (Gow et al. 2012); interestingly there seems to be an unidentified endogenous ligand present on T cells (Ariizumi et al. 2000b). β-glucan-containing particles such as zymosan and curdlan are potent Dectin-1 activators often used to probe Dectin-1 function in vitro, and the activity of these ligands can be competitively inhibited by the soluble β-glucans glucan phosphate and laminarin. Dectin-1 utilizes the signal transduction kinase Syk to activate MAPK, NFAT, and through the PKCδ-CARD9-Bcl10-MALT1 axis, NFκB (Gross et al. 2006; Goodridge et al. 2007; LeibundGut-Landmann et al. 2007; Sancho and Reis e Sousa 2012; Strasser et al. 2012). Furthermore, Dectin-1 can activate noncanonical NFκB subunits independently of Syk through the serine and threonine kinase Raf-1 (Gringhuis et al. 2009b), though it is unclear how Raf-1 interacts with the receptor itself. Interestingly, Dectin-1 signaling is only accomplished upon clustering into a "phagocytic synapse" from which the regulatory tyrosine phosphatases CD45 and CD148 are excluded (Goodridge et al. 2011).

Dectin-1 binds a range of fungal pathogens, and its importance to antifungal defense is evidenced by susceptibilities to *A. fumigatus*, *C. albicans*, and *P carinii* infections in Dectin-1-deficient mice. Dectin-1 is expressed primarily on cells of myeloid lineage, specifically macrophages, dendritic cells, monocytes, and neutrophils, although it is also found on an innate-like subset of T cells, γδ T cells (Martin et al. 2009). Dectin-1 engagement on myeloid cells initiates phagocytosis, respiratory burst, and release of inflammatory mediators including eicosanoids and cytokines/chemokines such as TNF-α, IL-1β, IL-6, IL-23, CCL2, CXCL1, and CCL3. These effects are mediated by the Syk/CARD9 signaling pathway in all cell types except for macrophages, in which Dectin-1 elicits phagocytosis independently of Syk (Herre et al. 2004). Indeed, in macrophages, Syk/CARD9 is only required for respiratory burst, and this response is restricted to a subset of cells that are primed to use this kinase (Underhill et al. 2005). Interestingly, myeloid usage of Syk/CARD9 is cell-type-specific, with only bone marrow-derived dendritic cells, peritoneal macrophages, alveolar macrophages, and GM-CSF or interferon (IFN)-γ primed bone marrow-derived macrophages being able to produce robust TNF-α following Dectin-1 stimulation (Goodridge et al. 2009a). Additionally, **dendritic cells stimulated by Dectin-1 activate naïve $CD4^+$ T cells and direct their polarization towards protective Th1/Th17 effector cells. Th17 immunity is crucial to antifungal defenses at mucosal sites of infection such as the oral mucosa** (Conti et al. 2009); furthermore, during pulmonary infection with

A. fumigatus, Dectin-1 suppresses IL-12 and IFN-γ production, favoring Th17 differentiation (Rivera et al. 2011). Interestingly, Dectin-1-stimulated dendritic cells instruct a percentage of regulatory T cells (Treg) to become IL-17 secreting effector cells (Osorio et al. 2008).

Th17 effector cells require IL-1β, IL-6, and IL-23 for polarization and maintenance; however, IL-1β is initially produced as a pro-peptide that requires cleavage to mature to its bioactive form. The inflammasome is a complex of cytoplasmic proteins responsible for the cleavage of pro-IL-1β, and Dectin-1 stimulation leads to the activation of the inflammasome and IL-1β production. Dectin-1-derived signals are translated to NLRs such as NLRP3 or NLR4C by an unknown mechanism that activates caspase-1 and initiates IL-1β processing; additionally, a direct link to IL-1β processing from Dectin-1 through a noncanonical inflammasome involving caspase-8 has also been described (Gringhuis et al. 2012).

The importance of Dectin-1 in antifungal immune defense is evidenced by human polymorphisms that lead to susceptibility to fungal infection. **One polymorphism in Dectin-1, Y238X**, generates an early stop codon, resulting in a truncated nonfunctional receptor. This mutation has been identified in patients with **recurrent mucosal candidiasis, and cells from these patients have poor cytokine responses to *C. albicans* and β-glucan** (Ferwerda et al. 2009). Interestingly, individuals with CARD9 polymorphisms have a more severe phenotype than that of Dectin-1 (Glocker et al. 2009). **CARD9 deficiency renders humans susceptible to infection with *Candida* and results in complete Th17 deficiency and altered Th1 responses** (LeibundGut-Landmann et al. 2007; Glocker et al. 2009). Reduced Th17 responses also correlate with susceptibility to mucocutaneous infections in Dectin-1-deficient humans (Ferwerda et al. 2009). Indeed, various defects in Th17 immunity, including mutations in STAT1, STAT3, IL-17, and IL17RA have been linked to susceptibility to mucocutaneous infections, especially chronic mucocutaneous candidiasis (CMC) (Ma et al. 2008; Milner et al. 2008; Liu et al. 2011; Puel et al. 2011; van de Veerdonk et al. 2011b). Furthermore, other diseases characterized by susceptibility to CMC, such as autoimmune polyendocrine syndrome 1, are also associated with alterations in Th17 immunity (Kisand et al. 2010; Puel et al. 2010). Recently, a polymorphism in Dectin-1 has also been linked to a severe form of ulcerative colitis, implicating a crucial role for this receptor in modulating fungal responses in the microbiome (Iliev et al. 2012).

2. Dectin-2

Dectin-2 was originally identified as an overexpressed transcript in a murine myeloid leukemia model (Fernandes et al. 1999), and was also identified as a dendritic cell-specific lectin in the subtractive cloning screen that identified Dectin-1 (Ariizumi et al. 2000a). Since the generation of an anti-Dectin-2 antibody, it has also been found on tissue macrophages and peripheral blood monocytes (Taylor et al. 2005). **Dectin-2 is selective and specific for α-mannans, such as those found in the fungal cell wall** (McGreal et al. 2006; Robinson et al. 2009). Dectin-2 is structurally similar to Dectin-1; however, its cytoplasmic tail is truncated and thus lacks an ITAM domain. Instead, Dectin-2 couples with the ITAM-containing FcRγ to activate Syk and, subsequently, the PKCδ-CARD9-Bcl10-MALT1 axis (Sato et al. 2006). The Dectin-2 CRD contains an EPN (Glu-Pro-Asn) motif that often indicates mannose specificity and is also found in the CLRs DC-SIGN, MBL, and MR. The gene encoding for Dectin-2 (*CLECf4n*) is also found within the NKC gene complex, in a cluster of genes adjacent to the Dectin-1 cluster. Genes encoded in the Dectin-2 cluster include another important fungal CLR, Mincle, as well as PRRs involved in cell turnover and homeostasis, such as BDCA-1, DCIR, and DCAR. Each of these receptors in the Dectin-2 cluster couple to FcRγ to initiate signaling, except for DCIR, whose cytoplasmic tail contains an inhibitory immunoreceptor (ITIM) signaling motif (Graham and Brown 2009).

Like Dectin-1, Dectin-2 also binds zymosan particles with high affinity; interestingly, however, when it comes to *C. albicans* Dectin-2 is thought to preferentially bind the hyphal morphology whereas Dectin-1 preferentially binds yeasts, although there is plenty of evidence to suggest that Dectin-1 will bind hyphae and

Dectin-2 yeasts (Gantner et al. 2005; Sato et al. 2006; Saijo and Iwakura 2011). In fact, cytokine production in response to *C. albicans* hyphae is only mildly abrogated in macrophages from Dectin-2-deficient mice, indicating that other receptors are clearly involved. Dectin-2 is known to bind a variety of fungal species, including *C. albicans*, unencapsulated *C. neoformans*, *H. capsulatum*, *P. brasiliensis*, and the dermatophyte species *Microsporum* and *Trichophyton* (Sancho and Reis e Sousa 2012). Ligation of Dectin-2 results in yeast phagocytosis and release of inflammatory cytokines, including TNF-α, IL-1β, IL-6, IL-12, and IL-23 (Robinson et al. 2009; Saijo et al. 2010). Dectin-2 signaling drives antifungal Th17 responses, and Dectin-2-deficient mice are impaired in Th17 immunity and are thus susceptible to *C. albicans* infection; however, Dectin-2 appears to collaborate with Dectin-1 to modulate Th1 immunity, rather than directly drive Th1 immunity on its own (Robinson et al. 2009; Saijo et al. 2010). Interestingly, Dectin-2 can also drive Th2 responses during allergy (Barrett et al. 2011). No human Dectin-2 polymorphisms have been identified to date.

3. DC-SIGN

The dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin receptor (DC-SIGN, CD209) in the human genome is found within a cluster of genes that encode for three related members of a "DC receptor" class, so named for their homology to DC-SIGN. These other members include CD23, a characteristic surface molecule of B cells, and SLectin, which is found on liver endothelium (Geijtenbeek et al. 2000). The murine DC-SIGN locus contains eight paralogues of CD209 (SIGNR1 to SIGNR8), though SIGNR6 is a pseudogene. SIGNR3 appears to be the closest relative to human DC-SIGN, as it is the only isoform that recognizes terminal mannose; however, SIGNR1 may also bind fungi (Powlesland et al. 2006). DC-SIGN is found on dendritic cells of the skin and mucosa and some subsets of tissue macrophages. Structurally, DC-SIGN contains a characteristic C-type lectin CRD with a mannan-binding EPN motif, transmembrane stalk, and intracellular tyrosine implicated in signaling. It functionally exists in the membrane as a tetramer, which lends a great amount of flexibility to ligand binding, and organizes itself into nanoclusters in the membrane (Cambi et al. 2004; Manzo et al. 2012). **DC-SIGN binds mannose and fucose linkages of diverse microbial origin, including fungi, bacteria, and viruses**. Though DC-SIGN contains an intracellular tyrosine it has not been directly associated with signaling; however, it does mediate phagocytosis of fungi, and plays a modulatory role in cytokine production. Like Dectin-1, DC-SIGN can activate noncanonical NFκB through Raf-1 (Gringhuis et al. 2007). Interestingly, DC-SIGN utilizes Raf-1 to collaborate with various TLRs to modulate cytokine signaling. Raf-1 activity results in acetylation of the p65 subunit of NFκB, but only after NFκB is previously activated by TLR3, TLR4, or TLR5. This NFκB acetylation leads to increased NFκB binding affinity at cytokine genes, including those coding for IL-6, IL-10, and IL-12. Thus, while Raf-1 activation by DC-SIGN is independent of TLRs, TLR signaling must first activate NFκB for Raf-1 to have its modulatory effects (Gringhuis et al. 2007). Furthermore, depending on whether mannose or fucose is presented, Raf-1 may be excluded from the signaling complex, resulting in differential cytokine expression. For example, Raf-1 is excluded upon recognition of *Helicobacter pylori*, a fucose-containing bacteria (Gringhuis et al. 2009a).

4. Mannose Receptor

Receptors with fungal mannose-binding capability have been described since the early 1980s, and, as such, the mannose receptor (MR, CD206, Clec13D) was **the first characterized fungus-binding receptor** (Jouin et al. 1981; Berton and Gordon 1983). MR is the prototypical member of the MR family of lectins that includes Dec205, Endo180, and phospholipase A2 receptor (PLA2R), which are all collagen-binding endocytic receptors involved in the clearance of endogenous ligands. MR is expressed on macrophages, inflammatory dendritic cells, and

some non-myeloid cells such as liver endothelium. **MR recognizes terminal mannose residues of α-mannan from a variety of microbial sources as well as putative endogenous ligands** (Linehan et al. 2001). The MR family of receptors has a unique structure compared to other CLRs, containing multiple CRD domains. MR itself has eight CRDs that bind carbohydrate moieties with varying specificity, although five of these (CRD4-8) are adequate to reproduce the binding affinity of the full-length molecule (Taylor et al. 1990, 1992). **MR does not contain any known consensus sequence for signal transduction, and no signaling pathways have been identified for it; therefore, MR probably exists as a modulatory receptor that facilitates the activities of canonical PRRs** (Martinez-Pomares 2012). The MR has been implicated in the recognition of several fungi in vitro, yet in mice this receptor appears to only be required for protective immunity to infections with *C. neoformans* (Lee et al. 2003; Dan et al. 2008). Although its signal transduction mechanisms are unknown, MR activation is known to promote the development of Th17 responses against *C. albicans*; however, MR knockout mice are not susceptible to infection with *C. albicans* or *P. carinii* (Lee et al. 2003; Swain et al. 2003; van de Veerdonk et al. 2009).

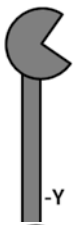
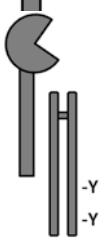
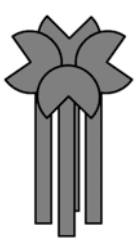
5. Mincle

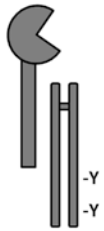
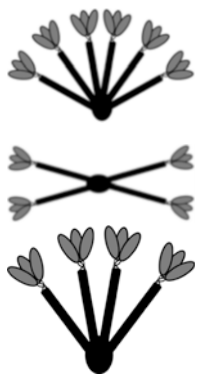
The macrophage-inducible C-type lectin, Mincle (Clec4e, Clecsf9), is located in the Dectin-2 cluster of the NKC gene complex and is structurally similar to Dectin-2. Mincle couples to Syk via the FcRγ to activate the PKCδ-CARD9-Bcl10-MALT1 axis, although Mincle does not induce phagocytosis, only cytokine production, in response to fungal ligands (Wells et al. 2008). Mincle is constitutively expressed at a low level on myeloid cells, and can be induced by LPS or cytokines such as IFN-γ. Although the FcRγ is not necessary for surface expression of Mincle, upregulation in response to LPS stimulation is abrogated in FcRγ-deficient mice (Yamasaki et al. 2008). Interestingly, the expression pattern of Mincle in response to *C. albicans* is similar to that of TLR2 (Wells et al. 2008). **Mincle recognizes α-mannan in the cell wall of *C. albicans*, *F. pedrosoi*, and *Malassezia* sp.**, is a receptor for mycobacterial cord factor, and plays a major homeostatic role in sensing necrosis, namely through recognition of the endogenous ligand SAP130 (Brown 2008; Bugarcic et al. 2008; Wells et al. 2008; Yamasaki et al. 2009). Using an NFAT-GFP reporter cell line, Mincle was found to specifically recognize several strains of *Malassezia* sp., and Mincle-deficient mice challenged with *Malassezia* produced less TNF-α, IL-10, CXCL1, and CXCL2 than wild-type mice (Yamasaki et al. 2009). Mincle is also necessary for proinflammatory TNFα responses to *F. pedrosoi*, particularly during TLR co-stimulation (see below) (Sousa Mda et al. 2011). Mincle-deficient mice are more susceptible to infection with *C. albicans*; however, there are no differences in survival between wild-type and Mincle-deficient mice when given a lethal dose of *C. albicans*, suggesting that Mincle plays a regulatory role for the receptor during *Candida* infection that can be compensated for (Wells et al. 2008). Although there is no data as yet for Mincle-mediated polarization of adaptive responses to fungi, it is known that Mincle is essential for driving Th17 responses to mycobacterial factors (Schoenen et al. 2010).

6. Collectins

Collectins are a family of soluble, secreted C-type lectins. Structurally, they contain the characteristic C-type lectin CRD, which is linked to a collagen-like arm, and individual subunits combine to form a functional multimeric protein (see Table 4.1). In mice, this class is composed of surfactant proteins A and D (SP-A, SP-D) and two mannose-binding lectins (MBL1 and MBL2), whereas in humans there are two SP-A isoforms and only one MBL. **Mannose and terminal mannan are the primary ligands that SP-A, SP-D, and MBL bind, and together these receptors have been found to bind nearly every known human fungal pathogen, including *A. fumigatus*, *B. dermatitidis*, *C. albicans*, *C. neoformans*, *H. capsulatum*, and *P. carinii*** (Brummer and Stevens 2010). Although lung surfactants are required for respiration by reducing surface tension at the

Table 4.1. C-type lectin receptors in antifungal Immunity

Receptor	Selected ligands	Signal transduction pathways	Major cell types expressing the receptor	Structure	Selected references
Dectin-1	β-1,3-Glucan *Aspergillus* *Candida* *Coccidioides* *Cryptococcus* (spores) *Pneumocystis*	Syk/CARD9 Raf-1 MAPK NFAT	Dendritic cells Macrophages Monocytes Neutrophils		Brown (2011) Brown et al. (2003) LeibundGut-Landmann et al. (2007) Taylor et al. (2007)
Dectin-2	α-Mannan *Candida* *Cryptococcus* (acapsular) *Histoplasma* *Paracoccidioides* *Trichophyton* *Microsporum*	Syk/CARD9 (via FcRγ)	Dendritic cells Macrophages Inflammatory monocytes		Sancho and Reis (2012) McGreal et al. (2006) Robinson et al. (2009) Saijo et al. (2010)
DC-SIGN	α-Mannan *Aspergillus* *Candida* *Paracoccidioides*	Raf-1	Dendritic cells Macrophages		Gringhuis et al. (2007) van den Berg et al. (2012)
MR	α-Mannan *Candida* *Cryptococcus* *Pneumocystis*	Unknown	Macrophages Inflammatory dendritic cells Liver epithelium		van de Veerdonk et al. (2009) Martinez-Pomares (2012) McGreal et al. (2005)

Mincle	α-Mannan *Candida* *Malassezia*	Syk/CARD9 (via FcRγ)	Macrophages		Brown (2008) Wells et al. (2008)
SP-A SP-D MBL	α-Mannan *Aspergillus* *Blastomyces* *Candida* *Cryptococcus* *Histoplasma* *Pneumocystis*	Complement (C1q) Modulates other PRR	Secreted by lung epithelium (SP-A, SP-D) Secreted by liver epithelium (MBL)		Kuroki et al. (2007) Brummer and Stevens (2010)

air–liquid interface, their immune role is to opsonize yeast cells or conidia, causing aggregation of the fungal particles and resulting in enhanced phagocytosis and oxidative burst by macrophages. Surfactant-opsonized zymosan particles are taken up by macrophages as aggregates, an effect that can be reversed by EDTA (Faro-Trindade et al. 2012). Uptake of surfactant-opsonized particles has been suggested to occur via interaction with opsonic receptors such as C1q (Eggleton et al. 2000), and cytokine responses are thought to occur in collaboration with other PRRs or a putative SP-A receptor (Gardai et al. 2003; Kuroki et al. 2007). However, recent studies have suggested that the only major function of surfactant in fungal recognition is particle aggregation (Faro-Trindade et al. 2012). SP-A and SP-D are produced by type II alveolar cells and Clara cells in the airways; MBL is produced in the liver and released into the plasma for distribution. MBL is an opsonic factor in the lectin pathway of complement activation. MBL recognizes antibody–antigen complexes and interacts with proteases such as C1r or MBL-associated serine proteases (MASPs), which then activate the lectin pathway of complement. Interestingly, low MBL levels have been associated with susceptibility to mycoses in humans (Mullighan et al. 2002; Granell et al. 2006). SP-D-deficient mice are susceptible to pulmonary *A. fumigatus* infection, which can be reversed by administration of exogenous SP-D; however, SP-A-deficient mice are more resistant to *A. fumigatus*, a result that is characterized by higher IL-4 to IFN-γ ratios (Madan et al. 2010).

C. NOD-Like Receptors

Nucleotide oligomerization domain (NOD)-like receptors (NLRs) are a family of more than 20 cytoplasmic proteins that regulate inflammatory and apoptotic processes, including the processing and activation of pro-IL-1β and pro-IL-18 in a multiprotein complex termed the "inflammasome" (Franchi et al. 2012). Both of these cytokines are essential for protective antifungal immunity, particularly for driving the development of protective Th1 and Th17 responses (van de Veerdonk et al. 2011a). **Two NLRs (NLRP3 and NLRC4) and their inflammasome components, including the adaptor molecule ASC and the protease caspase-1, are implicated in mediating IL-1β and IL-18 responses to fungi** (Netea et al. 2003; Vonk et al. 2006; Gross et al. 2009; Hise et al. 2009; Said-Sadier et al. 2010; van de Veerdonk et al. 2011a). Both NLRs are required for controlling mucosal infections with *Candida*, but only NLRP3 is involved in preventing dissemination (Hise et al. 2009; Tomalka et al. 2011). CLRs and TLRs can induce pro-IL-1β in response to fungi, but how the NLRs actually sense fungal pathogens is still unclear. Activation of the NLRP3 inflammasome in response to *A. fumigatus* and *C. albicans* requires Syk kinase, as well as respiratory burst and potassium efflux, which is suggestive of a direct involvement of CLRs (Gross et al. 2009; Hise et al. 2009; Said-Sadier et al. 2010). One inflammasome pathway, mediated by caspase-8 cleavage, is directly stimulated via Dectin-1 through Syk and does not require ASC or NLRs; instead caspase-8 is integrated into the CARD9-Bcl10-MALT1 complex via binding to MALT1 (Gringhuis et al. 2012) (Fig. 4.2).

D. Scavenger Receptors

Scavenger receptors are best known for their roles in atherosclerosis and Alzheimer's disease. During atherosclerosis, they bind and internalize oxidized polyanionic lipid products such as LDL, generating the classic "foamy" cell macrophage morphology and leading to enhanced plaque inflammation. In the case of Alzheimer's disease, it is believed that scavenger receptors such as CD36 recognize amyloid fibrils, resulting in protein secretion that exacerbates amyloid adducts. Scavenger receptors also bind a wide diversity of ligands and are crucial mediators of turnover, clearance, and homeostasis throughout the lifespan of an individual; **they also play a role in the recognition of microbe-derived ligands, including fungal PAMPs such as β-glucan** (Goodridge et al. 2009b). Scavenger

receptor A (SRA) isoforms I and II have been shown to mediate uptake of *C. albicans* yeasts in transfected CHO cells; however, macrophages from SRA-I/II-deficient mice show no defect in uptake, suggesting a redundant role for these receptors in fungal recognition (Wang and Chandawarkar 2010). Scavenger receptor class F, member 1 (SCARF) and CD36 have been shown to mediate uptake of *C. neoformans* and to modulate TLR2 signaling in response to fungal pathogens (Means et al. 2009).

E. Complement

Complement is an inducible cascade of factors that lead to pathogen opsonization, agglutination, and the assembly of a pore-forming complex in the membrane. The three pathways of complement activation (classical, lectin, and alternative) converge at the level of C3, generating the anaphylatoxins C3a and C5a and the membrane attack complex (MAC). The classical pathway begins with deposition of C1q onto antigen–antibody complexes; the lectin pathway with the binding of MBL to surface carbohydrates; and the alternative pathway by spontaneous activation of C3. **Fungi are potent activators of complement, resulting in opsonization, uptake, and recruitment of inflammatory cells, and this is essential for the clearance of fungi, as complement-deficient mice are susceptible to infection** (Kozel 1996). Specifically, mice deficient in C3 or A/J and DBA2 mice, which are naturally deficient in C5, are susceptible to infection with *C. albicans*, and C3 is implicated in mortality after very high doses of *Saccharomyces cerevisiae* in mice (Tsoni et al. 2009). Interestingly, **fungi are resistant to complement-mediated lysis by the MAC, therefore the crucial roles of complement appear to be opsonization, uptake by myeloid or other inflammatory cells, and regulation of inflammation**. Some complement components specifically recognize fungal PAMPs: **complement receptor 3 (CR3) possesses a lectin domain that binds β-glucan, and C3b and C3d are readily deposited on beads coated with β-1,6-glucan** (Rubin-Bejerano et al. 2007; Goodridge et al. 2009b).

III. Immune Modulation by Fungal Pattern Recognition Receptors

Fungal recognition by PRRs mobilizes innate immune cells to initiate antimicrobial responses, and skews the polarization of adaptive immunity towards protective Th1 and Th17 immunity. All of the signaling CLRs described above (Dectin-1, Dectin-2, DC-SIGN, MR, and Mincle) directly contribute to these processes, and their critical role in immunity is evidenced by increased susceptibilities to fungal infection in CLR-, Syk-, or CARD9-deficient mice (LeibundGut-Landmann et al. 2007; Saijo et al. 2007; Taylor et al. 2007; Wells et al. 2008; Robinson et al. 2009; Saijo et al. 2010). The individual role of TLRs in antifungal immunity is controversial; however, their modulatory activity on CLR function is critical, as will be discussed later in this chapter. Scavenger receptors appear to be ancillary phagocytic receptors, but may modulate TLR responses. NLRs initiate inflammasome processing of IL-1β, which is crucial for antifungal immunity; however, prior TLR or CLR engagement is required for NLR inflammasome activation against fungi. Interestingly, an inflammasome-activating pathway independent of NLRs and dependent on Dectin-1 has recently been described, circumventing NLR inflammasomes during fungal infection (Gringhuis et al. 2012). **Thus, it is apparent that CLRs are the critical receptor class for immunity to fungi, and the involvement of other PRRs modulates and refines these responses.**

Phagocytic cells are essential components of protective antifungal immunity and defects in their antimicrobial effects result in susceptibility to fungal infection. CLR mediate these functions and promote inflammatory responses during fungal infection. Dectin-1 and Dectin-2, for example, induce the production of inflammatory mediators, including eicosanoids, TNF, IL-1β, IL-6, IL-23, CCL2, CXCL1, and CCL3 (Underhill et al. 2005; Saijo et al. 2010; Suram et al. 2010). In mouse models of infection with *C. albicans*, loss of Dectin-1 or Dectin-2 results in a failure to mount protective inflammatory responses, defective neutrophil and monocyte recruitment, and a failure to control fungal

growth (Taylor et al. 2007; Saijo et al. 2010). The same observations have been made in Dectin-1-deficient mice infected with *P. carinii* or *A. fumigatus* (Saijo et al. 2007; Werner et al. 2009).

As we have already discussed, CLRs drive critical adaptive responses to fungi. All of the signaling CLRs that recognize fungi are capable of inducing or modulating Th1 and/or Th17 responses. **Although Th1 responses are essential for the control of systemic fungal infections, in part through the activation of phagocytes by the cytokine IFN-γ, Th17 responses appear to be required for mucosal protection** (Conti and Gaffen2010; Romani 2011). How Th17 responses drive protection in the mucosa is unclear, but is thought to involve IL-17-mediated neutrophil recruitment and IL-22-mediated induction of antimicrobial peptides from epithelial cells (Conti and Gaffen 2010). Th17 responses may also be involved in controlling systemic infections with some fungi, such as *Candida*, although there is evidence to suggest that these responses may contribute to pathology and susceptibility in certain settings (Zelante et al. 2007; Saijo et al. 2010). Interestingly, during pulmonary infection with *Aspergillus*, Dectin-1 suppresses IL-12 and IFN-γ production, favoring Th17 differentiation (Rivera et al. 2011).

Although it is unclear how Th17 immunity drives protection, there are significant insights into the mechanisms by which CLRs drive Th17 responses. Stimulation of Dectin-1, for example, induces dendritic cell maturation and the expression of polarizing cytokines such as IL-1β, IL-6, and IL-23, which promote Th17 differentiation (LeibundGut-Landmann et al. 2007). The activation of the NFκB subunit, c-Rel, by the CARD9-Bcl10-MALT1 complex induces the production of IL-1β and IL-23, and this is essential for Dectin-1- and Dectin-2-mediated Th17 differentiation (Gringhuis et al. 2011). In fact, Dectin-2 appears to selectively activate c-Rel, whereas Dectin-1 also activates other NFκB subunits, including the noncanonical RelB, which promotes both Th1 and Th17 responses in vitro (Gringhuis et al. 2009b; Gringhuis et al. 2011). Dectin-1-stimulated dendritic cells can convert a subset of Treg cells into IL-17 secreting effector T cells (Osorio et al. 2008). Furthermore, Dectin-1 (along with TLR2) can amplify MR-induced Th17 responses (van de Veerdonk et al. 2009).

CLRs and their signaling pathways play varied roles in the development of antifungal immune responses. Notably, **CARD9 deficiency renders both mice and humans susceptible to infection with *Candida* and results in ablated Th17 and altered Th1 responses** (LeibundGut-Landmann et al. 2007; Glocker et al. 2009). **Reduced Th17 responses also correlated with susceptibility to mucocutaneous infections in Dectin-1-deficient humans** (Ferwerda et al. 2009). In mice, deficiency of Dectin-1 or Dectin-2 results in susceptibility to infection with *Candida*, but only loss of Dectin-2 resulted in significant alterations in Th17 responses (Robinson et al. 2009; Saijo et al. 2010). Loss of both Dectin-1 and Dectin-2, however, also lead to profound reductions in Th1 responses, further demonstrating the importance of receptor cooperation, which will be discussed later in this chapter (Robinson et al. 2009). Interestingly, Dectin-2 is capable of inducing Th2 immunity in response to house-dust mite allergens (Barrett et al. 2011), but whether this receptor, or other CLRs, are able induce this type of response to fungi needs to be investigated, as Th2 immunity is generally considered to contribute to fungal susceptibility (Romani 2011).

Fungi, through the activities of CLRs, can also influence the function of γδ and iNKT cells. γδ T cells are potent innate sources of IL-17, and they produce this cytokine in response to IL-23 and IL-1β by directly ligating to the pathogen (Sutton et al. 2009). $CCR6^{+}$ γδ T cells express TLR1, TLR2, and Dectin-1, and triggering of these receptors directly induces IL-17 production in these cells; importantly, this IL-17 is produced in large amounts and is prior to the development of adaptive Th17 responses, potentially delivering a polarizing source of cytokine to drive early responses (Martin et al. 2009; Sutton et al. 2009). iNKT cells, in contrast, do not respond directly to fungi, although they are required for the control of fungal pathogens in vivo. Instead, IL-12 production mediated by Dectin-1 or TLRs enables self-reactive iNKT cells to produce IFN-γ (Cohen et al. 2011). Although Dectin-1 normally suppresses IL-12 production by dendritic cells, as discussed above, co-culture with iNKT cells restored production of this cytokine. Such iNKT responses could be initiated by several fungal species, including *A. fumigatus*,

C. albicans, *H. capsulatum*, and *Alternaria alternate* (Cohen et al. 2011). Thus, the CLR-mediated responses of γδ and iNKT cells may represent key early steps in the development of protective antifungal immune responses.

IV. Differential Recognition of Fungi and Immune Evasion

The fungal cell wall is a dynamic structure that is capable of major transformations during growth and/or morphological transition. Furthermore, the cell wall composition of individual strains can differ such that recognition by certain PRRs is variable. Strains of *C. albicans* are highly variable in their recognition by Dectin-1, Dectin-2, MR, or TLR4, as blockade of these receptors results in varying degrees of cytokine inhibition by phagocytes pulsed with *C. albicans* isolates. For some strains, multiple receptors contribute to cytokine induction whereas for others complete blockade is achieved with a single inhibitor (Netea et al. 2010; Gringhuis et al. 2011). To add to the complexity, *Candida* hyphae are preferentially recognized by Dectin-2 and only swollen or germinating *Aspergillus* spores are recognized by Dectin-1 (Gantner et al. 2005; Aimanianda et al. 2009). Exposed β-glucans become masked by mannan upon the formation of *Candida* hyphae, and by hydrophobins on the surface of resting *Aspergillus* conidia. These are clever strategies for immune evasion, allowing the organism to establish an invasive infection without recognition. Interestingly, the antifungal drug caspofungin has been demonstrated to unmask *C. albicans* β-glucan moieties in vitro and in vivo, allowing recognition by PRR. This unmasking takes place at subclinical doses of the drug and is preferential for the hyphal form (Wheeler et al. 2008). Likewise, *H. capsulatum* uses a layer of α-glucan to shield β-glucan moieties from detection. *C. neoformans* evades recognition by cloaking PAMPs with a polysaccharide capsule, which is able to expand during infection, blocking the ability of most PRR to recognize it; furthermore, *C. neoformans* can shed capsule components, diverting immune cells away from the yeast (Voelz and May 2010). Other mechanisms of evading immune recognition by *A. fumigatus*, *C. albicans*, or *C. neoformans* include the modulation of complement activation by inactivating or depleting complement proteins or secreting complement-degrading proteases (Zipfel et al. 2007). Understanding the roles of differential receptor binding and mechanisms that fungi use to evade immune recognition could lead to powerful clinical treatment options in the future.

V. Receptor Co-stimulation and Crosstalk

As we have seen, most fungi are recognized by more than one receptor. **In vivo, coordinated recognition is required for optimal antifungal responses to intact pathogens.** There are several examples where direct interactions between receptor families have been demonstrated to occur. Collaborative responses from Dectin-1 and TLR-2 were among the first such interactions described (Brown et al. 2003; Gantner et al. 2003), and now we know that Dectin-1 can cooperate with several MyD88-coupled TLRs such as TLR2, TLR4, TLR5, TLR7, and TLR9 to synergistically induce cytokines, including TNF, IL-10 and IL-23, while repressing others like IL-12 (Dennehy et al. 2008, 2009; Gerosa et al. 2008; Huang et al. 2009). MR-mediated production of IL-17 is dependent on TLR2 and Dectin-1, and DC-SIGN interacts with TLR3, TLR4, and TLR5 to augment cytokine responses to fungi (Nagaoka et al. 2005; Gringhuis et al. 2007; van de Veerdonk et al. 2009). How MR modulates these responses is still unclear, but DC-SIGN is thought to interact with TLR signal transduction at the level of gene transcription through the activities of Raf-1. MyD88-coupled TLRs induce the transcription factor NFκB; Raf-1 activity leads to phosphorylation of the NFκB subunit p65, and this increases NFκB binding affinity at cytokine genes, including IL-6, IL-10, and IL-12

(Gringhuis et al. 2007). Raf-1 activation by DC-SIGN is independent of TLRs; however, PRR signaling must first activate NFκB for Raf-1 to have its modulatory effects (van den Berg et al. 2012). Furthermore, **in an infection setting, eliciting crosstalk has demonstrated valuable treatment options for chronic fungal infection.** *F. pedrosoi* is recognized by CLRs, including Mincle, but a failure to modulate TLR activity results in defective inflammatory responses. However, exogenous administration of purified TLR2, TLR4, or TLR7 ligands restores the cooperative inflammatory responses and leads to pathogen clearance in mouse models (Sousa Mda et al. 2011). Furthermore, administration of heat-killed *E. coli* clears *P. carinii* infection in mice through the restoration of cooperative inflammatory responses (Empey et al. 2007).

VI. Conclusions

PRR are a diverse set of molecules that are crucial for initiating and orchestrating protective antifungal immunity. PRR initiate the binding and uptake of fungi, induce antifungal mediators and inflammation, and modulate adaptive responses. CLRs are emerging as the major receptor class for mediating antifungal immunity, and together this class of PRR recognizes nearly every known human fungal pathogen. CLRs are a genetically and structurally diverse group of receptors characterized by a common CLTD, and many are coupled to the signal transduction molecule Syk. Mice and humans deficient in Syk or its downstream signaling partner Card9 are highly susceptible to fungal disease, illustrating the central importance of these molecules for protective immunity. Interestingly, although mice deficient in the TLR adaptor MyD88 are also susceptible to fungal infections, humans with MyD88 polymorphisms are not predisposed. **Crucially, human polymorphisms that result in abrogated Th17 development, such as CARD9 deficiency, lead to susceptibility to fungi, demonstrating the importance of this adaptive response and the underlying roles of the CLRs.**

Although primary fungal ligands for PRR are cell wall structural carbohydrates such as β-glucan and α-mannan, the fungal cell wall is a dynamic structure, capable of major changes during morphological transition and can vary significantly between different strains of the same species. **Thus, the apparent redundancy in the recognition network for fungi allows for the recognition of many permutations in cell wall composition. In this vein, collaborations between PRR are thus crucial for optimal antifungal immunity and have a profound effect on shaping inflammation and adaptive responses;** this effect is demonstrated by the fact that the use of TLR activators to cure fungal disease is also dependent on CLRs. Crosstalk between various CLR and TLR receptors modulates cytokine release, thus shaping the development of adaptive immunity. Furthermore, CLR signals are also translated to NLRs for activation of the inflammasome, a crucial step in the differentiation of Th17 cells. The mechanisms by which PRRs collaborate is still an area of active investigation. Elucidating the details of cell wall variation and crosstalk between receptor classes will increase our understanding of the complexity in pattern recognition, and may lead to the development of tailored therapies for individual fungal diseases, as exemplified by the use of TLR stimulants in the treatment of chromoblastomycosis.

Acknowledgements We thank the Wellcome Trust, Wellcome Trust Strategic Award, and the Medical Research Council (UK) for funding.

References

Aimanianda V, Bayry J, Bozza S, Kniemeyer O, Perruccio K, Elluru SR, Clavaud C, Paris S, Brakhage AA, Kaveri SV, Romani L, Latge JP (2009) Surface hydrophobin prevents immune recognition of airborne fungal spores. Nature 460(7259):1117–1121

Ariizumi K, Shen GL, Shikano S, Ritter R 3rd, Zukas P, Edelbaum D, Morita A, Takashima A (2000a) Cloning of a second dendritic cell-associated C-type lectin (dectin-2) and its alternatively spliced isoforms. J Biol Chem 275(16):11957–11963

Ariizumi K, Shen GL, Shikano S, Xu S, Ritter R 3rd, Kumamoto T, Edelbaum D, Morita A, Bergstresser PR, Takashima A (2000b) Identification of a novel, dendritic cell-associated molecule, dectin-1, by

subtractive cDNA cloning. J Biol Chem 275 (26):20157–20167

Barrett NA, Rahman OM, Fernandez JM, Parsons MW, Xing W, Austen KF, Kanaoka Y (2011) Dectin-2 mediates Th2 immunity through the generation of cysteinyl leukotrienes. J Exp Med 208(3):593–604

Berton G, Gordon S (1983) Modulation of macrophage mannosyl-specific receptors by cultivation on immobilized zymosan. Effects on superoxide-anion release and phagocytosis. Immunology 49(4):705–715

Brown GD (2006) Dectin-1: a signalling non-TLR pattern-recognition receptor. Nat Rev Immunol 6(1):33–43

Brown GD (2008) Sensing necrosis with Mincle. Nat Immunol 9(10):1099–1100

Brown GD (2011) Innate antifungal immunity: the key role of phagocytes. Annu Rev Immunol 29:1–21

Brown GD, Gordon S (2001) Immune recognition. A new receptor for beta-glucans. Nature 413(6851):36–37

Brown GD, Herre J, Williams DL, Willment JA, Marshall ASJ, Gordon S (2003) Dectin-1 mediates the biological effects of beta-glucan. J Exp Med 197 (9):1119–1124

Brummer E, Stevens DA (2010) Collectins and fungal pathogens: roles of surfactant proteins and mannose binding lectin in host resistance. Med Mycol Off Publ Int Soc Hum Anim Mycol 48(1):16–28

Bugarcic A, Hitchens K, Beckhouse AG, Wells CA, Ashman RB, Blanchard H (2008) Human and mouse macrophage-inducible C-type lectin (Mincle) bind *Candida albicans*. Glycobiology 18 (9):679–685

Cambi A, de Lange F, van Maarseveen NM, Nijhuis M, Joosten B, van Dijk EM, de Bakker BI, Fransen JA, Bovee-Geurts PH, van Leeuwen FN, Van Hulst NF, Figdor CG (2004) Microdomains of the C-type lectin DC-SIGN are portals for virus entry into dendritic cells. J Cell Biol 164(1):145–155

Cohen NR, Tatituri RV, Rivera A, Watts GF, Kim EY, Chiba A, Fuchs BB, Mylonakis E, Besra GS, Levitz SM, Brigl M, Brenner MB (2011) Innate recognition of cell wall beta-glucans drives invariant natural killer T cell responses against fungi. Cell Host Microbe 10(5):437–450

Conti HR, Gaffen SL (2010) Host responses to *Candida albicans*: Th17 cells and mucosal candidiasis. Microbes Infect 12:518–527

Conti HR, Shen F, Nayyar N, Stocum E, Sun JN, Lindemann MJ, Ho AW, Hai JH, Yu JJ, Jung JW, Filler SG, Masso-Welch P, Edgerton M, Gaffen SL (2009) Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. J Exp Med 206(2):299–311

Dan JM, Kelly RM, Lee CK, Levitz SM (2008) Role of the mannose receptor in a murine model of *Cryptococcus neoformans* infection. Infect Immun 76(6):2362–2367

Dennehy KM, Ferwerda G, Faro-Trindade I, Pyz E, Willment JA, Taylor PR, Kerrigan A, Tsoni SV, Gordon S, Meyer-Wentrup F, Adema GJ, Kullberg BJ, Schweighoffer E, Tybulewicz V, Mora-Montes HM, Gow NA, Williams DL, Netea MG, Brown GD (2008) Syk kinase is required for collaborative cytokine production induced through Dectin-1 and Toll-like receptors. Eur J Immunol 38(2):500–506

Dennehy KM, Willment JA, Williams DL, Brown GD (2009) Reciprocal regulation of IL-23 and IL-12 following co-activation of Dectin-1 and TLR signaling pathways. Eur J Immunol 39(5):1379–1386

Eggleton P, Tenner AJ, Reid KB (2000) C1q receptors. Clin Exp Immunol 120(3):406–412

Empey KM, Hollifield M, Garvy BA (2007) Exogenous heat-killed *Escherichia coli* improves alveolar macrophage activity and reduces *Pneumocystis carinii* lung burden in infant mice. Infect Immun 75 (7):3382–3393

Faro-Trindade I, Willment JA, Kerrigan AM, Redelinghuys P, Hadebe S, Reid DM, Srinivasan N, Wainwright H, Lang DM, Steele C, Brown GD (2012) Characterisation of innate fungal recognition in the lung. PLoS One 7(4):e35675

Fernandes MJ, Finnegan AA, Siracusa LD, Brenner C, Iscove NN, Calabretta B (1999) Characterization of a novel receptor that maps near the natural killer gene complex: demonstration of carbohydrate binding and expression in hematopoietic cells. Cancer Res 59(11):2709–2717

Ferwerda B, Ferwerda G, Plantinga TS, Willment JA, van Spriel AB, Venselaar H, Elbers CC, Johnson MD, Cambi A, Huysamen C, Jacobs L, Jansen T, Verheijen K, Masthoff L, Morre SA, Vriend G, Williams DL, Perfect JR, Joosten LA, Wijmenga C, van der Meer JW, Adema GJ, Kullberg BJ, Brown GD, Netea MG (2009) Human dectin-1 deficiency and mucocutaneous fungal infections. N Engl J Med 361(18):1760–1767

Franchi L, Munoz-Planillo R, Nunez G (2012) Sensing and reacting to microbes through the inflammasomes. Nat Immunol 13(4):325–332

Gantner BN, Simmons RM, Canavera SJ, Akira S, Underhill DM (2003) Collaborative induction of inflammatory responses by Dectin-1 and Toll-like receptor 2. J Exp Med 197:1107–1117

Gantner BN, Simmons RM, Underhill DM (2005) Dectin-1 mediates macrophage recognition of *Candida albicans* yeast but not filaments. EMBO J 24(6):1277–1286

Gardai SJ, Xiao YQ, Dickinson M, Nick JA, Voelker DR, Greene KE, Henson PM (2003) By binding SIRPalpha or calreticulin/CD91, lung collectins act as dual function surveillance molecules to suppress or enhance inflammation. Cell 115(1):13–23

Geijtenbeek TB, Torensma R, van Vliet SJ, van Duijnhoven GC, Adema GJ, van Kooyk Y, Figdor CG (2000) Identification of DC-SIGN, a novel dendritic

cell-specific ICAM-3 receptor that supports primary immune responses. Cell 100(5):575–585

Gerosa F, Baldani-Guerra B, Lyakh LA, Batoni G, Esin S, Winkler-Pickett RT, Consolaro MR, De Marchi M, Giachino D, Robbiano A, Astegiano M, Sambataro A, Kastelein RA, Carra G, Trinchieri G (2008) Differential regulation of interleukin 12 and interleukin 23 production in human dendritic cells. J Exp Med 205:1447–1461

Glocker EO, Hennigs A, Nabavi M, Schaffer AA, Woellner C, Salzer U, Pfeifer D, Veelken H, Warnatz K, Tahami F, Jamal S, Manguiat A, Rezaei N, Amirzargar AA, Plebani A, Hannesschlager N, Gross O, Ruland J, Grimbacher B (2009) A homozygous CARD9 mutation in a family with susceptibility to fungal infections. N Engl J Med 361(18):1727–1735

Goodridge HS, Simmons RM, Underhill DM (2007) Dectin-1 stimulation by *Candida albicans* yeast or zymosan triggers NFAT activation in macrophages and dendritic cells. J Immunol 178(5):3107–3115

Goodridge HS, Shimada T, Wolf AJ, Hsu YM, Becker CA, Lin X, Underhill DM (2009a) Differential use of CARD9 by dectin-1 in macrophages and dendritic cells. J Immunol 182(2):1146–1154

Goodridge HS, Wolf AJ, Underhill DM (2009b) Beta-glucan recognition by the innate immune system. Immunol Rev 230(1):38–50

Goodridge HS, Reyes CN, Becker CA, Katsumoto TR, Ma J, Wolf AJ, Bose N, Chan AS, Magee AS, Danielson ME, Weiss A, Vasilakos JP, Underhill DM (2011) Activation of the innate immune receptor Dectin-1 upon formation of a 'phagocytic synapse'. Nature 472(7344):471–475

Gow NA, van de Veerdonk FL, Brown AJ, Netea MG (2012) *Candida albicans* morphogenesis and host defence: discriminating invasion from colonization. Nat Rev Microbiol 10(2):112–122

Graham LM, Brown GD (2009) The Dectin-2 family of C-type lectins in immunity and homeostasis. Cytokine 48(1–2):148–155

Granell M, Urbano-Ispizua A, Suarez B, Rovira M, Fernandez-Aviles F, Martinez C, Ortega M, Uriburu C, Gaya A, Roncero JM, Navarro A, Carreras E, Mensa J, Vives J, Rozman C, Montserrat E, Lozano F (2006) Mannan-binding lectin pathway deficiencies and invasive fungal infections following allogeneic stem cell transplantation. Exp Hematol 34 (10):1435–1441

Gringhuis SI, den Dunnen J, Litjens M, van Het Hof B, van Kooyk Y, Geijtenbeek TB (2007) C-type lectin DC-SIGN modulates Toll-like receptor signaling via Raf-1 kinase-dependent acetylation of transcription factor NF-kappaB. Immunity 26(5):605–616

Gringhuis SI, den Dunnen J, Litjens M, van der Vlist M, Geijtenbeek TB (2009a) Carbohydrate-specific signaling through the DC-SIGN signalosome tailors immunity to Mycobacterium tuberculosis, HIV-1 and Helicobacter pylori. Nat Immunol 10 (10):1081–1088

Gringhuis SI, den Dunnen J, Litjens M, van der Vlist M, Wevers B, Bruijns SC, Geijtenbeek TB (2009b) Dectin-1 directs T helper cell differentiation by controlling noncanonical NF-kappaB activation through Raf-1 and Syk. Nat Immunol 10 (2):203–213

Gringhuis SI, Wevers BA, Kaptein TM, van Capel TM, Theelen B, Boekhout T, de Jong EC, Geijtenbeek TB (2011) Selective C-Rel activation via Malt1 controls anti-fungal T(H)-17 immunity by dectin-1 and dectin-2. PLoS Pathog 7(1):e1001259

Gringhuis SI, Kaptein TM, Wevers BA, Theelen B, van der Vlist M, Boekhout T, Geijtenbeek TB (2012) Dectin-1 is an extracellular pathogen sensor for the induction and processing of IL-1beta via a noncanonical caspase-8 inflammasome. Nat Immunol 13(3):246–254

Gross O, Gewies A, Finger K, Schafer M, Sparwasser T, Peschel C, Forster I, Ruland J (2006) Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity. Nature 442(7103):651–656

Gross O, Poeck H, Bscheider M, Dostert C, Hannesschlager N, Endres S, Hartmann G, Tardivel A, Schweighoffer E, Tybulewicz V, Mocsai A, Tschopp J, Ruland J (2009) Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. Nature 459(7245):433–436

Herre J, Marshall AS, Caron E, Edwards AD, Williams DL, Schweighoffer E, Tybulewicz V, Reis e Sousa C, Gordon S, Brown GD (2004) Dectin-1 uses novel mechanisms for yeast phagocytosis in macrophages. Blood 104(13):4038–4045

Hise AG, Tomalka J, Ganesan S, Patel K, Hall BA, Brown GD, Fitzgerald KA (2009) An essential role for the NLRP3 inflammasome in host defense against the human fungal pathogen *Candida albicans*. Cell Host Microbe 5(5):487–497

Huang H, Ostroff GR, Lee CK, Wang JP, Specht CA, Levitz SM (2009) Distinct patterns of dendritic cell cytokine release stimulated by fungal beta-glucans and toll-like receptor agonists. Infect Immun 77 (5):1774–1781

Huysamen C, Brown GD (2009) The fungal pattern recognition receptor, Dectin-1, and the associated cluster of C-type lectin-like receptors. FEMS Microbiol Lett 290(2):121–128

Iliev ID, Funari VA, Taylor KD, Nguyen Q, Reyes CN, Strom SP, Brown J, Becker CA, Fleshner PR, Dubinsky M, Rotter JI, Wang HL, McGovern DP, Brown GD, Underhill DM (2012) Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis. Science 336 (6086):1314–1317

Jouin H, Staub AM, Alouf JE (1981) Isolation of an (O, H, Vi)-free immunoprotective antigenic fraction with mannose receptor-like activity from Salmonella typhi. J Infect Dis 143(1):106–113

Kisand K, Boe Wolff AS, Podkrajsek KT, Tserel L, Link M, Kisand KV, Ersvaer E, Perheentupa J, Erichsen MM, Bratanic N, Meloni A, Cetani F, Perniola R, Ergun-Longmire B, Maclaren N, Krohn KJ, Pura M, Schalke B, Strobel P, Leite MI, Battelino T, Husebye ES, Peterson P, Willcox N, Meager A (2010) Chronic mucocutaneous candidiasis in APECED or thymoma patients correlates with autoimmunity to Th17-associated cytokines. J Exp Med 207(2):299–308

Kozel TR (1996) Activation of the complement system by pathogenic fungi. Clin Microbiol Rev 9(1):34–46

Kuroki Y, Takahashi M, Nishitani C (2007) Pulmonary collectins in innate immunity of the lung. Cell Microbiol 9(8):1871–1879

Lee SJ, Zheng NY, Clavijo M, Nussenzweig MC (2003) Normal host defense during systemic candidiasis in mannose receptor-deficient mice. Infect Immun 71(1):437–445

LeibundGut-Landmann S, Gross O, Robinson MJ, Osorio F, Slack EC, Tsoni SV, Schweighoffer E, Tybulewicz V, Brown GD, Ruland J, Reis e Sousa C (2007) Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. Nat Immunol 8(6):630–638

Linehan SA, Martinez-Pomares L, da Silva RP, Gordon S (2001) Endogenous ligands of carbohydrate recognition domains of the mannose receptor in murine macrophages, endothelial cells and secretory cells; potential relevance to inflammation and immunity. Eur J Immunol 31(6):1857–1866

Liu L, Okada S, Kong XF, Kreins AY, Cypowyj S, Abhyankar A, Toubiana J, Itan Y, Audry M, Nitschke P, Masson C, Toth B, Flatot J, Migaud M, Chrabieh M, Kochetkov T, Bolze A, Borghesi A, Toulon A, Hiller J, Eyerich S, Eyerich K, Gulacsy V, Chernyshova L, Chernyshov V, Bondarenko A, Grimaldo RM, Blancas-Galicia L, Beas IM, Roesler J, Magdorf K, Engelhard D, Thumerelle C, Burgel PR, Hoernes M, Drexel B, Seger R, Kusuma T, Jansson AF, Sawalle-Belohradsky J, Belohradsky B, Jouanguy E, Bustamante J, Bue M, Karin N, Wildbaum G, Bodemer C, Lortholary O, Fischer A, Blanche S, Al-Muhsen S, Reichenbach J, Kobayashi M, Rosales FE, Lozano CT, Kilic SS, Oleastro M, Etzioni A, Traidl-Hoffmann C, Renner ED, Abel L, Picard C, Marodi L, Boisson-Dupuis S, Puel A, Casanova JL (2011) Gain-of-function human STAT1 mutations impair IL-17 immunity and underlie chronic mucocutaneous candidiasis. J Exp Med 208(8):1635–1648

Ma CS, Chew GY, Simpson N, Priyadarshi A, Wong M, Grimbacher B, Fulcher DA, Tangye SG, Cook MC (2008) Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. J Exp Med 205(7):1551–1557

Madan T, Reid KB, Clark H, Singh M, Nayak A, Sarma PU, Hawgood S, Kishore U (2010) Susceptibility of mice genetically deficient in SP-A or SP-D gene to invasive pulmonary aspergillosis. Mol Immunol 47 (10):1923–1930

Manzo C, Torreno-Pina JA, Joosten B, Reinieren-Beeren I, Gualda EJ, Loza-Alvarez P, Figdor CG, Garcia-Parajo MF, Cambi A (2012) The neck region of the C-type lectin DC-SIGN regulates its surface spatiotemporal organization and virus-binding capacity on antigen-presenting cells. J Biol Chem 287(46):38946–38955

Martin B, Hirota K, Cua DJ, Stockinger B, Veldhoen M (2009) Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. Immunity 31 (2):321–330

Martinez-Pomares L (2012). The mannose receptor. J Leukoc Biol 92:1177–1186

McGreal EP, Miller JL, Gordon S (2005) Ligand recognition by antigen-presenting cell C-type lectin receptors. Curr Opin Immunol 17(1):18–24

McGreal EP, Rosas M, Brown GD, Zamze S, Wong SY, Gordon S, Martinez-Pomares L, Taylor PR (2006) The carbohydrate-recognition domain of Dectin-2 is a C-type lectin with specificity for high mannose. Glycobiology 16(5):422–430

Means TK, Mylonakis E, Tampakakis E, Colvin RA, Seung E, Puckett L, Tai MF, Stewart CR, Pukkila-Worley R, Hickman SE, Moore KJ, Calderwood SB, Hacohen N, Luster AD, El Khoury J (2009) Evolutionarily conserved recognition and innate immunity to fungal pathogens by the scavenger receptors SCARF1 and CD36. J Exp Med 206(3):637–653

Milner JD, Brenchley JM, Laurence A, Freeman AF, Hill BJ, Elias KM, Kanno Y, Spalding C, Elloumi HZ, Paulson ML, Davis J, Hsu A, Asher AI, O'Shea J, Holland SM, Paul WE, Douek DC (2008) Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. Nature 452 (7188):773–776

Mullighan CG, Heatley S, Doherty K, Szabo F, Grigg A, Hughes TP, Schwarer AP, Szer J, Tait BD, Bik To L, Bardy PG (2002) Mannose-binding lectin gene polymorphisms are associated with major infection following allogeneic hemopoietic stem cell transplantation. Blood 99(10):3524–3529

Nagaoka K, Takahara K, Tanaka K, Yoshida H, Steinman RM, Saitoh S, Akashi-Takamura S, Miyake K, Kang YS, Park CG, Inaba K (2005) Association of SIGNR1 with TLR4-MD-2 enhances signal transduction by recognition of LPS in gram-negative bacteria. Int Immunol 17(7):827–836

Netea MG, van der Meer JW (2011) Immunodeficiency and genetic defects of pattern-recognition receptors. N Engl J Med 364(1):60–70

Netea MG, Vonk AG, van den Hoven M, Verschueren I, Joosten LA, van Krieken JH, van den Berg WB, Van der Meer JW, Kullberg BJ (2003) Differential role of IL-18 and IL-12 in the host defense against

disseminated *Candida albicans* infection. Eur J Immunol 33(12):3409–3417

Netea MG, Sutmuller R, Hermann C, Van der Graaf CA, Van der Meer JW, van Krieken JH, Hartung T, Adema G, Kullberg BJ (2004) Toll-like receptor 2 suppresses immunity against *Candida albicans* through induction of IL-10 and regulatory T cells. J Immunol 172(6):3712–3718

Netea MG, Brown GD, Kullberg BJ, Gow NA (2008) An integrated model of the recognition of *Candida albicans* by the innate immune system. Nat Rev Microbiol 6(1):67–78

Netea MG, Gow NA, Joosten LA, Verschueren I, van der Meer JW, Kullberg BJ (2010) Variable recognition of *Candida albicans* strains by TLR4 and lectin recognition receptors. Med Mycol Off Publ Int Soc Hum Anim Mycol 48(7):897–903

Osorio F, LeibundGut-Landmann S, Lochner M, Lahl K, Sparwasser T, Eberl G, Reis e Sousa C (2008) DC activated via dectin-1 convert Treg into IL-17 producers. Eur J Immunol 38(12):3274–3281

Powlesland AS, Ward EM, Sadhu SK, Guo Y, Taylor ME, Drickamer K (2006) Widely divergent biochemical properties of the complete set of mouse DC-SIGN-related proteins. J Biol Chem 281 (29):20440–20449

Puel A, Doffinger R, Natividad A, Chrabieh M, Barcenas-Morales G, Picard C, Cobat A, Ouachee-Chardin M, Toulon A, Bustamante J, Al-Muhsen S, Al-Owain M, Arkwright PD, Costigan C, McConnell V, Cant AJ, Abinun M, Polak M, Bougneres PF, Kumararatne D, Marodi L, Nahum A, Roifman C, Blanche S, Fischer A, Bodemer C, Abel L, Lilic D, Casanova JL (2010) Autoantibodies against IL-17A, IL-17F, and IL-22 in patients with chronic mucocutaneous candidiasis and autoimmune polyendocrine syndrome type I. J Exp Med 207 (2):291–297

Puel A, Cypowyj S, Bustamante J, Wright JF, Liu L, Lim HK, Migaud M, Israel L, Chrabieh M, Audry M, Gumbleton M, Toulon A, Bodemer C, El-Baghdadi J, Whitters M, Paradis T, Brooks J, Collins M, Wolfman NM, Al-Muhsen S, Galicchio M, Abel L, Picard C, Casanova JL (2011) Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. Science 332(6025):65–68

Reese TA, Liang HE, Tager AM, Luster AD, Van Rooijen N, Voehringer D, Locksley RM (2007) Chitin induces accumulation in tissue of innate immune cells associated with allergy. Nature 447(7140):92–96

Rivera A, Hohl TM, Collins N, Leiner I, Gallegos A, Saijo S, Coward JW, Iwakura Y, Pamer EG (2011) Dectin-1 diversifies *Aspergillus fumigatus*-specific T cell responses by inhibiting T helper type 1 CD4 T cell differentiation. J Exp Med 208(2):369–381

Robinson MJ, Osorio F, Rosas M, Freitas RP, Schweighoffer E, Gross O, Verbeek JS, Ruland J, Tybulewicz V, Brown GD, Moita LF, Taylor PR, Reis e Sousa C (2009) Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. J Exp Med 206(9):2037–2051

Rogers NC, Slack EC, Edwards AD, Nolte MA, Schulz O, Schweighoffer E, Williams DL, Gordon S, Tybulewicz VL, Brown GD, Reis e Sousa C (2005) Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. Immunity 22(4):507–517

Romani L (2011) Immunity to fungal infections. Nat Rev Immunol 11(4):275–288

Rubin-Bejerano I, Abeijon C, Magnelli P, Grisafi P, Fink GR (2007) Phagocytosis by human neutrophils is stimulated by a unique fungal cell wall component. Cell Host Microbe 2(1):55–67

Said-Sadier N, Padilla E, Langsley G, Ojcius DM (2010) *Aspergillus fumigatus* stimulates the NLRP3 inflammasome through a pathway requiring ROS production and the Syk tyrosine kinase. PLoS One 5(4):e10008

Saijo S, Iwakura Y (2011) Dectin-1 and Dectin-2 in innate immunity against fungi. Int Immunol 23 (8):467–472

Saijo S, Fujikado N, Furuta T, Chung SH, Kotaki H, Seki K, Sudo K, Akira S, Adachi Y, Ohno N, Kinjo T, Nakamura K, Kawakami K, Iwakura Y (2007) Dectin-1 is required for host defense against *Pneumocystis carinii* but not against *Candida albicans*. Nat Immunol 8(1):39–46

Saijo S, Ikeda S, Yamabe K, Kakuta S, Ishigame H, Akitsu A, Fujikado N, Kusaka T, Kubo S, Chung SH, Komatsu R, Miura N, Adachi Y, Ohno N, Shibuya K, Yamamoto N, Kawakami K, Yamasaki S, Saito T, Akira S, Iwakura Y (2010) Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against *Candida albicans*. Immunity 32(5):681–691

Sancho D, Reis e Sousa C (2012) Signaling by myeloid C-type lectin receptors in immunity and homeostasis. Annu Rev Immunol 30:491–529

Sato K, Yang XL, Yudate T, Chung JS, Wu J, Luby-Phelps K, Kimberly RP, Underhill D, Cruz PD Jr, Ariizumi K (2006) Dectin-2 is a pattern recognition receptor for fungi that couples with the Fc receptor gamma chain to induce innate immune responses. J Biol Chem 281(50):38854–38866

Schoenen H, Bodendorfer B, Hitchens K, Manzanero S, Werninghaus K, Nimmerjahn F, Agger EM, Stenger S, Andersen P, Ruland J, Brown GD, Wells C, Lang R (2010) Cutting edge: Mincle is essential for recognition and adjuvanticity of the mycobacterial cord factor and its synthetic analog trehalose-dibehenate. J Immunol 184(6):2756–2760

Sousa Mda G, Reid DM, Schweighoffer E, Tybulewicz V, Ruland J, Langhorne J, Yamasaki S, Taylor PR, Almeida SR, Brown GD (2011) Restoration of pattern recognition receptor costimulation to treat chromoblastomycosis, a chronic fungal infection of the skin. Cell Host Microbe 9(5):436–443

Strasser D, Neumann K, Bergmann H, Marakalala MJ, Guler R, Rojowska A, Hopfner KP, Brombacher F, Urlaub H, Baier G, Brown GD, Leitges M, Ruland J (2012) Syk kinase-coupled C-type lectin receptors engage protein kinase C-sigma to elicit Card9 adaptor-mediated innate immunity. Immunity 36 (1):32–42

Suram S, Gangelhoff TA, Taylor PR, Rosas M, Brown GD, Bonventre JV, Akira S, Uematsu S, Williams DL, Murphy RC, Leslie CC (2010) Pathways regulating cytosolic phospholipase A2 activation and eicosanoid production in macrophages by *Candida albicans*. J Biol Chem 285(40):30676–30685

Sutton CE, Lalor SJ, Sweeney CM, Brereton CF, Lavelle EC, Mills KH (2009) Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. Immunity 31(2):331–341

Swain SD, Lee SJ, Nussenzweig MC, Harmsen AG (2003) Absence of the macrophage mannose receptor in mice does not increase susceptibility to *Pneumocystis carinii* infection in vivo. Infect Immun 71(11):6213–6221

Taylor ME, Conary JT, Lennartz MR, Stahl PD, Drickamer K (1990) Primary structure of the mannose receptor contains multiple motifs resembling carbohydrate-recognition domains. J Biol Chem 265(21):12156–12162

Taylor ME, Bezouska K, Drickamer K (1992) Contribution to ligand binding by multiple carbohydrate-recognition domains in the macrophage mannose receptor. J Biol Chem 267(3):1719–1726

Taylor PR, Reid DM, Heinsbroek SE, Brown GD, Gordon S, Wong SY (2005) Dectin-2 is predominantly myeloid restricted and exhibits unique activation-dependent expression on maturing inflammatory monocytes elicited in vivo. Eur J Immunol 35 (7):2163–2174

Taylor PR, Tsoni SV, Willment JA, Dennehy KM, Rosas M, Findon H, Haynes K, Steele C, Botto M, Gordon S, Brown GD (2007) Dectin-1 is required for beta-glucan recognition and control of fungal infection. Nat Immunol 8(1):31–38

Tomalka J, Ganesan S, Azodi E, Patel K, Majmudar P, Hall BA, Fitzgerald KA, Hise AG (2011) A novel role for the NLRC4 inflammasome in mucosal defenses against the fungal pathogen *Candida albicans*. PLoS Pathog 7(12):e1002379

Tsoni SV, Kerrigan AM, Marakalala MJ, Srinivasan N, Duffield M, Taylor PR, Botto M, Steele C, Brown GD (2009) Complement C3 plays an essential role in the control of opportunistic fungal infections. Infect Immun 77(9):3679–3685

Underhill DM, Rossnagle E, Lowell CA, Simmons RM (2005) Dectin-1 activates Syk tyrosine kinase in a dynamic subset of macrophages for reactive oxygen production. Blood 106(7):2543–2550

van de Veerdonk FL, Marijnissen RJ, Kullberg BJ, Koenen HJ, Cheng SC, Joosten I, van den Berg WB, Williams DL, van der Meer JW, Joosten LA, Netea MG (2009) The macrophage mannose receptor induces IL-17 in response to *Candida albicans*. Cell Host Microbe 5(4):329–340

van de Veerdonk FL, Joosten LA, Shaw PJ, Smeekens SP, Malireddi RK, van der Meer JW, Kullberg BJ, Netea MG, Kanneganti TD (2011a) The inflammasome drives protective Th1 and Th17 cellular responses in disseminated candidiasis. Eur J Immunol 41 (8):2260–2268

van de Veerdonk FL, Plantinga TS, Hoischen A, Smeekens SP, Joosten LA, Gilissen C, Arts P, Rosentul DC, Carmichael AJ, Smits-van der Graaf CA, Kullberg BJ, van der Meer JW, Lilic D, Veltman JA, Netea MG (2011b) STAT1 mutations in autosomal dominant chronic mucocutaneous candidiasis. N Engl J Med 365(1):54–61

van den Berg LM, Gringhuis SI, Geijtenbeek TB (2012) An evolutionary perspective on C-type lectins in infection and immunity. Ann N Y Acad Sci 1253:149–158

Villamon E, Gozalbo D, Roig P, O'Connor JE, Fradelizi D, Gil ML (2004) Toll-like receptor-2 is essential in murine defenses against *Candida albicans* infections. Microbes Infect 6(1):1–7

Voelz K, May RC (2010) Cryptococcal interactions with the host immune system. Eukaryot Cell 9(6):835–846

von Bernuth H, Picard C, Jin Z, Pankla R, Xiao H, Ku CL, Chrabieh M, Mustapha IB, Ghandil P, Camcioglu Y, Vasconcelos J, Sirvent N, Guedes M, Vitor AB, Herrero-Mata MJ, Arostegui JI, Rodrigo C, Alsina L, Ruiz-Ortiz E, Juan M, Fortuny C, Yague J, Anton J, Pascal M, Chang HH, Janniere L, Rose Y, Garty BZ, Chapel H, Issekutz A, Marodi L, Rodriguez-Gallego C, Banchereau J, Abel L, Li X, Chaussabel D, Puel A, Casanova JL (2008) Pyogenic bacterial infections in humans with MyD88 deficiency. Science 321(5889):691–696

Vonk AG, Netea MG, van Krieken JH, Iwakura Y, van der Meer JW, Kullberg BJ (2006) Endogenous interleukin (IL)-1 alpha and IL-1 beta are crucial for host defense against disseminated candidiasis. J Infect Dis 193(10):1419–1426

Wang R, Chandawarkar RY (2010) Phagocytosis of fungal agents and yeast via macrophage cell surface scavenger receptors. J Surg Res 164(2): e273–e279

Wells CA, Salvage-Jones JA, Li X, Hitchens K, Butcher S, Murray RZ, Beckhouse AG, Lo YL, Manzanero S, Cobbold C, Schroder K, Ma B, Orr S, Stewart L, Lebus D, Sobieszczuk P, Hume DA, Stow J, Blanchard H, Ashman RB (2008) The macrophage-inducible C-type lectin, mincle, is an essential component of the innate immune response to *Candida albicans*. J Immunol 180 (11):7404–7413

Werner JL, Metz AE, Horn D, Schoeb TR, Hewitt MM, Schwiebert LM, Faro-Trindade I, Brown GD, Steele C (2009) Requisite role for the dectin-1 beta-glucan

receptor in pulmonary defense against *Aspergillus fumigatus*. J Immunol 182(8):4938–4946

Wheeler RT, Kombe D, Agarwala SD, Fink GR (2008) Dynamic, morphotype-specific *Candida albicans* beta-glucan exposure during infection and drug treatment. PLoS Pathog 4(12):e1000227

Wuthrich M, Deepe GS Jr, Klein B (2012) Adaptive immunity to fungi. Annu Rev Immunol 30:115–148

Yamasaki S, Ishikawa E, Sakuma M, Hara H, Ogata K, Saito T (2008) Mincle is an ITAM-coupled activating receptor that senses damaged cells. Nat Immunol 9(10):1179–1188

Yamasaki S, Matsumoto M, Takeuchi O, Matsuzawa T, Ishikawa E, Sakuma M, Tateno H, Uno J, Hirabayashi J, Mikami Y, Takeda K, Akira S, Saito T (2009) C-type lectin Mincle is an activating receptor for pathogenic fungus, *Malassezia*. Proc Natl Acad Sci USA 106(6):1897–1902

Zelante T, De Luca A, Bonifazi P, Montagnoli C, Bozza S, Moretti S, Belladonna ML, Vacca C, Conte C, Mosci P, Bistoni F, Puccetti P, Kastelein RA, Kopf M, Romani L (2007) IL-23 and the Th17 pathway promote inflammation and impair antifungal immune resistance. Eur J Immunol 37(10):2695–2706

Zipfel PF, Wurzner R, Skerka C (2007) Complement evasion of pathogens: common strategies are shared by diverse organisms. Mol Immunol 44(16): 3850–3857

5 Macrophages in the Immune Response Against *Cryptococcus*

Robert J. Evans[1], Robin C. May[1]

CONTENTS

I. Introduction

Cryptococcus neoformans is a pathogenic fungus that causes disease in humans and other mammals. The first recorded case of human *C. neoformans* infection was found in a subcutaneous skin lesion (Mitchell and Perfect 1995), but infection more commonly occurs in the lungs following inhalation. Although infection begins in the lungs, life-threatening symptoms only develop if the fungus disseminates to the central nervous system (CNS); fatal dissemination occurs almost exclusively in individuals with pre-existing immune deficiencies. In the decades following the discovery of *C. neoformans*, cases of fatal cryptococcosis were rare because immune deficiency syndromes were uncommon. Medical advances in the twenty-first century such as organ transplantation and cancer treatment (which require or result in immune deficiency) increased cryptococcosis rates slightly but it was not until the spread of HIV in the 1980s that *C. neoformans* truly emerged as a major global pathogen.

Cryptococcosis currently afflicts about one million people around the world annually, mainly within HIV-infected populations. In sub-Saharan Africa, where the HIV epidemic hit hardest, complications caused by cryptococcosis account for up to 44% of HIV-related deaths (Park et al. 2009). Ominously, the rise of cryptococcosis within immune-suppressed populations may foreshadow a wider emergence of disease within healthy populations in the future. Of particular note is an ongoing outbreak of infections in immunocompetent residents of the Pacific Northwest, caused by a particularly virulent lineage of *Cryptococcus gattii* – a newly designated *Cryptococcus* species closely related to *C. neoformans* (Bartlett et al. 2008; MacDougall et al. 2007; Byrnes et al. 2009, 2010).

Cryptococcus is a genus of basidiomycete, containing at least 40 recognised species. Almost all cases of human infection are caused by two species: *C. neoformans* and *C. gattii*. These two species have been further classified according to variations in the capsule polysaccharide (Fig. 5.1) between isolates (Ikeda et al. 1982). Variants are split into five serotypes: serotype A, otherwise known as *C. neoformans var. grubii*; serotype D, otherwise known as *C. neoformans var. neoformans*; serotypes B and C, which collectively make up the newly classified *C. gattii* species; and serotype AD, which is a hybrid of

[1]Institute of Microbiology & Infection and the School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK; e-mail: r.c.may@bham.ac.uk

Human Fungal Pathogens, 2nd Edition
The Mycota XII
O. Kurzai (Ed.)

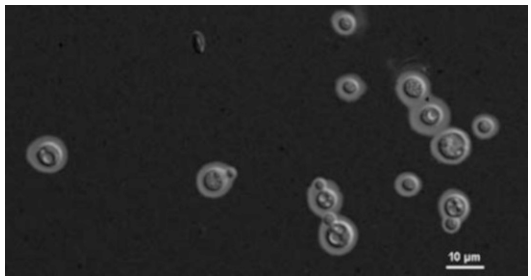


Fig. 5.1. India ink stain of *C. neoformans var. grubii* strain H99, revealing the characteristic polysaccharide capsule surrounding the fungal cells as a white halo. Note also that a number of the cells in the image are in the process of budding to produce new daughter cells

serotypes A and D (Lengeler et al. 2001). This chapter will mainly refer to *C. neoformans* serotypes A and D unless otherwise stated.

C. neoformans is primarily an environmental organism, which is widespread in soil, bird guano and rotting wood (Kronstad et al. 2011). *C. neoformans* can reproduce asexually, via budding, or sexually via a teleomorph form. The sexual (teleomorph) state is called *Filobasidiella neoformans* and forms in response to certain environmental and nutritional conditions. Sexual reproduction occurs between two distinct mating types, a and α, producing basidiospores, which may be the main infective vectors in cryptococcosis (Giles et al. 2009; Hull and Heitman 2002).

II. The Pathogenesis of Cryptococcosis

Cryptococcal infection in the lungs begins following inhalation of infectious cells or spores (Giles et al. 2009). There are no documented cases of human-to-human spread of cryptococcosis and thus infections are thought to result almost exclusively from environmental exposure. Following inhalation, *C. neoformans* initially colonises the extracellular alveolar space, where it comes into contact with the host innate immune system. As part of the innate immune response, alveolar macrophages migrate to the site of infection and attempt to clear the infection by engulfing fungal cells via phagocytosis (see Fig. 5.2). Following phagocytosis, however, ***C. neoformans* is able to survive and grow within the intracellular niche provided by the macrophage.** Initial respiratory infection occurs in both healthy and immunocompromised individuals, but **subsequent disseminated infection is far more likely if an immune deficiency is present.**

Disease outcome following *C. neoformans* infection depends on the strength of the host immune response. Broadly there are **three possible outcomes:**

1. Fungal cells in the alveolar space are phagocytosed by alveolar macrophages and infiltrating neutrophils; following phagocytosis the fungi are killed, resulting in **total clearance of infection.**
2. Fungal cells are phagocytosed, but not destroyed, by alveolar macrophages. The fungi reproduce in alveolar macrophages, eventually **disseminating to the CNS where they cause fatal meningoencephalitis.**
3. Fungal cells in the alveolar space are phagocytosed by alveolar macrophages; the immune system is unable to fully clear infection but manages **to contain the fungi within the lungs in a latent state within granulomatous structures.**

For *C. neoformans*, fatal disseminative disease (ii) occurs almost exclusively in individuals with pre-existing immune deficiencies whereas total clearance (i) and latent disease (iii) can manifest in individuals who are immune competent.

Epidemiological studies can never truly determine the extent of *C. neoformans* infection within immune competent populations because the majority of infections are asymptomatic and go unreported. To address this issue, studies have used anti-cryptococcal immunoglobulin titres within immune competent populations as a sign of past infection. These studies conclude that cryptococcal infection is common in the normal adult population (Deshaw and Pirofski 1995; Houpt et al. 1994) and most individuals are probably exposed to the fungus during childhood (Abadi and Pirofski 1999; Houpt et al. 1994). These conclusions seem reasonable considering the ubiquity of *C. neoformans* within

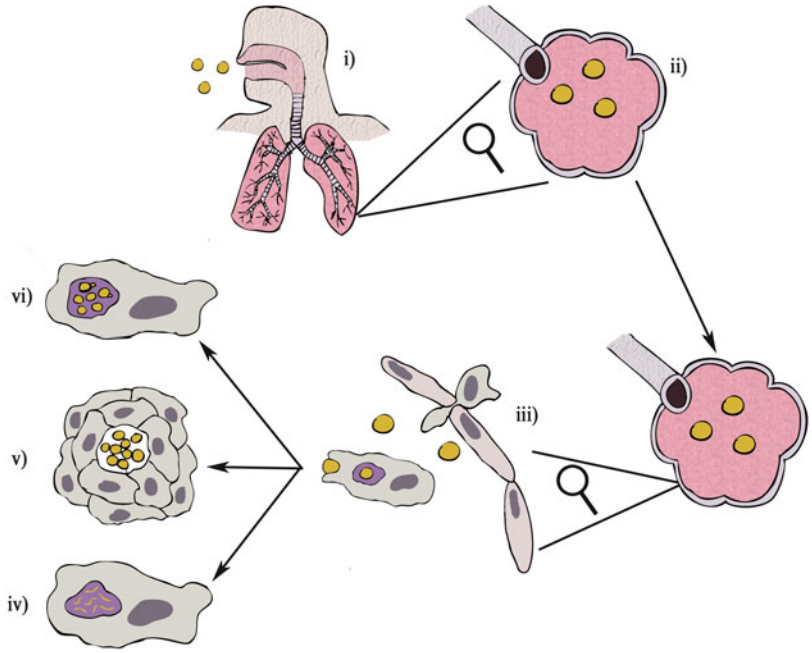


Fig. 5.2. *Cryptococcosis* pathogenesis. (*i*) Desiccated *C. neoformans* cells or infectious spores are breathed into the respiratory tract following environmental contact. (*ii*) The fungi move down the respiratory tract until they reach the alveoli where they initially grow extracellulary. (*iii*) Immune phagocytes such as macrophages are attracted to the site of infection and proceed to engulf extracellular fungi via phagocytosis. Following phagocytosis there are three possible outcomes for fungi in the macrophage: (*iv*) the macrophages succeeds in killing the invader and the fungus is destroyed; (*v*) the macrophage cannot kill the fungus but is able to keep it contained within a granuloma in a dormant state; or (*vi*) the macrophage cannot control fungal growth and the fungi begin to replicate within the macrophage, escape from the macrophage and may disseminate to the central nervous system

the natural environment, thus it is likely that **individuals encounter *C. neoformans* many times during their lifetime but are protected by a healthy immune system.**

Some studies show that latent infection contained by the immune system may re-emerge if an immune deficiency later develops. Evidence for this phenomenon comes from a study by Garcia-Hermoso et al., which determined the genetic origin of clinical *C. neoformans* isolates taken from a cohort of cryptococcosis sufferers in France. This analysis found that a number of immunocompromised African immigrants in the study, who had lived in France for many years before developing immune deficiency, were infected with *C. neoformans* strains originating in Africa. Garcia-Hermoso et al. concluded that these patients may have developed a latent form of cryptococcosis before moving to France and that the infection re-emerged when immune deficiency later developed (Garcia-Hermoso et al. 1999).

III. The Macrophage

Macrophages are innate immune phagocytic cells that patrol the body's tissues searching for signs of infection. When a macrophage detects a foreign cell, such as a pathogenic microorganism, it becomes activated and will attempt to engulf the invader via a process called phagocytosis (see Fig. 5.3). Phagocytosis begins when phagocytic receptors on the cell surface of the macrophage encounter ligands that are only found on foreign cells. Following receptor ligation, signalling pathways are activated inside the macrophage, which trigger cytoskeletal actin

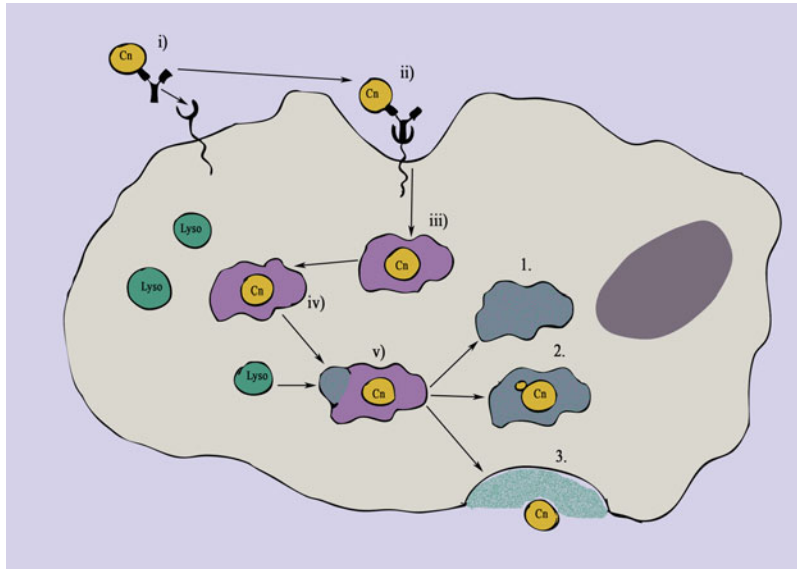


Fig. 5.3. Possible outcomes following *C. neoformans* phagocytosis. (*i*) Opsonised *C. neoformans* (in this case opsonised with antibody) binds to phagocytic receptors on the cell surface of the macrophage. (*ii*) Ligation of phagocytic receptors induces cytoskeletal rearrangements within the macrophage resulting in invagination of the membrane and the formation of phagocytic pseudopodia around the bound cell. (*iii*) *C. neoformans* is contained inside the macrophage within a membrane-bound compartment called the phagosome. (*iv*) Over time the phagosome matures, beginning with the recruitment of v-ATPase, NADPH oxidase and NOS to the phagosome membrane, resulting in the lowering of pH and the production of reactive oxygen species within the phagosome. (*v*) Phagosomal maturation culminates when lysosomes fuse with the phagosome; this releases digestive proteolytic enzymes into the phagosome (which is now termed the phagolysosome). There are three possible outcomes for *C. neoformans* following formation of the phagosome: (*1*) Biocidal conditions within the phagosome destroy the fungus, which is then digested; (*2*) *C. neoformans* resists the conditions within the phagolysosome and begins to replicate; or (*3*) *C. neoformans* escapes from the macrophage via a process called vomocytosis (either following, or independently of, replication)

rearrangements around the receptor, leading to internalisation of the particle into a membrane-bound compartment termed the phagosome. Over time, conditions within the phagosome are altered by the macrophage to create an environment that is destructive to microorganisms, a process called phagosomal maturation. Via a series of vesicle fusion events, membrane proteins such as NADPH oxidase, V-ATPase and NOS are delivered to the phagosome. Together, these create a highly antimicrobial environment, for instance via the production of reactive oxygen species (ROS) by the NADPH oxidase, or lowering of the phagosomal pH by the V-ATPase. Finally, the phagosome fuses with a specialised cytosolic vesicle called the lysosome, which contains an array of digestive enzymes. These digestive enzymes, which work best at low pH, kill any remaining organisms in the phagosome and then break up the dead cell into its constituent parts.

Phagocytosis can clear infection directly but it also triggers signals that enhance the immune response. Upon sensing infection, macrophages can release proinflammatory cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-1β; the inflammation that these cytokines produce attracts other immune cells to the site of infection, ultimately engaging the adaptive immune system if the infection is not cleared rapidly.

IV. Phagocytosis of *C. neoformans* by Macrophages

Macrophages must detect the presence of foreign organisms before they can initiate phagocytosis.

To facilitate detection, macrophages express an array of cell surface phagocytic receptors that bind to ligands present on foreign cells but not host cells. Phagocytic receptors can be categorised according to the nature of their ligands. Non-opsonic phagocytic receptors or pathogen recognition receptors (PRRs) bind to molecular ligands (pathogen-associated molecular patterns or PAMPs) such as polysaccharide residues or lipid species found on the surface of foreign cells. In contrast, opsonic receptors bind to opsonising proteins, such as antibodies or complement proteins, which coat foreign cells following activation of the humoral immune system.

Capsule expression has long been implicated in cryptococcal virulence since it was observed that *Cryptococcus* acapsular mutant strains are avirulent (Fromtling et al. 1982; Chang and Kwon-Chung 1994). The capsule has a number of properties that contribute to virulence during infection, but the most important seems to be its **anti-phagocytic quality**, which was first realised when acapsular strains were found to be phagocytosed by macrophages more readily than were capsular strains (Kozel and Gotschlich 1982; Cross and Bancroft 1995). Capsule growth can be stimulated by CO_2 levels and pH conditions similar to those found during host infection (Granger et al. 1985) and has also been observed to occur in vivo (Feldmesser et al. 2001). Synthesis of the capsule is controlled by at least four genes, designated *CAP64*, *CAP59*, *CAP10* and *CAP60* (Buchanan and Murphy 1998; Ma and May 2009). In a series of experiments, Chang et al. found that deletion of each CAP gene produced an acapsular mutant that lacked virulence until the gene was reconstituted (Chang et al. 1996; Chang and Kwon-Chung 1994, 1999, 1998). **The capsule is composed of two key polysaccharide components: glucuronoxylomannan (GXM) and galactoxymannan (GalXM)** (Bose et al. 2003), with a ratio of approximately 9:1 by mass in favour of GXM (Idnurm et al. 2005).

One of the main ways that the capsule protects *C. neoformans* against phagocytosis is by providing a shroud around the cell, which interferes with macrophage detection. This shrouding effect blocks uptake via both opsonic and non-opsonic phagocytic receptors although it appears to have a greater inhibitory effect on non-opsonic uptake because non-opsonised *C. neoformans* cells are almost completely protected from phagocytosis (Mukherjee et al. 1996).

A. Non-opsonic Uptake

Phagocytosis of yeast-like fungi that do not produce a polysaccharide capsule, such as *Candida albicans* and *Saccharomyces cerevisiae,* often occurs via non-opsonic phagocytic receptors such as Dectin-1 and the Mannose receptor (MR) (Gantner et al. 2005; Brown and Gordon 2001; Porcaro et al. 2003; Giaimis et al. 1993). Both phagocytic receptors bind to cell wall constituents found in the fungal cell wall; Dectin-1 binds to β-1,3- and β-1,6-linked glucan residues (Brown and Gordon 2003) whereas MR binds to mannoproteins. **Macrophages can easily phagocytose acapsular *C. neoformans* mutants via Dectin-1 or MR ligation, but they struggle to phagocytose encapsulated cells** (Cross and Bancroft 1995). In wild-type capsular strains, the ligands for each receptor are obscured by the overlying capsule, thus it is thought that non-opsonic phagocytic receptors such as Dectin-1 and MR may contribute to the phagocytosis of recently inhaled cryptococcal cells (which have a minimal capsule), but once capsule growth is stimulated non-opsonic uptake becomes redundant.

B. Opsonic Uptake

Under most conditions, the cryptococcal capsule conceals cell wall ligands detected by non-opsonic receptors so **the main route of phagocytosis by macrophages is via opsonic phagocytic receptors** (Mukherjee et al. 1996). Opsonisation is more effective at facilitating the detection and phagocytosis of *C. neoformans* because opsonins bind to capsular components and thus are more accessible to macrophage phagocytic receptors than the non-opsonic receptor ligands. Two main classes of opsonic phagocytic receptors are expressed by macrophages: **complement receptors**, which recognise activated complement proteins, and **Fc receptors**, which recognise the Fc regions of immunoglobulin (antibody) molecules.

Complement can be activated via two pathways – the classical pathway and the alternative pathway. Classical activation requires the presence of antigen-specific immunoglobulin bound to the surface of the foreign cell, whereas alternative activation does not require antigen specificity. Antibody produced during cryptococcal infection is thought to be ineffective at activating classical complement cascades (Houpt et al. 1994) and thus **the alternative complement cascade is considered more important to opsonisation during infection**. Regardless of the route of activation, the end result of the complement cascade is the deposition of the complement fragment C3b, which can be recognised and phagocytosed by the macrophage complement receptor CR3.

Although complement deposition facilitates macrophage detection of *C. neoformans,* the pathogen is still able to reduce the efficiency of complement-mediated uptake by modifying the makeup of its capsule. Consequently, cryptococcal cells producing a capsule that is thick (Zaragoza et al. 2003) or has a low density (Gates and Kozel 2006) are harder for macrophages to detect, since both of these properties increase the likelihood that complement proteins will bind within the capsule, where they are obscured, as opposed to at the surface.

The production of antigen-specific antibody against a newly encountered pathogen requires activation of the adaptive immune response, although secondary exposure to an antigen evokes a quicker response due to the persistence of immunological memory. Antibodies can improve the phagocytic uptake of foreign cells in two ways once they have bound to the cell. They can amplify complement opsonisation by activating the classical complement pathway, but they can also directly stimulate phagocytosis via ligation of macrophage Fc receptors, which bind to the non-variable region of antigen-bound antibodies. **During *C. neoformans* infection, most antibody produced is against capsular components such as GXM. Anti-capsular GXM antibody titres are often used as markers of acquired immunity to *C. neoformans* in healthy individuals** (Deshaw and Pirofski 1995; Houpt et al. 1994); however, the protective role of anti-GXM antibodies during infection is debateable. For example, a 1994 study by Houpt et al. found that the sera of healthy subjects contained IgG and IgM antibodies reactive against cryptotoccal GXM, but that these antibodies were ineffective at activating the classical complement cascade (Houpt et al. 1994).

C. Capsule-Independent Antiphagocytic Factors

The capsule is a major antiphagocytic mechanism deployed by *C. neoformans*, but capsule-independent antiphagocytic mechanisms also exist. A study by Liu et al. identified a GATA family transcription factor called **Gat201**, which appears to control such a mechanism (Liu et al. 2008). A recent study of Gat201 revealed that knockout of its gene affected the transcription of ~1,100 genes in *C. neoformans*, although only 62 of these genes are thought to be regulated by direct binding of Gat201. **Phenotypic analysis of the *GAT201* knockout strain showed reduced capsule size and increased uptake by macrophages.** The susceptibility to phagocytosis observed was greater than that of acapsular mutants alone, suggesting that capsule-independent antiphagocytic mechanisms are also controlled by Gat201 potentially via two downstream genes – *BLP1* and *GAT204* (Chun et al. 2011).

One capsule-independent antiphagocytic mechanism is the secreted protein **APP1**. This protein is expressed by *C. neoformans* during infection. APP1 can block complement-dependent phagocytosis by binding to and subsequently **blocking macrophage CR3 receptors** (Luberto et al. 2003).

D. Titan Cell Formation

Several studies have reported the presence of abnormally large cryptococcal cells in the lungs during in vivo infection (Zaragoza et al. 2010; Cruickshank et al. 1973; Feldmesser et al. 2001; Love et al. 1985) that appear to be **resistant to phagocytosis** (Okagaki et al. 2010). These giant cells are now recognised as a distinct cell morphology during infection and are now often

called "Titan cells" in the literature. The size of reported Titan cells differs between studies and ranges from around **25 μm to 100 μm, making their volume up to 900 times greater than that of normal cryptococci** (Zaragoza et al. 2010). The underlying mechanism or stimuli behind Titan cell development is not known, although the presence of macrophages, or spent media used to grow macrophages, can induce the morphology (Okagaki et al. 2010) suggesting that interactions with the macrophage play at least some role in the process.

Titan cells appear to be more resistant to phagocytosis than normally sized cells. The most obvious defence a Titan cell has against a macrophage is its physical size. However, recent work has suggested that the presence of **Titan cells during infection may also protect neighbouring wild-type cells from phagocytosis, suggesting the presence of a secreted inhibitory factor** (Okagaki et al. 2010; Okagaki and Nielsen 2012).

In summary, the detection and phagocytosis of *C. neoformans* cells is an important part of the immune response against the fungus. To protect itself from phagocytosis, *C. neoformans* relies heavily on its polysaccharide capsule but also deploys a range of other antiphagocytic strategies. Such mechanisms appear effective at helping some cells evade phagocytosis, but nonetheless the infection of macrophages in vitro and in vivo is often observed, indicating that phagocytosis of *C. neoformans* is far from rare.

V. Life Within the Phagosome

To grow inside a macrophage following phagocytosis, an intracellular pathogen must successfully avoid the killing mechanisms that develop in the phagosome during phagosomal maturation. A number of mechanisms are employed by pathogens to do this, including inhibition of V-ATPase to prevent phagosomal acidification as seen with *Mycobacterium tuberculosis* infection (Huynh and Grinstein 2007) and escape from the phagosome into the cytoplasm as seen with *Listeria monocytogenes* infection (Hamon et al. 2006). In contrast to such pathogens, *C. neoformans* is able to survive within the phagosome seemingly without modifying the maturation process. Instead, it is able to **resist and overcome the destructive conditions it encounters in the phagosome and can even grow within this harsh environment**.

The first barrier to cryptococcal growth in mammalian macrophages is the higher temperature within the host than in the environment. The complete molecular mechanisms that allow *C. neoformans* to grow at 37 °C are not fully known, but a number of potential pathways have been implicated. For instance, higher temperatures induce upregulation of TSP1 and TSP2, which together lead to higher levels of the polysaccharide trehalose, protecting cellular proteins from denaturation (Petzold et al. 2006).

Inside the phagosome, the largest barrier to cryptococcal growth is phagosomal maturation, i.e. the production of oxygen free radicals accompanied by the acidification of the phagosome. During infection most *C. neoformans* strains synthesise **melanin**, which is a dark-coloured pigment and an antioxidant (Wang et al. 1995) that is thought to protect the fungi from reactive oxygen species. In addition to melanin, **capsule enlargement** during macrophage infection may also be protective against oxygen free radicals by creating a barrier between the cell body and phagosomal contents (Zaragoza et al. 2008); furthermore, GXM within the capsule may act as an antioxidant (Monari et al. 2006; Vecchiarelli et al. 1996). Interestingly, however, the phagosomal acidification that occurs during maturation may actually be beneficial to *C. neoformans* because neutralising phagosomal pH can block intracellular replication (Levitz et al. 1999).

While in the phagosome, *C. neoformans* secretes a number of proteins that potentially influence the macrophage. One such protein, which is well known to contribute to cryptococcal virulence, is the enzyme **phospholipase B (Plb)**. Plb is a lipid-modifying enzyme that has multiple enzymatic activities. It possesses phospholipase B activity, which can cleave the ester bonds linking the glycerol headgroup in

a phospholipid to the two fatty acid chains at both the sn1 (phospholipase A1 activity) and sn2 positions (phospholipase A2 activity) to produce a free fatty acid and a lysophospholipase. As well phospholipase B activity, Plb also has lysophospholipase and lysophospholipid transacetylase activity; these two activities modify the lysophospholipid products of sn1 or sn2 cleavage, resulting in further degradation or re-synthesis of the original substrate (Shea et al. 2006).

Previous studies have reported that Plb-deficient strains of *C. neoformans* are avirulent during murine infection, displaying reduced fungal burden in the lungs, possibly due to a reduced ability to proliferate within macrophages (unpublished observations from our group), and a lower propensity to disseminate to the central nervous system (Chayakulkeeree et al. 2011; Noverr et al. 2003; Chen et al. 1997; Cox et al. 2001). The molecular mechanism behind Plb-mediated virulence is not fully known. However, a compelling theory is that Plb activity metabolises *Cryptococcus* or macrophage-derived phospholipids to produce arachidonic acid (AA), which is a precursor to immunoregulatory eicosanoids. Members of the eicosanoid family include prostaglandins, leukotrienes and lipoxins. Both macrophages (Harizi et al. 2008) and *C. neoformans* itself (Noverr et al. 2001) can produce eicosanoids, meaning that either or both types of cell could potentially make use of the AA produced.

Eicosanoids might aid *C. neoformans* survival by directly suppressing the antimicrobial mechanisms of the infected macrophage; in addition they may also alter the immune response during cryptococcosis at a wider level. For example, PGE_2 (one of the most ubiquitous prostaglandins during inflammation) can produce a Th2 $CD4^+$-biased immune response by downregulating IL-12 release from macrophages (van der Pouw Kraan et al. 1995). With this in mind, it has been observed that the development of **Th1 adaptive immune responses is protective against *C. neoformans* infection, whereas the Th2 adaptive immune response is not** (Hoag et al. 1997; Voelz et al. 2009; Wormley et al. 2007).

VI. Escape from the Macrophage

Pathogens that are specialised in the parasitism of macrophages often possess mechanisms that allow them to escape the intracellular niche when the time is right. *C. neoformans* is no different in this respect and possesses an escape mechanism called non-lytic phagosomal extrusion or vomocytosis.

Vomocytosis is a mechanism first observed by our group (Ma et al. 2006) and others (Alvarez and Casadevall 2006) that allows *C. neoformans* to escape from infected macrophages without causing host cell lysis. Exiting without causing harm to the macrophage is relatively rare for a phagocytic escape mechanism and is beneficial to *C. neoformans* because it produces lower levels of inflammation than host cell lysis. The cellular events driving vomocytosis are not fully understood, although it appears to occur via fusion of *Cryptococcus*-loaded phagosomes with the macrophage outer membrane following prior permeabilisation of the phagosome membrane (Johnston and May 2010). Interestingly, host cells appear to attempt to block vomocytosis by initiating repeated actin polymerisation/depolymerisation cycles (actin flashing) around the phagosome following permeabilisation (Johnston and May 2010).

VII. Macrophages as a "Trojan Horse"

Dissemination from the lungs to the CNS is a crucial escalation point during cryptococcosis because CNS infection is almost always fatal unless treated. **To enter the CNS, *C. neoformans* must cross the blood–brain barrier (BBB),** whose normal function is to prevent such passage. There are **two main routes** that *C. neoformans* potentially uses to cross the BBB: (**a**) extracellular *C. neoformans* cells in the blood during fungemia **pass independently across the BBB** by an as-yet-undefined mechanism or (**b**) **macrophages infected with *C. neoformans*** in the lungs pass back into circulation and subsequently cross the BBB and disgorge their fungal cargo – this is popularly termed the "Trojan horse" theory of dissemination (Fig. 5.4).

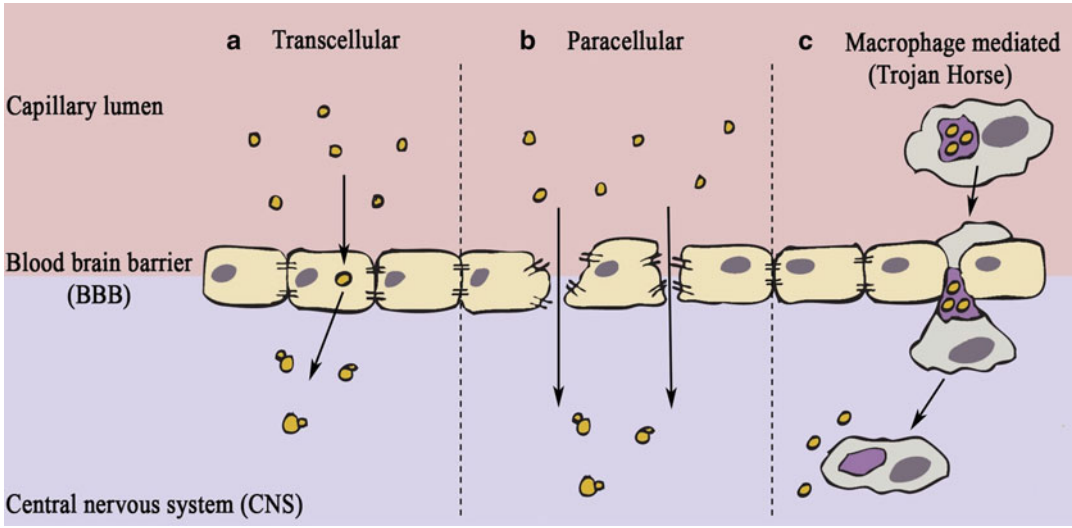


Fig. 5.4. Possible routes of dissemination across the blood brain barrier for *C. neoformans*. There are three routes that *C. neoformans* is thought to use to cross the blood–brain barrier (BBB). (*a*) Transcellular route: fungal cells pass directly through endothelial cells lining capillaries in the brain and are taken up at the apical side and exit on the basal side. (*b*) Paracellular route: fungal cells pass between endothelial cells following disruption of cell-to-cell contacts; this disruption could be due to factors produced by the fungi or to inflammation. (*c*) "Trojan horse" dissemination: macrophages infected with *C. neoformans* cells pass from the circulation through the BBB via diapedesis; the fungi within the macrophage then escape into the extracellular space

Evidence to support both routes of entrance can be found in the literature, suggesting that the mechanisms may not be mutually exclusive and in fact may co-exist. In this respect, a study that examined disseminative cryptococcosis in an in vivo mouse model found that cryptococcal cells could be found within the brain both extracellulary or associated with monocytes and endothelial cells (Chrétien et al. 2002). Intravital imaging of *C. neoformans* in mouse brain capillaries found that extracellular fungi in the capillary lumen could cross the epithelial layer following sudden stopping. In this study, arrest in the lumen appeared to be purely via mechanic trapping when the size of *Cryptococcus* cells exceeded that of the capillary, whereas crossing of the epithelial layer was an active process requiring fungal viability (Shi et al. 2010).

In support of the Trojan horse theory, infected macrophages from the alveolar space can re-enter circulation and transport *C. neoformans* to other parts of the body whereas infected macrophages injected directly into the blood lead to increased fungal burden in the brain (Charlier et al. 2009).

VIII. How Has the *C. neoformans*–Macrophage Interaction Evolved?

A significant challenge for the field is to explain why an environmental opportunistic pathogen that is not dependent on a host has nonetheless evolved a battery of virulence factors that seem specific for mammalian hosts. The most likely explanation is that ***C. neoformans* did not evolve its virulence factors to cause infections in animals but rather that such factors were selected in response to attack by soil predators**. Considerable support for this model comes from investigations looking at the interaction between *C. neoformans* and soil-dwelling amoebae such as *Acanthamoeba castellanii* and *Dictyostelium discoideum*. Both of these species can engulf C. *neoformans* and, excitingly, many aspects of the *Cryptococcus*/macrophage relationship, e.g. intracellular proliferation and the production of

exopolysaccharides, were mirrored in the amoebal host following phagocytosis. Further confirmation of the similarities between the two models were provided by the observations that avirulent mutants in the macrophage, such as acapsular or phospholipase-deficient cryptococcal strains, were also more vulnerable to predation (Steenbergen et al. 2001; Steenbergen and Casadevall 2003; Casadevall et al. 2003).

IX. Conclusion

To summarise, the role of the macrophage during antifungal immune responses is of central importance. Not only is the macrophage responsible for detecting infection and shaping subsequent immune responses, it is also required to directly kill and clear fungal cells. The behaviour of the macrophage during *C. neoformans* infection is of even greater importance than for most fungal infections because while the macrophage seeks to clear infection the fungus itself relies on the macrophage as safe niche for intracellular proliferation and possibly as a vehicle for CNS dissemination.

References

Abadi J, Pirofski L (1999) Antibodies reactive with the cryptococcal capsular polysaccharide glucuronoxylomannan are present in sera from children with and without human immunodeficiency virus infection. J Infect Dis 180:915–919

Alvarez M, Casadevall A (2006) Phagosome extrusion and host-cell survival after *Cryptococcus neoformans* phagocytosis by macrophages. Curr Biol 16:2161–2165

Bartlett KH, Kidd SE, Kronstad JW (2008) The emergence of *Cryptococcus gattii* in British Columbia and the Pacific Northwest. Curr Infect Dis Rep 10:58–65

Bose I, Reese AJ, Ory JJ, Janbon G, Doering TL (2003) A yeast under cover: the capsule of *Cryptococcus neoformans*. Eukaryot Cell 2:655–663

Brown GD, Gordon S (2001) Immune recognition. A new receptor for beta-glucans. Nature 413:36–37

Brown GD, Gordon S (2003) Fungal beta-glucans and mammalian immunity. Immunity 19:311–315

Buchanan KL, Murphy JW (1998) What makes *Cryptococcus neoformans* a pathogen? Emerg Infect Dis 4:71–83

Byrnes EJ, Bildfell RJ, Frank SA, Mitchell TG, Marr KA, Heitman J (2009) Molecular evidence that the range of the Vancouver Island outbreak of *Cryptococcus gattii* infection has expanded into the Pacific Northwest in the United States. J Infect Dis 199:1081–1086

Byrnes EJ III, Li W, Lewit Y, Ma H, Voelz K, Ren P, Carter DA, Chaturvedi V, Bildfell RJ, May RC, Heitman J (2010) Emergence and Pathogenicity of Highly Virulent *Cryptococcus gattii* Genotypes in the Northwest United States. PLoS Pathog 6:e1000850

Casadevall A, Steenbergen JN, Nosanchuk JD (2003) 'Ready made' virulence and 'dual use' virulence factors in pathogenic environmental fungi–the *Cryptococcus neoformans* paradigm. Curr Opin Microbiol 6:332–337

Chang YC, Kwon-Chung KJ (1994) Complementation of a capsule-deficient mutation of *Cryptococcus neoformans* restores its virulence. Mol Cell Biol 14:4912–4919

Chang YC, Kwon-Chung KJ (1998) Isolation of the third capsule-associated gene, CAP60, required for virulence in *Cryptococcus neoformans*. Infect Immun 66:2230–2236

Chang YC, Kwon-Chung KJ (1999) Isolation, characterization, and localization of a capsule-associated gene, CAP10, of *Cryptococcus neoformans*. J Bacteriol 181:5636–5643

Chang YC, Penoyer LA, Kwon-Chung KJ (1996) The second capsule gene of *Cryptococcus neoformans*, CAP64, is essential for virulence. Infect Immun 64:1977–1983

Charlier C, Nielsen K, Daou S, Brigitte M, Chretien F, Dromer F (2009) Evidence of a role for monocytes in dissemination and brain invasion by *Cryptococcus neoformans*. Infect Immun 77:120–127

Chayakulkeeree M, Johnston SA, Oei JB, Lev S, Williamson PR, Wilson CF, Zuo X, Leal AL, Vainstein MH, Meyer W, Sorrell TC, May RC, Djordjevic JT (2011) SEC14 is a specific requirement for secretion of phospholipase B1 and pathogenicity of *Cryptococcus neoformans*. Mol Microbiol 80:1088–1101

Chen SC, Muller M, Zhou JZ, Wright LC, Sorrell TC (1997) Phospholipase activity in *Cryptococcus neoformans*: a new virulence factor? J Infect Dis 175:414–420

Chrétien F, Lortholary O, Kansau I, Neuville S, Gray F, Dromer F (2002) Pathogenesis of cerebral *Cryptococcus neoformans* infection after fungemia. J Infect Dis 186:522–530

Chun CD, Brown JC, Madhani HD (2011) A major role for capsule-independent phagocytosis-inhibitory mechanisms in mammalian infection by *Cryptococcus neoformans*. Cell Host Microbe 9:243–251

Cox GM, Mcdade HC, Chen SC, Tucker SC, Gottfredsson M, Wright LC, Sorrell TC, Leidich SD, Casadevall A, Ghannoum MA, Perfect JR (2001) Extracellular phospholipase activity is a virulence

factor for *Cryptococcus neoformans*. Mol Microbiol 39:166–175

Cross CE, Bancroft GJ (1995) Ingestion of acapsular *Cryptococcus neoformans* occurs via mannose and beta-glucan receptors, resulting in cytokine production and increased phagocytosis of the encapsulated form. Infect Immun 63:2604–2611

Cruickshank JG, Cavill R, Jelbert M (1973) *Cryptococcus neoformans* of unusual morphology. Appl Microbiol 25:309–312

Deshaw M, Pirofski LA (1995) Antibodies to the *Cryptococcus neoformans* capsular glucuronoxylomannan are ubiquitous in serum from HIV+ and HIV− individuals. Clin Exp Immunol 99:425–432

Feldmesser M, Kress Y, Casadevall A (2001) Dynamic changes in the morphology of *Cryptococcus neoformans* during murine pulmonary infection. Microbiology 147:2355–2365

Fromtling RA, Shadomy HJ, Jacobson ES (1982) Decreased virulence in stable, acapsular mutants of *Cryptococcus neoformans*. Mycopathologia 79:23–29

Gantner BN, Simmons RM, Underhill DM (2005) Dectin-1 mediates macrophage recognition of *Candida albicans* yeast but not filaments. EMBO J 24:1277–1286

Garcia-Hermoso D, Janbon G, Dromer F (1999) Epidemiological evidence for dormant *Cryptococcus neoformans* infection. J Clin Microbiol 37:3204–3209

Gates MA, Kozel TR (2006) Differential localization of complement component 3 within the capsular matrix of *Cryptococcus neoformans*. Infect Immun 74:3096–3106

Giaimis J, Lombard Y, Fonteneau P, Muller CD, Levy R, Makaya-Kumba M, Lazdins J, Poindron P (1993) Both mannose and beta-glucan receptors are involved in phagocytosis of unopsonized, heat-killed *Saccharomyces cerevisiae* by murine macrophages. J Leukoc Biol 54:564–571

Giles SS, Dagenais TR, Botts MR, Keller NP, Hull CM (2009) Elucidating the pathogenesis of spores from the human fungal pathogen *Cryptococcus neoformans*. Infect Immun 77:3491–3500

Granger DL, Perfect JR, Durack DT (1985) Virulence of *Cryptococcus neoformans*. Regulation of capsule synthesis by carbon dioxide. J Clin Invest 76:508–516

Hamon M, Bierne H, Cossart P (2006) Listeria monocytogenes: a multifaceted model. Nat Rev Microbiol 4:423–434

Harizi H, Corcuff JB, Gualde N (2008) Arachidonic-acid-derived eicosanoids: roles in biology and immunopathology. Trends Mol Med 14:461–469

Hoag KA, Lipscomb MF, Izzo AA, Street NE (1997) IL-12 and IFN-gamma are required for initiating the protective Th1 response to pulmonary cryptococcosis in resistant C.B-17 mice. Am J Respir Cell Mol Biol 17:733–739

Houpt DC, Pfrommer GS, Young BJ, Larson TA, Kozel TR (1994) Occurrences, immunoglobulin classes, and biological activities of antibodies in normal human serum that are reactive with *Cryptococcus neoformans* glucuronoxylomannan. Infect Immun 62:2857–2864

Hull CM, Heitman J (2002) Genetics of *Cryptococcus neoformans*. Annu Rev Genet 36:557–615

Huynh KK, Grinstein S (2007) Regulation of vacuolar pH and its modulation by some microbial species. Microbiol Mol Biol Rev 71:452–462

Idnurm A, Bahn YS, Nielsen K, Lin X, Fraser JA, Heitman J (2005) Deciphering the model pathogenic fungus *Cryptococcus neoformans*. Nat Rev Microbiol 3:753–764

Ikeda R, Shinoda T, Fukazawa Y, Kaufman L (1982) Antigenic characterization of *Cryptococcus neoformans* serotypes and its application to serotyping of clinical isolates. J Clin Microbiol 16:22–29

Johnston SA, May RC (2010) The human fungal pathogen *Cryptococcus neoformans* escapes macrophages by a phagosome emptying mechanism that is inhibited by arp2/3 complex-mediated actin polymerisation. PLoS Pathog 6:e1001041

Kozel TR, Gotschlich EC (1982) The capsule of cryptococcus neoformans passively inhibits phagocytosis of the yeast by macrophages. J Immunol 129:1675–1680

Kronstad JW, Attarian R, Cadieux B, Choi J, D'souza CA, Griffiths EJ, Geddes JM, Hu G, Jung WH, Kretschmer M, Saikia S, Wang J (2011) Expanding fungal pathogenesis: *Cryptococcus* breaks out of the opportunistic box. Nat Rev Microbiol 9:193–203

Lengeler KB, Cox GM, Heitman J (2001) Serotype AD strains of *Cryptococcus neoformans* are diploid or aneuploid and are heterozygous at the mating-type locus. Infect Immun 69:115–122

Levitz SM, Nong SH, Seetoo KF, Harrison TS, Speizer RA, Simons ER (1999) Cryptococcus neoformans resides in an acidic phagolysosome of human macrophages. Infect Immun 67:885–890

Liu OW, Chun CD, Chow ED, Chen C, Madhani HD, Noble SM (2008) Systematic genetic analysis of virulence in the human fungal pathogen *Cryptococcus neoformans*. Cell 135:174–188

Love GL, Boyd GD, Greer DL (1985) Large *Cryptococcus neoformans* isolated from brain abscess. J Clin Microbiol 22:1068–1070

Luberto C, Martinez-Mariño B, Taraskiewicz D, Bolaños B, Chitano P, Toffaletti DL, Cox GM, Perfect JR, Hannun YA, Balish E, Del Poeta M (2003) Identification of App1 as a regulator of phagocytosis and virulence of *Cryptococcus neoformans*. J Clin Invest 112:1080–1094

Ma H, May RC (2009) Virulence in *Cryptococcus* species. Adv Appl Microbiol 67:131–190

Ma H, Croudace JE, Lammas DA, May RC (2006) Expulsion of live pathogenic yeast by macrophages. Curr Biol 16:2156–2160

Macdougall L, Kidd SE, Galanis E, Mak S, Leslie MJ, Cieslak PR, Kronstad JW, Morshed MG,

Bartlett KH (2007) Spread of *Cryptococcus gattii* in British Columbia, Canada, and detection in the Pacific Northwest, USA. Emerg Infect Dis 13:42–50

Mitchell TG, Perfect JR (1995) Cryptococcosis in the era of AIDS–100 years after the discovery of *Cryptococcus neoformans*. Clin Microbiol Rev 8:515–548

Monari C, Bistoni F, Vecchiarelli A (2006) Glucuronoxylomannan exhibits potent immunosuppressive properties. FEMS Yeast Res 6:537–542

Mukherjee S, Feldmesser M, Casadevall A (1996) J774 murine macrophage-like cell interactions with *Cryptococcus neoformans* in the presence and absence of opsonins. J Infect Dis 173:1222–1231

Noverr MC, Phare SM, Toews GB, Coffey MJ, Huffnagle GB (2001) Pathogenic yeasts *Cryptococcus neoformans* and *Candida albicans* produce immunomodulatory prostaglandins. Infect Immun 69:2957–2963

Noverr MC, Cox GM, Perfect JR, Huffnagle GB (2003) Role of PLB1 in pulmonary inflammation and cryptococcal eicosanoid production. Infect Immun 71:1538–1547

Okagaki LH, Nielsen K (2012) Titan cells confer protection from phagocytosis in *Cryptococcus neoformans* infections. Eukaryot Cell 11:820–826

Okagaki LH, Strain AK, Nielsen JN, Charlier C, Baltes NJ, Chrétien F, Heitman J, Dromer F, Nielsen K (2010) Cryptococcal cell morphology affects host cell interactions and pathogenicity. PLoS Pathog 6:e1000953

Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM (2009) Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. AIDS 23:525–530

Petzold EW, Himmelreich U, Mylonakis E, Rude T, Toffaletti D, Cox GM, Miller JL, Perfect JR (2006) Characterization and regulation of the trehalose synthesis pathway and its importance in the pathogenicity of *Cryptococcus neoformans*. Infect Immun 74:5877–5887

Porcaro I, Vidal M, Jouvert S, Stahl PD, Giaimis J (2003) Mannose receptor contribution to *Candida albicans* phagocytosis by murine E-clone J774 macrophages. J Leukoc Biol 74:206–215

Shea JM, Henry JL, Del Poeta M (2006) Lipid metabolism in *Cryptococcus neoformans*. FEMS Yeast Res 6:469–479

Shi M, Li SS, Zheng C, Jones GJ, Kim KS, Zhou H, Kubes P, Mody CH (2010) Real-time imaging of trapping and urease-dependent transmigration of *Cryptococcus neoformans* in mouse brain. J Clin Invest 120:1683–1693

Steenbergen JN, Casadevall A (2003) The origin and maintenance of virulence for the human pathogenic fungus *Cryptococcus neoformans*. Microbes Infect 5:667–675

Steenbergen JN, Shuman HA, Casadevall A (2001) *Cryptococcus neoformans* interactions with amoebae suggest an explanation for its virulence and intracellular pathogenic strategy in macrophages. Proc Natl Acad Sci USA 98:15245–15250

van der Pouw Kraan TC, Boeije LC, Smeenk RJ, Wijdenes J, Aarden LA (1995) Prostaglandin-E2 is a potent inhibitor of human interleukin 12 production. J Exp Med 181:775–779

Vecchiarelli A, Retini C, Monari C, Tascini C, Bistoni F, Kozel TR (1996) Purified capsular polysaccharide of *Cryptococcus neoformans* induces interleukin-10 secretion by human monocytes. Infect Immun 64:2846–2849

Voelz K, Lammas DA, May RC (2009) Cytokine signaling regulates the outcome of intracellular macrophage parasitism by *Cryptococcus neoformans*. Infect Immun 77:3450–3457

Wang Y, Aisen P, Casadevall A (1995) *Cryptococcus neoformans* melanin and virulence: mechanism of action. Infect Immun 63:3131–3136

Wormley FL, Perfect JR, Steele C, Cox GM (2007) Protection against cryptococcosis by using a murine gamma interferon-producing *Cryptococcus neoformans* strain. Infect Immun 75: 1453–1462

Zaragoza O, Taborda CP, Casadevall A (2003) The efficacy of complement-mediated phagocytosis of *Cryptococcus neoformans* is dependent on the location of C3 in the polysaccharide capsule and involves both direct and indirect C3-mediated interactions. Eur J Immunol 33:1957–1967

Zaragoza O, Chrisman CJ, Castelli MV, Frases S, Cuenca-Estrella M, Rodríguez-Tudela JL, Casadevall A (2008) Capsule enlargement in *Cryptococcus neoformans* confers resistance to oxidative stress suggesting a mechanism for intracellular survival. Cell Microbiol 10:2043–2057

Zaragoza O, García-Rodas R, Nosanchuk JD, Cuenca-Estrella M, Rodríguez-Tudela JL, Casadevall A (2010) Fungal cell gigantism during mammalian infection. PLoS Pathog 6:e1000945

6 T Cell Responses in Fungal Infections

Cristina Cunha[1], Franco Aversa[2], Luigina Romani[1], Agostinho Carvalho[1]

CONTENTS

I. Introduction: Dynamics of the Host–Fungus Interaction

The past two decades have seen an unprecedented number of fungal diseases (Fisher et al. 2012). The fungal kingdom is characterized by enormous biodiversity, with over 70,000 known species and an estimated 1.5 million species, 150–400 of which have already been associated with human and/or animal disease. In general, pathogenic fungi (e.g., *Aspergillus fumigatus, Cryptococcus neoformans, Pneumocystis jirovecii* and the thermally dimorphic *Histoplasma capsulatum, Paracoccidioides brasiliensis, Coccidioides immitis* and *posadasii, Blastomyces dermatitidis,* and *Sporothrix schenckii*) are distinguished from commensals (e.g., *Malassezia* spp. and *Candida albicans*) by their strategies for survival and replication within a host, or for transmission from a host, that eventually lead to **cellular and tissue damage** (Romani 2011). Pathogenicity tactics can vary a great deal and often define unique signatures for specific fungal species, as these involve particular mechanisms for gaining access to the host, adhering to and colonizing a niche, evading immune defenses, and multiplying (Rappleye and Goldman 2008). Indeed, disease onset is often critically dependent on the ability of fungi to **reversibly switch morphotypes** in infection, a trait that, on the other hand, has forced the host immune system to continuously evolve its repertoire of cross-regulatory and overlapping antifungal responses at different body sites (Romani 2011). Thus, in the context of a dynamic host–fungus interaction, the strategies used by the host to limit fungal infectivity are necessarily assorted in order to cope with the multitude of fungal survival strategies; in retaliation, fungi have developed their own elaborate tactics to evade or modulate host defenses and to survive.

Given the advances in medical care witnessed in the last few decades, specifically

[1]Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Polo Unico Sant'Andrea delle Fratte, 06132 Perugia, Italy; e-mail: agostinho.carvalho@unipg.it; aacarvalho2008@gmail.com
[2]Department of Clinical and Experimental Medicine, University of Parma, via Gramsci 14, 43126 Parma, Italy

Human Fungal Pathogens, 2nd Edition
The Mycota XII
O. Kurzai (Ed.)

regarding transplantation and cancer treatment, the number of immunocompromised patients has risen, resulting in an increased incidence of fungal diseases (Kontoyiannis et al. 2010; Pappas et al. 2010). For this reason, opportunistic fungi, such as *Candida* and *Aspergillus* spp. have become major concerns in clinical care of immunocompromised patients. Despite the ability of fungi to survive and persevere within the human host, the truth is that fungal diseases in immunocompetent hosts are fairly uncommon, indicating that fungi have evolved particular adaptation mechanisms that allow them to persist relatively unnoticed by the host's immune system (Cooney and Klein 2008). This "peaceful" coexistence may digress into overt disease under conditions of immune deregulation, which may modify the environmental conditions perceived by the fungus. **Fungi are very proficient at sensing their surroundings and in responding to cues that promote their survival in changing environments.** One such example is the sensing of mammalian interleukin (IL)-17A, which triggers virulence by increasing fungal adhesion and filamentous growth, leading to enhanced biofilm formation and resistance to local antifungal defenses (Zelante et al. 2012). Besides the immunologically dynamic context, fungi have often to sustain extreme environmental abiotic stress conditions, the adaptation to which relies on profound metabolic changes (Grahl et al. 2011).

Thus, in the absence of a relatively inert immune response, fungal adaptation to hostile surroundings may trigger complex genomic microevolution (Odds and Jacobsen 2008; Magditch et al. 2012) and structural and metabolic adjustments that, by "awakening" host immunity, may paradoxically contribute to disease. Although such adaptation mechanisms may improve fungal fitness, they can nonetheless provide important insights into potential therapeutic targets (Richie et al. 2009).

Given that existing antifungal therapy is often toxic and ineffective, there is currently a pressing demand for the development of antifungal vaccination strategies (Cassone and Casadevall 2012; Iannitti et al. 2012). For this purpose, a clear understanding of the mechanisms of adaptive immunity is ultimately required to foster the development of vaccines or strategies aiming at modulating the host's immune response. The permanent interaction between the host and fungi and the commensal relationship with some of them may pose significant challenges in eliciting durable protective immunity, owing to repeated exposure or sensitivity to fungal antigens. A balanced host–fungus relationship and the concomitant **fine tuning of pro- and anti-inflammatory signaling** to allow host survival irrespective of pathogen elimination is a prerequisite for coexistence and requires the concerted actions of both innate and adaptive immune systems (Romani 2011). Therefore, generation of antifungal immunity presents a challenge that relies on a precarious equilibrium between pathogen clearance and tissue damage restriction, while preserving the host microbiota ecology.

This chapter focuses on new findings on adaptive immunity to the major medically important fungi and emphasizes how dendritic cells (DCs), through the discrimination of fungal molecular patterns, prime responses that nurture and shape the differentiation of T cell subsets. Also discussed is the contribution of T cell subsets to resistance and tolerance mechanisms of antifungal immune protection and how these mechanisms can be exploited for effective antifungal vaccine design.

II. Resistance and Tolerance Mechanisms in Antifungal Protection

The immune system protects from infections primarily by detecting and eliminating invading pathogens through a variety of host resistance effector mechanisms (Romani 2011; Medzhitov et al. 2012). Resistance is meant to reduce pathogen burden during infection through innate and adaptive immune mechanisms, whereas tolerance mitigates the substantial cost to host fitness of resistance. Even in the absence of overt tissue damage, resistance mechanisms commonly occur at a **cost to nor-**

mal tissue function, thus causing immunopathology. This means that the optimal immune response is determined by the balance between efficient pathogen clearance and an acceptable level of immunopathology.

Inflammation is an essential process required for immune resistance, particularly at mucosal tissues, during the transition from the rapid innate to the slower adaptive response. However, the downside of this powerful mechanism of protection against fungi is the collateral damage to the host. These side effects may be more devastating than infection itself. Thus, the ability to tolerate a pathogen's presence is a distinct host defense strategy that may have evolved to favor protective mechanisms without pathogen killing (Romani 2011; Medzhitov et al. 2012). A plethora of tolerance mechanisms, although not as well known as resistance mechanisms, protect the host from immune- or pathogen-induced damage (Cobbold et al. 2010; Saraiva and O'Garra 2010). Therefore, the term "tolerance" is semantically used here to refer to the multitude of anti-inflammatory mechanisms, including immunological tolerance (i.e., unresponsiveness to self-antigens). At this stage, however, whether "unwanted" immune responses against "self" environmental antigens and commensal microorganisms occur is not clearly defined, although there is evidence that fungal sensitization contributes to auto-reactivity against self-antigens due to shared epitopes with homologous fungal allergens (Zeller et al. 2008).

T helper (Th)1 and Th17 cells, which provide antifungal resistance, and T regulatory (Treg) cells, which limit the inflammation-associated deleterious effects, crucially contribute to the activation and preservation of these two disparate antifungal mechanisms. **Combined deficiency of the Th1 and Th17 pathways predisposes to fungal diseases** (van de Veerdonk et al. 2011), thus emphasizing the important role played by both pathways in protection against fungi (Moraes-Vasconcelos et al. 2005; Romani 2011; Hardison and Brown 2012). Thus, the Th1/Th17 pathways and Treg cells, capable of fine-tuning protective antimicrobial immunity to lessen harmful immune pathology, are key components of the current view of immunity to fungi. The enzyme **indoleamine 2,3-dioxygenase 1 (IDO1)** and its downstream catabolites sustain this delicate balance by providing the host with adequate protective immune mechanisms without necessarily eliminating the pathogen or causing undesirable tissue damage (Zelante et al. 2009). As a result of their ability to induce differentiation of Treg cells and inhibit Th17 cells, IDO1 is critical to cell lineage commitment in experimental fungal infections and contributes to the overall outcome of inflammation, allergy, and Th17-driven inflammation in these infections. Under these circumstances, the Th17 pathway, by inhibiting tryptophan catabolism, may instead favor pathology and provides evidence accommodating the apparently paradoxical association of chronic inflammation with fungal disease (Romani et al. 2008b).

III. Fungi and Inflammation: Evolving Concepts

As in autoimmunity and chronic inflammation, an imbalance between pro- and anti-inflammatory signals may prevent successful host–fungal interaction, thus leading to infection and disease (Romani and Puccetti 2007). Indeed, despite the occurrence of severe fungal infections in immunocompromised patients, **clinical evidence indicates that fungal disease also occurs in the setting of a heightened inflammatory response**, in which immunity occurs at the expense of host damage and pathogen eradication (Perfect 2012). Although inflammation is an essential component of the protective response, fungi have evolved ways to exploit and subvert it, thereby affecting their ability to persist in the host and pathogenicity (Romani and Puccetti 2008). A hyperinflammatory response does, in fact, enhance the virulence of some fungi. This is well illustrated by the commensal lifestyle of *Malassezia* spp. in normal skin, possibly due to the downregulation of inflammation via tumor growth factor-β1 (TGF-β1) and IL-10 (Ashbee 2006). In contrast, in atopic dermatitis and psoriasis, the skin barrier enhances release of allergens and molecules involved in hyperproliferation, cell migration, and disease exacerbation. Additional fungal diseases are also important examples of such dichotomy. For example, in **chronic mucocutaneous candidiasis (CMC)**, *C. albicans* yeasts

persist in recurring lesions of the skin, nails, and mucous membranes (Lilic 2002). Although CMC has occasionally been associated with autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (a condition of dysfunctional T cell activity), evidence has highlighted the contribution of deregulated inflammation and immune responses to disease pathogenesis (Liu et al. 2011; Puel et al. 2011; van de Veerdonk et al. 2011). As already mentioned, fungal sensing of IL-17A is a newly described mechanism by which host inflammation may favor fungal infectivity and promote the transition from fungal commensalism to infection (Zelante et al. 2012). **Thus, commensals or ubiquitous fungi have evolved a contingency-based system during co-evolution to guarantee their persistence in an inflammatory host environment.**

The main implication of these findings is that, at least in specific clinical settings, it is a heightened inflammatory response that probably compromises a patient's ability to eradicate infection and not an "intrinsic" susceptibility to infection that determines a state of chronic or intractable diseases (Romani and Puccetti 2007). The conceptual principle highlighting a truly bipolar nature of the inflammatory process in infection is best exemplified by the occurrence of severe fungal infections in patients with chronic granulomatous disease (Romani et al. 2008a) or with immune reconstitution syndrome, an entity characterized by localized and systemic inflammatory reactions and worsening disease in opportunistic and non-opportunistic infections that are associated with immunological recovery (Gupta and Singh 2011; Perfect 2012). Additionally, a high incidence of fungal infections and sensitization to *Aspergillus* spp. has been described in the **hyper-IgE syndrome**, in which increased levels of pro-inflammatory gene transcripts have been found (Antachopoulos et al. 2007; Holland et al. 2007). These observations suggest that an inflammatory loop hampering a patient's capacity to counter infection seems to be at work, at least in specific clinical settings. The manipulation of this loop may offer strategies to control or prevent exacerbations of these diseases.

IV. Activation of Antifungal Immunity

A. Dendritic Cells

Immunity to fungi is a dynamic interplay between every arm of the immune system. Innate immune mechanisms are used by the host to respond to a range of fungal pathogens in an acute and conserved fashion (as discussed in chapter "Receptor-Ligand Interactions in Fungal Infections"). The induction of innate immunity shapes the development of the adaptive immune response. As sentinels of the immune system, DCs are responsible for sampling antigenic material in the environment, shaping T cell responses through secretion of cytokines, and priming T cells via antigen presentation (Steinman 2012). Priming of T cells by DCs is mediated by pathogen-associated antigens on major histocompatibility complex class I (MHC-I) or MHC class II (MHC-II) molecules for priming of CD8+ or CD4+ T cells, respectively. After priming of naïve T cells, the response is generally described as Th1, Th2, Th17, or Treg (described in detail in a later section B) based on the pattern of cytokine production. **Thus, the ability to control the fate of the immune response makes DCs both central to balancing antifungal immunity and a prime target for vaccination strategies.**

For years, DC biologists have oscillated between two apparently opposing concepts: functional specialization of DC subsets (division of labor) and plasticity (multitasking). More recently, a third hypothesis is gathering support: crosstalk between functionally distinct DC subsets. This reveals a previously unappreciated hierarchy of organization within the DC system, and provides a conceptual framework for understanding how cooperation between functionally distinct, yet plastic, DC subsets can shape adaptive immunity and immunological memory (Pulendran et al. 2008).

1. The Role of DCs in Antifungal Immunity

DCs are uniquely proficient at decoding the fungus-associated antigens and translating them into qualitatively different adaptive T cell immune responses (Romani et al. 2002; Romani 2011; Roy and Klein 2012; Wuthrich et al. 2012). DCs have the exceptional competence to initiate distinct adaptive antifungal immune responses as a result of cooperation between subsets (Romani and Puccetti 2006; Romani et al. 2008a) and activation of distinct intracellular signaling pathways (Bonifazi et al. 2009, 2010). The **functional plasticity** of DCs is mostly defined by the discriminative recognition of different fungal species and morphotypes by the full range of pattern recognition receptors (PRRs). As a matter of fact, whole−genome transcriptional analysis of fungus-pulsed DCs revealed the presence of a specific transcriptional program governing fungal recognition (Rizzetto and Cavalieri 2010). Among DC subsets, **plasmacytoid (p) DCs have a prominent role in fungal infections.** Not only are pDCs rapidly recruited and activated in response to fungi at mucosal sites, such as the lung (Bonifazi et al. 2010; Ramirez-Ortiz et al. 2011; Carvalho et al. 2012; De Luca et al. 2012) and gut (De Luca et al. 2007; Bonifazi et al. 2009), but infusion of pDCs in bone marrow-transplanted mice has been found to trigger Th1/Treg cell priming, eventually leading to fungal growth restriction, limited inflammatory pathology and, interestingly, transplantation tolerance (Romani et al. 2006). More recently, Toll-like receptor (TLR) 3-dependent recognition of fungal RNA by $CD8^+$ DCs induced potent cytotoxic $CD8^+$ T cell responses to *A. fumigatus*, both in mice and humans (Carvalho et al. 2012). TLR3 deficiency renders mice highly susceptible to aspergillosis, and a *TLR3* mutation affecting the ability of $CD8^+$ DCs to cross-present antigens renders hematopoietic stem cell-transplanted patients more susceptible to invasive aspergillosis.

The activation of distinct signaling pathways in DCs **translates recognition of fungi into distinct inflammatory and adaptive immune responses** (Bonifazi et al. 2009, 2010). The screening of signaling pathways in DCs through a systems biology approach was exploited for the development of therapeutics to attenuate inflammation in experimental fungal infections and diseases. In vivo targeting inflammatory [phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR)] or anti-inflammatory [signal transducer and activator of transcription (STAT3)/IDO1] DC pathways by intranasally delivered small interfering RNA (siRNA) modified resistance and tolerance to infection. Thus, the screening of signaling pathways in DCs through a systems biology approach may be exploited for the development of siRNA therapeutics to attenuate inflammation in respiratory fungal infections and diseases (Bonifazi et al. 2010).

DCs are now being exploited to improve **vaccine efficacy** (Steinman 2008). The potential use of tolerogenic pDCs as negative cellular vaccines to induce experimental transplantation tolerance has been suggested (Turnquist and Thomson 2008).

Over recent years, experimental models have shown that it is possible to exploit the mechanisms that normally maintain immune homeostasis and tolerance to self-antigens to induce tolerance to allo-antigens (Waldmann and Cobbold 2004; Martinic and von Herrath 2006). Like natural tolerance, transplantation tolerance is achieved through control of T cell reactivity by central and peripheral mechanisms of tolerance.

Fungus-pulsed DCs or RNA-transfected DCs acted as potent fungal vaccines in experimental hematopoietic stem cell transplantation (Bozza et al. 2003), a model in which autologous reconstitution of host stem cells is greatly reduced due to the benefit of a long-term, donor-type chimerism in more than 95% of the mice and low incidence of graft rejection. **Protection was associated with myeloid and T cell recovery, the activation of $CD4^+$ Th1 lymphocytes, and the concomitant IL-10-driven Treg cells.** Thus, tolerogenic DCs proved to be pivotal in the generation of some form of dominant regulation that ultimately controlled inflammation, pathogen immunity, and tolerance in transplant recipients eventually leading to prevention of graft-versus-host reaction

and reduction of aspergillosis incidence rates. These results, along with the finding that fungus-pulsed DCs could reverse T cell anergy of patients with fungal diseases, further supports the utility of targeting DCs for antifungal vaccination strategies (Bozza et al. 2004).

2. Metabolic Regulation of DC Plasticity in Response to Fungi

A wealth of evidence indicates that acquisition of an immunogenic or tolerogenic phenotype is a trait of a specific subset or lineage of DCs, but that it is an environmentally acquired feature. In this regard, the tryptophan metabolic pathway pivotally contributes to DC regulation, such that tolerance and Treg induction can be mediated by IDO1-expressing DCs (Orabona et al. 2004) (see below). In response to fungi, IDO1 expression was found to confer tolerogenic properties to DCs (Zelante et al. 2009) such that *C. albicans*-pulsed, IDO1-expressing gut DCs ameliorated experimental colitis (Bonifazi et al. 2009). By subverting the morphotype-specific program of activation of DCs, environmental factors and fungi themselves qualitatively affect DC functioning and Th/Treg cell selection in vivo, ultimately impacting on fungal virulence. **Thus, the current view accommodates the concept of virulence as an important component of fungal fitness within the plasticity of immune responses orchestrated by DCs.** Indeed, impaired DC maturation and function have been associated with disease in patients with CMC (Ryan et al. 2008).

B. T Cell Responses in Fungal Infection

Studies on nonmammalian hosts have provided means to examine the molecular elements of fungal virulence and host innate immunity (Fuchs and Mylonakis 2006; Mylonakis et al. 2007; Peleg et al. 2008). In higher organisms, however, innate sensing mechanisms are capable of distinguishing different fungal morphotypes and are hard-wired to activate distinct adaptive immune responses with protective and nonprotective functions against the different fungal species. It has been suggested that a memory-based immune mechanism may have evolved in vertebrates to **accommodate colonization by symbiotic microbes while retaining the capacity to oppose their infectivity** (McFall-Ngai 2007). This suggests that the adaptive immune system has co-evolved with ubiquitous or commensal fungi, with a price to be paid for this permissiveness. Stimulation of antigen-presenting macrophages, DCs, and, more recently, epithelial cells (ECs) leads to activation and recruitment of lymphocytes and the development of Th cell−specific antifungal responses. There is **extensive plasticity** in T cell responses to fungi (Fig. 6.1). 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. Once committed, T cells express effector functions largely, but not exclusively, through release of cytokines, most notably interferon (IFN)-γ, tumor necrosis factor (TNF)-α and IL-17/IL-22, which 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 (Romani 2011).

1. Th1 Cells

Generation of a dominant Th1 response driven by IL-12 is essentially **required for the expression of protective immunity** to fungi and is compulsory for the design of effective antifungal vaccines (Spellberg et al. 2008). Through the release of the signature cytokine IFN-γ and by helping the production of opsonizing antibodies, induction of Th1 cells is instrumental for the optimal activation of phagocytes at sites of infection. Of interest, Th1 cell-based cross-protection for different fungi has been recently demonstrated to be achievable using a single immunogenic epitope from the *A. fumigatus* cell wall, thus providing attractive opportunities for immunotherapeutic strategies (Stuehler et al. 2011). The failure to deliver

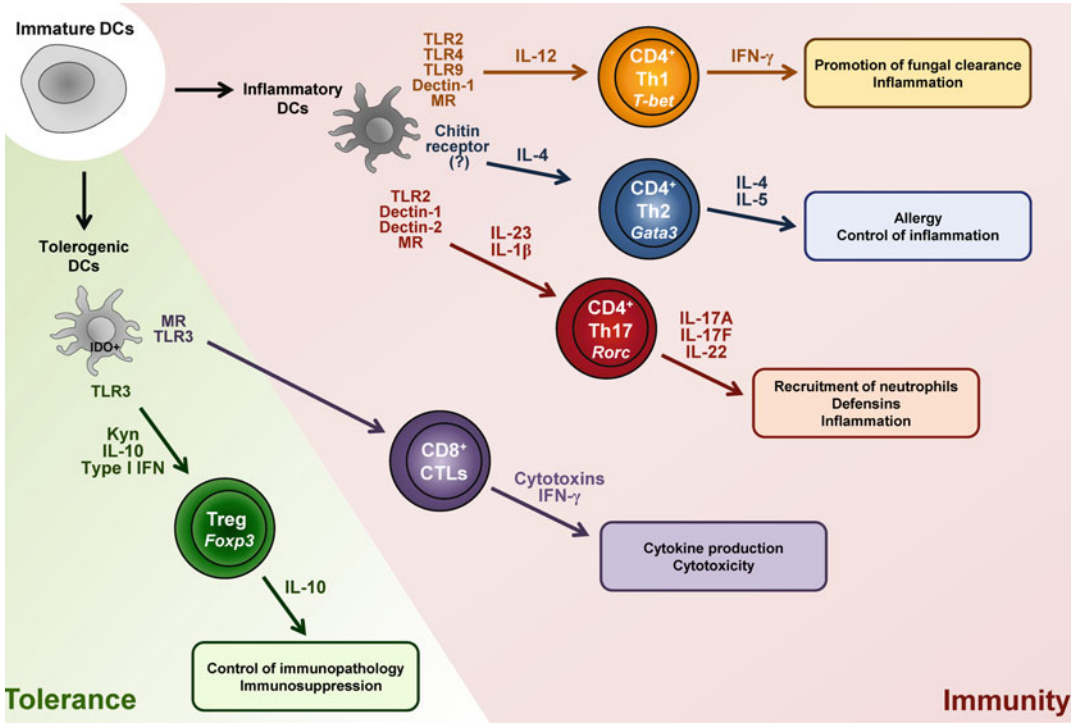


Fig. 6.1 T cell activation in fungal infection. Dendritic cells (DCs) are at the crossroads of antifungal immunity and tolerance because both inflammatory and tolerogenic DCs may arise from their common immature progenitors. Following fungal recognition by specific pattern recognition receptors, inflammatory DCs may trigger the expression of subset-associated transcription factors and differentiation of naïve $CD4^+$ T cells into T helper 1 (Th1), Th2, or Th17 cells, which may induce cytokine secretion and activation of specific antifungal immunity programs. On the other hand, plasmacytoid DCs may activate $CD8^+$ cytotoxic lymphocytes (CTLs) that, by displaying direct fungicidal activity, also contribute to antifungal immunity. In addition, indoleamine 2,3-dioxygenase 1 (IDO1)-competent tolerogenic DCs produce kynurenines (Kyn) and cytokines that contribute to the expansion of interleukin (IL)-10-secreting T regulatory (Treg) cells, crucial for the maintenance of tolerance to the fungus. *MR* mannose receptor, *IFN* interferon, *TLR* Toll-like receptor, *T-bet* T-box expressed in T cells, *Gata3* GATA-binding protein 3, *Rorc* retinoic acid receptor (RAR)-related orphan receptor C

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 (Romani 2011).

As noted above, Th cell skewing is determined by the way that DCs respond to the combination of fungal-derived TLR and C-type lectin receptor (CLR) signaling. Interestingly, dectin-1-mediated signals can alter the Th1 profile of $CD4^+$ T cell responses to fungal infection by decreasing the production of IL-12 and IFN-γ in innate cells and consequent expression of T-box expressed in T cells (T-bet) in *A. fumigatus*-specific $CD4^+$ T cells, thus enabling Th17 differentiation (Rivera et al. 2011).

Further supporting the pivotal role of Th1 cell activation, immunological studies on patients with polar forms of paracoccidioidomycosis demonstrate an association between Th1-biased reactivity and asymptomatic and mild forms of infection, as opposed to the correlation of Th2 cell responses with severe disease and poor prognosis. Thus, the finding that estradiol favors Th1-type immunity may explain why paracoccidioidomycosis is much more frequent in men than in women (Pinzan et al. 2010).

2. Th2 Cells

Progressive fungal infections, especially those acquired through the respiratory tract, are eventually associated with a shift in dominance from Th1 to Th2. IL-4 and IL-13 provide the

most potent proximal signals for commitment of naïve T cells to the Th2 cell lineage. Despite often occurring coincidentally with vigorous Th1 responses, Th2 reactivity results in a net effect of **poor control of the fungal burden** because it induces alternatively activated macrophages that are permissive for intracellular fungal growth and may be impervious to signaling by IFN-γ (Voelz et al. 2009). In addition, Th2 reactivity alters pulmonary physiology such that airway resistance increases, thus compounding the severity of infection and contributing to fungus-associated allergic responses (Jain et al. 2009). Accordingly, attenuating Th2 responses to *A. fumigatus* in cystic fibrosis patients with allergic bronchopulmonary aspergillosis (Kreindler et al. 2010) or limiting IL-4 production in experimental models of histoplasmosis (Szymczak and Deepe 2009) restored antifungal resistance.

For the vast majority of fungi, Th2 responses are associated with **pathogenic allergic responses**. The contradiction is pneumocystosis, in which a Th2 cell-dependent humoral immunity affords some degree of protection (Rapaka et al. 2010). In addition, alternatively activated macrophages driven by IL-13 and amplified by IL-33 displayed enhanced fungicidal activity regardless of the antibody-dependent effect, a finding that further supports a contribution of Th2 cells to fungal clearance (Bhatia et al. 2011).

3. Th17 Cells

Over the past several years, the Th1/Th2 dichotomy has been replaced by the belief that the "fates" for developing $CD4^+$ T cells and associated cytokines are more flexible than formerly anticipated (Zhou et al. 2009). Th17 cells are a separate lineage of effector Th cells contributing to immune pathogenesis previously attributed to the Th1 lineage (Kaufmann and Kuchroo 2009). Th17 cells have an important function in the host response against extracellular pathogens, but they are also associated with the pathogenesis of many autoimmune and allergic disorders. It is now well accepted that Th17 cell activation occurs in fungal infections (Hernandez-Santos and Gaffen 2012), mainly through the spleen tyrosine kinase/caspase recruitment domain-containing protein 9 (Syk/CARD9), myeloid differentiation primary response protein 88 (MyD88), and mannose receptor (MR), signaling pathways in DCs and macrophages leading to the production of unique cytokines such as IL-17, IL-17F, and IL-22. This signaling is inhibited by Raf-1 kinase and TIR domain–containing adapter–inducing interferon-beta (TRIF)/type I IFN pathways, indicating that the molecular pathways defining activation or inhibition of Th17 cells are present downstream of both CLRs and TLRs. Indeed, the central role of Th17 cells in antifungal immunity is supported by studies reporting Mendelian susceptibility to fungal infections of individuals with inborn errors of dectin-1, CARD9, STAT1, STAT3 and, specifically, IL-17 immunity (Ferwerda et al. 2009; Glocker et al. 2009; Puel et al. 2011; van de Veerdonk et al. 2011) (Table 6.1). In experimental fungal infections, however, IL-17-dependent immunity has been reported to be either essential (Huang et al. 2004; Saijo et al. 2007; Conti et al. 2009; Wuthrich et al. 2011) or not (Lin et al. 2009; De Luca et al. 2010b; Hardison et al. 2010). This suggests that the protective or detrimental effects of this pathway may **depend on the stage and site of infection**, probably influenced by environmental stimuli that induce cells to produce cytokines of the IL-17 family, including IL-22 (see below). In this regard, recent evidence has demonstrated that Th17 cells can be segregated into pathogenic or non-pathogenic cells, depending on whether they produce granulocyte macrophage colony stimulating factor (GM-CSF) or IL-10, respectively (Codarri et al. 2011; El-Behi et al. 2011). These Th17 phenotypes are still to be described in the context of fungal diseases (Wuthrich et al. 2012).

Th17 cells are present in the fungus-specific T cell memory repertoire in humans (Acosta-Rodriguez et al. 2007; Bozza et al. 2009) and mediate some (Wuthrich et al. 2011), but not all (De Luca et al. 2012), vaccine-induced protection in mice. Interestingly, as in mice (De Luca et al. 2012), human host defense against *A. fumigatus* relies on Th1 rather than Th17 cell responses (Chai et al. 2010), and CMC patients (with or without autosomal dominant

Table 6.1. Mendelian susceptibility to fungal diseases

Primary immunodeficiency	Molecular/cellular defect	Reported fungal disease(s)	Associated gene	Mode of Mendelian inheritance
AD-HIES	Defective STAT3–dependent signaling (e.g., impaired generation of Th17 cells and signaling by IL-17R, IL-22R, and IL-23R)	CMC, histoplasmosis, cryptococcosis, and coccidioidomycosis	*STAT3*	Autosomal dominant
APECED	Loss of central tolerance, with persisting auto-reactive T cells and autoantibodies to cytokines (e.g., IL-17 and IL-22)	CMC	*AIRE*	Autosomal recessive
CARD9 deficiency	Defective function of signalosomes for dectin-1, dectin-2 and other PRRs	CMC	*CARD9*	Autosomal recessive
DOCK8 deficiency	Impaired T cell activation and survival	CMC	*DOCK8*	Autosomal recessive
Hyper-IgM syndrome (HIGM)	Impaired co-stimulation of T cells and monocytes	Histoplasmosis and cryptococcosis	*CD40L*	X-linked recessive
IL-12/IL-23 deficiency	Impaired development of Th17 cells	CMC	*IL12B*	Autosomal recessive
			IL12RB1	Autosomal recessive
IL-12/IFNG deficiency (also termed MSMD)	Impaired activation of macrophage intracellular killing	Paracoccidioidomycosis and coccidioidomycosis	*IL12RB1*	Autosomal recessive
			IFNGR1	Autosomal dominant
			STAT1	Autosomal dominant
IL-17 deficiency	Impaired development of Th17 cells	CMC	*IL17F*	Autosomal dominant
			IL17RA	Autosomal recessive
SCID	Impaired generation of T cells, with or without concomitant B and NK lymphocytopenia	CMC	*IL2RG*	X-linked
			JAK3	Autosomal recessive
			IL7R	Autosomal recessive
			CD3D	Autosomal recessive
			CD3E	Autosomal recessive
			RAG1	Autosomal recessive
			RAG2	Autosomal recessive
			DCLRE1C	Autosomal recessive
			CD45	Autosomal recessive
STAT1 deficiency	Defective STAT1-dependent signaling (e.g., impaired generation of Th1 and Th17 cells and signaling by IL-12R and IL-23R)	CMC	*STAT1*	Autosomal dominant
STAT1 gain-of-function	Increased STAT1-dependent cellular responses and impaired IL-17 immunity	CMC	*STAT1*	Autosomal dominant
TYK2 deficiency	Impaired receptor signaling (e.g. IL-23R)	CMC	*TYK2*	Autosomal recessive

MSMD syndrome has been described to occur in patients with mutations in *IL12B*, *IFNGR2*, *NEMO*, and *TYK2*, yet no susceptibility to fungal disease was reported. Similarly, HIGM has also been linked with mutations in *CD40*, *AID*, *UNG*, and *NEMO*, but without association with fungal diseases. Modes of inheritance are in regard to those reported with the relevant fungal diseases. *AD-HIES* autosomal dominant hyper-IgE (Job's) syndrome; *APECED* autoimmune polyglandular endocrinopathy candidiasis ectodermal dystrophy; *CMC* chronic mucocutaneous candidiasis; *PRR* pattern recognition receptor; *SCID* severe combination immunodeficiency; *MSMD* Mendelian susceptibility to mycobacterial disease

hyper-IgE syndrome) have defective Th17 and Th1 cell responses (Ryan et al. 2008; van de Veerdonk et al. 2011). This could be explained by the notion that Th17 cells, although found early during the initiation of an immune response, are involved in a broad range of both Th1- and Th2-type responses.

Indeed, a role for Th17 cells in supporting Th1 cell responses has been shown in experimental mucosal candidiasis (Conti et al. 2009; De Luca et al. 2010b). In addition, in experimental aspergillosis, enhanced Th2 cell responses and fungal allergy are observed in the absence of IL-17A receptor signaling (our unpublished observations). Thus, these findings point to an important regulatory function of the Th17 cell pathway in promoting Th1-type and restraining Th2-type immunity.

It is intriguing that Th17 cell responses are dampened by *C. albicans* (Cheng et al. 2010) and that failure to do so eventually results in chronic inflammation and failure to resolve the infection (Zelante et al. 2007; Loures et al. 2009). In this regard, it is fascinating that fungi are able to sense mammalian IL-17 in their surrounding environment and turn on molecular programs that result in enhanced virulence and survival aptitude (Zelante et al. 2012).

The mechanisms that link inflammation to chronic infection may lie in an inability to control inflammation following IL-17A-dependent neutrophil mobilization, thus preventing optimal protection and favoring fungal persistence. Thus, the Th17 cell pathway could be involved in the immunopathogenesis of chronic fungal disease, in which persistent fungal antigens may promote immune deregulation, as demonstrated in patients with autoimmune polyendocrine syndrome type 1 and in the absence of autoimmune regulator (AIRE), in which excessive Th17-type responses to fungi have been observed (Ahlgren et al. 2011).

As noted above, Th17 cells can concomitantly synthesize IL-22, a member of the IL-10 family of cytokines, which has been shown to play a more important role than IL-17 in host defense in the lung and gut (Zenewicz and Flavell 2008). Our recent findings suggest that **the IL-23/IL-22/defensins pathway is crucially involved in the control of fungal growth at mucosal and nonmucosal sites in both candidiasis and aspergillosis**, particularly in conditions of Th1 deficiency. Interestingly, memory IL-22$^+$ CD4$^+$ cells specific for *C. albicans* are present in humans (Liu et al. 2009) and are defective in patients with CMC (Eyerich et al. 2008). Thus, further tweaking the Th17 model, Th17 cells may exert their protective role in fungal infections through IL-22. Indeed, IL-22 has recently been demonstrated to be required for the control of *C. albicans* growth at mucosal sites in the absence of Th1 and Th17 cells (De Luca et al. 2010b). Specifically, IL-22 produced by NKp46$^+$ innate lymphoid cells expressing the aryl hydrocarbon receptor directly targeted intestinal ECs and induced STAT3 phosphorylation and release of S100 calcium binding protein A8 (S100A8) and S100A9, peptides known to have antifungal activity and anti-inflammatory effects. **Thus, in the relative absence of protective Th1/Treg, IL-22$^+$ Th17 cells may fulfill the role of a protective response that exploits primitive effector defense mechanisms of antifungal resistance, as demonstrated also for experimental bacterial diseases** (Aujla et al. 2008). Consistent with this role for IL-22, patients with autosomal dominant hyper-IgE syndrome, owing to dominant-negative mutations of STAT3, have a defective Th17 cell response to *C. albicans* (Milner et al. 2008). Accordingly, *C. albicans*-specific IL-22$^+$ CD4$^+$ memory T cells are present in healthy individuals (Liu et al. 2009) but are lacking in CMC patients (Eyerich et al. 2010), an observation pointing to IL-22 production in the mucosa as a primitive mechanism of resistance against fungi under conditions of limited inflammation. Of interest, dectin-1-mediated production of IL-22 in the lung has also been demonstrated to contribute to early innate immune resistance to *A. fumigatus* (Gessner et al. 2012), although it paradoxically promoted lung inflammation and immunopathology during persistent fungal exposure in an allergy model (Lilly et al. 2012). These seemingly discrepant findings further add to the complexity of IL-22 function in antifungal mucosal immunity and point to the existence of regulatory events leading to its production that depend on the stage and site of infection.

4. Treg Cells

During infection, the immune response must eliminate the fungus while limiting infection-associated costs to host fitness and restoring a homeostatic environment. Treg cells, by means of their anti-inflammatory activity, play a central role in this process. In experimental fungal infections, inflammatory immunity and immune tolerance in the respiratory or gastrointestinal mucosa have been shown to be controlled by the coordinated activation of different Treg cell subsets. Because Treg cell responses may handicap the efficacy of protective immunity, **Treg cell activity decreases host tissue damage but may conversely promote fungal persistence** (Romani and Puccetti 2006) and, eventually, immunosuppression (Ferreira et al. 2010). Some cells with this function, such as $CD4^+$ $Foxp3^+$ natural Tregs (nTregs), exist regardless of the presence of infectious stimuli, whereas others may be induced as a consequence of infection (iTregs) or in conditions of impaired co-stimulatory signaling and in the presence of deactivating cytokines and drugs. This scenario is crucially exemplified in experimental aspergillosis, in which inflammation was controlled at an early stage by nTregs suppressing neutrophils whereas, later tolerogenic iTregs inhibited Th2 cells and prevented fungal allergy (Montagnoli et al. 2006).

As already discussed, a reciprocal relationship has been described between the development of $Foxp3^+$ Treg and effector Th17 cells, so that naïve T cell activation in the presence of innate stimuli redirects iTreg generation to Th17 generation. In this regard, $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells have been recently found to promote IL-17 upregulation and contribute to suppression of mucosal candidiasis in vivo (Pandiyan et al. 2011). Thus, by controlling the quality and magnitude of effector innate and adaptive responses, the spectrum of Treg cell skills may go from protective tolerance and immune homeostasis to dominant effector activities. Furthermore, this suggests that this degree of interaction between fungi and the host immune system determines whether a fungus is perceived as commensal or pathogen, and that this definition may evolve constantly.

5. Indoleamine 2,3-Dioxygenase 1 is a Critical Regulator of Tolerance to Fungi

IDO1 is an IFN-γ-inducible intracellular enzyme that catalyzes the catabolism of tryptophan (Puccetti and Grohmann 2007; Mellor and Munn 2008). The effects of IDO1 activity are tryptophan deficiency, excess tryptophan breakdown products (kynurenines), and consumption of reactive oxygen species. IDO1 and kynurenines serve many roles in fungal infections. A number of studies have established that the proper control of infection and associated inflammatory reactions require IDO1 induction and consequent production of tryptophan metabolites with immunoregulatory activities, contributing to the maintenance of the Treg/Th17 balance (Romani et al. 2008b). As already mentioned, IDO1-expressing DCs are regarded as regulatory DCs specialized in antigen-specific deletional tolerance or induction of $CD4^+$ $CD25^+$ Treg cells. These findings disclose a mutual interaction between DCs and Treg cells for the **preservation of immunological tolerance**. Indeed, IDO1 blockade greatly exacerbated experimental fungal infections and the associated inflammatory pathology, and swept away resistance to re-infection, 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 (Romani and Puccetti 2006). IDO1 expression is paradoxically upregulated in patients with allergy or autoimmune inflammation, a finding pointing to the occurrence of a homeostatic mechanism to halt ongoing inflammation (Grohmann et al. 2007). During experimental fungal allergy, modulation of tryptophan catabolism via the glucocorticoid-induced tumor necrosis factor receptor (GITR) and its ligand, GITRL, inhibited Th2 cell responses and allergy and induced the expression of $Foxp3^+$ Treg cells through IDO1-dependent mechanisms (Grohmann et al. 2007), a finding pointing to the potential

relevance of IDO1 in the anti-inflammatory action of corticosteroids. As already mentioned, a reciprocal antagonistic relationship exists between IDO1 and the Th17 pathway, with IDO1 restraining Th17 responses and IL-17A inhibiting IDO1 (Zelante et al. 2007).

Recent evidence indicates that the **non-hematopoietic compartment** also contributes to tolerance to fungi via IDO1 (Cunha et al. 2010; de Luca et al. 2010a). ECs are key players in tolerance to respiratory pathogens via an IFN-γ–IDO1 axis culminating in the inhibition of Th17 cell responses (Desvignes and Ernst 2009; de Luca et al. 2010a). IDO1 overexpression in airway ECs was found to restrain $CD4^+$ T cell activation to *A. fumigatus*, an activity that was nevertheless dispensable in the presence of IDO1-expressing tolerogenic DCs. However, IDO1 induction in ECs could compensate for the lack of IDO1 on hematopoietic cells (Paveglio et al. 2011). The expression of IDO1 on ECs occurred through the TLR3/TRIF-dependent pathway, a finding consistent with the abundant expression of TLR3 both intracellularly and on the cell surface of ECs. The failure to activate IDO1 probably accounted for the lack of tolerance to the fungus observed in experimental stem cell transplantation in conditions in which either the recipient or the donor (or even more when both) were TRIF- or TLR3-deficient (de Luca et al. 2010a).

Overall, these data shed light on pathways of immune resistance and tolerance to the fungus that probably take place in a hematopoietic stem cell transplantation setting. It appears that protective tolerance to the fungus is achieved through a TLR3/TRIF-dependent pathway activating Th1/Treg cells via IDO1 expressed on both the hematopoietic and non-hematopoietic compartments. In contrast, the MyD88 pathway provides antifungal resistance, i.e., the ability to restrict fungal growth through defensins and, probably, other effector mechanisms (de Luca et al. 2010a). However, the ability of mice to clear the fungus in the relative absence of the MyD88 pathway (Bretz et al. 2008) clearly indicates redundancies and hierarchy in antifungal mechanisms of resistance. Ultimately, the finding that both *C. albicans* (De Luca et al. 2007) and *A. fumigatus* (de Luca et al. 2010a), two major human fungal pathogens, exploit the TRIF/IDO1-dependent pathway at the interface with the mammalian hosts indicates that the **exploitation of tolerance mechanisms is an advantageous option.**

V. Immune Memory and Antifungal Vaccines

A successful vaccination relies on the elicitation of pathogen-specific immune memory that mediates long-term protection from infection or disease. Given the plethora of fungal ligands present at the cell surface, as well as those that become available to immune sensing upon processing of the fungus by phagocytic cells, it is clear that vaccine-induced protection to attenuated fungal strains occurs through distinct PRRs and downstream signaling adapters (Wuthrich et al. 2011; De Luca et al. 2012). **For instance, Th17-induced acquisition of vaccine immunity to live attenuated strains of *B. dermatitidis*, *H. capsulatum*, and *C. posadasii* was found to require MyD88 signaling (Wuthrich et al. 2011), whereas Th1-induced protection to *A. fumigatus* relied on TRIF (De Luca et al. 2012).** Of interest, vaccination with purified *A. fumigatus* antigens was found to be dependent on the MyD88 pathway in the presence of the appropriate adjuvant (Carvalho et al. 2012; De Luca et al. 2012), a finding pointing to the crucial role of adjuvants in promoting T cell differentiation along specific effector pathways. Thus, fungal innate sensing is one critical step in mounting immune responses, eventually defining appropriate effector responses to maximize protection (Levitz and Golenbock 2012).

Although $CD4^+$ Th1 cells have been historically considered the cornerstone of cell-mediated defense against intracellular fungi (Cassone and Casadevall 2012; Iannitti et al. 2012), $CD8^+$ T cells have also earned a place in this category (Cutler et al. 2007). Indeed, in a mouse model of vaccination against blastomycosis, both the numbers and function of protective antifungal memory $CD8^+$ T cells were maintained, even in the absence of $CD4^+$ T cell help (Nanjappa et al. 2012b). Under these circumstances, a distinct lineage of $CD8^+$ T cells able to produce IL-17 (Tc17 cells) has been found to be nonredundant for vaccine immunity to fungal

infection by mediating protection in a **neutrophil-dependent manner** (Nanjappa et al. 2012a).

The persistence of immunological memory and how it relates to vaccination strategies is a question of central importance. Memory T cells are derived from normal T cells that have learned how to overcome a pathogen by "remembering" the strategy used to defeat previous infections (Sallusto et al. 2010). In addition to central memory T cells present in secondary lymphoid organs, which scrutinize the presence of remote pathogens via DCs, effector memory T cells reside in peripheral non-lymphoid tissues such as the skin and mucosa. The latter are heterogeneous in terms of homing receptor expression and effector function and comprise the Th1, Th2, Th17, and Treg cells and cytotoxic T lymphocytes. Memory $CD8^+$ cytotoxic T cells are also induced in fungal infections (Nanjappa et al. 2012b) and exhibit a pleiotropic activity by mediating protection via production of IFN-γ and cytolytic activity against fungus-laden cells or the fungus itself (Carvalho et al. 2012; De Luca et al. 2012). As such, $CD8^+$ T cells, especially if long-lasting, are regarded as ideal candidates for expansion at mucosal surfaces by vaccination strategies.

The **nature of the antifungal vaccine, the route of antigen delivery, and the mode of antigen routing and presentation** are important for determining the success of a fungal vaccine. Indeed, recent evidence has highlighted striking differences in antigen presentation pathways in DCs leading to the activation of $CD4^+$ or $CD8^+$ T cells (De Luca et al. 2012). Memory $CD4^+$ T cells were activated by purified antigens from *A. fumigatus* that were routed to the endosome/lysosome-dependent MHC-II presentation pathway via MyD88 with the involvement of distinct upstream PRRs. En route to lysosomes, purified antigens were also targeted to the mildly acidic stable early endosomal compartment where uptake by MR led to presentation on MHC-I molecules and Th1 polarization. Consistently, mannosylation has been reported to significantly enhance antifungal $CD8^+$ T cell priming (Luong et al. 2007). In contrast to soluble antigens, phagocytosed cells or particulate antigens activated CD8-dependent memory through a pathway relying on TLR3/TRIF signaling (De Luca et al. 2012). Similar to the situation for *C. neoformans* (Wozniak and Levitz 2008), phagocytosed cells were routed to the late endosome/lysosome compartment and also to the rat sarcoma (RAS)-related protein (Rab)14^+ compartment, which is known to limit routing of antigens from early endosomes to the acidic lysosomal environment, thus limiting antigen degradation and favoring cross-presentation (Saveanu et al. 2009). The escape of fungal cells from the endosomal/lysosomal compartment to the $Rab14^+$ compartment occurs through TLR3-dependent autophagy. Accordingly, $CD8^+$ T cell memory to conidia was abrogated when autophagy was defective (e.g., in the absence of TLR3) or under conditions of defective endosomal alkalinization (e.g., in NADPH deficiency) (Savina et al. 2006) (Fig. 6.2). In these conditions, long-lasting antifungal protection and disease control was successfully achieved upon vaccination with purified fungal antigens that activate $CD4^+$ T cells. Thus, $CD8^+$ T cells can provide antifungal memory in $CD4^+$ T cell deficiency and vice versa. These data highlight how understanding memory at a basic level, including information obtained from suitable animal models, may be exploited to personalize vaccination strategies against fungal diseases. Refinement of these approaches could lead to antifungal vaccines and adjuvants tailored to the different target populations, administered either alone or in combination with immunomodulators targeting antigen trafficking and presentation pathways. In this regard, promoting autophagy restores defective $CD8^+$ T cell memory (De Luca et al. 2012), a finding that broadens the role of autophagy in adaptive immunity to include response to vaccines.

VI. Final Remarks

The control of inflammation leading to tolerance, the molecular bases of immune regulation and deregulation, and the way in which commensal but opportunistic fungal pathogens can switch from a "friendly" affinity with the host to

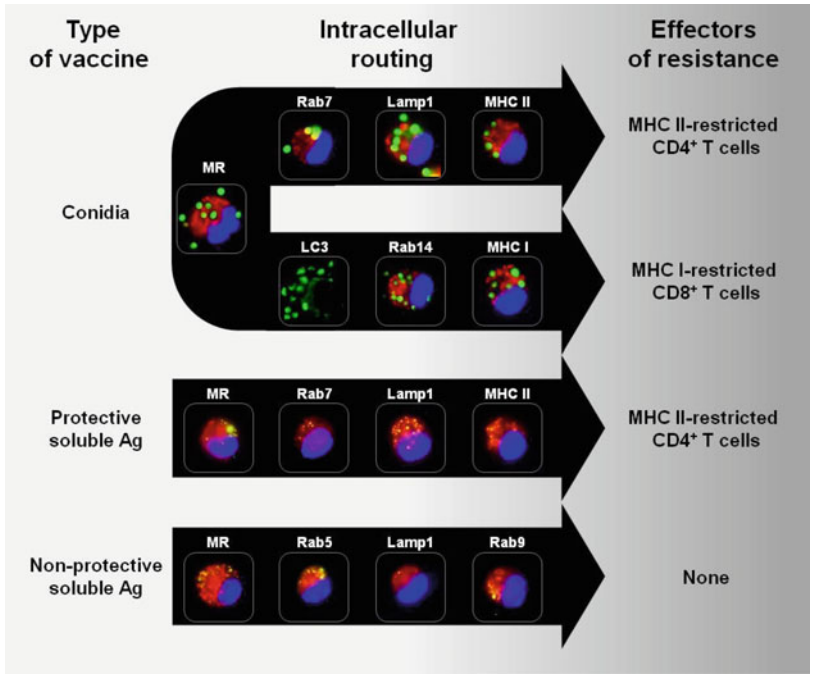


Fig. 6.2. En route to antifungal vaccines. Antifungal vaccines undergo specific antigen presentation pathways leading to the activation of $CD4^+$ and $CD8^+$ T cells. Shown are results from co-localization studies using live conidia of *A. fumigatus* or protective and nonprotective soluble antigens (Ag). Memory, major histocompatibility complex (MHC) class II-restricted $CD4^+$ T cells were activated by live conidia and purified Ags that were routed to the endosome/lysosome-dependent MHC class II presentation pathway. En route to lysosomes, purified fungal Ags also targeted to the mildly acidic stable early endosomal compartment and to MHC class II^+ organelles. In contrast, phagocytosed cells activated MHC class I-restricted $CD8^+$ T cells upon routing to the $Rab14^+$ compartment, known to favor cross-presentation, and to MHC class I^+ organelles. Of interest, the escape of fungal cells from endosomal/lysosomal degradation to the $Rab14^+$ compartment occurred through activation of autophagy. *MR* mannose receptor, *LAMP1* lysosomal-associated membrane protein 1, *LC3* microtubule-associated protein 1A/1B-light chain 3, *Rab14* rat sarcoma (RAS)–related protein (Rab) 14

a pathological relationship by evading or subverting host inflammation, are challenging issues in the field of medical mycology and infection-related immunological disorders. A related question is how and whether the fungal microbiota contributes to the regulation of inflammation in health and disease. By the use of multidisciplinary approaches based on whole-genome immunogenetics, cutting-edge "omics" techniques, advanced bioinformatics, and systems biology applied to immune profiling, it will be possible to challenge existing paradigms in the fields of fungal immunopathology, thereby leading to the discovery of "commensal signatures" for the fungal biota and the development of therapeutic approaches for mucosal and systemic fungal diseases.

Acknowledgments We thank Cristina Massi-Benedetti for editorial assistance. The authors' work was supported by the ERC Advanced Grant FUNMETA (ERC-2011-AdG-293714). Cristina Cunha was supported by the Fundação para a Ciência e Tecnologia, Portugal (contract SFRH/BD/65962/2009). We sincerely apologize to all those colleagues whose important work is not cited because of space considerations.

References

Acosta-Rodriguez EV, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia A, Sallusto F, Napolitani G (2007) Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. Nat Immunol 8:639–646

Ahlgren KM, Moretti S, Lundgren BA, Karlsson I, Ahlin E, Norling A, Hallgren A, Perheentupa J, Gustafs-

son J, Rorsman F, Crewther PE, Ronnelid J, Bensing S, Scott HS, Kampe O, Romani L, Lobell A (2011) Increased IL-17A secretion in response to *Candida albicans* in autoimmune polyendocrine syndrome type 1 and its animal model. Eur J Immunol 41:235–245

Antachopoulos C, Walsh TJ, Roilides E (2007) Fungal infections in primary immunodeficiencies. Eur J Pediatr 166:1099–1117

Ashbee HR (2006) Recent developments in the immunology and biology of *Malassezia* species. FEMS Immunol Med Microbiol 47:14–23

Aujla SJ, Chan YR, Zheng M, Fei M, Askew DJ, Pociask DA, Reinhart TA, Mcallister F, Edeal J, Gaus K, Husain S, Kreindler JL, Dubin PJ, Pilewski JM, Myerburg MM, Mason CA, Iwakura Y, Kolls JK (2008) IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. Nat Med 14:275–281

Bhatia S, Fei M, Yarlagadda M, Qi Z, Akira S, Saijo S, Iwakura Y, Van Rooijen N, Gibson GA, St Croix CM, Ray A, Ray P (2011) Rapid host defense against *Aspergillus fumigatus* involves alveolar macrophages with a predominance of alternatively activated phenotype. PLoS One 6:e15943

Bonifazi P, Zelante T, D'angelo C, De Luca A, Moretti S, Bozza S, Perruccio K, Iannitti RG, Giovannini G, Volpi C, Fallarino F, Puccetti P, Romani L (2009) Balancing inflammation and tolerance in vivo through dendritic cells by the commensal *Candida albicans*. Mucosal Immunol 2:362–374

Bonifazi P, D'angelo C, Zagarella S, Zelante T, Bozza S, De Luca A, Giovannini G, Moretti S, Iannitti RG, Fallarino F, Carvalho A, Cunha C, Bistoni F, Romani L (2010) Intranasally delivered siRNA targeting PI3K/Akt/mTOR inflammatory pathways protects from aspergillosis. Mucosal Immunol 3:193–205

Bozza S, Perruccio K, Montagnoli C, Gaziano R, Bellocchio S, Burchielli E, Nkwanyuo G, Pitzurra L, Velardi A, Romani L (2003) A dendritic cell vaccine against invasive aspergillosis in allogeneic hematopoietic transplantation. Blood 102:3807–3814

Bozza S, Montagnoli C, Gaziano R, Rossi G, Nkwanyuo G, Bellocchio S, Romani L (2004) Dendritic cell-based vaccination against opportunistic fungi. Vaccine 22:857–864

Bozza S, Clavaud C, Giovannini G, Fontaine T, Beauvais A, Sarfati J, D'angelo C, Perruccio K, Bonifazi P, Zagarella S, Moretti S, Bistoni F, Latge JP, Romani L (2009) Immune sensing of *Aspergillus fumigatus* proteins, glycolipids, and polysaccharides and the impact on Th immunity and vaccination. J Immunol 183(4):2407–2414

Bretz C, Gersuk G, Knoblaugh S, Chaudhary N, Randolph-Habecker J, Hackman RC, Staab J, Marr KA (2008) MyD88 signaling contributes to early pulmonary responses to *Aspergillus fumigatus*. Infect Immun 76:952–958

Carvalho A, De Luca A, Bozza S, Cunha C, D'angelo C, Moretti S, Perruccio K, Iannitti RG, Fallarino F, Pierini A, Latge JP, Velardi A, Aversa F, Romani L (2012) TLR3 essentially promotes protective class I-restricted memory CD8(+) T-cell responses to *Aspergillus fumigatus* in hematopoietic transplanted patients. Blood 119:967–977

Cassone A, Casadevall A (2012) Recent progress in vaccines against fungal diseases. Curr Opin Microbiol 15:427–433

Chai LY, Van De Veerdonk F, Marijnissen RJ, Cheng SC, Khoo AL, Hectors M, Lagrou K, Vonk AG, Maertens J, Joosten LA, Kullberg BJ, Netea MG (2010) Anti-Aspergillus human host defence relies on type 1 T helper (Th1), rather than type 17 T helper (Th17), cellular immunity. Immunology 130:46–54

Cheng SC, Van De Veerdonk F, Smeekens S, Joosten LA, Van Der Meer JW, Kullberg BJ, Netea MG (2010) *Candida albicans* dampens host defense by downregulating IL-17 production. J Immunol 185:2450–2457

Cobbold SP, Adams E, Nolan KF, Regateiro FS, Waldmann H (2010) Connecting the mechanisms of T-cell regulation: dendritic cells as the missing link. Immunol Rev 236:203–218

Codarri L, Gyulveszi G, Tosevski V, Hesske L, Fontana A, Magnenat L, Suter T, Becher B (2011) RORgammat drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. Nat Immunol 12:560–567

Conti HR, Shen F, Nayyar N, Stocum E, Sun JN, Lindemann MJ, Ho AW, Hai JH, Yu JJ, Jung JW, Filler SG, Masso-Welch P, Edgerton M, Gaffen SL (2009) Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. J Exp Med 206:299–311

Cooney NM, Klein BS (2008) Fungal adaptation to the mammalian host: it is a new world, after all. Curr Opin Microbiol 11:511–516

Cunha C, Di Ianni M, Bozza S, Giovannini G, Zagarella S, Zelante T, D'angelo C, Pierini A, Pitzurra L, Falzetti F, Carotti A, Perruccio K, Latge JP, Rodrigues F, Velardi A, Aversa F, Romani L, Carvalho A (2010) Dectin-1 Y238X polymorphism associates with susceptibility to invasive aspergillosis in hematopoietic transplantation through impairment of both recipient- and donor-dependent mechanisms of antifungal immunity. Blood 116:5394–5402

Cutler JE, Deepe GS Jr, Klein BS (2007) Advances in combating fungal diseases: vaccines on the threshold. Nat Rev Microbiol 5:13–28

De Luca A, Montagnoli C, Zelante T, Bonifazi P, Bozza S, Moretti S, D'angelo C, Vacca C, Boon L, Bistoni F,

Puccetti P, Fallarino F, Romani L (2007) Functional yet balanced reactivity to *Candida albicans* requires TRIF, MyD88, and IDO-dependent inhibition of Rorc. J Immunol 179:5999–6008

De Luca A, Bozza S, Zelante T, Zagarella S, D'angelo C, Perruccio K, Vacca C, Carvalho A, Cunha C, Aversa F, Romani L (2010a) Non-hematopoietic cells contribute to protective tolerance to *Aspergillus fumigatus* via a TRIF pathway converging on IDO. Cell Mol Immunol 7:459–470

De Luca A, Zelante T, D'angelo C, Zagarella S, Fallarino F, Spreca A, Iannitti RG, Bonifazi P, Renauld JC, Bistoni F, Puccetti P, Romani L (2010b) IL-22 defines a novel immune pathway of antifungal resistance. Mucosal Immunol 3:361–373

De Luca A, Iannitti RG, Bozza S, Beau R, Casagrande A, D'angelo C, Moretti S, Cunha C, Giovannini G, Massi-Benedetti C, Carvalho A, Boon L, Latge JP, Romani L (2012) CD4(+) T cell vaccination overcomes defective cross-presentation of fungal antigens in a mouse model of chronic granulomatous disease. J Clin Invest 122:1816–1831

Desvignes L, Ernst JD (2009) Interferon-gamma-responsive nonhematopoietic cells regulate the immune response to Mycobacterium tuberculosis. Immunity 31:974–985

El-Behi M, Ciric B, Dai H, Yan Y, Cullimore M, Safavi F, Zhang GX, Dittel BN, Rostami A (2011) The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. Nat Immunol 12:568–575

Eyerich K, Foerster S, Rombold S, Seidl HP, Behrendt H, Hofmann H, Ring J, Traidl-Hoffmann C (2008) Patients with chronic mucocutaneous candidiasis exhibit reduced production of Th17-associated cytokines IL-17 and IL-22. J Invest Dermatol 128:2640–2645

Eyerich K, Eyerich S, Hiller J, Behrendt H, Traidl-Hoffmann C (2010) Chronic mucocutaneous candidiasis, from bench to bedside. Eur J Dermatol 20:260–265

Ferreira MC, De Oliveira RT, Da Silva RM, Blotta MH, Mamoni RL (2010) Involvement of regulatory T cells in the immunosuppression characteristic of patients with paracoccidioidomycosis. Infect Immun 78:4392–4401

Ferwerda B, Ferwerda G, Plantinga TS, Willment JA, Van Spriel AB, Venselaar H, Elbers CC, Johnson MD, Cambi A, Huysamen C, Jacobs L, Jansen T, Verheijen K, Masthoff L, Morre SA, Vriend G, Williams DL, Perfect JR, Joosten LA, Wijmenga C, Van Der Meer JW, Adema GJ, Kullberg BJ, Brown GD, Netea MG (2009) Human dectin-1 deficiency and mucocutaneous fungal infections. N Engl J Med 361:1760–1767

Fisher MC, Henk DA, Briggs CJ, Brownstein JS, Madoff LC, Mccraw SL, Gurr SJ (2012) Emerging fungal threats to animal, plant and ecosystem health. Nature 484:186–194

Fuchs BB, Mylonakis E (2006) Using non-mammalian hosts to study fungal virulence and host defense. Curr Opin Microbiol 9:346–351

Gessner MA, Werner JL, Lilly LM, Nelson MP, Metz AE, Dunaway CW, Chan YR, Ouyang W, Brown GD, Weaver CT, Steele C (2012) Dectin-1-dependent interleukin-22 contributes to early innate lung defense against *Aspergillus fumigatus*. Infect Immun 80:410–417

Glocker EO, Hennigs A, Nabavi M, Schaffer AA, Woellner C, Salzer U, Pfeifer D, Veelken H, Warnatz K, Tahami F, Jamal S, Manguiat A, Rezaei N, Amirzargar AA, Plebani A, Hannesschlager N, Gross O, Ruland J, Grimbacher B (2009) A homozygous CARD9 mutation in a family with susceptibility to fungal infections. N Engl J Med 361:1727–1735

Grahl N, Puttikamonkul S, Macdonald JM, Gamcsik MP, Ngo LY, Hohl TM, Cramer RA (2011) In vivo hypoxia and a fungal alcohol dehydrogenase influence the pathogenesis of invasive pulmonary aspergillosis. PLoS Pathog 7:e1002145

Grohmann U, Volpi C, Fallarino F, Bozza S, Bianchi R, Vacca C, Orabona C, Belladonna ML, Ayroldi E, Nocentini G, Boon L, Bistoni F, Fioretti MC, Romani L, Riccardi C, Puccetti P (2007) Reverse signaling through GITR ligand enables dexamethasone to activate IDO in allergy. Nat Med 13:579–586

Gupta AO, Singh N (2011) Immune reconstitution syndrome and fungal infections. Curr Opin Infect Dis 24:527–533

Hardison SE, Brown GD (2012) C-type lectin receptors orchestrate antifungal immunity. Nat Immunol 13:817–822

Hardison SE, Wozniak KL, Kolls JK, Wormley FL Jr (2010) Interleukin-17 is not required for classical macrophage activation in a pulmonary mouse model of *Cryptococcus neoformans* infection. Infect Immun 78:5341–5351

Hernandez-Santos N, Gaffen SL (2012) Th17 cells in immunity to *Candida albicans*. Cell Host Microbe 11:425–435

Holland SM, Deleo FR, Elloumi HZ, Hsu AP, Uzel G, Brodsky N, Freeman AF, Demidowich A, Davis J, Turner ML, Anderson VL, Darnell DN, Welch PA, Kuhns DB, Frucht DM, Malech HL, Gallin JI, Kobayashi SD, Whitney AR, Voyich JM, Musser JM, Woellner C, Schaffer AA, Puck JM, Grimbacher B (2007) STAT3 mutations in the hyper-IgE syndrome. N Engl J Med 357:1608–1619

Huang W, Na L, Fidel PL, Schwarzenberger P (2004) Requirement of interleukin-17A for systemic anti-*Candida albicans* host defense in mice. J Infect Dis 190:624–631

Iannitti RG, Carvalho A, Romani L (2012) From memory to antifungal vaccine design. Trends Immunol 33:467–474

Jain AV, Zhang Y, Fields WB, Mcnamara DA, Choe MY, Chen GH, Erb-Downward J, Osterholzer JJ, Toews GB, Huffnagle GB, Olszewski MA (2009) Th2 but not Th1 immune bias results in altered lung functions in a murine model of pulmonary *Cryptococcus neoformans* infection. Infect Immun 77:5389–5399

Kaufmann SH, Kuchroo VK (2009) Th17 cells. Microbes Infect 11:579–583

Kontoyiannis DP, Marr KA, Park BJ, Alexander BD, Anaissie EJ, Walsh TJ, Ito J, Andes DR, Baddley JW, Brown JM, Brumble LM, Freifeld AG, Hadley S, Herwaldt LA, Kauffman CA, Knapp K, Lyon GM, Morrison VA, Papanicolaou G, Patterson TF, Perl TM, Schuster MG, Walker R, Wannemuehler KA, Wingard JR, Chiller TM, Pappas PG (2010) Prospective surveillance for invasive fungal infections in hematopoietic stem cell transplant recipients, 2001-2006: overview of the Transplant-Associated Infection Surveillance Network (TRANSNET) Database. Clin Infect Dis 50:1091–1100

Kreindler JL, Steele C, Nguyen N, Chan YR, Pilewski JM, Alcorn JF, Vyas YM, Aujla SJ, Finelli P, Blanchard M, Zeigler SF, Logar A, Hartigan E, Kurs-Lasky M, Rockette H, Ray A, Kolls JK (2010) Vitamin D3 attenuates Th2 responses to *Aspergillus fumigatus* mounted by CD4+ T cells from cystic fibrosis patients with allergic bronchopulmonary aspergillosis. J Clin Invest 120:3242–3254

Levitz SM, Golenbock DT (2012) Beyond empiricism: informing vaccine development through innate immunity research. Cell 148:1284–1292

Lilic D (2002) New perspectives on the immunology of chronic mucocutaneous candidiasis. Curr Opin Infect Dis 15:143–147

Lilly LM, Gessner MA, Dunaway CW, Metz AE, Schwiebert L, Weaver CT, Brown GD, Steele C (2012) The beta-glucan receptor dectin-1 promotes lung immunopathology during fungal allergy via IL-22. J Immunol 189:3653–3660

Lin L, Ibrahim AS, Xu X, Farber JM, Avanesian V, Baquir B, Fu Y, French SW, Edwards JE Jr, Spellberg B (2009) Th1-Th17 cells mediate protective adaptive immunity against Staphylococcus aureus and *Candida albicans* infection in mice. PLoS Pathog 5:e1000703

Liu Y, Yang B, Zhou M, Li L, Zhou H, Zhang J, Chen H, Wu C (2009) Memory IL-22-producing CD4+ T cells specific for *Candida albicans* are present in humans. Eur J Immunol 39:1472–1479

Liu L, Okada S, Kong XF, Kreins AY, Cypowyj S, Abhyankar A, Toubiana J, Itan Y, Audry M, Nitschke P, Masson C, Toth B, Flatot J, Migaud M, Chrabieh M, Kochetkov T, Bolze A, Borghesi A, Toulon A, Hiller J, Eyerich S, Eyerich K, Gulacsy V, Chernyshova L, Chernyshov V, Bondarenko A, Grimaldo RM, Blancas-Galicia L, Beas IM, Roesler J, Magdorf K, Engelhard D, Thumerelle C, Burgel PR, Hoernes M, Drexel B, Seger R, Kusuma T, Jansson AF, Sawalle-Belohradsky J, Belohradsky B, Jouanguy E, Bustamante J, Bue M, Karin N, Wildbaum G, Bodemer C, Lortholary O, Fischer A, Blanche S, Al-Muhsen S, Reichenbach J, Kobayashi M, Rosales FE, Lozano CT, Kilic SS, Oleastro M, Etzioni A, Traidl-Hoffmann C, Renner ED, Abel L, Picard C, Marodi L, Boisson-Dupuis S, Puel A, Casanova JL (2011) Gain-of-function human STAT1 mutations impair IL-17 immunity and underlie chronic mucocutaneous candidiasis. J Exp Med 208:1635–1648

Loures FV, Pina A, Felonato M, Calich VL (2009) TLR2 is a negative regulator of Th17 cells and tissue pathology in a pulmonary model of fungal infection. J Immunol 183:1279–1290

Luong M, Lam JS, Chen J, Levitz SM (2007) Effects of fungal N- and O-linked mannosylation on the immunogenicity of model vaccines. Vaccine 25:4340–4344

Magditch DA, Liu TB, Xue C, Idnurm A (2012) DNA mutations mediate microevolution between host-adapted forms of the pathogenic fungus *Cryptococcus neoformans*. PLoS Pathog 8:e1002936

Martinic MM, Von Herrath MG (2006) Control of graft-versus-host disease by regulatory T cells: which level of antigen specificity? Eur J Immunol 36:2299–2303

Mcfall-Ngai M (2007) Adaptive immunity: care for the community. Nature 445:153

Medzhitov R, Schneider DS, Soares MP (2012) Disease tolerance as a defense strategy. Science 335:936–941

Mellor AL, Munn DH (2008) Creating immune privilege: active local suppression that benefits friends, but protects foes. Nat Rev Immunol 8:74–80

Milner JD, Brenchley JM, Laurence A, Freeman AF, Hill BJ, Elias KM, Kanno Y, Spalding C, Elloumi HZ, Paulson ML, Davis J, Hsu A, Asher AI, O'shea J, Holland SM, Paul WE, Douek DC (2008) Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. Nature 452:773–776

Montagnoli C, Fallarino F, Gaziano R, Bozza S, Bellocchio S, Zelante T, Kurup WP, Pitzurra L, Puccetti P, Romani L (2006) Immunity and tolerance to *Aspergillus* involve functionally distinct regulatory T cells and tryptophan catabolism. J Immunol 176:1712–1723

Moraes-Vasconcelos D, Grumach AS, Yamaguti A, Andrade ME, Fieschi C, De Beaucoudrey L, Casanova JL, Duarte AJ (2005) *Paracoccidioides brasiliensis* disseminated disease in a patient with inherited deficiency in the beta1 subunit of the interleukin (IL)-12/IL-23 receptor. Clin Infect Dis 41:e31–e37

Mylonakis E, Casadevall A, Ausubel FM (2007) Exploiting amoeboid and non-vertebrate animal model systems to study the virulence of human pathogenic fungi. PLoS Pathog 3:e101

Nanjappa SG, Heninger E, Wuthrich M, Gasper DJ, Klein BS (2012a) Tc17 cells mediate vaccine immunity against lethal fungal pneumonia in immune deficient hosts lacking CD4+ T cells. PLoS Pathog 8:e1002771

Nanjappa SG, Heninger E, Wuthrich M, Sullivan T, Klein B (2012b) Protective antifungal memory CD8(+) T cells are maintained in the absence of CD4(+) T cell help and cognate antigen in mice. J Clin Invest 122:987–999

Odds FC, Jacobsen MD (2008) Multilocus sequence typing of pathogenic *Candida* species. Eukaryot Cell 7:1075–1084

Orabona C, Grohmann U, Belladonna ML, Fallarino F, Vacca C, Bianchi R, Bozza S, Volpi C, Salomon BL, Fioretti MC, Romani L, Puccetti P (2004) CD28 induces immunostimulatory signals in dendritic cells via CD80 and CD86. Nat Immunol 5:1134–1142

Pandiyan P, Conti HR, Zheng L, Peterson AC, Mathern DR, Hernandez-Santos N, Edgerton M, Gaffen SL, Lenardo MJ (2011) CD4(+)CD25(+)Foxp3(+) regulatory T cells promote Th17 cells in vitro and enhance host resistance in mouse *Candida albicans* Th17 cell infection model. Immunity 34:422–434

Pappas PG, Alexander BD, Andes DR, Hadley S, Kauffman CA, Freifeld A, Anaissie EJ, Brumble LM, Herwaldt L, Ito J, Kontoyiannis DP, Lyon GM, Marr KA, Morrison VA, Park BJ, Patterson TF, Perl TM, Oster RA, Schuster MG, Walker R, Walsh TJ, Wannemuehler KA, Chiller TM (2010) Invasive fungal infections among organ transplant recipients: results of the Transplant-Associated Infection Surveillance Network (TRANSNET). Clin Infect Dis 50:1101–1111

Paveglio SA, Allard J, Foster Hodgkins SR, Ather JL, Bevelander M, Campbell JM, Whittaker Leclair LA, Mccarthy SM, Van Der Vliet A, Suratt BT, Boyson JE, Uematsu S, Akira S, Poynter ME (2011) Airway epithelial indoleamine 2,3-dioxygenase inhibits CD4+ T cells during *Aspergillus fumigatus* antigen exposure. Am J Respir Cell Mol Biol 44:11–23

Peleg AY, Tampakakis E, Fuchs BB, Eliopoulos GM, Moellering RC Jr, Mylonakis E (2008) Prokaryote-eukaryote interactions identified by using caenorhabditis elegans. Proc Natl Acad Sci USA 105:14585–14590

Perfect JR (2012) The impact of the host on fungal infections. Am J Med 125:S39–S51

Pinzan CF, Ruas LP, Casabona-Fortunato AS, Carvalho FC, Roque-Barreira MC (2010) Immunological basis for the gender differences in murine *Paracoccidioides brasiliensis* infection. PLoS One 5: e10757

Puccetti P, Grohmann U (2007) IDO and regulatory T cells: a role for reverse signalling and non-canonical NF-kappaB activation. Nat Rev Immunol 7:817–823

Puel A, Cypowyj S, Bustamante J, Wright JF, Liu L, Lim HK, Migaud M, Israel L, Chrabieh M, Audry M, Gumbleton M, Toulon A, Bodemer C, El-Baghdadi J, Whitters M, Paradis T, Brooks J, Collins M, Wolfman NM, Al-Muhsen S, Galicchio M, Abel L, Picard C, Casanova JL (2011) Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. Science 332:65–68

Pulendran B, Tang H, Denning TL (2008) Division of labor, plasticity, and crosstalk between dendritic cell subsets. Curr Opin Immunol 20:61–67

Ramirez-Ortiz ZG, Lee CK, Wang JP, Boon L, Specht CA, Levitz SM (2011) A nonredundant role for plasmacytoid dendritic cells in host defense against the human fungal pathogen *Aspergillus fumigatus*. Cell Host Microbe 9:415–424

Rapaka RR, Ricks DM, Alcorn JF, Chen K, Khader SA, Zheng M, Plevy S, Bengten E, Kolls JK (2010) Conserved natural IgM antibodies mediate innate and adaptive immunity against the opportunistic fungus *Pneumocystis* murina. J Exp Med 207:2907–2919

Rappleye CA, Goldman WE (2008) Fungal stealth technology. Trends Immunol 29:18–24

Richie DL, Hartl L, Aimanianda V, Winters MS, Fuller KK, Miley MD, White S, Mccarthy JW, Latge JP, Feldmesser M, Rhodes JC, Askew DS (2009) A role for the unfolded protein response (UPR) in virulence and antifungal susceptibility in *Aspergillus fumigatus*. PLoS Pathog 5:e1000258

Rivera A, Hohl TM, Collins N, Leiner I, Gallegos A, Saijo S, Coward JW, Iwakura Y, Pamer EG (2011) Dectin-1 diversifies *Aspergillus fumigatus*-specific T cell responses by inhibiting T helper type 1 CD4 T cell differentiation. J Exp Med 208:369–381

Rizzetto L, Cavalieri D (2010) A systems biology approach to the mutual interaction between yeast and the immune system. Immunobiology 215:762–769

Romani L (2011) Immunity to fungal infections. Nat Rev Immunol 11:275–288

Romani L, Puccetti P (2006) Protective tolerance to fungi: the role of IL-10 and tryptophan catabolism. Trends Microbiol 14:183–189

Romani L, Puccetti P (2007) Controlling pathogenic inflammation to fungi. Expert Rev Anti Infect Ther 5:1007–1017

Romani L, Puccetti P (2008) Immune regulation and tolerance to fungi in the lungs and skin. Chem Immunol Allergy 94:124–137

Romani L, Bistoni F, Puccetti P (2002) Fungi, dendritic cells and receptors: a host perspective of fungal virulence. Trends Microbiol 10:508–514

Romani L, Bistoni F, Perruccio K, Montagnoli C, Gaziano R, Bozza S, Bonifazi P, Bistoni G, Rasi G, Velardi A, Fallarino F, Garaci E, Puccetti P (2006) Thymosin alpha1 activates dendritic cell tryptophan catabolism and establishes a regulatory environment for balance of inflammation and tolerance. Blood 108:2265–2274

Romani L, Fallarino F, De Luca A, Montagnoli C, D'angelo C, Zelante T, Vacca C, Bistoni F, Fioretti MC, Grohmann U, Segal BH, Puccetti P (2008a) Defective tryptophan catabolism underlies inflammation in mouse chronic granulomatous disease. Nature 451:211–215

Romani L, Zelante T, De Luca A, Fallarino F, Puccetti P (2008b) IL-17 and therapeutic kynurenines in pathogenic inflammation to fungi. J Immunol 180:5157–5162

Roy RM, Klein BS (2012) Dendritic cells in antifungal immunity and vaccine design. Cell Host Microbe 11:436–446

Ryan KR, Hong M, Arkwright PD, Gennery AR, Costigan C, Dominguez M, Denning D, Mcconnell V, Cant AJ, Abinun M, Spickett GP, Lilic D (2008) Impaired dendritic cell maturation and cytokine production in patients with chronic mucocutanous candidiasis with or without APECED. Clin Exp Immunol 154:406–414

Saijo S, Fujikado N, Furuta T, Chung SH, Kotaki H, Seki K, Sudo K, Akira S, Adachi Y, Ohno N, Kinjo T, Nakamura K, Kawakami K, Iwakura Y (2007) Dectin-1 is required for host defense against *Pneumocystis* carinii but not against *Candida albicans*. Nat Immunol 8:39–46

Sallusto F, Lanzavecchia A, Araki K, Ahmed R (2010) From vaccines to memory and back. Immunity 33:451–463

Saraiva M, O'garra A (2010) The regulation of IL-10 production by immune cells. Nat Rev Immunol 10:170–181

Saveanu L, Carroll O, Weimershaus M, Guermonprez P, Firat E, Lindo V, Greer F, Davoust J, Kratzer R, Keller SR, Niedermann G, Van Endert P (2009) IRAP identifies an endosomal compartment required for MHC class I cross-presentation. Science 325:213–217

Savina A, Jancic C, Hugues S, Guermonprez P, Vargas P, Moura IC, Lennon-Dumenil AM, Seabra MC, Raposo G, Amigorena S (2006) NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells. Cell 126:205–218

Spellberg B, Ibrahim AS, Lin L, Avanesian V, Fu Y, Lipke P, Otoo H, Ho T, Edwards JE Jr (2008) Antibody titer threshold predicts anti-candidal vaccine efficacy even though the mechanism of protection is induction of cell-mediated immunity. J Infect Dis 197:967–971

Steinman RM (2008) Dendritic cells and vaccines. Proc (Bayl Univ Med Cent) 21(1):3–8. PMID 18209746

Steinman RM (2012) Decisions about dendritic cells: past, present, and future. Annu Rev Immunol 30:1–22

Stuehler C, Khanna N, Bozza S, Zelante T, Moretti S, Kruhm M, Lurati S, Conrad B, Worschech E, Stevanovic S, Krappmann S, Einsele H, Latge JP, Loeffler J, Romani L, Topp MS (2011) Cross-protective TH1 immunity against *Aspergillus fumigatus* and *Candida albicans*. Blood 117:5881–5891

Szymczak WA, Deepe GS Jr (2009) The CCL7-CCL2-CCR2 axis regulates IL-4 production in lungs and fungal immunity. J Immunol 183:1964–1974

Turnquist HR, Thomson AW (2008) Taming the lions: manipulating dendritic cells for use as negative cellular vaccines in organ transplantation. Curr Opin Organ Transplant 13:350–357

Van De Veerdonk FL, Plantinga TS, Hoischen A, Smeekens SP, Joosten LA, Gilissen C, Arts P, Rosentul DC, Carmichael AJ, Smits-Van Der Graaf CA, Kullberg BJ, Van Der Meer JW, Lilic D, Veltman JA, Netea MG (2011) STAT1 mutations in autosomal dominant chronic mucocutaneous candidiasis. N Engl J Med 365:54–61

Voelz K, Lammas DA, May RC (2009) Cytokine signaling regulates the outcome of intracellular macrophage parasitism by *Cryptococcus neoformans*. Infect Immun 77:3450–3457

Waldmann H, Cobbold S (2004) Exploiting tolerance processes in transplantation. Science 305:209–212

Wozniak KL, Levitz SM (2008) *Cryptococcus neoformans* enters the endolysosomal pathway of dendritic cells and is killed by lysosomal components. Infect Immun 76:4764–4771

Wuthrich M, Gern B, Hung CY, Ersland K, Rocco N, Pick-Jacobs J, Galles K, Filutowicz H, Warner T, Evans M, Cole G, Klein B (2011) Vaccine-induced protection against 3 systemic mycoses endemic to North America requires Th17 cells in mice. J Clin Invest 121:554–568

Wuthrich M, Deepe GS Jr, Klein B (2012) Adaptive immunity to fungi. Annu Rev Immunol 30:115–148

Zelante T, De Luca A, Bonifazi P, Montagnoli C, Bozza S, Moretti S, Belladonna ML, Vacca C, Conte C, Mosci P, Bistoni F, Puccetti P, Kastelein RA, Kopf M, Romani L (2007) IL-23 and the Th17 pathway promote inflammation and impair antifungal immune resistance. Eur J Immunol 37:2695–2706

Zelante T, Fallarino F, Bistoni F, Puccetti P, Romani L (2009) Indoleamine 2,3-dioxygenase in infection: the paradox of an evasive strategy that benefits the host. Microbes Infect 11:133–141

Zelante T, Iannitti RG, De Luca A, Arroyo J, Blanco N, Servillo G, Sanglard D, Reichard U, Palmer GE, Latge JP, Puccetti P, Romani L (2012) Sensing of mammalian IL-17A regulates fungal adaptation and virulence. Nat Commun 3:683

Zeller S, Glaser AG, Vilhelmsson M, Rhyner C, Crameri R (2008) Immunoglobulin-E-mediated reactivity to self antigens: a controversial issue. Int Arch Allergy Immunol 145:87–93

Zenewicz LA, Flavell RA (2008) IL-22 and inflammation: leukin' through a glass onion. Eur J Immunol 38:3265–3268

Zhou L, Chong MM, Littman DR (2009) Plasticity of CD4+ T cell lineage differentiation. Immunity 30:646–655

7 Molecular Mechanisms of *Histoplasma* Pathogenesis

Chad A. Rappleye[1]

CONTENTS

I. Introduction

Histoplasma capsulatum is a primary fungal pathogen that causes respiratory and systemic disease. **The fungus is endemic to regions of North, Central, and South America as well as parts of Africa.** In the USA, *Histoplasma* causes more hospitalizations than all other endemic fungi (Chu et al. 2006; Baddley et al. 2011). Serology studies indicate that up to 80% of individuals in endemic areas of the USA have been infected (Edwards et al. 1969), yielding estimates of 200,000 infections annually (Ajello 1971). The severity of histoplasmosis is determined by the dose and by the immunological state of the host, with the majority of infections being self-limiting upon activation of cell-mediated immunity.

Histoplasmosis is initially a respiratory infection that results from inhalation of *Histoplasma* conidia. The infectious conidia are produced by and released from the environmental (mycelial) form of the fungus. Within the mammalian lung, elevated temperatures cause conidia to differentiate into the pathogenic yeast form. *Histoplasma* is thus a thermally dimorphic fungus with exclusively separate mycelial and yeast forms. Beyond fungal morphology, ***Histoplasma*'s dimorphism corresponds to alternate lifestyles, with the mycelia deriving nourishment from decaying organic matter but the yeast parasitizing host cells. *Histoplasma*'s dimorphism also reflects substantial transcriptional changes, which include expression of genes necessary for pathogenesis.**

Most fungi that cause human disease are opportunistic pathogens, their diseases being restricted to hosts with defects in innate and/or adaptive immunity. In contrast, ***Histoplasma* infections are not restricted to immunocompromised or immunosuppressed hosts.** Of clinical cases, less than half are associated with other comorbidities or known immunocompromising conditions (Chu et al. 2006; Baddley et al. 2011). The innate immune system is entirely unable to control *Histoplasma* infections until activation by cells of the adaptive immune system. **In the absence of cell-mediated immunity, *Histoplasma* infections nearly always progress to life-threatening, progressive disseminated histoplasmosis.** Even with T cell activation, *Histoplasma* infections may not be completely cleared and latent organisms can persist as a

[1]Department of Microbiology, Department of Microbial Infection and Immunity, Ohio State University, 43210 Columbus, OH, USA; e-mail: rappleye.1@osu.edu

Human Fungal Pathogens, 2nd Edition
The Mycota XII
O. Kurzai (Ed.)

reservoir for reactivation histoplasmosis (Jain et al. 2006; Allen and Deepe 2006). This greater virulence of *Histoplasma* compared to opportunistic fungi indicates that *Histoplasma* must produce specific virulence factors that enable it to defeat immune defenses. Within the mammalian host, ***Histoplasma* is almost exclusively an intracellular pathogen with yeasts predominantly found in host macrophages.** As will be discussed in molecular detail, *Histoplasma*'s ability to reside within these immune cells that normally eliminate invading fungi is a function of (1) avoidance of detection by the host and (2) negation of host antimicrobial defenses.

Dissection of the molecular mechanisms that promote *Histoplasma* pathogenesis relies upon identification and characterization of the underlying virulence factors. Functional genetic studies have only recently been possible with molecular advances to eliminate or deplete specific gene functions. These include the development of a gene knockout strategy for a fungus with vanishingly low rates of homologous recombination (Sebghati et al. 2000), methodologies to deplete gene functions by RNA interference (RNAi) (Rappleye et al. 2004), and insertional mutagenesis techniques using Agrobacterium-mediated transformation (Sullivan et al. 2002; Youseff et al. 2009; Youseff and Rappleye 2012). In addition, modern platform technologies such as gene expression profiling (Hwang et al. 2003, 2012; Nittler et al. 2005) and proteomics (Albuquerque et al. 2008; Winters et al. 2008; Holbrook et al. 2011) have greatly enhanced identification and selection of candidate molecules for understanding *Histoplasma* virulence.

The dimorphism of *Histoplasma*, and the built-in ability to correlate expression patterns specifically with the pathogenic phase, has helped propel virulence factor identification. Logically, factors uniquely produced by pathogenic-phase cells have been considered prime candidates that specifically increase the fitness of this phase. Because **differentiation of *Histoplasma* into the yeast phase is required for establishment of disease** (Medoff et al. 1986; Nemecek et al. 2006), yeast-phase factors are probably produced to facilitate *Histoplasma* pathogenesis. Although not virulence factors per se, a number of regulators of the transition to the yeast phase in response to mammalian body temperature have been identified. Transition to the yeast phase requires a histidine signaling kinase (Drk1) (Nemecek et al. 2006). In addition, the Ryp1 transcriptional regulator, which is related to the *Candida* Wor1 differentiation factor, binds to DNA upstream of yeast-phase genes and activates expression of approximately 740 genes of the yeast-phase regulon (Nguyen and Sil 2008). Two additional proteins, Ryp2 and Ryp3, are related to Velvet A-type regulators of filamentous fungi and are required for Ryp1 accumulation and for yeast-phase differentiation (Webster and Sil 2008). How the thermal stimulus is transduced into signals that involve these pathways is still unknown. Nevertheless, these yeast-phase-regulating factors contribute to pathogenesis indirectly by activating expression of many of the virulence mechanisms discussed below. Indeed, most virulence factors identified to date are characterized by yeast-phase-specific expression, and their functions tailored to ensuring *Histoplasma* survival as the yeasts interact with a fully functional host immune system.

II. Avoidance of Host Defenses

A. Adhesion to and Entry into Phagocytes

Infection of phagocytes involves intimate association of *Histoplasma* cells with the host cell surface to promote internalization, yet survival requires yeasts to minimize activation of phagocyte defense mechanisms (Fig. 7.1). Initial adherence of *Histoplasma* to macrophages is mediated by binding to macrophage heteromeric transmembrane β-integrins, chiefly the CD18-family complement receptors (CR): LFA-1 (CD11a+CD18), CR3/Mac-1 (CD11b+CD18), or CR4 (CD11c+CD18). Antibodies that block complement receptors substantially reduce attachment and phagocytosis of *Histoplasma* yeasts and conidia by macrophages, although blocking individual alpha subunits causes less impairment than blocking CD18 due to expression of multiple complement receptors on macrophages (Bullock and Wright 1987; Newman et al. 1990; Lin et al. 2010). Although *Histoplasma's* association with

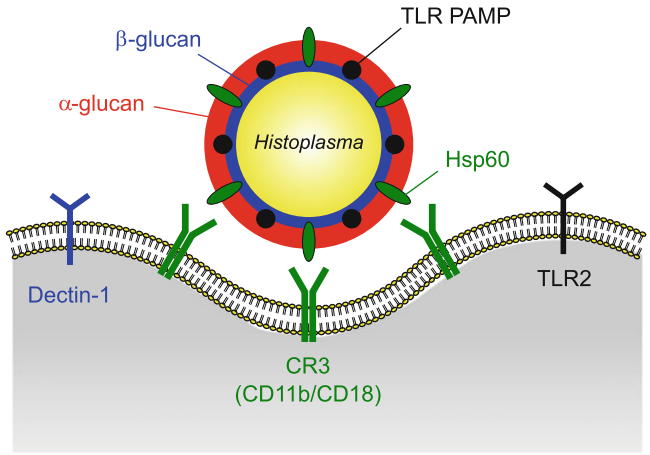


Fig. 7.1. Molecular mechanisms of detection avoidance during interaction of *Histoplasma* yeasts with macrophages. Adherence and phagocytosis of yeasts into macrophages is mediated by cell-wall-localized Hsp60 binding to CD18-family receptors (e.g., CR3) in the macrophage plasma membrane. At the same time, *Histoplasma* yeasts avoid detection by the macrophage β-glucan receptor Dectin-1 by concealing fungal β-glucans beneath an α-glucan polysaccharide layer in the yeast cell wall. In addition, macrophage TLR2 receptors do not detect undefined TLR2 PAMPs present in the yeast cell wall

macrophages is mediated by complement receptors, binding is not dependent on complement opsonization of yeasts (Bullock and Wright 1987). **The cognate adhesin protein on yeasts that binds to CR3 is heat-shock protein 60 (Hsp60)** (Long et al. 2003). How Hsp60, a normally intracellular stress-response protein, becomes localized to the extracellular surface of yeasts is unknown. Consistent with its extracellular localization, antibodies to Hsp60 can opsonize yeast and promote Fc-dependent phagocytosis (Guimarães et al. 2009). Furthermore, vaccination with Hsp60 is protective to mice (Gomez et al. 1995; Deepe et al. 1996). In contrast to macrophages, attachment of *Histoplasma* yeasts to dendritic cells is facilitated by yeast cyclophilin A binding to dendritic cell-expressed VLA-5 receptors (Gildea et al. 2001; Gomez et al. 2008). No adhesin-receptor pairing has been determined yet for the interaction of yeasts with polymorphonuclear neutrophils (PMNs). Complement receptors seem unlikely candidates because surface expression of complement receptors on PMNs is minimal until PMNs are stimulated (Sengeløv et al. 1993; Videm and Strand 2004).

Binding to complement receptors for adherence also triggers internalization of yeasts into macrophages. Intracellular residence in macrophages enhances the virulence of *Histoplasma* by protecting yeast cells from pulmonary collectins, specifically surfactant proteins A (SP-A) and D (SP-D) (McCormack et al. 2003). Recent work has shown that SP-A and SP-D have direct fungicidal activity on *Histoplasma* by permeabilizing the yeast cells. SP-A knockout mice have increased susceptibility to *Histoplasma* infection in vivo (McCormack et al. 2003). This suggests that **phagocytic uptake is not only tolerated by *Histoplasma* but important for full virulence because it provides a shelter from soluble host defense molecules**. Unlike Fc-mediated phagocytosis of antibody-opsonized particles, complement receptor-mediated phagocytosis is generally noninflammatory unless accompanied by other stimulating signals (Aderem 2003).

B. Concealment of Pro-inflammatory Fungal Patterns

To reduce potential pro-inflammatory host responses, *Histoplasma* yeasts minimize cell wall β-glucan detection by concealing it beneath a layer of α-glucan. Macrophage-expressed

Dectin-1 is a critical pattern recognition receptor (PRR) for detection of fungal cell wall β-glucan and stimulates the antifungal responses of phagocytes (Goodridge et al. 2009; Drummond and Brown 2011). Dectin-1 detection of *Histoplasma* yeasts is necessary for pro-inflammatory cytokine production (Rappleye et al. 2007; Lin et al. 2010). However, most *Histoplasma* species synthesize α-linked glucan (α-glucan) in addition to β-glucans, and these two polysaccharides are organized in the yeast cell wall such that the β-glucans are concealed beneath the α-glucan layer (Rappleye et al. 2007). Functionally, **production of the yeast-specific α-glucan polysaccharide effectively masks the normally immunostimulatory cell wall β-glucans** (Fig. 7.1). Yeast cells unable to synthesize α-glucan are readily detected by Dectin-1 and these yeasts are severely attenuated in vivo (Rappleye et al. 2004, 2007).

Synthesis of α-glucan requires the function of an α-(1,3)-glucan synthase (Ags1) as well as an α-(1,4)-amylase (Amy1) (Rappleye et al. 2004; Marion et al. 2006). One species of *Histoplasma* (the North American type 2; NAm 2) lacks detectable α-glucan yet remains virulent. This species appears to express an alternate means of preventing β-glucan detection through an unknown mechanism (Edwards et al. 2011a).

Thus, the α-glucan polysaccharide produced by most species of *Histoplasma* facilitates stealthy entry of *Histoplasma* yeast into macrophages, thereby enhancing yeast survival.

In addition to preventing Dectin-1 detection, ***Histoplasma* yeast cells do not trigger Toll-like receptors (TLRs) expressed on the macrophage surface.** The most relevant TLRs that recognize fungal pathogen-associated molecular patterns (PAMPs) are TLR2 and TLR4. One possibility is that *Histoplasma* yeasts lack TLR ligands altogether. Consistent with this possibility are in vitro studies in which TLR2 and TLR4 do not play a role in macrophage interactions with *Histoplasma* yeasts (Lin et al. 2010). Similarly, TLR2 does not contribute to the interferon (IFN)-β response of macrophages to *Histoplasma* conidia (Inglis et al. 2010). On the other hand, biochemical fractions of *Histoplasma* yeast cell walls can stimulate lipid body formation by macrophages (which is correlated with leukotriene production), and this is dependent on TLR2, indicating that cell wall fractions contain PAMP(s) recognized by TLR2 (Sorgi et al. 2009). Together, these data suggest that *Histoplasma* yeast have TLR ligands but that they are hidden from immune cells in intact, wild-type yeasts in much the same way that cell wall β-glucans are veiled (Fig. 7.1). All of these studies used cultured phagocytes so the role of TLRs in vivo remains undetermined. It is unknown whether α-glucan might also be the cell wall factor that masks the TLR ligands.

III. Negation of Host Defenses

A. Elimination of Antimicrobial Reactive Oxygen

Interaction of *Histoplasma* yeasts with phagocytes variably induces production of reactive oxygen species (ROS), which *Histoplasma* must detoxify in order to survive. Stimulatory signals, including PRR recognition of PAMPs, cytokines, and other phagocytic signals, trigger assembly of the NADPH-phagocyte oxidase (phox), a multimeric protein complex that produces the initial ROS, superoxide (O_2^-). In response to *Histoplasma* yeasts, PMNs readily produce ROS (Schaffner et al. 1986; Schnur and Newman 1990; Kurita et al. 1991a, b) but macrophages only produce significant ROS upon cytokine activation (Eissenberg and Goldman 1987; Wolf et al. 1987, 1989; Fleischmann et al. 1990; Wolf and Massof 1990). Regardless, *Histoplasma* survives phagocyte-derived superoxide in vitro and in vivo by expression of a yeast-phase-specific, **extracellular superoxide dismutase, Sod3** (Youseff et al. 2012). *Histoplasma* Sod3 is both secreted and anchored on the yeast cell wall, positioning it optimally to destroy phagocyte-derived superoxide (Fig. 7.2). Intracellular superoxide dismutase (i.e., *Histoplasma* Sod1) does not provide protection against extracellular superoxide because the charge on superoxide renders this ROS unable to pass through biological membranes. Without Sod3, *Histoplasma* virulence is significantly attenuated and *Histoplasma* yeast survival against PMNs and

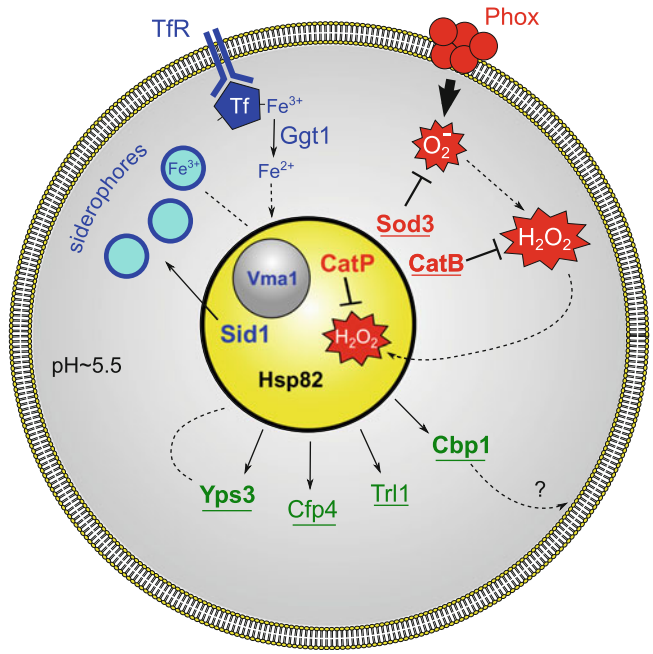


Fig. 7.2. Mechanisms facilitating *Histoplasma* yeast intracellular survival and proliferation within macrophage phagosomes. Demonstrated virulence factors are indicated in *bold* and those with pathogenic-phase-specific expression are *underlined*. *Histoplasma* yeasts extinguish phagocyte-produced ROS by production of extracellular oxidative stress defense factors (*red*). Assembly of the multimeric phagocyte NADPH-oxidase complex (Phox) in the phagosomal membrane leads to production of antimicrobial superoxide. This toxic ROS is eliminated by the extracellular *Histoplasma* superoxide dismutase (Sod3). Peroxide molecules are similarly destroyed by extracellular catalase (CatB). Residual peroxide that escapes CatB destruction and enters the yeast cell is eliminated by an intracellular catalase (CatP). *Histoplasma* yeasts have at least two strategies for essential iron acquisition (*blue*). Host iron is brought to the phagosome via transferrin after binding to the transferrin receptor (TfR). In one strategy, *Histoplasma* yeasts secrete a γ-glutamyl transferase (Ggt1), which utilizes GSH to reduce ferric to ferrous iron, promoting iron release from transferrin and allowing for import into yeast cells. In a second strategy, the *Histoplasma* ornithine monooxygenase (Sid1) initiates siderophore synthesis and siderophores are secreted into the phagosomal lumen to chelate iron. The action of the vacuolar proton-ATPase, of which Vma1 is a central subunit, maintains iron homeostasis in *Histoplasma* yeasts. *Histoplasma* yeasts also secrete several novel factors (*green*) with undefined roles in intracellular residence. Cbp1, a calcium-binding protein, is abundantly secreted by yeast cells and may interact with phagosomal lipids. Yps3 is a secreted factor that also binds to cell wall chitin. *Histoplasma* yeasts produce an extracellular thioredoxin reductase-like protein (Trl1) and the Cfp4 glycoprotein during infection of macrophages. Additionally, yeasts utilize the stress-response Hsp82 protein to ameliorate intracellular stresses

activated macrophages is reduced to similar levels as for wild-type *Candida*. Thus, **Sod3-dependent dismutation of host-derived superoxide contributes to the greater virulence of *Histoplasma* compared to opportunistic fungi.**

In addition to Sod3, *Histoplasma* yeasts express a yeast-phase-specific, **extracellular catalase, CatB.** CatB is equivalent to M-antigen, a major diagnostic exoantigen for histoplasmosis (Hamilton et al. 1990; Zancopé-Oliveira et al. 1999). Given the ability of CatB to destroy peroxide, many have assumed that CatB is essential to *Histoplasma* virulence. However, CatB-deficient *Histoplasma* yeasts are fully virulent (Holbrook et al. 2013). This is due to functional redundancy with the **intracellular *Histoplasma* catalase, CatP** (Holbrook et al. 2013). Production of either catalase is sufficient to protect *Histoplasma* from host-produced ROS. Nonetheless, expression of dual catalases may provide a more effective means of protecting ROS-sensitive intracellular targets (Fig. 7.2);

the phagocyte-derived ROS is efficiently destroyed by extracellular CatB before peroxide can enter the cell and affect intracellular targets. CatP, as an intracellular ROS defense, provides additional protection against any peroxide that escapes destruction by CatB. The combined activities of Sod3 and the dual catalase system effectively negate phagocyte-produced reactive oxygen defenses, thereby enabling yeast survival during infection.

B. Resistance to Reactive Nitrogen Compounds

Host defenses against fungal infections also include production of reactive nitrogen species (RNS), particularly by cytokine-activated phagocytes. Depletion or inhibition of the pro-inflammatory cytokines IFN-γ, TNF-α, and GM-CSF increases susceptibility to *Histoplasma* infection. This effect is correlated with impaired production of reactive nitrogen compounds, implicating RNS as one of the host defense mechanisms *Histoplasma* must combat (Lane et al. 1994; Allendoerfer and Deepe 1998; Deepe et al. 1999). Consistent with this, inhibition of RNS production by phagocytes in culture decreases their ability to control *Histoplasma* yeasts (Nakamura et al. 1994; Lane et al. 1994). However, the effect of phagocyte RNS on *Histoplasma* yeast is fungistatic, not fungicidal, suggesting that *Histoplasma* has defenses against the lethal effects of RNS. As candidate factors mediating this, 59 genes are upregulated in *Histoplasma* yeasts specifically in response to RNS treatment (Nittler et al. 2005). One of the induced genes is a mycelial-expressed nitric oxide reductase (Nor1) that, when ectopically expressed in yeasts, provides a slight benefit in vitro to yeast grown in the presence of nitric oxide. Whether Nor1 is necessary for the RNS resistance of *Histoplasma* yeasts remains to be determined.

C. Stress Response

In addition to ROS- and RNS-based host defenses, *Histoplasma* yeasts must contend with other stresses related to intracellular growth and host infection. A **heat-shock protein (Hsp82)**, which is homologous to proteins of the 90 kDa stress-response family, **facilitates intracellular growth as well as full virulence in vivo** (Edwards et al. 2011b). Although it is assumed that heat-shock proteins are essential for thermally dimorphic fungal adaptations, yeasts with reduced Hsp82 function grow normally at mammalian body temperature (37 °C). At febrile temperatures, *hsp82*-mutant yeasts are more inhibited than wild-type yeast cells. Thus, Hsp82 is not required for yeast growth at 37 °C but instead ameliorates stresses associated with infection that are not limited to high temperature growth.

IV. Essential Nutrients for Intracellular Growth

A. Iron Acquisition

Like all cells, *Histoplasma* yeasts require iron for essential life processes and, during infection, must acquire this element from the host. Host iron restriction, enhanced by IFN-γ that is produced during cell-mediated immunity or administration of iron chelators, restricts *Histoplasma* replication (Lane et al. 1991; Newman et al. 1994, 1995). To obtain limited iron, *Histoplasma* relies upon a number of iron acquisition strategies. Maintenance of the phagosomal pH at around 5.5 is thought to allow release of one of the two iron atoms from transferrin, yet this pH is not acidic enough for activation of lysosomal hydrolases. In addition, *Histoplasma* yeasts produce and secrete iron-scavenging hydroxamate siderophores, which can acquire iron from holotransferrin (Howard et al. 2000) (Fig. 7.2). Recent genetic studies have confirmed the importance of siderophore production in *Histoplasma* virulence; the **loss of siderophore production reduces *Histoplasma* yeast growth under iron-limiting conditions in vitro, and impairs yeast replication in cultured macrophages** by 50–75% (Hwang et al. 2008; Hilty et al. 2011).

Conflicting evidence exists for the requirement of siderophores in vivo. In one study, loss of siderophore production in the genetically tractable Panama strain

does not impair infection and replication of *Histoplasma* in murine lungs until 15 days post infection (Hwang et al. 2008). On the other hand, depletion of siderophores in the NAm 2 strain caused a nearly 100-fold reduction in lung fungal burden at 7 days post infection (Hilty et al. 2011). This discrepancy may result from the operation of alternative iron acquisition mechanisms in some *Histoplasma* strains. In support of this, the genome of the Panama strain, but not the NAm 2 strain, contains *FET3* and *FTR1* genes that encode a high-affinity iron transport system (Hilty et al. 2011).

Although iron is an essential cofactor, excess iron has detrimental effects on cells, and consequently iron acquisition must be tightly regulated. Iron limitation induces both transcriptional (Hwang et al. 2008) and protein level changes (Winters et al. 2008) in *Histoplasma* yeast. A cluster of genes repressed by the iron-responsive GATA transcription factor, Sre1, encode enzymes responsible for siderophore synthesis in response to iron limitation (Hwang et al. 2008, 2012). The enzyme ornithine monooxygenase (encoded by the *SID1* gene) that catalyzes the first committed step in siderophore biosynthesis is included in this iron-regulated gene cluster. Iron homeostasis in *Histoplasma* also depends on vacuolar pH because loss of the vacuolar ATPase subunit Vma1 or treatment with chemicals that block vacuolar acidification (e.g., bafilomycin) impair growth under iron-limiting conditions (Hilty et al. 2008).

Histoplasma yeasts preferentially utilize ferrous iron (Zarnowski et al. 2008b) and have multiple ferric ion reducing systems (Fig. 7.2). Each of these ferric reducing systems is extracellular, consistent with reduction of ferric ions to promote release from extracellular siderophores and host iron-carrying molecules such as transferrin. Early biochemical studies indicated that yeasts produce three extracellular ferric reductases, two soluble and one cell-associated (Timmerman and Woods 1999, 2001). The secreted glutathione-dependent ferric reductase has now been identified as a γ-glutamyl transferase (Ggt1), which is produced by *Histoplasma* yeast cells and catalyzes a two-step, pH-independent reduction of ferric iron (Zarnowski et al. 2008a) Depletion of Ggt1 by RNAi impairs the ability of *Histoplasma* yeasts to replicate and kill cultured phagocytes but the requirement for Ggt1 in vivo remains to be determined.

B. De Novo Vitamin Biosynthesis

Growth of *Histoplasma* yeasts in the nutrient-limiting phagosome of phagocytes requires de novo vitamin synthesis. Growth in chemically defined, minimal media confirms that ***Histoplasma* yeasts can synthesize the essential cofactors riboflavin, niacin, pantothenate, pyridoxine, biotin, and folate** (McVeigh and Morton 1965). On the other hand, mammals are unable to synthesize these vitamins and must acquire them from dietary sources. Yeast cells unable to synthesize their own riboflavin are severely compromised for intracellular growth (Garfoot et al. 2013). These yeasts are similarly unable to replicate following lung infection, indicating that dietary vitamins of the host are unavailable to intracellular yeasts in vivo, therefore mandating that *Histoplasma* yeasts synthesize them de novo. This suggests that targeting vitamin biosynthetic enzymes is a viable strategy for development of new therapeutics.

V. Secretion of Novel Factors

To influence the outcome of the host–pathogen interaction, *Histoplasma* yeasts secrete several novel factors with as yet unidentified functions (Fig. 7.2). Each of these factors is preferentially expressed by pathogenic-phase cells compared to nonpathogenic mycelia, arguing that their function is fundamentally linked to pathogenesis. The secreted **Cbp1 protein was the first virulence factor identified** and, without it, yeasts are significantly attenuated in their ability to establish respiratory infection (Sebghati et al. 2000). Originally described as a calcium-binding protein (Batanghari and Goldman 1997), Cbp1 has nanomolar affinity for calcium (Beck et al. 2008). The Cbp1 protein is a homodimer that is held in a compact structure by

intramolecular disulfide bonds, making Cbp1 highly resistant to protease treatment, a characteristic that suits it well for function in the macrophage phagosome (Beck et al. 2008). Indeed, Cbp1 is secreted into the lumen of the phagosome by yeast cells (Kügler et al. 2000). The α-helices of Cbp1 have structural similarity to lipid-binding saposin-family proteins, which suggests that Cbp1 interacts with host membranes or glycolipids of the phagosome (Beck et al. 2009).

Histoplasma yeasts secrete the **Yps3 protein**, although production is restricted to the North American (NAm 2) phylogenetic group (Keath et al. 1989; Keath and Abidi 1994; Bohse and Woods 2007a). Restricted production results from transcriptional regulation because replacement of the promoter of the *YPS3* gene with a constitutive promoter restores production of Yps3 in *Histoplasma* cells that normally do not synthesize it (Bohse and Woods 2007a). Yps3 has homology to the Bad1 *Blastomyces* adhesin protein and, like Bad1, Yps3 is both secreted and associated with the cell wall through interactions with chitin (Weaver et al. 1996; Bohse and Woods 2005). However, Yps3 does not share the adhesin functions of Bad1 and, although Yps3 has internal tandem repeats, Yps3 lacks the tryptophan-enriched tandem repeat domains of Bad1 that have immunomodulatory function. Functionally, Yps3 is dispensable for pulmonary colonization, but depletion of Yps3 by RNAi impairs the ability of yeasts to establish disseminated infections (Bohse and Woods 2007b). At the cellular level, no function has yet been identified for either soluble or cell-associated Yps3.

A more systematic effort to identify the proteins comprising the secreted proteome of *Histoplasma* yeasts has revealed additional factors with potential roles in *Histoplasma* virulence (Holbrook et al. 2011). The secreted proteome includes the Sod3, CatB, and Cbp1 factors described above. Additional prominent factors in the extracellular fraction include glycanases (exo- and endoglucanases, Exg1 and Eng1, respectively) as well as a chitinase (Chs1), which probably contribute to yeast cell wall remodeling. The secreted proteome has at least five novel culture filtrate proteins (Cfp). Chief among these is Cfp4, a copiously produced, heavily glycosylated factor uniquely expressed by yeast-phase cells. *Histoplasma* yeasts also secrete a thioredoxin reductase-like protein (Trl1), which is distinct from intracellular thioredoxin reductases. Although no functional role has been determined for the glycanases, Cfp4, or Trl1, expression of each factor is linked to the pathogenic phase and expression is maintained during infection (Holbrook et al. 2011). Given that these factors share characteristics common to the vast majority of demonstrated virulence factors (i.e., extracellular localization and pathogenic-phase regulated expression), the identified secreted proteome is a rich source of candidate factors that promote *Histoplasma* virulence. In addition to the canonical eukaryotic secretion pathway, *Histoplasma* also secretes vesicles (Albuquerque et al. 2008), which may be a secondary means of delivering factors to the extracellular environment. Nevertheless, not all secreted factors are necessarily important for virulence, as shown by the lack of any attenuation when the secreted dipeptidyl peptidase (DppIV) is depleted (Cooper and Woods 2009).

VI. Conclusions

Histoplasma capsulatum's success as a pathogen of mammals is due first and foremost to its ability to survive host immune defenses. The intracellular nature of *Histoplasma* pathogenesis provides a shelter from soluble immune molecules but also presents challenges because phagocytes are the primary effector cells for elimination of fungi. The virulence factors recently identified though molecular genetic advancements in *Histoplasma* reveal **a twofold strategy used by yeast cells to overcome this challenge: avoidance of host defenses and neutralization of antifungal reactive oxygen**. Stealthy entry into macrophages relies upon binding of yeasts to complement receptors while at the same time using an α-glucan polysaccharide to conceal fungal molecules from the phagocyte pattern recognition receptors. Protection from phagocyte-derived reactive oxygen

critically depends on expression of ROS-destroying enzymes by *Histoplasma* yeasts. These major mechanisms all employ factors that are characterized by pathogenic-phase-specific production as these tasks are unique requirements for the pathogenic phase. Furthermore, the extracellular localization of these factors positions them to directly affect the host–pathogen interaction. Identification of additional factors that share these two features will probably reveal additional mechanisms of *Histoplasma* pathogenesis.

Once the task of survival has been met, *Histoplasma* pathogenesis depends on acquisition of essential nutrients for growth and replication. Identification of siderophore production and a number of iron reductases that are necessary for full virulence highlight the central role iron restriction plays in host defense and the mechanisms employed by *Histoplasma* yeasts to attain this critical element. Beyond iron, we know little about the metabolism of yeasts within the nutrient-poor phagosome, but continued identification of genes required for intracellular replication should help decipher the metabolic process and the intracellular resources exploited by *Histoplasma* to enable intracellular growth. Various novel factors such as Cbp1 and the Cfp proteins will require additional characterization to uncover the roles they play in pathogenesis. Nonetheless, their yeast-phase-specific expression and their secretion from yeasts suggests that they will somehow affect the host cell or host cell defenses. Mechanistic studies on these factors and application of functional tests on additional candidates discovered will continue to reveal the mechanisms underlying *Histoplasma*'s success as an intracellular primary fungal pathogen.

References

Aderem A (2003) Phagocytosis and the inflammatory response. J Infect Dis 187:S340–S345

Ajello L (1971) Distribution of *Histoplasma capsulatum* in the United States. In: Ajello L, Chick EW, Furcolow MF (eds) Histoplasmosis. Charles C. Thomas, Springfield, pp. 103–22

Albuquerque PC, Nakayasu ES, Rodrigues ML, Frases S, Casadevall A, Zancope-Oliveira RM, Almeida IC, Nosanchuk JD (2008) Vesicular transport in *Histoplasma capsulatum*: an effective mechanism for trans-cell wall transfer of proteins and lipids in ascomycetes. Cell Microbiol 10:1695–1710

Allen HL, Deepe GS (2006) B cells and CD4−CD8− T cells are key regulators of the severity of reactivation histoplasmosis. J Immunol 177:1763–1771

Allendoerfer R, Deepe GS (1998) Blockade of endogenous TNF-α exacerbates primary and secondary pulmonary histoplasmosis by differential mechanisms. J Immunol 160:6072–6082

Baddley JW, Winthrop KL, Patkar NM, Delzell E, Beukelman T, Xie F, Chen L, Curtis JR (2011) Geographic distribution of endemic fungal infections among older persons, United States. Emerg Infect Dis 17:1664–1669

Batanghari JW, Goldman WE (1997) Calcium dependence and binding in cultures of *Histoplasma capsulatum*. Infect Immun 65:5257–5261

Beck MR, DeKoster GT, Hambly DM, Gross ML, Cistola DP, Goldman WE (2008) Structural features responsible for the biological stability of *Histoplasma*'s virulence factor CBP. Biochemistry 47:4427–4438

Beck MR, DeKoster GT, Cistola DP, Goldman WE (2009) NMR structure of a fungal virulence factor reveals structural homology with mammalian saposin B. Mol Microbiol 72:344–353

Bohse ML, Woods JP (2005) Surface localization of the Yps3p protein of *Histoplasma* capsulatum. Eukaryot Cell 4:685–693

Bohse ML, Woods JP (2007a) Expression and interstrain variability of the YPS3 gene of *Histoplasma capsulatum*. Eukaryot Cell 6:609–615

Bohse ML, Woods JP (2007b) RNA interference-mediated silencing of the YPS3 gene of *Histoplasma capsulatum* reveals virulence defects. Infect Immun 75:2811–2817

Bullock WE, Wright SD (1987) Role of the adherence-promoting receptors, CR3, LFA-1, and p150,95, in binding of *Histoplasma capsulatum* by human macrophages. J Exp Med 165:195–210

Chu JH, Feudtner C, Heydon K, Walsh TJ, Zaoutis TE (2006) Hospitalizations for endemic mycoses: a population-based national study. Clin Infect Dis 42:822–825

Cooper KG, Woods JP (2009) Secreted dipeptidyl peptidase IV activity in the dimorphic fungal pathogen *Histoplasma capsulatum*. Infect Immun 77:2447–2454

Deepe GS Jr, Gibbons R, Brunner GD, Gomez FJ (1996) A protective domain of heat-shock protein 60 from *Histoplasma capsulatum*. J Infect Dis 174:828–834

Deepe GS, Gibbons R, Woodward E (1999) Neutralization of endogenous granulocyte-macrophage colony-stimulating factor subverts the protective immune response to *Histoplasma capsulatum*. J Immunol 163:4985–4993

Drummond RA, Brown GD (2011) The role of Dectin-1 in the host defence against fungal infections. Curr Opin Microbiol 14:392–399

Edwards LB, Acquaviva FA, Livesay VT, Cross FW, Palmer CE (1969) An atlas of sensitivity to tuberculin, PPD-B, and histoplasmin in the United States. Am Rev Respir Dis 99(Suppl):1–132

Edwards JA, Alore EA, Rappleye CA (2011a) The yeast-phase virulence requirement for α-glucan synthase differs among *Histoplasma capsulatum* chemotypes. Eukaryot Cell 10:87–97

Edwards JA, Zemska O, Rappleye CA (2011b) Discovery of a role for Hsp82 in *Histoplasma* virulence through a quantitative screen for macrophage lethality. Infect Immun 79:3348–3357

Eissenberg LG, Goldman WE (1987) *Histoplasma capsulatum* fails to trigger release of superoxide from macrophages. Infect Immun 55:29–34

Fleischmann J, Wu-Hsieh B, Howard DH (1990) The intracellular fate of *Histoplasma capsulatum* in human macrophages is unaffected by recombinant human interferon-gamma. J Infect Dis 161:143–145

Garfoot AG, Zemska O, Rappleye CA (2013) Histoplasma capsulatum depends on de novo vitamin biosynthesis for intraphagosomal proliferation. Manuscript submitted

Gildea LA, Morris RE, Newman SL (2001) *Histoplasma capsulatum* yeasts are phagocytosed via very late antigen-5, killed, and processed for antigen presentation by human dendritic cells. J Immunol 166:1049–1056

Gomez FJ, Allendoerfer R, Deepe GS (1995) Vaccination with recombinant heat shock protein 60 from *Histoplasma capsulatum* protects mice against pulmonary histoplasmosis. Infect Immun 63:2587–2595

Gomez FJ, Pilcher-Roberts R, Alborzi A, Newman SL (2008) *Histoplasma capsulatum* cyclophilin a mediates attachment to dendritic cell VLA-5. J Immunol 181:7106–7114

Goodridge HS, Wolf AJ, Underhill DM (2009) Beta-glucan recognition by the innate immune system. Immunol Rev 230:38–50

Guimarães AJ, Frases S, Gomez FJ, Zancopé-Oliveira RM, Nosanchuk JD (2009) Monoclonal antibodies to heat shock protein 60 alter the pathogenesis of *Histoplasma capsulatum*. Infect Immun 77:1357–1367

Hamilton AJ, Bartholomew MA, Figueroa J, Fenelon LE, Hay RJ (1990) Evidence that the M antigen of *Histoplasma capsulatum* var. capsulatum is a catalase which exhibits cross-reactivity with other dimorphic fungi. J Med Vet Mycol 28:479–485

Hilty J, Smulian AG, Newman SL (2008) The *Histoplasma capsulatum* vacuolar ATPase is required for iron homeostasis, intracellular replication in macrophages and virulence in a murine model of histoplasmosis. Mol Microbiol 70:127–139

Hilty J, George Smulian A, Newman SL (2011) *Histoplasma capsulatum* utilizes siderophores for intracellular iron acquisition in macrophages. Med Mycol 49:633–642

Holbrook ED, Edwards JA, Youseff BH, Rappleye CA (2011) Definition of the extracellular proteome of pathogenic-phase *Histoplasma capsulatum*. J Proteome Res 10:1929–1943

Holbrook ED, Smolnycki KA, Youseff BH, Rappleye CA (2013) Redundant catalases detoxify phagocyte reactive oxygen and facilitate *Histoplasma capsulatum* pathogenesis. Infect Immun 81(7):2334–2346

Howard DH, Rafie R, Tiwari A, Faull KF (2000) Hydroxamate siderophores of *Histoplasma capsulatum*. Infect Immun 68:2338–2343

Hwang L, Hocking-Murray D, Bahrami AK, Andersson M, Rine J, Sil A (2003) Identifying phase-specific genes in the fungal pathogen *Histoplasma capsulatum* using a genomic shotgun microarray. Mol Biol Cell 14:2314–2326

Hwang LH, Mayfield JA, Rine J, Sil A (2008) *Histoplasma* requires *SID1*, a member of an iron-regulated siderophore gene cluster, for host colonization. PLoS Pathog 4:e1000044

Hwang LH, Seth E, Gilmore SA, Sil A (2012) SRE1 regulates iron-dependent and-independent pathways in the fungal pathogen *Histoplasma capsulatum*. Eukaryot Cell 11:16–25

Inglis DO, Berkes CA, Murray DRH, Sil A (2010) Conidia but not yeast cells of the fungal pathogen *Histoplasma capsulatum* trigger a type I interferon innate immune response in murine macrophages. Infect Immun 78:3871–3882

Jain VV, Evans T, Peterson MW (2006) Reactivation histoplasmosis after treatment with anti-tumor necrosis factor α in a patient from a nonendemic area. Respir Med 100:1291–1293

Keath EJ, Abidi FE (1994) Molecular cloning and sequence analysis of yps-3, a yeast-phase-specific gene in the dimorphic fungal pathogen *Histoplasma capsulatum*. Microbiology 140:759–767

Keath EJ, Painter AA, Kobayashi GS, Medoff G (1989) Variable expression of a yeast-phase-specific gene in *Histoplasma capsulatum* strains differing in thermotolerance and virulence. Infect Immun 57:1384–1390

Kügler S, Young B, Miller VL, Goldman WE (2000) Monitoring phase-specific gene expression in *Histoplasma capsulatum* with telomeric GFP fusion plasmids. Cell Microbiol 2:537–547

Kurita N, Brummer E, Yoshida S, Nishimura K, Miyaji M (1991a) Antifungal activity of murine polymorphonuclear neutrophils against *Histoplasma capsulatum*. J Med Vet Mycol 29:133–143

Kurita N, Terao K, Brummer E, Ito E, Nishimura K, Miyaji M (1991b) Resistance of *Histoplasma capsulatum* to killing by human neutrophils. Evasion of oxidative burst and lysosomal-fusion products. Mycopathologia 115:207–213

Lane TE, Wu-Hsieh BA, Howard DH (1991) Iron limitation and the gamma interferon-mediated antihistoplasma state of murine macrophages. Infect Immun 59:2274–2278

Lane TE, Wu-Hsieh BA, Howard DH (1994) Antihistoplasma effect of activated mouse splenic macrophages involves production of reactive nitrogen intermediates. Infect Immun 62:1940–1945

Lin J-S, Huang J-H, Hung L-Y, Wu S-Y, Wu-Hsieh BA (2010) Distinct roles of complement receptor 3, Dectin-1, and sialic acids in murine macrophage interaction with *Histoplasma* yeast. J Leukoc Biol 88:95–106

Long KH, Gomez FJ, Morris RE, Newman SL (2003) Identification of heat shock protein 60 as the ligand on *Histoplasma capsulatum* that mediates binding to CD18 receptors on human macrophages. J Immunol 170:487–494

Marion CL, Rappleye CA, Engle JT, Goldman WE (2006) An α-(1,4)-amylase is essential for α-(1,3)-glucan production and virulence in *Histoplasma capsulatum*. Mol Microbiol 62:970–983

McCormack FX, Gibbons R, Ward SR, Kuzmenko A, Wu H, Deepe GS (2003) Macrophage-independent fungicidal action of the pulmonary collectins. J Biol Chem 278:36250–36256

McVeigh I, Morton K (1965) Nutritional studies of *Histoplasma capsulatum*. Mycopathol Mycol Appl 25:294–308

Medoff G, Sacco M, Maresca B, Schlessinger D, Painter A, Kobayashi GS, Carratu L (1986) Irreversible block of the mycelial-to-yeast phase transition of *Histoplasma capsulatum*. Science (New York) 231:476–479

Nakamura LT, Wu-Hsieh BA, Howard DH (1994) Recombinant murine gamma interferon stimulates macrophages of the RAW cell line to inhibit intracellular growth of *Histoplasma capsulatum*. Infect Immun 62:680–684

Nemecek JC, Wüthrich M, Klein BS (2006) Global control of dimorphism and virulence in fungi. Science 312:583–588

Newman SL, Bucher C, Rhodes J, Bullock WE (1990) Phagocytosis of *Histoplasma capsulatum* yeasts and microconidia by human cultured macrophages and alveolar macrophages. Cellular cytoskeleton requirement for attachment and ingestion. J Clin Invest 85:223–230

Newman SL, Gootee L, Brunner G, Deepe GS (1994) Chloroquine induces human macrophage killing of *Histoplasma capsulatum* by limiting the availability of intracellular iron and is therapeutic in a murine model of histoplasmosis. J Clin Invest 93:1422–1429

Newman SL, Gootee L, Stroobant V, van der Goot H, Boelaert JR (1995) Inhibition of growth of *Histoplasma capsulatum* yeast cells in human macrophages by the iron chelator VUF 8514 and comparison of VUF 8514 with deferoxamine. Antimicrob Agents Chemother 39:1824–1829

Nguyen VQ, Sil A (2008) Temperature-induced switch to the pathogenic yeast form of *Histoplasma capsulatum* requires Ryp1, a conserved transcriptional regulator. Proc Natl Acad Sci USA 105:4880–4885

Nittler MP, Hocking-Murray D, Foo CK, Sil A (2005) Identification of *Histoplasma capsulatum* transcripts induced in response to reactive nitrogen species. Mol Biol Cell 16:4792–4813

Rappleye CA, Engle JT, Goldman WE (2004) RNA interference in *Histoplasma capsulatum* demonstrates a role for α-(1,3)-glucan in virulence. Mol Microbiol 53:153–165

Rappleye CA, Eissenberg LG, Goldman WE (2007) *Histoplasma capsulatum* α-(1,3)-glucan blocks innate immune recognition by the β-glucan receptor. Proc Natl Acad Sci USA 104:1366–1370

Schaffner A, Davis CE, Schaffner T, Markert M, Douglas H, Braude AI (1986) In vitro susceptibility of fungi to killing by neutrophil granulocytes discriminates between primary pathogenicity and opportunism. J Clin Invest 78:511–524

Schnur RA, Newman SL (1990) The respiratory burst response to *Histoplasma capsulatum* by human neutrophils. Evidence for intracellular trapping of superoxide anion. J Immunol 144:4765–4772

Sebghati TS, Engle JT, Goldman WE (2000) Intracellular parasitism by *Histoplasma capsulatum*: fungal virulence and calcium dependence. Science (New York) 290:1368–1372

Sengeløv H, Kjeldsen L, Diamond MS, Springer TA, Borregaard N (1993) Subcellular localization and dynamics of Mac-1 (alpha m beta 2) in human neutrophils. J Clin Invest 92:1467–1476

Sorgi CA, Secatto A, Fontanari C, Turato WM, Belangér C, de Medeiros AI, Kashima S, Marleau S, Covas DT, Bozza PT, Faccioli LH (2009) *Histoplasma capsulatum* cell wall β-Glucan induces lipid body formation through CD18, TLR2, and dectin-1 receptors: correlation with leukotriene B4 generation and role in HIV-1 infection. J Immunol 182:4025–4035

Sullivan TD, Rooney PJ, Klein BS (2002) Agrobacterium tumefaciens integrates transfer DNA into single chromosomal sites of dimorphic fungi and yields homokaryotic progeny from multinucleate yeast. Eukaryot Cell 1:895–905

Timmerman MM, Woods JP (1999) Ferric reduction is a potential iron acquisition mechanism for *Histoplasma capsulatum*. Infect Immun 67:6403–6408

Timmerman MM, Woods JP (2001) Potential role for extracellular glutathione-dependent ferric reductase in utilization of environmental and host ferric compounds by *Histoplasma capsulatum*. Infect Immun 69:7671–7678

Videm V, Strand E (2004) Changes in neutrophil surface-receptor expression after stimulation with FMLP, endotoxin, interleukin-8 and activated complement compared to degranulation. Scand J Immunol 59:25–33

Weaver CH, Sheehan KC, Keath EJ (1996) Localization of a yeast-phase-specific gene product to the cell wall in *Histoplasma capsulatum*. Infect Immun 64:3048–3054

Webster RH, Sil A (2008) Conserved factors Ryp2 and Ryp3 control cell morphology and infectious spore formation in the fungal pathogen *Histoplasma capsulatum*. Proc Natl Acad Sci USA 105:14573–14578

Winters MS, Spellman DS, Chan Q, Gomez FJ, Hernandez M, Catron B, Smulian AG, Neubert TA, Deepe GS (2008) *Histoplasma capsulatum* proteome response to decreased iron availability. Proteome Sci 6:36

Wolf JE, Massof SE (1990) In vivo activation of macrophage oxidative burst activity by cytokines and amphotericin B. Infect Immun 58:1296–1300

Wolf JE, Kerchberger V, Kobayashi GS, Little JR (1987) Modulation of the macrophage oxidative burst by *Histoplasma capsulatum*. J Immunol 138:582–586

Wolf JE, Abegg AL, Travis SJ, Kobayashi GS, Little JR (1989) Effects of *Histoplasma capsulatum* on murine macrophage functions: inhibition of macrophage priming, oxidative burst, and antifungal activities. Infect Immun 57:513–519

Youseff BH, Rappleye CA (2012) RNAi-based gene silencing using a GFP sentinel system in *Histoplasma capsulatum*. In: Brand AC, MacCallum DM (eds) Host-fungus interactions. Methods and protocols, methods in molecular biology, vol 845. Humana Press, New York, pp 151–164. http://link.springer.com/protocol/10.1007/978-1-61779-539-8_10

Youseff BH, Dougherty JA, Rappleye CA (2009) Reverse genetics through random mutagenesis in *Histoplasma capsulatum*. BMC Microbiol 9:236

Youseff BH, Holbrook ED, Smolnycki KA, Rappleye CA (2012) Extracellular superoxide dismutase protects *Histoplasma* yeast cells from host-derived oxidative stress. PLoS Pathog 8:e1002713

Zancopé-Oliveira RM, Reiss E, Lott TJ, Mayer LW, Deepe GS (1999) Molecular cloning, characterization, and expression of the M Antigen of *Histoplasma capsulatum*. Infect Immun 67:1947–1953

Zarnowski R, Cooper KG, Brunold LS, Calaycay J, Woods JP (2008a) *Histoplasma capsulatum* secreted γ-glutamyltransferase reduces iron by generating an efficient ferric reductant. Mol Microbiol 70:352–368

Zarnowski R, Dobrzyn A, Ntambi JM, Woods JP (2008b) Ferrous, but not ferric, iron maintains homeostasis in *Histoplasma capsulatum* triacylglycerides. Curr Microbiol 57:153–157

8 Visualizing Immune Responses in Fungal Infections: Established and Novel Methods

Mike Hasenberg[1], Sven Krappmann[2], Matthias Gunzer[1]

CONTENTS

I. Introduction

A large variety of fungal species belong to the normal flora of human skin and mucosal tissues and can colonize hosts throughout life. Furthermore, due to environmental interaction, e.g., breathing of ordinary air, people come into contact with huge numbers of fungal elements (Latge 1999). Interestingly, under normal conditions neither colonization nor environmental exposure cause recognizable health problems (Latge 1999). This is due to permanent mutual control by other members of the microbiome (Peleg et al. 2010) and also by the effective barrier function and immune system of the host (Hasenberg et al. 2011a, 2013). However, when either the barrier is broken or the immune system loses its grip, environmental or commensal fungi can infect the host, a condition that is one of the most dangerous threats to human health (Romani 2011; Denning and Hope 2010).

In the clinical daily routine, one of the key problems of mycological infections is the difficulty in clearly and rapidly identifying a fungal infection and differentiating it from other microbial infections. In the face of these problems, a typical therapeutic regime with risk patients suffering from fever of unknown origin is prophylactic treatment with antibiotics, followed by antimycotics if the fever has not vanished after 3–7 days (Freifeld et al. 2011).

Thus, obviously, the lack of a definitive and specific diagnosis can lead to inadequate therapy that may compromise the patient's health without addressing the real problem. Reasons for this therapeutic failure are the poor accessibility of adequate patient samples, imprecise detection tools, or the lack of information on the precise localization and extent of fungal growth.

Whole-body imaging approaches have the potential to address many of these problems at once: techniques like **X-ray chest scans** including **computed tomography (CT)** are able to detect fungal masses in lungs. However, these images are often difficult to interpret such that a fungal mass cannot easily be differentiated from "shadows" caused by other lung afflictions (Muller et al. 2002). A much better soft-tissue contrast, allowing a more precise estimation of fungal masses, can be obtained by **magnetic**

[1]Institute for Experimental Immunology and Imaging, University Duisburg-Essen, University Hospital, Hufelandstrasse 55, 45147 Essen, Germany; e-mail: Matthias.gunzer@uni-due.de
[2]Microbiology Institute – Clinical Microbiology, Immunology and Hygiene, University Hospital of Erlangen and Friedrich-Alexander-Universität Erlangen-Nürnberg, Wasserturmstrasse 3/5, 91054 Erlangen, Germany

Human Fungal Pathogens, 2nd Edition
The Mycota XII
O. Kurzai (Ed.)

resonance imaging (MRI), but still without a definitive identification of a fungal species. Specificity for an inflammatory process can be brought in to a certain extent with **positron emission tomography (PET)** by using metabolic tracers such as [^{18}F]fluorodeoxy-D-glucose (FDG) (Seshadri et al. 2007), but signals generated in this imaging approach can also be obscured or modulated by completely unrelated inflammatory processes such as cancer (Shrikanthan et al. 2005). Thus, more specific tracers such as siderophores, which are used by fungi to trap iron from the host, can be functionalized for **PET** or **PET/MRI** and provide more specific signals, at least in animal models (Petrik et al. 2012). Molecular imaging approaches also have the opportunity to detect "reactivity" or "shadow" anywhere in the body, even at sites normally not sampled for conventional analysis. However, today no available technology, not even FDG-PET imaging, is able to specifically detect the pathogen or the immune response against it in the clinical routine, despite the strong need for such a diagnostic tool.

In addition to diagnostic imaging in clinical applications, another important field of work is experimental imaging. Of course, any clinical application has been developed from preclinical studies in vitro or in suitable animal models. However, experimental imaging also aims at elucidating the basic cell-biological mechanisms behind the obtained data that help to explain the course of the disease or the response of the host towards the fungal attack. Experimental imaging aims to develop techniques that look closely at the immune response of a host, either alone or in combination with imaging of the fungus. Specifically, **complex light** and **confocal microscopy** approaches have allowed an understanding of the dynamics underlying phagocytic responses towards fungal elements in vitro (Behnsen et al. 2007a; Bruns et al. 2010). **Flow cytometry**, in contrast, is able to very rapidly image many cells/pathogen in a short period of time; however, with little resolution. Nevertheless this technology is extremely powerful for answering certain questions on fungal biology. A recent addition to the armament of fungal imagers is **intravital 2-photon microscopy**, which allows observation of immune cells while they are phagocytosing fungal elements or producing neutrophil extracellular traps (NETs). This is a powerful immunological weapon against microbial spreading in intact tissue (Bruns et al. 2010) or in vivo. New developments in the field of light microscopy have brought about very remarkable increases in resolution, such that today light microscopy can resolve structures down to 20–30 nm in size. The key developments here are **stimulated emission depletion (STED)**, **structured illumination (SIM)** and **localization microscopy (STORM/PALM)** (Coltharp and Xiao 2012). To obtain the highest possible resolution, **scanning electron microscopy (SEM)** of surface structures (Behnsen et al. 2007a; Hasenberg et al. 2013) or **transmission electron microscopy (TEM)** of fungal elements internalized by phagocytes has been applied (Ibrahim-Granet et al. 2003). Finally, there is a specific very powerful whole-body imaging approach that so far has only been used in experimental systems, namely **luminescence imaging** (Donat et al. 2012).

Generally, any imaging approach has to be validated for its sensitivity, specificity, and cost. An important issue is, of course, if the question being asked by the clinician or researcher can be answered by the chosen imaging approach. In order to help potential users to decide on a successful imaging strategy for their relevant projects, we have summarized the imaging modalities mentioned above. We have sorted them into the categories of clinical and experimental imaging. Each method is introduced by a brief technical description, followed by specific examples of their use, and concluding with a discussion of the sensitivity, specificity, and cost as well as suitability for a given question. The overall aim of this list is to provide interested users with a rational basis for the choice of a suitable imaging approach. Based on our own experimental experience, most samples for fungal imaging center around the mold *Aspergillus fumigatus*. But of course, all discussed systems can in principle also be used for other fungal species and, where appropriate, we will also mention some examples of these.

II. Experimental Scientific Imaging

We start our excursion with modern imaging systems that are employed in scientific laboratories all over the world to visualize cellular processes in (fungal) infections. The overall goal of these techniques is the generation of imaging data that build the basis for understanding the complex interactions between host immune cells and fungal pathogens under diverse infection conditions. For the development of therapeutic strategies it is of high importance to understand which immune cell types are involved in the antimicrobial fight and with which kinetics. To optimize antifungal treatments clinicians need to know which organs are colonized, at which time point in the course of an infection, and which treatments might display promising effects against the infectious threat. All these parameters can be investigated by the huge variety of experimental in vitro and in vivo imaging approaches.

A. (Time-Lapse) Brightfield/Widefield Microscopy

Due to the inherent complexity of the immune response against fungal infections, researchers initially tend to keep their experiments as simple as possible before more complicated systems are explored. A very common way is to reduce the environmental conditions to the level of single cells or at least to explanted tissues, resulting in focused questions. These entities can subsequently be the focus of a variety of different imaging techniques, starting with a basic light microscope.

The major component of a brightfield microscope is a light source that emits transmission light, which passes the microscopic sample. The diffracted light is finally gathered by an objective, typically positioned opposite the light source. The objective generates the magnified picture of the observed sample, which is then further magnified by the eyepiece. **Combined magnifications of objective and eyepiece in commercial systems reach 40× to 1,000×, with a theoretical maximum resolution of ~200 nm, based on the physics of diffraction** (Hell 2007) (see section on electron microscopy). **The obtained picture has a bright background (hence the name) from the transmission light and the structures of the specimen appear darker due to light absorption.** Because the transmission light fully illuminates the specimen over a wide angle (in contrast to a spot illumination), this technique is **also called widefield microscopy**.

Around 1730, the Italian botanist Pietro Antonio Micheli used such a microscope to catalog molds (Michelio 1729). He was the first to illustrate the fungal genus *Aspergillus* and, more than 100 years later in 1863, the physician Georg W. Fresenius introduced *A. fumigatus* as a new species able to cause severe lung infections (Schmidt 1998). His work was also based on transmission microscopy studies of infected human lung samples.

Although this visualization technique is very old it is still used heavily in modern experimental setups, but now extended by a number of crucial inventions. One central progress was the enhancement of contrast by various optical phenomena such as cross-polarized light, dark field, phase contrast, and differential interference contrast (DIC). Together with very light-sensitive detectors (digital complementary metal–oxide–semiconductor; CMOS) or CCD (charge-coupled device) cameras and objectives with a high numerical aperture, the quality of micrographs has significantly increased over time. Current widefield microscope systems all reach the theoretical resolution barrier of 200 nm and are therefore well suited to visualize cellular behavior and even subcellular processes. The application range of widefield systems was further enlarged when these microscopes were equipped for the detection of fluorescence signals. **By employing widefield fluorescent illumination of pre-labeled biological samples it is possible to specifically detect different cell types, cellular compartments, or metabolic products/states inside of cells.** Like other fluorescence-based methods, the specificity is realized by the use of fluorochrome-coupled monoclonal antibodies, separate pre-labeling of different cells with intracellular fluorescent dyes, or by a change in the fluorescent properties of chemical compounds

during intra- or extracellular reactions (Hasenberg et al. 2011b). A very prominent example is the detection of intracellular calcium ions. Ca^{2+} is a major regulator of central signaling pathways during cell activation, including cellular movement. In order to detect these activation signals, living cells can be loaded with calcium chelating agents such as Fura-2, a polyamino carboxylic acid. This fluorescent dye has excitation maxima at 340 and 380 nm and the ratio of its emissions at these wavelengths directly allows the calculation of the free calcium ion concentration inside a cell (Waibler et al. 2008).

Ca^{2+} imaging can only be done in living cells and requires the observation of cells over a period of time with repetitive imaging. Other dynamic processes like phagocytosis can also only be thoroughly studied if live cell imaging is employed (Behnsen et al. 2007a; Sasse et al. 2013). This so-called **time-lapse widefield microscopy** requires appropriate conditions for the isolated cells and fungal elements under investigation. Modern systems are therefore equipped with fully automated climate chambers that allow the regulation of important vital parameters such as temperature, humidity, and CO_2 concentration. As soon as these key settings are well controlled, cellular behavior can be analyzed under conditions that mimic the natural environment regarding these parameters. Upgraded to this level, widefield microscopes can efficiently be used to answer a variety of questions in the field of fungal biology. They can bring light into unknown processes of fungal life cycles and growth, such as the formation of the Spitzenkörper in growing hyphae (Steinberg 2007), and they are well suited to study immune responses towards fungal morphotypes such as phagocytic events and cellular contact formation under in vitro conditions (Behnsen et al. 2007b; Sasse et al. 2013).

On the other hand, widefield microscopy has clear limitations. As soon as a biological sample is composed of more than one cell layer, the ensuing immense light diffraction negatively influences the system's resolution. In the case of fluorescence imaging, light coming from out-of-focus areas of the sample blurs the image and can even make the generation of useful images impossible. **Therefore, a golden rule for obtaining appropriate image material from transmission light microscopy is to keep the samples as thin as possible.** However, if the experimental question does not allow the use of extremely thin samples or the goal is to maintain multicellular layers for physiological reasons, a powerful alternative approach is confocal laser scanning microscopy (CLSM).

B. Confocal Laser Scanning Microscopy

CLSM is a specific kind of fluorescence light microscopy. The basic idea behind this technique is the illumination of the microscopic specimen in a very narrow spatial volume (x, y, and z dimensions) per time point by using a point source of light rather than a widefield illumination. Typically, this point source is a laser of suitable color. A full picture of the sample is then generated by a scanning process in which the exciting light beam is driven line by line and plane by plane over the whole sample volume, resulting in an optical sectioning. As laser light is a very bright and thin beam this is an optimal light source for this application. However, due to the fact that the sample is not illuminated in its entirety at once, imaging with the eye or a camera is not possible. Instead, the light emitted from a given spot of laser-activated fluorochromes is gathered in real-time by sensitive detectors (typically photomultiplier tubes, PMTs) during the acquisition procedure to generate one pixel of a final image. Here, the use of several independent PMTs and suitable filters also allows the generation of multicolor images. **Taking the *x*, *y*, and *z* coordinates together with the emitted light intensities at each illuminated position, the operation software of these microscopes is able to calculate a final three-dimensional (3D) fluorescence picture of the entire sample.**

To minimize diffraction effects, which negatively influence the resolution of light microscopes in thicker samples, CLSMs are equipped with another decisive feature. A so-called **pinhole** in the light path of the emission light eliminates all light that results from the excitation of fluorochromes in out-of-focus areas in the z-direction. Therefore, these fluorescence

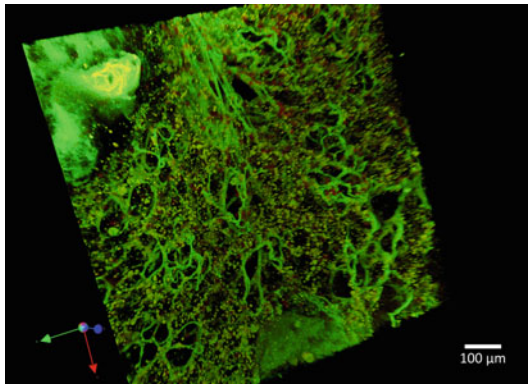


Fig. 8.1. CLSM 3D reconstruction of an *A. fumigatus*-infected lung. This picture has been taken from an infected and subsequently explanted murine lung by use of confocal laser scanning microscopy (CLSM). A 3D rendering was done based on the microscopic sample sections. The organ-specific structures such as alveoli were detected by their autofluorescence signal. The *small red clouds* are so-called neutrophil extracellular traps (NETs), which are composed of extracellular DNA fibers and were released during the neutrophilic immune reaction towards the fungal pathogen. NETs were stained with a DNA-specific dye

signals cannot contribute to the digital calculation of the micrograph at a given position in *z* and as a result the final picture displays much less background noise and can provide a sharp section through a thick sample.

Although this technique does not provide a gain in resolution, the resulting images are usually of a much better quality than comparable widefield fluorescence data from the same sample because they **almost completely lack out-of-focus haze**. In addition, by generating images at different positions in *z*, a CLSM is able to generate series of image planes that result in a real 3D picture of the observed sample (Fig. 8.1). This 3D information is absolutely necessary for all analyses in which the definite spatial localization of certain structures is of importance. With respect to the analysis of the phagocytic uptake of *A. fumigatus* spores by immune cells for example, it is essential to assess from all dimensions around a particular phagocyte whether associated fungal particles have really been ingested or are only attached to the surface of the cell (Mech et al. 2011).

In order to provide a certain flexibility regarding the parallel detection of different fluorescence colors, common CLSM systems are equipped with several lasers and a combination of adjustable excitation and emission filter sets. Very recent machines have been released that use white lasers as light source and can very quickly and precisely tune acousto-optical beam splitters and filters on both the excitation as well as emission side. These systems allow the use of almost all fluorophores that possess a spectral excitation/emission peak in the visible light spectrum.

One disadvantage of note is the speed of image acquisition in a CLSM. As every single two-dimensional (2D) picture is based on the scanning process of the whole specimen in one plane, it takes a certain time until the required digital information for one image is obtained (in contrast to a widefield system in which the picture is recorded by the camera as soon as the specimen is illuminated by the transmission light). Although it is possible to analyze cellular behavior using CLSM (Gunzer et al. 2004), fast moving cells sometimes generate problems when their tracks are the question of the analysis. These problems become even bigger as soon as the cellular movements are analyzed in four dimensions (3D over time). However, novel developments such as resonant scanners and extremely fast and sensitive detectors have helped to partly overcome these problems. Today, the efficiency of light generation from the sample, rather than the slow detection speed of the CLSM, is the key factor limiting the recording speed of a given system.

C. Electron Microscopy

In 1873, Ernst Abbé published a landmark equation that easily correlates the wave nature of light or electrons and the lens properties, thereby calculating the resolving power of a given optical system.

$d = 0.61 \frac{\lambda}{n \cdot \sin a}$	d	Resolution
	λ	Wavelength of the light source
	n	Refractive index of the medium
	α	Aperture angle of the objective

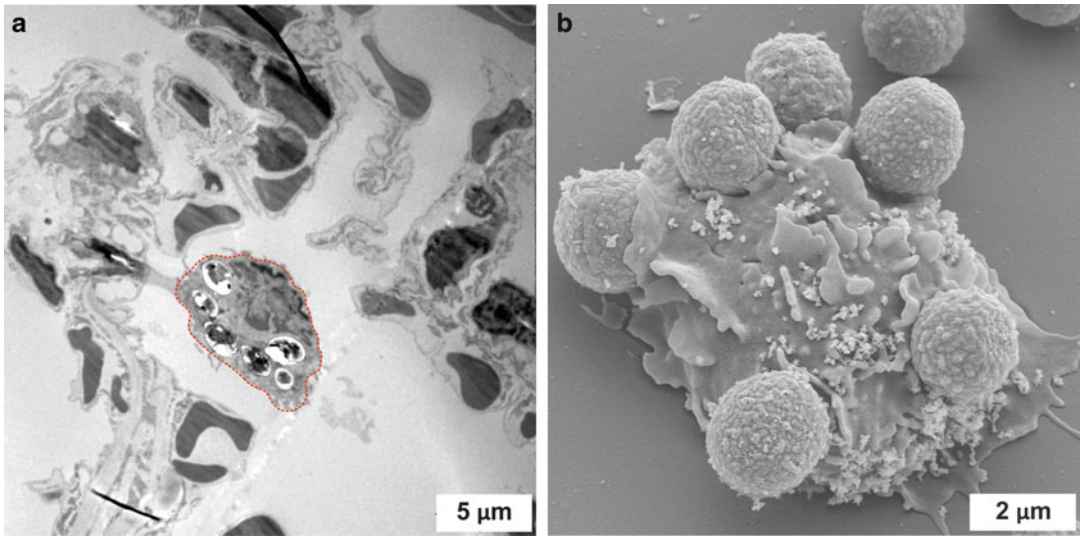


Fig. 8.2. Different appearance of TEM and SEM images. (**a**) Transmission electron microscopy (TEM) image of the situation in an *A. fumigatus*-infected mouse lung. An alveolar macrophage (outlined in *red*) scans the alveolar lumen and has already taken up six fungal spores. (**b**) The scanning electron micrograph (SEM) visualizes the in vitro co-incubation of isolated murine neutrophils and spores of the mold *A. fumigatus* after 4 h. It is nicely observable that the immune cell has started to internalize some of the attached fungal particles. *Scale bars* are depicted in *white*. The pictures were taken in collaboration with Prof. Manfred Rohde at the Helmholtz Center for Infectious Diseases in Braunschweig, Germany

The value d defines the smallest distance between two objects such that they can still be detectable as two separate entities. Thus, the smaller the value for d, the better resolution a microscope has. The equation clearly shows that d is highly dependent on the wavelength λ, where short wavelengths are obviously desirable. For normal light microscopy, light in the range of the visible light spectrum (>380 nm) is usually used, resulting in a maximum resolution of around 200 nm. The wavelength of an electron beam is dramatically smaller. **In modern electron microscopy (EM) systems, values around 2 pm are achievable, suggesting a theoretical resolution of ~1 pm. However, the practical resolution with these machines is estimated to be ~0.1 nm due to aberration errors of the electron lenses.** This is still good enough to see individual atoms (Meyer et al. 2008).

Inside these microscopes, an electron beam is generated by the emission of electrons from a glowing cathode. These electrons are subsequently accelerated in a high vacuum by a tunable anode and aligned by a system of electronically charged lenses. **In the field of electron microscopy, two principally different major technologies are known: transmission EM (TEM) and scanning EM (SEM)** (Koning and Koster 2009).

In TEM, the ray of electrons is shone through an extremely thin sample of interest (Fig. 8.2a). Upon interaction with the atoms in the sample, the electrons are scattered and/or absorbed. A detection device behind the sample is then able to generate a magnified image of the modified electron beam that can be observed on a screen or with a camera. TEM thus generates highly resolved images of thin sections of samples. In contrast, SEM uses an electron beam that is scanned over the surface of a fully intact 3D specimen and releases secondary electrons from its surface. These are measured by a detector and generate one pixel of a digital image. By scanning over the surface of the sample very detailed 3D images of surfaces can be obtained (Fig. 8.2b). We have used this approach to document the details of phagocyte contacts to *A. fumigatus* spores (Behnsen et al. 2007a) as well as the fine structures of DNA NETs with

these fungal elements (Bruns et al. 2010). Others have used SEM and TEM to document the uptake of *Aspergillus* spores by alveolar macrophages (Ibrahim-Granet et al. 2003).

Although TEM images give an extremely good resolution of cellular structures, they are typically only 2D. A fairly recent addition to the technology now allows the generation of 3D reconstructions from TEM images without the need for serial sections. This technique is called **electron tomography (ET)**. Here, the thin sample is placed on a rotating device and a series of TEM images from different angles is taken, which then allows reconstruction of the 3D network of observed structures with unprecedented detail (Koning and Koster 2009). This technology has already been used to study the ultrastructure of fungal elements (Hohmann-Marriott et al. 2006). It would be extremely interesting to also use ET for the study of host–pathogen interactions.

Thus, while the advantage of EM obviously lies in its extreme resolving power, the downside is its inability to image live specimens. Furthermore, EM requires extensive sample preparation and a lot of experience both in the use of the microscopes as well as in interpretation of the data. Nevertheless, without doubt the electron microscope has opened to human eyes the fascinating ultrastructural world, which has always intrigued scientists and laymen and will continue to do so in the future. The inability of EM to image live specimens is partly overcome, albeit not with the same resolving power, with new methods in optical microscopy (see next section).

D. Super-Resolution Light Microscopy

For more than 100 years the obtainable maximum resolution of optical microscopes was accepted to be ~200 nm, based on Abbés rules of diffraction (Hell 2009) (see also the previous section on electron microscopy). However, since the 1990s a number of innovative advances have been made that allow resolutions of up to ten times higher using currently available commercial light microscopy systems. Here, we will discuss three key developments in the field: stimulated emission depletion, structured illumination microscopy, and localization microscopy. Unfortunately, not much use has been made of these developments in experimental mycology so far. For a fine review of the use of these novel systems in the general microbiology field the reader is referred to (Coltharp and Xiao 2012). Future work should thus aim at exploiting these techniques for investigating fungal infections and the interaction of fungi with the vertebrate immune system in unprecedented detail.

STED (stimulated emission depletion): STED is principally an advanced method of CLSM. However, rather than using just one blue exciting laser spot, the dimensions of which are limited to the 200 nm size defined by Abbé optics, **STED projects a second, red-colored, doughnut shaped depletion beam over the exciting beam**. This depletion beam has a hole in the middle, where no laser light is emitted. All fluorescence hit by the depletion laser is immediately quenched again due to a process called stimulated depletion. The key feature of STED is that the hole in the center of the doughnut can be adjusted to be significantly smaller than 200 nm, based on the power and the optical shaping of the laser. Thus, the combination of excitation spot and depletion doughnut allows the generation of a much smaller spot of residual excitation than could be obtained with a focused excitation laser alone (Eggeling et al. 2009). **This small spot of residual excitation increases the optical resolution in the *x*,*y*-plane by a factor of four to eight.** In modern commercial systems, this is indeed achieved by the click of a mouse on the control software. The advantage in resolution is bought at the expense of the very bright light of the depletion beam. Thus, STED imaging is prone to rapid bleaching and phototoxicity, although novel developments such as gated STED reduce this problem (Vicidomini et al. 2011). Furthermore, not all dyes are suitable for STED imaging and there is only the possibility to simultaneously image two colors as opposed to other multicolor options. Finally, STED only increases the resolution in the *x*,*y*-plane, while resolution in the *z*-direction is not changed.

SIM (structured illumination microscopy): SIM uses the projection of a known grating structure into the imaging plane that is recorded together with the emitted fluorescence. By interference with the emitted fluorescence, so-called moiré fringes are generated. These contain additional structural information about the sample that cannot be simply extracted by optical resolution but needs complex mathematical operations (Fourier transformations) to obtain an image. As a result, **SIM increases the resolution in all three directions of space by a factor of at least two** (Schermelleh et al. 2008; Gustafsson 2005). Thus, although not as good as STED, the principal advantage of SIM is the fact that it works with any fluorescent dye and does not use high intensities of light. Thus, it can also be used for long-term time-lapse imaging (Fiolka et al. 2012).

Localization microscopy (PALM/STORM): These approaches exploit the ability of fluorescent dyes to emit light only for short amounts of time, whereby two adjacent molecules rarely emit simultaneously. However, this is typically not seen because conventional fluorescent images are an integrated fluorescence signal of many molecules permanently illuminated over a long time. But, when a given field of fluorescent molecules is excited with stroboscopic light rather than continuous illumination, at one stroboscopic pulse only a fraction of molecules will fluoresce. When the next stroboscopic pulse hits the system, a separate fraction of molecules will glow. If, by this approach, a large series (typically several thousand images) of stroboscopic images is taken, for statistical reasons all available fluorescent molecules will have glowed at least once. The important feature is that by this approach two directly adjacent molecules will not shine on the same frame of the series, but only on separate frames. This fact allows their **optical separation as two molecules, rather than as one larger fluorescent entity**. The image generated by this method is not a classical picture like that made by a PMT. Instead, from each light spot detected in the series of images the exact center of the generating fluorescent spot will be calculated from fitting the Gaussian light distribution with nanometer precision. This calculated molecular position will be artificially set as the position of a positive pixel whose superimposition with all other found spot positions generates the final super-resolution image. As a result, **localization images have a "pointillist" appearance but their resolution (or rather, their precision in exactly localizing the source of a fluorescence signal) in the *x*,*y*-plane can reach values of 20 nm or better** (Coltharp and Xiao 2012; Hell 2007). To generate this extreme resolution, PALM/STORM requires very specific dyes and experimental conditions (such as stringent buffer conditions on fixed samples). This renders the technology rather complicated and limited in its use. Nevertheless, commercial systems are available and if the requests by researchers rise, it is expected that suppliers will respond with developments towards greater ease of use.

E. Flow Cytometry

A very powerful approach for analyzing host–fungal interactions is flow cytometry. Here, rather than imaging a few cells or fungal elements with very high resolution, many cells (typically many thousands) are imaged in a short time with only limited resolution. Flow cytometry uses a constant flow of liquid droplets to enclose individual cells or cell pairs. These cell-containing droplets subsequently cross one or more laser beams. By hitting intra-and extracellular structures, the laser light is diffracted and scattered light is produced. Light that is diffracted on the surface of the spherical particles is then detected by a spectral detector in a 180° angle relative to the excitation beam (forward scatter, FSC). The diffraction angle allows the calculation of the cell size so that this detector (i) counts all cells that pass the detection optics and (ii) assesses their individual relative size. A second detector is located (side scatter, SSC) at a 90° angle. The amount of light detected here directly correlates with the amount of intracellular structures (granules) of a cell or cell–cell pair.

In addition, cells can be labeled with a variety of fluorescent agents that bind to their surface or intracellularly. If, for example, a fluorescently labeled monoclonal antibody is used

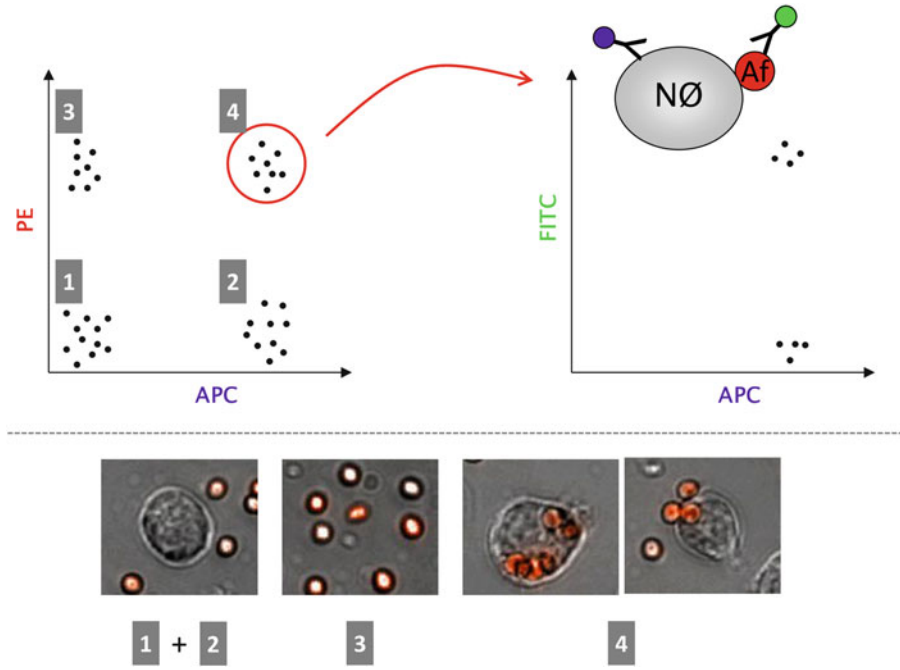


Fig. 8.3. Quantification of phagocytic events by flow cytometry. Example of how phagocytic events can be analyzed by a flow cytometric approach. The background of this experiment were mice that had been infected intratracheally with genetically modified fungal spores, expressing the red fluorescent protein tdTomato. After an incubation time of several hours, the immunological infiltrate was washed out of the lung by a bronchoalveolar lavage followed by monoclonal antibody staining specific for neutrophil granulocytes (clone 1A8 coupled to the fluorescent dye allophycocyanine, APC). After flow cytometric analysis, four clearly distinguishable cell populations are visible by plotting the APC fluorescence versus the tdTomato signal detected in the PE channel (see *left* part of Fig. 8.1): Population *1* constitutes cells that are not neutrophils and that are not co-localized with any fungal spores. Cell population *2* consists of neutrophils that are also not in contact with the fungal particles. The majority of population *3* is most likely composed of free fungal spores but also other (immune) cells that are in contact with the spores are found here. Cell population *4* is the group of interest, where many neutrophils are found that have internalized at least one or more spores. These cells are double-positive for both APC as well as the tdTomato signal. However, especially for neutrophils, it is well known that these cells, besides phagocytosing fungal morphotypes, also attach these entities to their surface, thereby collecting a large number of them. This complex is of course also double-positive for both types of fluorescence and hence generates a potential source of error for this quantitative approach. One way to eliminate false positive events is to add a second antibody (labeled with the green fluorophore fluorescein isothiocyanate, FITC) specific for the fungal spores (AF). Neutrophils (Nϕ) that have spores attached to their surface are then additionally stained green so that these events can be excluded from the analysis. Please note that the image only shows theoretical flow cytometry data for reasons of simplicity. Real dot plots contain many hundreds to thousands of dots

that can bind to a specific surface structure of a cell, this cell type can be selectively labeled in a heterogeneous cell suspension (Fig. 8.3). **By running many thousands of cells through the flow cytometer and counting all labeled cells, the relative number of these cells in a mixture can be easily obtained.** Introduced as principal technology in 1969 (Hulett et al. 1969), flow cytometry has revolutionized many scientific fields, especially immunology. Importantly, **in addition to imaging cells, flow cytometry can also be used to physically sort them according to predetermined parameters of scatter and fluorescence** (fluorescence activated cell sorting, FACS). There are many applications in the field of antifungal immunology that can benefit from the technique, e.g., measuring the genomic response of sorted epithelial cells after

phagocytosis of *Aspergillus* spores (Gomez et al. 2010) or quantifying the organ-specific immune response after invasive candidiasis (Lionakis et al. 2011), the response of mast cells towards the fungal cell wall component zymosan (Yang and Marshall 2009), or the uptake and killing of conidia by diverse phagocytyes (Jhingran et al. 2012).

In general, any approach that allows the fluorescent labeling of fungal elements and immune cells separately, in vitro, within true infection models, or even from patient samples, is able to be analyzed on a large scale by flow cytometry.

A critical limitation of flow cytometry is that it is not able to show individual events directly. Instead they are transformed into dot plots or histograms as output graphs. But, if for example, phagocytosis is the investigated process, flow cytometry is hardly able to distinguish whether co-associated fungal elements and immune cells are only attached to each other's surfaces or whether a phagocyte has entirely internalized the pathogen. A novel invention called **imaging flow cytometry** has changed this. Here, in principle, the same hardware setup as inside a flow cytometer is used. However, rather than just recording the total fluorescence of an event passing the laser beams, it is photographed by a fast, multicolor camera (Barteneva et al. 2012). At first glance the resulting data look like conventional flow cytometry plots but they allow **a detailed look at each single dot of a dot plot as a photographic image**. Here, phagocytosis can easily be distinguished from simple attachment. This technology has been used to show that treatment of mice with the drug sulfasalazine during pulmonary pneumocystosis enhances fungal uptake by alveolar macrophages and leads to more rapid clearing of the fungal infection (Wang et al. 2010). Thus flow cytometry, with its ability to rapidly scan and/or sort cells in large numbers, and imaging flow cytometry are two very potent imaging technologies, both in experimental as well as clinical mycology.

However, although important observations can be made in a reduced environmental complexity in vitro, one has to keep in mind that an in vitro situation never fully represents the in vivo environment of a living organism. Therefore, it is always important to think about experimental approaches that allow the in vivo observation of the investigated phenomena. This is more complicated to establish and often limited in its application possibilities but the results obtained from intravital imaging experiments deliver more meaningful results. In fact, due to the increasing availability of in vivo imaging techniques, observations made purely in vitro are nowadays often considered as potentially suffering from artifact. As a result, researchers wishing to publish work on dynamic cellular processes today are often asked to verify their in vitro data in an in vivo setup. To help find the right approach and judge the feasibility of a reviewer's comment, we will now discuss different systems that can generate intravital imaging data.

F. Intravital 2-Photon Microscopy

The principal construction of an intravital 2-photon (2-P) microscope is similar to the CLSM, as discussed above. Thus, a laser beam is scanned over the sample and the generated fluorescence is detected by PMTs and reconstructed into a full multicolor picture (Niesner et al. 2008a; Niesner et al. 2007). However, the difference lies in the use of the laser and the physics behind the generation of the fluorescent signal. For the generation of fluorescence, a fluorophore needs to absorb an incoming photon that has the correct wavelength so that the delocalized electrons of the fluorophore can be lifted from a ground state to an excited state. Upon return to the ground state, the electrons emit a fluorescence photon that is slightly redshifted in relation to the exciting photon. Hence, the color of fluorescence is slightly redder than the color of excitation. Typical fluorophores need excitation wavelengths in the visible spectrum. However, the principal problem of visible light photons is that they cannot penetrate very deeply into thick tissue due to massive diffraction. As a result, intravital microscopy with visible light using a conventional CLSM is possible, but does not reach large depths in tissue (Gunzer et al. 2004; Stoll et al. 2002). Importantly, wavelengths between 800 and 1,200 nm (near infrared) have excellent

tissue penetration capabilities and thus would be perfect for imaging in vivo (Konig 2000). However, conventional fluorophores cannot be excited by these photons because they possess too little energy.

This is where the 2-photon process comes into play. If laser radiation of near-infrared color is very intense, one fluorophore can be hit at almost the same time by two or more of these red photons. Then, they combine their energy on the surface of the fluorophore and excite it to emit normal fluorescence. At the same time, this allows the use of the red laser wavelengths that give good tissue penetration. The enormous intensity of laser light required for 2-PM is generated by the use of pulsed rather than continuous wave lasers (as in conventional CLSM). Pulsed lasers (typically titanium–sapphire solid-state lasers) have immense amounts of photons in extremely short-lived pulses (10^{-13} s pulse duration). When these are highly focused by the optics of the 2-PM system, the energy at the focal spot is high enough to allow the 2-P process to occur at a meaningful frequency that enables imaging (Germain et al. 2006). At the same time, the system is inherently confocal as no out-of-focus fluorescence is generated because the extreme level of photon flux required for the excitation of fluorescence is only reached at the center of the focal spot of the optics.

2-PM is the most widely used technology for high-resolution intravital imaging in experimental animals and we have made extensive use of the technology to investigate antifungal immunity (Hasenberg et al. 2011c; Nitschke et al. 2008; Bruns et al. 2010). But, 2-PM offers more than just the ability to image fluorescent structures deep inside scattering tissue. One powerful approach is **fluorescence lifetime imaging (FLIM)**. For FLIM, not only the intensity of fluorescence as such, but also its decay characteristics after a single excitation are measured. This can be nicely employed to study the characteristics of endogenous fluorescent molecules such as NAD(P)H. This important coenzyme has a characteristic 2-P excitation at 760 nm and an emission peak at around 460 nm. Within a cell (e.g. a neutrophil granulocyte), NAD(P)H can exist as the free coenzyme. This has a fluorescence lifetime of ~430 ps. However, there are more than 100 enzymes known that can bind NAD(P)H as a coenzyme, and when bound to a protein the lifetime of NAD(P)H changes quite substantially. We have shown that this feature is able to very specifically detect the NAD(P)H molecules inside of a neutrophil granulocyte because they are bound to NAD(P)H oxidase, the key enzyme for the generation of fungitoxic reactive oxygen species (Niesner et al. 2008b). When bound to the oxidase, the lifetime of NAD(P)H was stretched to 3.6 ns. Spots with this lifetime showed a characteristic enrichment at sites where neutrophils touched fungal elements and could be selectively quenched with the addition of inhibitors of the oxidase (Niesner et al. 2008b). Because there are many more enzymes to which NAD(P)H can bind, it is tempting to speculate that each one shows a characteristic lifetime of the associated NAD(P)H molecule and thus can be identified by FLIM without the need for additional staining. In addition, other endogenous fluorophores (e.g., FAD) exist in cells and are able to be investigated by FLIM. Thus, together with the ability for intravital imaging, 2-PM and FLIM are powerful tools for the investigation of host–fungal interactions in vivo.

G. Whole-Body Imaging

Although being very potent for high-resolution imaging at organ level, 2-PM is also limited in its penetration/detection depth. Depending on the type of tissue, several hundred micrometers can be achieved, but it is not possible to scan through larger organs or even whole intact animals. Thus, it is not possible to use the 2-PM technique for whole-body imaging, e.g., if the position or infectious spread of a fungus is to be monitored inside of an experimental animal. To close this gap, different companies offer so-called whole-body imaging systems for small animal research. The first machines that entered the market used the **detection of bioluminescence signals with very sensitive CCD cameras** as their key function. Bioluminescence makes use of luciferases, which are enzymes

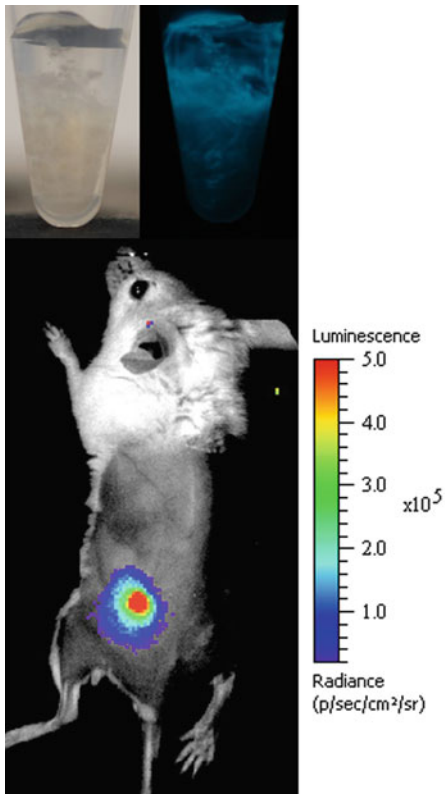


Fig. 8.4. Imaging cutaneous aspergillosis by bioluminescent imaging. In an immunosuppressed mouse infected with 10^4 conidia of a bioluminescent *A. fumigatus* reporter strain that displays the *Gaussia princeps* luciferase on its surface (*top*), localization of the fungal pathogen can be followed in the living animal in a longitudinal fashion. The substrate coelenterazine was applied subcutaneously and emitted photons were detected via a CCD camera to yield a bioluminescence signal that correlates to the fungal burden in the infected tissue (*bottom*)

made by insects, corals, or copepods. By the breakdown of their substrates (e.g., luciferin or coelentaerazine), luciferase is able to generate photons that can be detected with sensitive cameras, even if they are generated deep inside the tissue. The big advantage of this enzyme system lies in the spontaneous release of photons during the chemical reaction without an exciting light source that also would have to pass through the surrounding tissue. Such bioluminescent reporter systems **allow for real-time imaging and longitudinal infection studies, thereby reducing the number of monitored animals and decreasing experimental variability**. However, in contrast to bacterial bioluminescent reporter systems, eukaryotic systems require the addition of an extrinsic substrate. Accordingly, substrate stability, tissue penetration, and uptake by the pathogen are limiting factors for bioluminescence imaging of fungal infections. Based on pioneering studies in the human commensal *Candida albicans* (Doyle et al. 2006; Enjalbert et al. 2009), we have expressed the *Gaussia princeps* copepod luciferase on the surface of *A. fumigatus* to demonstrate that cutaneous aspergillosis is clearly detectable in infected animals (Fig. 8.4) and dependent on the innate immune system for growth control (Donat et al. 2012). Others have shown that pulmonary and systemic infections with *A. fumigatus* can also be monitored by this method when expressing the firefly luciferase from *Photinus pyralis* intracellularly (Brock et al. 2008; Jouvion et al. 2012; Ibrahim-Granet et al. 2010). Meanwhile, such whole-body scanners also make use of the **detection of fluorescence signals**. With the introduction of a variety of red and far-red dyes it became possible to excite these fluorophores from the outside with light of a very long wavelength. This relatively energy-poor light is able to penetrate biological tissue very efficiently, thereby enabling the use of fluorescent probes for this application. Bioluminescent fungal strains interacting in vivo with fluorescently labeled immune cells may therefore provide a deeper understanding of the intimate host–pathogen interplay during infection.

III. Clinical Imaging

Imaging fungal infections is not only a decisive tool for academic research but it is also a central technology for modern clinical routine in wards treating patients at risk of infection. Thus, it is important to consider the available options that help clinicians to clearly identify a fungal infection and, more importantly, to distinguish it from other situations that might cause inflammatory states in patients.

The main arms of clinical imaging are **ultrasound**, **X-ray** (conventional and **computed tomography,** CT), magnetic resonance imaging (**MRI**), and contrasting methods using radioisotopes, especially **PET** and scintigraphy. All of these techniques have been used in fungal diagnosis, even though MRI and CT have become the central methods in routine clinical application.

A. Ultrasound

Ultrasound uses sound waves with intensities above 16 KHz that are radiated from a transducer into the body. They are echoed back from reflecting tissues and bodily liquids or gases with different efficiencies, allowing them to be structurally distinguished upon re-detection in the transducer. Gases and bone are very effective echoing structures, whereas blood and other liquids give poor echoes. Thus, blood and other liquids will give black pixels in an ultrasound image while bone and gases give white pixels, thereby allowing the reconstruction of an irradiated tissue with useful resolution. The advantages of ultrasound are that it is fast, generates tissue sectioning images on the fly, and is without ionizing radiation. Thus, ultrasound is considered harmless. The disadvantages are the relatively low resolution and absence of specific contrasting methods, except Doppler sonography for visualizing flow. Collectively, sonography can be used for diagnosis of fungal infections such as in the urinary tract (Kauffman et al. 2011) or the heart (Ronco et al. 2010), but due to the limitations of the technology ultrasound has not found entry into routine application.

B. X-Ray and Computed Tomography

X-ray uses highly intense photons of wavelengths between 10^{-8} and 10^{-12} m. They can cross bones and tissues easily at full thickness, and upon traveling through different structures are more or less efficiently absorbed. After transfer through the body they hit a detection device (photosensitive plates or CCD cameras in modern digital X-ray systems). Structures with high absorptive potential (especially bones) will give white pixels (because only a few X-ray photons can cross the bone and blacken the photosensitive device), whereas soft tissue typically gives gray to black pixels. A conventional X-ray image is a 2D projection of the 3D tissues crossed by the X-ray beam during its journey through the body. This does not give information on the position of structures in space. Therefore, the more advanced method of CT was developed. Here, a patient is analyzed by X-rays that come from different directions in space to generate a depth-encoded section through the body. For a full CT section, X-rays are projected to from a complete 360° circle around the patient and then step-wise along the body length axis to generate a series of sections. Images are no longer directly generated by a detection device but instead need extensive computing efforts to generate precisely located volume-pixels (voxels). The advantage over conventional X-ray is the much better soft-tissue contrast and the 3D information of the investigated organ.

The disadvantage of X-ray in general and of CT in particular is that they use ionizing radiation and thus constitute a carcinogenic potential. Nevertheless, **the easy tissue penetration and excellent contrasting capabilities have made X-ray and CT the most used methods for the diagnosis of fungal infections, especially in the case of invasive aspergillosis** (Brown et al. 2012). However, despite their frequent use, the specific diagnosis of a fungal infection using X-ray or CT is very difficult and prone to error. Typically, radiologists search for a so-called **halo sign** (HS) or **reverse halo sign** (RHS) (Georgiadou et al. 2011; Marchiori et al. 2012) in the lung of patients with suspected fungal infection. These are optical features within a CT image with a central bright area surrounded by a ring of "ground glass opacity" (HS) or the reverse version of these optical features (RHS). Histopathologically, the ground glass structure represents hemorrhage of the lung tissue, while the bright area corresponds to necrosis of the infected lung tissue (Georgiadou et al. 2011). The problem with this approach is twofold: first, as can

be deduced from the description of the HS/RHS, its unequivocal identification is not easy and requires the inspection of CT images by a well-trained expert with a lot of experience in this field. Second, and maybe even more important, HS and RHS are by no means specific for invasive aspergillosis or a fungal infection in general. Instead, since their publication, identical signatures have been found in CT images associated with bacterial or parasitic infections but also in neoplastic disease or systemic disorders such as Wegener's granulomatosis or sarcoidosis (Georgiadou et al. 2011). Thus, **despite their superb optical contrast in human tissue, neither X-ray nor CT are 100% robust tools for the clinical diagnosis of fungal infections**. Yet, next to MRI (see below) they are the best available imaging technology for this purpose to date and will therefore continue to be used until their replacement by better approaches.

C. Magnetic Resonance Imaging

From its physical background, magnetic resonance imaging (MRI) is a very complicated method, the detailed description of which is far beyond the scope of this article. The interested reader is referred to some excellent reviews on the subject (Moonen et al. 1990; Friston 2009). In short, the technology uses extremely strong external magnetic fields to orient the spin axes of atoms, especially hydrogen atoms, in the body of the patient along the orientation of the magnetic field lines. Then, an additional radio frequency electrical field is induced for a short time, which causes a deflection of the spin axis of the nuclei. The rotation of the nuclei with a deflected spin axis around the general orientation of the external magnetic field (precession) induces an electrical signal whose decay with time can be measured (T2 value). In addition, the spin axis slowly returns to its orientation along the outer magnetic field lines and the precession is lost (relaxation time, T1 value). The relaxation time can also be measured. Importantly, the signal strength and the time to full relaxation is dependent on the environment of the atom and thus allows the extraction of information on the surroundings of where the atoms are measured. Using complicated mathematics, the different measured relaxation times and signal intensities allow a picture of the different surroundings of hydrogen atoms within the body to be drawn. Fat tissue, for example, is very bright, whereas the air-filled lung is almost black. It is possible to enhance the contrast, e.g. of blood vessels, by the application of contrasting agents such as gadolinium or gadolinium-labeled particles.

The general advantage of MRI is the lack of ionizing radiation and thus the method has much lower to absent inherent toxicity as compared to X-ray, CT, or imaging based on radionuclides (see below). In addition, **MRI provides an excellent soft tissue contrast** whereby, e.g., a kidney can be reconstructed with the individual nephrons clearly visible whereas CT images show a kidney without much internal structure. The downside of MRI is the enormous technical effort, meaning that only specialized centers can mount the necessary resources. **MRI is equally potent in detecting pulmonary fungal infections as CT. Ideally, both methods can be combined to provide a more robust diagnosis** (Muller et al. 2002). **The significant power of MRI comes with the diagnosis of cerebral fungal infections** (Mullins 2011) where, depending on the measurement used (T1 or T2 weighted), a significant contrast can be obtained that completely circumscribes the fungal lesion inside the brain (Gabelmann et al. 2007; Brown et al. 2012). Nevertheless, **MRI does not allow the specific identification of a fungal infection based on exactly defined parameters**. Instead, like CT, it requires the eyes of very experienced radiology experts and the diagnosis "fungal infection" is based on their interpretation of the imaging data rather than on a non-disputable independent parameter.

D. Positron Emission Tomography

PET uses the ability of some radionuclides to generate and emit positrons, the antiparticles of electrons. When positrons hit an electron in the vicinity, the two particles are annihilated,

which results in the generation of two gamma quanta that each possess the characteristic energy of 511 keV and are emitted at exactly a 180° angle from their origin of generation. The PET detector is able to measure these gamma quanta and also their relative arrival time at the two opposite positions on a ring-shaped collection of detectors around a patient. By correlating these events, it is possible to reconstruct the exact position of the radionuclide inside of the patient's body, from where the positron originated.

The power of the approach is the ability to locate anything associated with the radionuclide inside of a patient. Thus, **by designing tracer molecules that have the ability to localize at clinically important sites inside of a patient and loading these tracers with PET-suitable radionuclides, it is possible to exactly position the radionuclides at this site and therefore to clearly diagnose the presence of this structure inside of the patient.** A typical example used extensively in oncology is FDG. Glucose enriches at sites of heavy cellular metabolism, which are particularly frequent in fast growing tumors. Thus, a patient suffering from a metastasized tumor will enrich FDG both in the primary tumor and also in the metastases, allowing their localization via PET. This principle has led to the widespread use of FDG-PET in oncology (Jones and Price 2012). Despite its success in that field, PET imaging has not been used extensively so far in infection diagnosis and less so in fungal diagnosis (Walker et al. 2007), although it is being discussed (Limper et al. 2011). Interestingly, infection can also compromise oncological PET imaging because a compound such as FDG is not able to distinguish increased metabolic activity due to cancer growth or due to locally enriched immune cells fighting an infectious focus (Sandherr et al. 2001).

Thus, currently there is no tool available in the clinic that allows the specific labeling of fungal elements in a patient's body with a PET tracer and so allow the unequivocal detection of the infection. However, experimental approaches have demonstrated the principal ability of PET to specifically detect fungal infections using a ^{68}Ga-labeled siderophore (Petrik et al. 2012; 2010). Other approaches make use of the intrinsic ability of immune cells to localize places of fungal infection in the body (Bruns et al. 2010) by loading leukocytes with PET tracers and applying them as infection-specific agents. However, the success of this approach has been limited and it has not found widespread application in the clinical routine (Gardet et al. 2010; Dumarey et al. 2006). Other approaches use the radiolabeling of anti-granulocyte antibodies to locate infections in humans, which also sounds promising but is not widely used (Graute et al. 2010). All these approaches would, however, also experience analogous limitations in terms of specificity compared to FDG-PET.

Despite having the ability for highly specific labeling of infectious foci inside of a patient with optimized tracers, PET suffers from limitations in resolution. This is why **modern PET always comes as a combined modality such as PET/CT or, more recently, PET/MRI** (Pichler et al. 2008; Judenhofer et al. 2008). Here, the superb resolution of imaging with CT or MRI is combined with the molecular information yielded from PET. This has proven successful in a clinical study, which also showed the ability of FDG-PET to follow up on the therapeutic success of antifungal therapy (Hot et al. 2011). Thus, **in principle, PET in combination with a high-resolution imaging modality has enormous power to be used as a specific imaging approach for the rapid, non-invasive and definitive diagnosis of fungal infections.**

Next to these obvious advantages, PET suffers from a number of technical drawbacks. First, it uses radioactive tracers that have the intrinsic capability to induce cancer. Next, the need to handle potentially short-lived radioisotopes requires their production on site in expensive cyclotrons. Finally, it requires extensive experience in a clinical center with nuclear medicine and in the management of patients under these regimes. Thus, in the near future any such applications will only be possible in large dedicated centers. Nevertheless, the potential of the technology should continue to be harvested by driving the development of novel molecular tracers that can specifically identify fungal elements throughout the

Table 8.1. Comparison of imaging technologies discussed in this chapter

	Experimental imaging									Clinical imaging				
	Widefield microscopy	CLSM	Electron microscopy	STED	SIM	Localization microscopy	Flow cytometry	Intravital 2-PM	Whole-body imaging	Ultrasound	X-ray and CT	MRI	PET	Combined PET/MRI
Purchase considerations														
Purchase Costs	●●●	●●	●●	●●	●●	●●	●●	●●	●●●	●●●	●	●	●	●
Running Costs	●●●	●●	●●	●●	●●	●●	●●	●●	●●●	●●●	●	●	●	●
System size	●●●	●●	●●	●●	●●	●●	●●●	●●	●●●	●●●	●	●	●	●
Requirements on Installation Site	●●●	●●	●●●	●●	●●	●●	●●●	●●	●●●	●●●	●	●	●	●
Application considerations														
Ease of use	●●●	●●●	●●	●●	●●●	●●	●●	●●	●●●	●●	●	●	●	●
Intravital Application	●	●●	n.a.	●	n.a.	n.a.	n.a.	●●	●●●	●●●	●●●	●●●	●●●	●●●
Flexibility regarding animal type	●●	●●	n.a.	●●	n.a.	n.a.	n.a.	●●	●●	●●●	●●●	●●●	●●●	●●●
In vitro application	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●	n.a.	n.a.	n.a.	●	n.a.
Live cell Observation	●●●	●●	n.a.	●●	●●	n.a.	●●●	●●	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
3D information	●	●●●	●●	●●●	●●●	●	●	●●●	●	●●	n.a./ ●●●	●●●	●	●●●
Biomarker Detection (sensitivity)	●●	●●	●	●●	●●	●●	●●●	●●	●	●	n.a.	●	●●	●●
Parameter considerations														
Optical resolution	●●	●●	●●●+	●●●	●●	●●●	●	●●	●	●	●	●●	●	●●
3D sectioning	●	●●●	●●	●●●	●●●	●	●	●●	●	●●	n.a./ ●●●	●●●	●	●●●
Time resolution	●●●	●●	n.a.	●●	●●	n.a.	●●●	●●	●●	●●●	n.a./●●	●●	●	●
Data size	●●	●●	●●	●●	●●	●●	●●●	●●	●●●	●●●	●	●	●	●

n.a. non-applicable, ● poor, ●● good, ●●● optimal, + indicates even better

patient's body. A particularly well-suited tool for this would be monoclonal antibodies, which have already shown their potential for the specific detection of fungal infections in experimental systems. Monoclonal antibodies would not only allow the unequivocal diagnosis of the disease using PET but would also be able to highly selectively deliver aggressive ionizing radiation to the fungus as a treatment (Bryan et al. 2012).

IV. Summary and Outlook

In summary, we have given a general overview of existing imaging technologies that can be used efficiently to show fungal pathogens alone or during their interaction with the host immune system (see Table 8.1). The approaches have been used widely in experimental systems and clinically. Future work should exploit the capabilities of novel high-resolution imaging regimes in studying host–fungal interaction in experimental systems. Also, improvements in whole-body intravital microscopy to give better tissue penetration and resolution are highly desirable. **Clinical imaging currently suffers from a dearth in specific tools that allow the unequivocal identification of a fungal infection. Thus, research in this field should aim at developing useful novel molecular tracers.** With a number of exciting concepts at hand, some of which we have briefly mentioned here, we expect a bright future ahead of us for the imaging of fungal infections, both in experimental and clinical systems. Novel developments will then hopefully not only quench our academic thirst but also help patients suffering from life-threatening infections to obtain fast and specific treatment.

References

Barteneva NS, Fasler-Kan E, Vorobjev IA (2012) Imaging flow cytometry: coping with heterogeneity in biological systems. J Histochem Cytochem 60:723–733

Behnsen J, Narang P, Hasenberg M, Gunzer F, Bilitewski U, Klippel N, Rohde M, Brock M, Brakhage AA, Gunzer M (2007) Environmental dimensionality controls the interaction of phagocytes with the pathogenic fungi *Aspergillus fumigatus* and *Candida albicans*. PLoS Pathog 3:e13

Brock M, Jouvion G, Droin-Bergere S, Dussurget O, Nicola MA, Ibrahim-Granet O (2008) Bioluminescent Aspergillus fumigatus, a new tool for drug efficiency testing and in vivo monitoring of invasive aspergillosis. Appl Environ Microbiol 74:7023–7035

Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, White TC (2012) Hidden killers: human fungal infections. Sci Transl Med 4:165rv13

Bruns S, Kniemeyer O, Hasenberg M, Aimanianda V, Nietzsche S, Thywißen A, Jeron A, Latge JP, Brakhage AA, Gunzer M (2010) Production of extracellular traps against *Aspergillus fumigatus in vitro* and in infected lung tissue is dependent on invading neutrophils and influenced by Hydrophobin RodA. PLoS Pathog 6:e1000873

Bryan RA, Guimaraes AJ, Hopcraft S, Jiang Z, Bonilla K, Morgenstern A, Bruchertseifer F, Del PM, Torosantucci A, Cassone A, Nosanchuk JD, Casadevall A, Dadachova E (2012) Toward developing a universal treatment for fungal disease using radioimmunotherapy targeting common fungal antigens. Mycopathologia 173:463–471

Coltharp C, Xiao J (2012) Superresolution microscopy for microbiology. Cell Microbiol 14:1808–1818

Denning DW, Hope WW (2010) Therapy for fungal diseases: opportunities and priorities. Trends Microbiol 18:195–204

Donat S, Hasenberg M, Schafer T, Ohlsen K, Gunzer M, Einsele H, Löffler J, Beilhack A, Krappmann S (2012) Surface display of Gaussia princeps luciferase allows sensitive fungal pathogen detection during cutaneous aspergillosis. Virulence 3:51–61

Doyle TC, Nawotka KA, Kawahara CB, Francis KP, Contag PR (2006) Visualizing fungal infections in living mice using bioluminescent pathogenic Candida albicans strains transformed with the firefly luciferase gene. Microb Pathog 40:82–90

Dumarey N, Egrise D, Blocklet D, Stallenberg B, Remmelink M, Den MV, Van Simaeys G, Jacobs F, Goldman S (2006) Imaging infection with 18F-FDG-labeled leukocyte PET/CT: initial experience in 21 patients. J Nucl Med 47:625–632

Eggeling C, Ringemann C, Medda R, Schwarzmann G, Sandhoff K, Polyakova S, Belov VN, Hein B, von Middendorff C, Schonle A, Hell SW (2009) Direct observation of the nanoscale dynamics of membrane lipids in a living cell. Nature 457:1159–1162

Enjalbert B, Rachini A, Vediyappan G, Pietrella D, Spaccapelo R, Vecchiarelli A, Brown AJ, d'Enfert C (2009) A multifunctional, synthetic Gaussia princeps luciferase reporter for live imaging of Candida albicans infections. Infect Immun 77:4847–4858

Fiolka R, Shao L, Rego EH, Davidson MW, Gustafsson MG (2012) Time-lapse two-color 3D imaging of live cells with doubled resolution using structured illumination. Proc Natl Acad Sci USA 109:5311–5315

Freifeld AG, Bow EJ, Sepkowitz KA, Boeckh MJ, Ito JI, Mullen CA, Raad II, Rolston KV, Young JA, Wingard JR, Infectious Diseases Society of America (2011) Clinical practice guideline for the use of antimicrobial agents in neutropenic patients with cancer: 2010 update by the Infectious Diseases Society of America. Clin Infect Dis 52:427–431

Friston KJ (2009) Modalities, modes, and models in functional neuroimaging. Science 326:399–403

Gabelmann A, Klein S, Kern W, Kruger S, Brambs HJ, Rieber-Brambs A, Pauls S (2007) Relevant imaging findings of cerebral aspergillosis on MRI: a retrospective case-based study in immunocompromised patients. Eur J Neurol 14:548–555

Gardet E, Addas R, Monteil J, Le GA (2010) Comparison of detection of F-18 fluorodeoxyglucose positron emission tomography and 99mTc-hexamethylpropylene amine oxime labelled leukocyte scintigraphy for an aortic graft infection. Interact Cardiovasc Thorac Surg 10:142–143

Georgiadou SP, Sipsas NV, Marom EM, Kontoyiannis DP (2011) The diagnostic value of halo and reversed halo signs for invasive mold infections in compromised hosts. Clin Infect Dis 52:1144–1155

Germain RN, Miller MJ, Dustin ML, Nussenzweig MC (2006) Dynamic imaging of the immune system: progress, pitfalls and promise. Nat Rev Immunol 6:497–507

Gomez P, Hackett TL, Moore MM, Knight DA, Tebbutt SJ (2010) Functional genomics of human bronchial epithelial cells directly interacting with conidia of Aspergillus fumigatus. BMC Genomics 11:358

Graute V, Feist M, Lehner S, Haug A, Muller PE, Bartenstein P, Hacker M (2010) Detection of low-grade prosthetic joint infections using 99mTc-antigranulocyte SPECT/CT: initial clinical results. Eur J Nucl Med Mol Imaging 37:1751–1759

Gunzer M, Weishaupt C, Hillmer A, Basoglu Y, Friedl P, Dittmar KE, Kolanus W, Varga G, Grabbe S (2004) A spectrum of biophysical interaction modes between T cells and different antigen presenting cells during priming in 3-D collagen and in vivo. Blood 104:2801–2809

Gustafsson MG (2005) Nonlinear structured-illumination microscopy: wide-field fluorescence imaging with theoretically unlimited resolution. Proc Natl Acad Sci USA 102:13081–13086

Hasenberg M, Behnsen J, Krappmann S, Brakhage A, Gunzer M (2011a) Phagocyte responses towards Aspergillus fumigatus. Int J Med Microbiol 301:436–444

Hasenberg M, Köhler A, Bonifatius S, Borucki K, Riek-Burchardt M, Achilles J, Männ L, Baumgart K, Schraven B, Gunzer M (2011b) Rapid immunomagnetic negative enrichment of neutrophil granulocytes from murine bone marrow for functional studies in vitro and in vivo. PLoS One 6:e17314

Hasenberg M, Köhler A, Bonifatius S, Jeron A, Gunzer M (2011c) Direct observation of phagocytosis and NET-formation by neutrophils in infected lungs using 2-photon microscopy. J Vis Exp 52:e2659. doi:10.3791/2659

Hasenberg M, Stegemann-Koniszewski S, Gunzer M (2013) Cellular immune reactions in the lung. Immunol Rev 251:189–214

Hell SW (2007) Far-field optical nanoscopy. Science 316:1153–1158

Hell SW (2009) Microscopy and its focal switch. Nat Methods 6:24–32

Hohmann-Marriott MF, Uchida M, van de Meene AM, Garret M, Hjelm BE, Kokoori S, Roberson RW (2006) Application of electron tomography to fungal ultrastructure studies. New Phytol 172:208–220

Hot A, Maunoury C, Poiree S, Lanternier F, Viard JP, Loulergue P, Coignard H, Bougnoux ME, Suarez F, Rubio MT, Mahlaoui N, Dupont B, Lecuit M, Faraggi M, Lortholary O (2011) Diagnostic contribution of positron emission tomography with [18 F] fluorodeoxyglucose for invasive fungal infections. Clin Microbiol Infect 17:409–417

Hulett HR, Bonner WA, Barrett J, Herzenberg LA (1969) Cell sorting: automated separation of mammalian cells as a function of intracellular fluorescence. Science 166:747–749

Ibrahim-Granet O, Philippe B, Boleti H, Boisvieux-Ulrich E, Grenet D, Stern M, Latge JP (2003) Phagocytosis and intracellular fate of *Aspergillus fumigatus* conidia in alveolar macrophages. Infect Immun 71:891–903

Ibrahim-Granet O, Jouvion G, Hohl TM, Droin-Bergere S, Philippart F, Kim OY, Adib-Conquy M, Schwendener R, Cavaillon JM, Brock M (2010) In vivo bioluminescence imaging and histopathopathologic analysis reveal distinct roles for resident and recruited immune effector cells in defense against invasive aspergillosis. BMC Microbiol 10:105

Jhingran A, Mar KB, Kumasaka DK, Knoblaugh SE, Ngo LY, Segal BH, Iwakura Y, Lowell CA, Hamerman JA, Lin X, Hohl TM (2012) Tracing conidial fate and measuring host cell antifungal activity using a reporter of microbial viability in the lung. Cell Rep 2:1762–1773

Jones T, Price P (2012) Development and experimental medicine applications of PET in oncology: a historical perspective. Lancet Oncol 13:e116–e125

Jouvion G, Brock M, Droin-Bergere S, Ibrahim-Granet O (2012) Duality of liver and kidney lesions after systemic infection of immunosuppressed and immunocompetent mice with Aspergillus fumigatus. Virulence 3:43–50

Judenhofer MS, Wehrl HF, Newport DF, Catana C, Siegel SB, Becker M, Thielscher A, Kneilling M, Lichy MP, Eichner M, Klingel K, Reischl G, Widmaier S, Rocken M, Nutt RE, Machulla HJ, Uludag K, Cherry SR, Claussen CD, Pichler BJ (2008) Simultaneous PET-MRI: a new approach for functional and morphological imaging. Nat Med 14:459–465

Kauffman CA, Fisher JF, Sobel JD, Newman CA (2011) Candida urinary tract infections–diagnosis. Clin Infect Dis 52(Suppl 6):S452–S456

Konig K (2000) Multiphoton microscopy in life sciences. J Microsc 200(Pt 2):83–104

Koning RI, Koster AJ (2009) Cryo-electron tomography in biology and medicine. Ann Anat 191:427–445

Latge JP (1999) *Aspergillus fumigatus* and aspergillosis. Clin Microbiol Rev 12:310–350

Limper AH, Knox KS, Sarosi GA, Ampel NM, Bennett JE, Catanzaro A, Davies SF, Dismukes WE, Hage CA, Marr KA, Mody CH, Perfect JR, Stevens DA (2011) An official American thoracic society statement: treatment of fungal infections in adult pulmonary and critical care patients. Am J Respir Crit Care Med 183:96–128

Lionakis MS, Lim JK, Lee CC, Murphy PM (2011) Organ-specific innate immune responses in a mouse model of invasive candidiasis. J Innate Immun 3:180–199

Marchiori E, Zanetti G, Hochhegger B, Irion KL, Carvalho AC, Godoy MC (2012) Reversed halo sign on computed tomography: state-of-the-art review. Lung 190:389–394

Mech F, Thywissen A, Guthke R, Brakhage AA, Figge MT (2011) Automated image analysis of the host-pathogen interaction between phagocytes and Aspergillus fumigatus. PLoS One 6:e19591

Meyer JC, Girit CO, Crommie MF, Zettl A (2008) Imaging and dynamics of light atoms and molecules on graphene. Nature 454:319–322

Michelio PA (1729) Nova plantarum genera iuxta tournefortii methodum disposita. Bernardo Paperini, Florence

Moonen CT, van Zijl PC, Frank JA, Le BD, Becker ED (1990) Functional magnetic resonance imaging in medicine and physiology. Science 250:53–61

Muller FM, Trusen A, Weig M (2002) Clinical manifestations and diagnosis of invasive aspergillosis in immunocompromised children. Eur J Pediatr 161:563–574

Mullins ME (2011) Emergent neuroimaging of intracranial infection/inflammation. Radiol Clin North Am 49:47–62

Niesner RA, Andresen V, Neumann J, Spiecker H, Gunzer M (2007) The power of single- and multibeam 2-photon microscopy for high-resolution and high-speed deep tissue and intravital imaging. Biophys J 93:2519–2529

Niesner RA, Andresen V, Gunzer M (2008a) Intravital 2-photon microscopy – focus on speed and time resolved imaging modalities. Immunol Rev 221:7–25

Niesner RA, Narang P, Spiecker H, Andresen V, Gericke KH, Gunzer M (2008b) Selective detection of NADPH oxidase in polymorphonuclear cells by means of NAD(P)H-based fluorescence lifetime imaging. J Biophys 2008: 602639

Nitschke C, Garin A, Kosco-Vilbois M, Gunzer M (2008) 3-D and 4-D imaging of immune cells in vitro and in vivo. Histochem Cell Biol 130:1053–1062

Peleg AY, Hogan DA, Mylonakis E (2010) Medically important bacterial-fungal interactions. Nat Rev Microbiol 8:340–349

Petrik M, Haas H, Dobrozemsky G, Lass-Flörl C, Helbok A, Blatzer M, Dietrich H, Decristoforo C (2010) 68 Ga-siderophores for PET imaging of invasive pulmonary aspergillosis: proof of principle. J Nucl Med 51:639–645

Petrik M, Franssen GM, Haas H, Laverman P, Hortnagl C, Schrettl M, Helbok A, Lass-Flörl C, Decristoforo C (2012) Preclinical evaluation of two (68)Ga-siderophores as potential radiopharmaceuticals for Aspergillus fumigatus infection imaging. Eur J Nucl Med Mol Imaging 39:1175–1183

Pichler BJ, Judenhofer MS, Pfannenberg C (2008) Multimodal imaging approaches: PET/CT and PET/MRI. Handb Exp Pharmacol 185:109–132

Romani L (2011) Immunity to fungal infections. Nat Rev Immunol 11:275–288

Ronco F, Simsir S, Czer L, Luo H, Siegel RJ (2010) Incidental finding by two-dimensional echocardiography of a mycotic pseudoaneurysm of the ascending aorta after orthotopic heart transplantation. J Am Soc Echocardiogr 23:580–583

Sandherr M, von Schilling C, Link T, Stock K, von Bubnoff N, Peschel C, Avril N (2001) Pitfalls in imaging Hodgkin's disease with computed tomography and positron emission tomography using fluorine-18-fluorodeoxyglucose. Ann Oncol 12:719–722

Sasse C, Hasenberg M, Weyler M, Gunzer M, Morschhäuser J (2013) White-opaque switching of Candida albicans allows immune evasion in an environment-dependent fashion. Eukaryotic Cell, 12(1):50–58

Schermelleh L, Carlton PM, Haase S, Shao L, Winoto L, Kner P, Burke B, Cardoso MC, Agard DA, Gustafsson MG, Leonhardt H, Sedat JW (2008) Subdiffraction multicolor imaging of the nuclear periphery with 3D structured illumination microscopy. Science 320:1332–1336

Schmidt A (1998) Gerog Fresenius and the species Aspergillus fumigatus. Mycoses 41(Suppl 2):89–91

Seshadri N, Kaur B, Balan K (2007) Disseminated cryptococcosis: detection by F-18 FDG PET. Clin Nucl Med 32:476–478

Shrikanthan S, Aydin A, Dhurairaj T, Alavi A, Zhuang H (2005) Intense esophageal FDG activity caused by Candida infection obscured the concurrent primary esophageal cancer on PET imaging. Clin Nucl Med 30:695–697

Steinberg G (2007) Hyphal growth: a tale of motors, lipids, and the Spitzenkorper. Eukaryot Cell 6:351–360

Stoll S, Delon J, Brotz TM, Germain RN (2002) Dynamic imaging of T cell-dendritic cell interactions in lymph nodes. Science 296:1873–1876

Vicidomini G, Moneron G, Han KY, Westphal V, Ta H, Reuss M, Engelhardt J, Eggeling C, Hell SW (2011) Sharper low-power STED nanoscopy by time gating. Nat Methods 8:571–573

Waibler Z, Sender LY, Merten C, Hartig R, Kliche S, Gunzer M, Reichardt P, Kalinke U, Schraven B (2008) Signaling signatures and functional

properties of anti-human CD28 superagonistic antibodies. PLoS One 3:e1708

Walker RC, Jones-Jackson LB, Martin W, Habibian MR, Delbeke D (2007) New imaging tools for the diagnosis of infection. Future Microbiol 2:527–554

Wang J, Gigliotti F, Bhagwat SP, George TC, Wright TW (2010) Immune modulation with sulfasalazine attenuates immunopathogenesis but enhances macrophage-mediated fungal clearance during Pneumocystis pneumonia. PLoS Pathog 6: e1001058

Yang Z, Marshall JS (2009) Zymosan treatment of mouse mast cells enhances dectin-1 expression and induces dectin-1-dependent reactive oxygen species (ROS) generation. Immunobiology 214:321–330

9 Mucosal Immunology in *Candida albicans* Infection

Günther Weindl[1], Julian R. Naglik[2], David L. Moyes[2], Martin Schaller[3]

CONTENTS

I. Introduction

The mucosal epithelium has immense importance in host defence and immune surveillance because it is the primary cell layer that initially encounters the majority of microorganisms. The most important ability of the immune system is to discriminate between friend and foe, a property that is essential for maintaining immune homeostasis. This specialized interaction will result in either passive coexistence between microbe and host, as in the case of commensal microbes, or in a violation of the mucosal barrier and subsequent cell injury, as in the case of microbial pathogens. The cells that comprise the innate immune response are primarily phagocytes, including neutrophils and macrophages, and the cells that line the epithelial mucosa. Originally, it was thought that the epithelium serves only as a passive barrier against invading pathogens. Barrier function alone is usually adequate to restrain commensal microbes, but is often insufficient to protect against microbial pathogens. **However, recently it has become apparent that epithelial cells are capable of triggering an immune response similar to cells of the myeloid lineage, thus playing a crucial role in the active recognition of microbes.** Accordingly, the oral epithelium is able to secrete a variety of defence effector molecules (Diamond et al. 2008) and to orchestrate an immune inflammatory response to activate myeloid cells in the submucosal layers to clear the invading pathogens (Cutler and Jotwani 2006).

Recognition of *Candida albicans* (*C. albicans*) by the innate host defence system is mediated by pattern-recognition receptors (PRRs) from the Toll-like receptor (TLR), C-type lectin receptor (CLR) and NOD-like receptor (NLR) families (Bryant and Fitzgerald 2009; Netea et al. 2008; Roeder et al. 2004). To date, most investigations have focused on the interaction of *C. albicans* with macrophages and on systemic infections. At present we understand little about how the oral mucosa regulates itself in the context of fungal infections, although recent studies have progressed our understanding of pathogen recognition and signalling mechanisms in oral mucosa and epithelial cells. This chapter will discuss

[1]Institute of Pharmacy (Pharmacology and Toxicology), Freie Universität Berlin, 14195 Berlin, Germany
[2]Department of Oral Immunology, Clinical and Diagnostic Sciences, King's College London Dental Institute, King's College London, SE1 9RT London, UK
[3]Department of Dermatology, Eberhard Karls University Tübingen, 72076 Tübingen, Germany; e-mail: martin.schaller@med.uni-tuebingen.de

Human Fungal Pathogens, 2nd Edition
The Mycota XII
O. Kurzai (Ed.)

recent advances in our understanding of immune responses against *C. albicans* in the oral mucosa.

II. *C. albicans* and Mucosal Surfaces

The frequency of mucosal and cutaneous fungal infections is increasing worldwide, with oral candidiasis being the most common human fungal infection especially in early and later life (Samaranayake et al. 2009). Oral candidiasis is a common opportunistic infection of the oral cavity and presents a challenge for immunologically competent and immunodeficient patients alike. Various clinical presentations are traditionally divided into **acute** and **chronic forms**. Acute pseudomembranous candidiasis (mucosal candidiasis, oral thrush) presents with stippled (later confluent) white plaques that can be wiped off, on bright red and lightly bleeding mucosa (Fig. 9.1). Chronic atrophic candidiasis (denture-related stomatitis) is associated with erythema and oedema of the oral mucosa, often found on the fitting surfaces of dentures. Also belonging to the group of oral candidiasis are perlèche, candidal leukoplakia (chronic hyperplastic candidiasis), angular cheilitis and chronic mucocutaneous candidiasis (CMC), a rare infection that is associated with immune deficiency. Life-threatening systemic infection is generally limited to severely immunocompromised patients, such as neutropenic patients, often after nosocomial infection. The number of invasive fungal infections as a proportion of all nosocomial infections doubled during a 10-year period in the USA and the UK (Beck-Sague and Jarvis 1993; Lamagni et al. 2001). In immunocompetent patients, predisposing factors are responsible for infection or even chronic recurrent mucocutaneous candidiasis. **Oropharyngeal and vaginal infections** are the most common manifestation; predisposing factors include antibiotic, glucocorticosteroid and hormone therapies, as well as diabetes mellitus and infections such as HIV and AIDS. Around 80% of all fungal infections are caused by *Candida*, typically *C. albicans* (Ruhnke 2006). However, non-*C. albicans* spp., such as *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, *Candida guilliermondii* and *Candida krusei* are also pathogenic to humans and have emerged as important opportunistic pathogens in the oral mucosa (Li et al. 2007; Samaranayake et al. 2009).

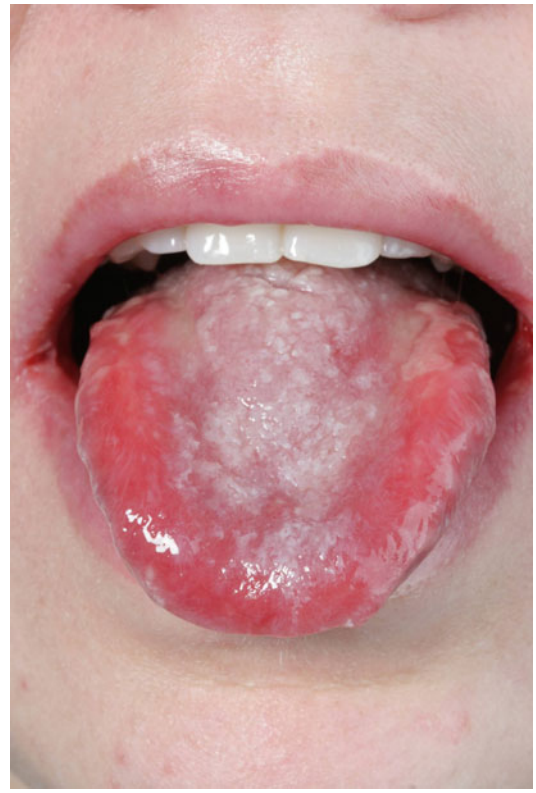


Fig. 9.1. Clinical presentation of oral *C. albicans* infections with erythematous patches and *white* pseudomembranes on the tongue of a patient with chronic mucocutaneous candidosis

C. albicans interacts with epithelial cells via the processes of adherence, invasion and induction of cell damage (Naglik et al. 2011). Virulence factors are crucial in determining the role of opportunistic pathogens in infections. Important virulence factors expressed by *C. albicans* include dimorphism, the ability to grow in either yeast or filamentous form, adhesions factors, phenotypic switching, thigmotropism (ability to identify intercellular junctions at the mucosal surface by contact sensing and their targeted penetration) and secretion of hydrolytic enzymes such as lipase, phospholipase and proteinase (reviewed in Calderone and Fonzi 2001; Hube and Naglik 2001; Schaller et al. 2005). The interaction between virulence factors of *C. albicans* and host defence mechanisms plays a central role in

determining whether colonization remains harmless or leads to infection of the epithelium and, possibly, systemic infection.

III. Epithelial Recognition and Signalling Detection Mechanisms

The innate immune system recognizes conserved pathogen associated molecular patterns (PAMPs), which represent broad groups of microbial species rather than a single specific species, through germline-encoded proteins, such as PRRs (Janeway and Medzhitov 2002). Recognition of *C. albicans* by the innate host defence system is mediated by PRRs of the TLR, CLR and nucleotide-binding domain, leucine-rich repeat (NLR) protein families (Franchi et al. 2010; Gauglitz et al. 2012; Naglik and Moyes 2011; Netea et al. 2008; Roeder et al. 2004; Weindl et al. 2010; Willment and Brown 2008). **PRRs are expressed by various cells in the oral mucosa, including polymorphonuclear leukocytes (PMNs), dendritic cells (DCs), monocytes, macrophages, B cells, T cells and epithelial cells.** Activation of PRRs leads to downstream signalling through pathways that induce transcription factors such as nuclear factor (NF)-κB, followed by enhanced transcription of antimicrobial peptides, cytokines, chemokines and co-stimulatory molecules. As such, **PRRs are critical mediators between innate and adaptive immune responses**. The relative contribution of specific PAMPs and their corresponding receptors in the oral mucosa remains to be elucidated in more detail.

A. Toll-Like Receptors in Epithelial Recognition of *C. albicans*

To date, most data regarding PRRs in the oral mucosa are available for TLRs. TLRs are a family of evolutionarily conserved receptors that react to bacterial, viral or fungal antigens or to endogenous factors released during cell injury. The capacity to recognize a variety of common microbial antigens and endogenous factors indicates that a primary function of TLRs is to act as sentinel receptors to alert the innate immune system to infection or tissue damage (Takeda et al. 2003; see also chapter "Receptor–Ligand Interactions in Fungal Infections" by Hardison and Brown).

To date, the TLR family comprises 10 members in humans (TLR1–TLR10) and 12 in the mouse (TLR1–TLR9 and TLR11–TLR13). All TLRs are characterized as type I transmembrane receptors with an extracellular leucine-rich repeat domain and a cytoplasmic tail with high similarity to the type 1 interleukin (IL)-1 receptor. The leucine-rich repeat domains of TLRs bind different microbial components (PAMPs), including bacteria cell wall molecules such as lipopolysaccharide and peptidoglycan, proteins (e.g. flagellin), as well as double-or single-stranded RNA of viruses or unmethylated CpG DNA. Ligation of TLRs leads to activation of a protease cascade, inducing transcription factors such as NF-kB and interferon regulatory factor (IRF)-3 and IRF-7 followed by enhanced transcription of antimicrobial peptides, cytokines, chemokines and co-stimulatory molecules. As such, TLRs function as critical mediators between innate and adaptive immune responses.

Several members of the TLR family are expressed constitutively in oral epithelial cells, healthy epithelial tissue (Beklen et al. 2008; Mahanonda and Pichyangkul 2007) and oral mucosa biopsies from patients with oral candidiasis (Ali et al. 2008). Previously, using a model of **oral reconstituted human epithelium** (RHE), we and others studied a number of different aspects of host–*Candida* interactions (de Boer et al. 2010; Moyes et al. 2010; Naglik et al. 2008; Schaller et al. 1998, 1999, 2002, 2004; Schaller and Weindl 2009; Wagener et al. 2012a; Weindl et al. 2007). Although the model consists of transformed cells (TR146; oral buccal carcinoma cell line) (Rupniak et al. 1985), all natural major markers of the epithelial basement membrane and of epithelial differentiation are expressed. More importantly, despite the artificiality of the model it behaves like human in vivo epithelium when treated with pathogens and pharmacologically active agents (Schaller and Weindl 2009) and it **mimics the clinical setting of *C. albicans* infections in the oral cavity** (Wilson et al. 2009). Analysis of the oral RHE by real-time RT-PCR demonstrated a high degree of similarity in TLR expression profiles between the oral RHE and buccal epithelial samples isolated from healthy individuals (Weindl et al. 2007). In the oral RHE model,

all TLR gene transcripts except TLR7 at a low level are constitutively expressed. Similarly, in samples from healthy individuals, all TLRs except TLR5 and TLR7 are detected. The most commonly expressed TLR genes in vivo appear to be those encoding TLR1, TLR2, TLR4 and TLR8, with TLR1 being the most highly expressed gene.

Increased expression of TLR2 and TLR4 has previously been observed in inflamed gingival epithelial tissues (Sugawara et al. 2006). The immunohistochemical expression of nine TLRs (TLR1 to TLR9) was demonstrated in a series of sections from chronic hyperplastic candidiasis, leukoplakia and healthy tissue (Ali et al. 2008). Although oral epithelial cells express TLRs, no studies have yet demonstrated TLR upregulation upon stimulation with *C. albicans*. Heat-killed *C. albicans* cells failed to modulate epithelial TLR expression (Pivarcsi et al. 2003). Similarly, in our infection model of oral candidiasis, both heat-killed and viable *C. albicans* cells were unable to upregulate epithelial TLR expression despite the fungus causing clear signs of mucosal damage (Weindl et al. 2007). With regard to commensal organisms, it has been suggested that rapid responsiveness by epithelial TLRs may be detrimental to the host by causing an immune overreaction (Strober 2004). Thus, one possible explanation for the lack of direct TLR upregulation by *C. albicans* could be because the fungus is usually a harmless colonizer of oral mucosal surfaces in approximately 40% of healthy individuals (Arendorf and Walker 1979) and **may even actively downregulate epithelial responses** by unknown mechanisms (our unpublished data). In addition, during the carrier state, it would serve little purpose for the host to activate a TLR-mediated inflammatory response when it is not required.

B. C-Type Lectin Receptors in Epithelial Recognition of *C. albicans*

In contrast to TLRs, much less is known about the expression of CLRs in the oral cavity. The CLRs are a large superfamily of proteins characterized by C-type lectin-like domains (Zelensky and Gready 2005). Importantly, these receptors mediate fungal binding, uptake and killing and also contribute to the initiation and/or modulation of the immune response to fungi (Hardison and Brown 2012; Netea et al. 2008; Willment and Brown 2008). The two most important receptors in epithelial recognition of *C. albicans* are the mannose receptor (MR) and dectin-1. For details on CLRs see chapter "Receptor–Ligand Interactions in Fungal Infections" by Hardison and Brown.

Mannose Receptor

The MR (CD206) is a prototypical type I (group VI) transmembrane protein and is mainly expressed by macrophages, as well as by DCs (Taylor et al. 2005b). After carbohydrate recognition, the receptor mediates internalization of pathogens by phagocytosis, induction of NF-κB activation and the production of numerous defensive cytokines (Gazi et al. 2011; Netea et al. 2006; Taylor et al. 2005a; van de Veerdonk et al. 2009b). More recently it has been shown, that the MR induces IL-17 production by *Candida* mannan in the absence of mitogenic stimulation even more potently than Gram-negative bacteria (van de Veerdonk et al. 2009b).

Dectin-1

Dectin-1 is a type II transmembrane receptor and belongs to the natural killer cell receptor-like CLRs (Brown 2006). The extracellular carbohydrate recognition domain (CRD) selectively binds β-glucan polymers, a major component of yeast and mycobacterial cell walls, and mediates the phagocytosis of zymosan particles and intact yeast (Herre et al. 2004; Underhill et al. 2005). Dectin-1 also synergizes with TLR2- and TLR4-induced signals inducing tumour necrosis factor alpha (TNFα), IL-10, transforming growth factor-β and maturation of DCs (Brown et al. 2003; Dillon et al. 2006; Gantner et al. 2003).

Currently, there are no data published on the role of **mannose receptor (MR)** in localized *Candida* infections. The receptor is expressed in keratinocytes (Szolnoky et al. 2001) and oral epithelial cells (Wagener et al. 2012b). However, gene expression analysis in the oral RHE model showed no significant differences upon infection with *C. albicans* (our unpublished data). In oral epithelial cells, MR blocking did not alter cytokine secretions levels of IL-6, IL-8 and GM-CSF upon stimulation with *Candida* cell wall components (Wagener et al. 2012b).

The function of **dectin-1** in mucosal candidiasis has not been fully established, but several studies suggest that dectin-1 might play a crucial

role in the mucosal immunity against *Candida*, at least in the intestine. Myeloid lineage cells in the intestinal tract express dectin-1, and the outgrowth of *Candida* in the digestive tract from dectin-1-deficient mice was disproportionally high, leading to occlusion and contributing to the increased mortality (Reid et al. 2004; Taylor et al. 2002, 2007). Furthermore, dectin-1 is paramount for IL-17 induction by *Candida* (Leibundgut-Landmann et al. 2007; Osorio et al. 2008). Patients with an impaired IL-17 production caused by STAT3 (signal transducer and activator of transcription 3) mutations (hyper-IgE syndrome) and CMC have recurrent *Candida* infections (Eyerich et al. 2008; Ma et al. 2008; Milner et al. 2008). Previous studies have failed to demonstrate dectin-1 expression in epithelial cells from the gastrointestinal tract (Rice et al. 2005), lung (Evans et al. 2005; Lee et al. 2009b) and gingiva (Laube et al. 2008). However, **epidermal keratinocytes appear to express functional dectin-1** (Lee et al. 2009a) and its expression can also be induced by mycobacteria in airway epithelial cells (Lee et al. 2009b). We have demonstrated that dectin-1 is expressed in the oral RHE but gene expression is not inducible by *C. albicans* and dectin-1 ligands did not stimulate cytokine secretion (Moyes et al. 2010; Wagener et al. 2012b). This suggests that dectin-1 plays only a minor role in oral epithelial cell detection of *C. albicans* and that other PRRs might contribute to the interaction between the epithelial cells and *C. albicans* PAMPs.

As for dectin-2, DC-SIGN and Mincle, these receptors seem not to be expressed in oral epithelial cells (our unpublished data).

C. NOD-Like Receptors in Epithelial Recognition of *C. albicans*

Oral epithelial cells express members of the NOD-like receptor (NLR) family, NLRC1 (NOD1) and NLRC2 (NOD2), and stimulation with synthetic ligands strongly increased expression of antimicrobial molecules whereas proinflammatory cytokines were not induced (Sugawara et al. 2006; Uehara et al. 2005, 2007; Uehara and Takada 2008).

NLRs are a family of intracellular immune receptors characterized by leucine-rich repeats and a nucleotide-binding domain. Like TLRs, NLRs recognize microbial products, as well as other intracellular danger signals, thus triggering host defence pathways through the activation of the NF-kB response and inflammatory caspases (Martinon et al. 2009). Several members of the NLR family, including NLRP3 (also known as NALP3 and cryopyrin), form large multiprotein complexes, termed the inflammasome, which in turn activate caspase-1 leading to the processing and secretion of IL-1β and IL-18 (Bryant and Fitzgerald 2009). Recent reports link IL-1β production induced by *C. albicans* to the NLRP3 and NLRC4 inflammasome (Gross et al. 2009; Hise et al. 2009; Joly et al. 2009; Kumar et al. 2009; Tomalka et al. 2011; van de Veerdonk et al. 2009a, 2011). Of note, TLR2, dectin-1 and NLRP3 were shown to be crucial for protection against dissemination of *Candida* in a murine model of oral mucosal infection (Hise et al. 2009). However, at present it is not known to what extent oral epithelial cells contribute to the observed defence mechanism or how important these mechanisms are in local, mucosal anti-*Candida* responses.

Although *C. albicans* is not recognized by NLRC1 and NLRC2 (van der Graaf et al. 2006), two other NLR members, **NLRP3 and NLRC4, might have an important function in the host defence against mucosal *Candida* infections** (Hise et al. 2009; Tomalka et al. 2011). Interestingly, NLRP3 is strongly expressed by keratinocytes in non-keratinizing epithelia such as oral cavity and oesophagus (Kummer et al. 2007). The potential role of NLRP3 in oral epithelial cells is further supported by studies showing increased IL-1β and IL-18 levels upon stimulation with *C. albicans* (Mostefaoui et al. 2004; Rouabhia et al. 2002; Schaller et al. 2004; Tardif et al. 2004; Weindl et al. 2007). NLRC4 or NLRP3 deficiency results in strongly reduced pro-inflammatory and antimicrobial peptide responses in a murine model of oral *C. albicans* infection, but only NLRP3 plays an important role in preventing systemic dissemination (Tomalka et al. 2011).

D. Discrimination Between Pathogenic and Commensal State

Recent reviews have addressed how epithelial cells interact with *Candida* and discussed the epithelial detection mechanisms that might enable mucosal tissues to discriminate between the "pathogenic" or "commensal" state of *C. albicans* (Moyes and Naglik 2011; Naglik et al. 2011). Briefly, *C. albicans* recognition activates two main epithelial signalling pathways, the MAPK and NF-κB pathways (Moyes et al. 2010). In oral epithelial cells, *C. albicans* hyphae (but not yeast) specifically activated the MAPK-p38 signalling pathway, which results in c-Fos transcription factor activation and the induction of proinflammatory cytokines. **Importantly, this hypha-mediated response is highly dependent on fungal burdens.** This indicates that MAPK-p38/c-Fos activation may constitute a "danger response" mechanism that is kept in check to permit immune quiescence in the presence of low *C. albicans* burdens but immune activation when *C. albicans* burdens increase and become hyphal. The MAPK–p38/c-Fos pathway is only activated by hypha-forming *Candida* species (*C. albicans* and *C. dubliniensis*, but not *C. tropicalis*, *C. glabrata*, *C. parapsilosis* or *C. krusei*) (Moyes et al. 2012) and is also present in human vaginal epithelial cells (Moyes et al. 2011). Therefore, this MAPK–p38/c-Fos pathway may represent a mechanism enabling different epithelial tissues to "recognise" dangerous *Candida* hyphae, thereby potentially identifying when this commensal fungus has become pathogenic. In is not yet known how hyphae activate this MAPK–p38/c-Fos response mechanism but it appears to be independent of cell wall polysaccharides including β-glucan, chitin and mannan (Murciano et al. 2011).

IV. Immune Responses Induced by *C. albicans* at Mucosal Surfaces

A. Modulation of Innate Immune Responses

During the period of colonization, extensive fungal growth is limited through release of antimicrobial peptides from epithelial cells or due to existence of other bacteria of the microbial flora. In this stage of colonization without clinical symptoms and signs of inflammation, neither *C. albicans* nor the host induce an inflammatory cytokine response. However, when these homeostatic conditions are disturbed, for instance due to antibiotic therapy or immunosuppression, superficial or even systemic infections may occur.

Oral candidiasis is a common opportunistic infection associated with HIV infection and may occur in 50% of untreated HIV-positive subjects and 90% of AIDS patients (Challacombe and Naglik 2006). Thus, it is tempting to speculate whether TLRs play a role in the development of opportunistic infections during HIV infection. In fact, in HIV-infected patients the risk of developing active tuberculosis (Ferwerda et al. 2007) and the occurrence of serious co-infections (Papadopoulos et al. 2010) positively correlates with the presence of common **TLR4 polymorphisms.** In contrast, polymorphisms of TLR2 or TLR4 do not influence susceptibility to oropharyngeal candidiasis in HIV-infected patients (Plantinga et al. 2010). However, functional studies were performed on leukocytes with heat-killed *C. albicans*, thus the relative contribution of TLRs on oral epithelial cells remains unclear.

1. Epithelial Cytokine Responses

During oral infection with *Candida* species, particularly with *C. albicans*, oral epithelial cells secrete a large number of cytokines, which maintain a central role in the protection against fungal organisms (Dongari-Bagtzoglou and Fidel 2005; Schaller et al. 2002). The cytokines are involved in enhancement of proliferation, activation and fungicidal activity of immune cells. In general, proinflammatory cytokines (IL-1α, IL-1β, IL-6, IL-8, TNFα, GM-CSF and others) regulate leukocyte trafficking (Eversole et al. 1997) and/or activate a strong antifungal response by oral epithelial cells (Schaller et al. 2002, 2004; Weindl et al. 2007). Our data support the hypothesis that *C. albicans* infection induces an epithelial cytokine pattern that may favour a chemotactic and Th1-type immune response and an environmental switch from an anti- to a

proinflammatory milieu. However, epithelial cytokine production induced by *C. albicans* has not yet been associated with specific PRRs. *C. albicans* strongly induce GM-CSF and IL-8 in human oral epithelial cells and in three-dimensional models (Dongari-Bagtzoglou and Kashleva 2003; Weindl et al. 2007). Recent data indicate that TLR4 is not involved in *Candida*-induced GM-CSF and IL-8 production in epithelial cells (Li and Dongari-Bagtzoglou 2009; Weindl et al. 2007). The **adhesion receptor CDw17** (lactosylceramide) might be responsible, at least partially, for GM-CSF activation mediated by NF-kB (Li and Dongari-Bagtzoglou 2009). Further studies are necessary to identify the receptors that mediate the epithelial cytokine response to *C. albicans*.

In response to infection and inflammatory stimuli, epithelial cells are also capable of inducing antimicrobial peptides such as **defensins, cathelicidins and histatins** (Diamond et al. 2008), which control *C. albicans* growth and infection. Among these peptides, human β-defensin-2 (hBD-2), hBD-3 and cathelicidin (LL-37) exhibit potent anti-candidal properties (Schneider et al. 2005). In this regard, we observed that the regulation of expression of the antimicrobial peptides hBD-1, hBD-2 and hBD-3 in the oral RHE also correlated with the degree of tissue damage caused by *C. albicans* (our unpublished results). These observations support the hypothesis of an active host-fungus interaction at the epithelial surface and provide insight into the molecular events leading to recruitment and activation of immune cells by the oral epithelium.

Recently, specific human gene mutations and polymorphisms have been linked to signal pathways resulting in susceptibility to *C. albicans*. STAT3 mutations identified in patients with hyper-IgE syndrome have been attributed to a defective IL-17 production and a diminished Th17 response, resulting in recurrent mucosal *Candida* infections (Eyerich et al. 2008; Ma et al. 2008; Milner et al. 2008). Similarly, deficiency in dectin-1 signalling pathways have been linked to CMC (Ferwerda et al. 2009; Glocker et al. 2009). Both studies, however, need to be interpreted with caution because both patient groups showed mucocutaneous manifestations, whereas the functional studies were performed on leukocytes. It remains to be proven whether the key mechanisms in these cases of severe candidiasis consist of impaired dectin-1 signalling at the epithelial level or impaired leukocyte activation of epithelium, mediated through cytokines such as IL-17 and IL-22. In addition to STAT3, dectin-1, IL-17RA and IL-17F polymorphisms, other polymorphisms affecting STAT1, DOCK8, TYK2, CARD9 and AIRE also appear to predispose patients to *Candida* infections (Plantinga et al. 2012).

2. Interaction with Immune Cells

Polymorphonuclear leukocytes (PMNs) are a central component of the innate immune response. In many mucosal infections and inflammatory disorders, the combination of epithelial injury, disease activity and symptoms parallel PMN infiltration of the mucosa. Similarly, during oral *Candida* infections, transepithelial migration of PMNs is believed to play a crucial role in the clearance of infection and in epithelial homeostasis (Fidel 2002). During infection, local production of multiple cytokines and chemokines induces the **recruitment and activation of PMNs from the vascular compartment to the oral mucosa**. IL-8 and GM-CSF are key chemokines involved in the recruitment of PMNs to sites of infection (Godaly et al. 2001; Reaves et al. 2005) and are rapidly activated by exposure to proinflammatory cytokines including IL-1α, IL-1β, IL-6 and TNFα (Yamashiro et al. 2001). The oral epithelium produces high levels of IL-8 and GM-CSF in response to *C. albicans* infection (Schaller et al. 2002, 2004; Weindl et al. 2007), which might explain the substantial migration of PMNs into *Candida*-infected oral mucosal sites. Addition of PMNs to our in vitro model of oral candidiasis enhanced a Th1-type immune response [interferon (IFN)-γ, TNFα], downregulated the expression of the Th2-type cytokine IL-10 and was associated with protection against *Candida*-induced tissue damage (Schaller et al. 2004). PMNs could protect the epithelium from *C. albicans*-induced cell injury via a process that was independent of

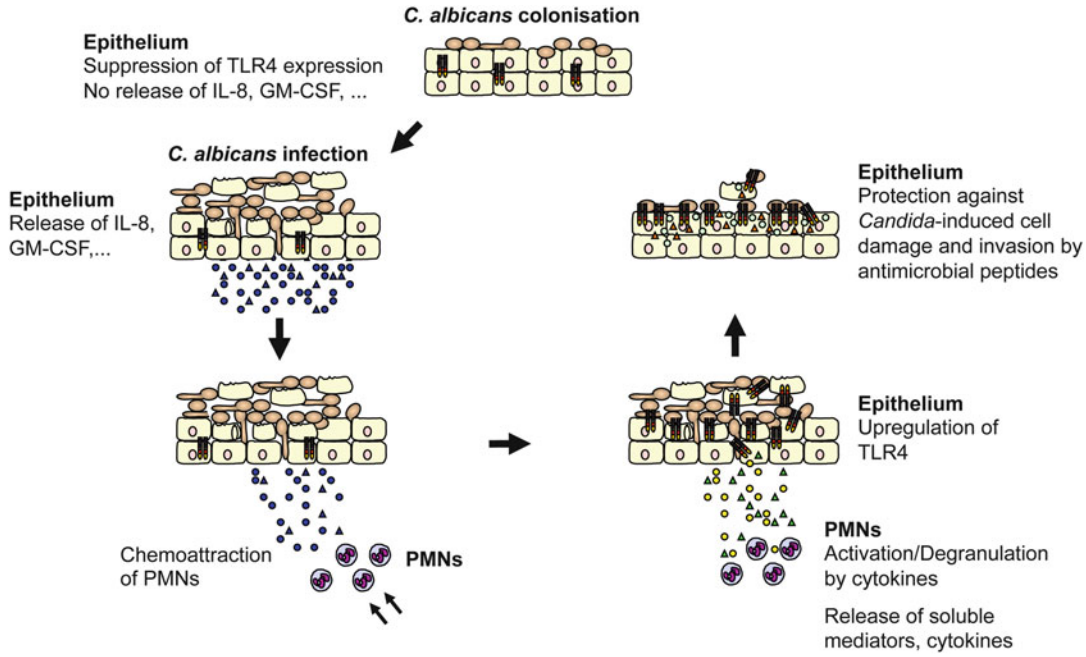


Fig. 9.2. Model of TLR4-mediated and PMN-dependent antifungal defence by the oral epithelium. Epithelial cells control fungal cell growth and invasion. During colonization of the oral epithelium, *C. albicans* suppresses TLR4 expression and does not induce cytokine production. Infection, particularly in immunocompromised patients, leads to increased cytokine secretion that recruits and stimulates PMNs at the site of infection. After recruitment, several cytokines, especially TNFα, are directly involved in initiating the subsequent PMN-mediated upregulation of epithelial TLR4 via a process that does not require PMN infiltration of the mucosal tissues. Finally, epithelial TLR4 directly protects the oral mucosa from fungal invasion and cell injury by production of antimicrobial peptides. *Circles* and *triangles* represent secreted cytokines, antimicrobial peptides and other soluble mediators by the oral epithelium or PMNs, respectively. TLR4 expression is depicted in the oral epithelium (Modified with permission from Weindl et al. 2010, 2011)

phagocytosis, PMN transmigration or physical PMN–epithelial cell contact. Interestingly, the immunological crosstalk between *C. albicans*-infected oral epithelium and PMNs causes **PMN-mediated upregulation of epithelial TLR4** (Weindl et al. 2007). Furthermore, epithelial **TLR4 is directly responsible for protecting the mucosal surface** from fungal invasion and cell injury. Noteworthy, cytokines such as IL-1α, IL-1β, IL-6, IL-8, macrophage inflammatory protein (MIP)-1β, monocyte chemoattractant protein (MCP)-1 and GM-CSF seem not to have an essential function in direct host defence against invading fungi, even in the presence of PMNs. Incubation of the RHE with neutralizing antibodies had a minimal effect on epithelial cell damage induced by *C. albicans*, and strong TLR4 upregulation was still observed after PMN addition. In contrast, incubation of the supplemented PMNs with all neutralizing antibodies, except GM-CSF, led to strongly reduced TLR4 mRNA expression. Thus, cytokines are crucial for the activation of PMNs and/or are released from the PMNs, which in turn results in upregulation of epithelial TLR4 and protection from fungal invasion (Fig. 9.2). Among the cytokines, **TNFα showed the most potent effect**, which confirms the important role of this cytokine in host defence against opportunistic fungal infections (Filler et al. 2005). Absence of this cytokine strongly impairs neutrophil recruitment and effective phagocytosis of *C. albicans* (Netea et al. 1999).

In an in vitro model of oesophageal candidiasis, co-incubation of PMNs with *C. albicans* strongly upregulated hBD-2 and hBD-3 in

oesophageal cells compared to PMNs or *C. albicans* alone (Steubesand et al. 2009). We recently observed that the addition of PMNs to the *Candida*-infected oral RHE model not only strongly upregulates epithelial TLR4 expression, but also promotes the release of LL-37, which correlated directly with protection (our unpublished data). **The protective effect of LL-37 was abolished by the addition of TLR4-specific neutralizing antibodies and by TLR4 knockdown by RNA interference (RNAi), demonstrating the direct role of LL-37 in the protective process.** We confirmed the protective role of LL-37 by exogenous addition, which reduced *C. albicans*-induced cell damage in the absence of PMNs. It is known that membrane permeabilization of *C. albicans* drives the antimicrobial activity of cathelicidins such as LL-37, although the precise mechanisms for *Candida* cell killing have not yet been identified (den Hertog et al. 2005; López-García et al. 2005). In addition, LL-37 also modulates TLR-induced cytokine responses in epithelial cells (Filewod et al. 2009). The detailed role of LL-37 in the protection from *Candida*-induced cell damage is currently under investigation. In summary, increased PMN-dependent production of antimicrobial peptides by epithelial cells could contribute to the protective effect and further underlines the important role for PMNs in clearance of experimental oral candidiasis.

B. Mucosal T Cell Responses

Activation of the innate immune system by *C. albicans* induces the secretion of a variety of proinflammatory cytokines and the expression of co-stimulatory molecules. It is generally accepted that induction of a Th1-type cellular response is crucial for the defence against *C. albicans* (Fidel et al. 1997; Romani 1999; Schaller et al. 2004). In contrast, a Th2 cellular response is considered non-protective because it induces a class-switch to non-opsonizing antibody subclasses and IgE (Clemons and Stevens 2001; Savolainen et al. 1996). Investigation of the role of Th17 in mediating the immune response has shown that Th17 memory cells are induced by *Candida* hyphae (Acosta-Rodriguez et al. 2007; Zhou et al. 2008). In a murine model, IL-17AR knockout mice had an increased susceptibility to systemic (Huang et al. 2004) and oropharyngeal candidiasis (OPC) (Conti et al. 2009). On the other hand, deleterious effects of IL-17 inflammatory activities have also been demonstrated (Bozza et al. 2008; De Luca et al. 2007; Zelante et al. 2007). **Patients with an impaired IL-17 production suffer from mucosal *C. albicans* infections in hyper-IgE syndrome and CMC** (Eyerich et al. 2008; Ma et al. 2008; Milner et al. 2008). Furthermore, in cases of autoimmunity with neutralizing antibodies to Th17 cytokines (IL-17A, IL-17F and IL-22), there is an increased incidence of CMC (Kisand et al. 2010). In contrast to Th cells, regulatory T (Treg) cells suppress inflammatory responses in disseminated *C. albicans*, resulting in higher susceptibility in mice (Netea et al. 2004; Sutmuller et al. 2006). However, the tolerization-inducing effects of Treg cells seem to be beneficial at mucosal sites (De Luca et al. 2007; Vignali et al. 2008). Treg cells may also promote Th17 cells in vitro and enhance host resistance in experimental oral candidiasis in mice (Pandiyan et al. 2011).

Th17 cells are a distinct lineage from Th1 and Th2 cells, and are characterized by the release of IL-17A and IL-17F, IL-22 and IL-26. Receptors for IL-17A and IL-17F (Il-17Ra and IL-17Rc) are present in several cell types, including antigen presenting cells and epithelial cells (Gaffen 2009; Xie et al. 2000). In contrast, receptors for IL-22 and IL-26 appear to be localized to the epithelium (Sheikh et al. 2004; Wolk et al. 2004; Xie et al. 2000). Very little is known about the role of IL-26 during mucosal infection because rodents do not express this cytokine. During colonization of the oral cavity with *C. albicans*, IL-17 receptor signalling is essential for defence (Conti et al. 2009). **Interestingly, Th17-deficient (IL-23p19$^{-/-}$) and IL-17R-deficient (IL-17RA$^{-/-}$) mice experienced severe OPC, whereas Th1-deficient (IL-12p35$^{-/-}$) mice showed low fungal burdens and no apparent sign of disease.** Furthermore, neutrophil recruitment was impaired in IL-23p19$^{-/-}$ and IL-17RA$^{-/-}$, but not IL-12$^{-/-}$, mice, and T cell receptor αβ cells were more important than γδ cells. On the other hand, mice deficient in the Th17 cytokine IL-22 were only mildly

susceptible to OPC, indicating that IL-17 rather than IL-22 is crucial in defence against oral candidiasis. Gene profiling of oral mucosal tissue showed strong induction of Th17 signature genes, including those encoding β-defensin-3 and CXC chemokines. hBD-3 has candidacidal activity in vitro (Vylkova et al. 2006) and saliva from wild-type mice, but not IL-17RA$^{-/-}$ mice has candidacidal activity, indicating that IL-17 also controls *C. albicans* proliferation by promoting secretion of antimicrobial peptides (Conti et al. 2009). However, more work is needed to understand whether Th17 responses also govern the response to oral candidiasis in humans, because it is unclear whether mouse and human diseases have the same aetiology.

In humans, OPC has diverse aetiologies ranging from antimicrobial use, to immune dysregulation associated with advanced HIV infection, to mutations in autoimmune regulator genes. Patients with CMC show differences in cytokine production, including IL-23, depending on the aetiology of the disorder (Ryan et al. 2008). HIV infection and host genetics are likely to be important variables in Th17 expression, but environmental factors, particularly those that affect the microbiota, could also influence Th cell polarization. Although microbe-specific motifs could induce Th polarization, common microbial PAMPs that induce Th1 or Th17 may also be present. Thus, a diverse group of microbial ligands could induce different responses, and immunity towards pathogens could be less specific than previously thought.

Together the data indicate optimal protection against (chronic) mucosal *Candida* infections by Th1, Th17 and regulatory T cells. An effective Th1 and antibody (humoral) response are crucial for defence against disseminated *Candida* infections. In the case of localized *Candida* infections, however, more work is needed to decipher the relative contributions of innate and cell-mediated immunity.

V. Conclusion

In the past decade, our understanding of the events and mechanisms involved in host mucosal responses to fungal pathogens has grown considerably. Originally, the role of epithelial cells in these events was considered to be relatively unimportant, but it is becoming increasingly apparent that they are key players in initiating protective mucosal immunity via the production of antimicrobial peptides and immunological crosstalk with local immune cells. Equally, the elucidation of an epithelial-based mechanism for detecting hyphae and potentially identifying "pathogenic" *C. albicans* has confirmed the importance of epithelial cells in mediating protective mucosal mechanisms and in commensal–pathogen discrimination. These advances provide important insights into the complex mechanisms by which appropriate innate and acquired immune responses are initiated and how this affects the pathogenesis of fungal infections.

Acknowledgements This work was supported by the BMBF (0315409B), Deutsche Forschungsgemeinschaft (Sch897/1-3, graduate college 685) and by a NIDCR grant R01 DE017514-01. J.N. and D.M are funded by the MRC (MR-J008303-1) and BBSRC (BB-J016411-1).

References

Acosta-Rodriguez EV, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia A, Sallusto F, Napolitani G (2007) Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. Nat Immunol 8:639–646

Ali A, Natah S, Konttinen Y (2008) Differential expression of Toll-like receptors in chronic hyperplastic candidosis. Oral Microbiol Immunol 23:299–307

Arendorf TM, Walker DM (1979) Oral candidal populations in health and disease. Br Dent J 147:267–272

Beck-Sague C, Jarvis WR (1993) Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980–1990. National Nosocomial Infections Surveillance System. J Infect Dis 167:1247–1251

Beklen A, Hukkanen M, Richardson R, Konttinen YT (2008) Immunohistochemical localization of Toll-like receptors 1–10 in periodontitis. Oral Microbiol Immunol 23:425–431

Bozza S, Zelante T, Moretti S, Bonifazi P, DeLuca A, D'Angelo C, Giovannini G, Garlanda C, Boon L, Bistoni F, Puccetti P, Mantovani A, Romani L (2008) Lack of Toll IL-1R8 exacerbates Th17 cell responses in fungal infection. J Immunol 180:4022–4031

Brown GD (2006) Dectin-1: a signalling non-TLR pattern-recognition receptor. Nat Rev Immunol 6:33–43

Brown GD, Herre J, Williams DL, Willment JA, Marshall AS, Gordon S (2003) Dectin-1 mediates the biological effects of beta-glucans. J Exp Med 197:1119–1124

Bryant C, Fitzgerald KA (2009) Molecular mechanisms involved in inflammasome activation. Trends Cell Biol 19:455–464

Calderone RA, Fonzi WA (2001) Virulence factors of *Candida albicans*. Trends Microbiol 9:327–335

Challacombe SJ, Naglik JR (2006) The effects of HIV infection on oral mucosal immunity. Adv Dent Res 19:29–35

Clemons KV, Stevens DA (2001) Overview of host defense mechanisms in systemic mycoses and the basis for immunotherapy. Semin Respir Infect 16:60–66

Conti HR, Shen F, Nayyar N, Stocum E, Sun JN, Lindemann MJ, Ho AW, Hai JH, Yu JJ, Jung JW, Filler SG, Masso-Welch P, Edgerton M, Gaffen SL (2009) Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. J Exp Med 206:299–311

Cutler CW, Jotwani R (2006) Dendritic cells at the oral mucosal interface. J Dent Res 85:678–689

de Boer AD, de Groot PW, Weindl G, Schaller M, Riedel D, Diez-Orejas R, Klis FM, de Koster CG, Dekker HL, Gross U, Bader O, Weig M (2010) The *Candida albicans* cell wall protein Rhd3/Pga29 is abundant in the yeast form and contributes to virulence. Yeast 27:611–624

De Luca A, Montagnoli C, Zelante T, Bonifazi P, Bozza S, Moretti S, D'Angelo C, Vacca C, Boon L, Bistoni F, Puccetti P, Fallarino F, Romani L (2007) Functional yet balanced reactivity to *Candida albicans* requires TRIF, MyD88, and IDO-dependent inhibition of Rorc. J Immunol 179:5999–6008

den Hertog AL, van Marle J, van Veen HA, Van't Hof W, Bolscher JG, Veerman EC, Nieuw Amerongen AV (2005) Candidacidal effects of two antimicrobial peptides: histatin 5 causes small membrane defects, but LL-37 causes massive disruption of the cell membrane. Biochem J 388:689–695

Diamond G, Beckloff N, Ryan LK (2008) Host defense peptides in the oral cavity and the lung: similarities and differences. J Dent Res 87:915–927

Dillon S, Agrawal S, Banerjee K, Letterio J, Denning TL, Oswald-Richter K, Kasprowicz DJ, Kellar K, Pare J, van Dyke T, Ziegler S, Unutmaz D, Pulendran B (2006) Yeast zymosan, a stimulus for TLR2 and dectin-1, induces regulatory antigen-presenting cells and immunological tolerance. J Clin Invest 116:916–928

Dongari-Bagtzoglou A, Fidel PL (2005) The host cytokine responses and protective immunity in oropharyngeal candidiasis. J Dent Res 84:966–977

Dongari-Bagtzoglou A, Kashleva H (2003) Granulocyte-macrophage colony-stimulating factor responses of oral epithelial cells to *Candida albicans*. Oral Microbiol Immunol 18:165–170

Evans SE, Hahn PY, McCann F, Kottom TJ, Pavlovic ZV, Limper AH (2005) Pneumocystis cell wall beta-glucans stimulate alveolar epithelial cell chemokine generation through nuclear factor-kappaB-dependent mechanisms. Am J Respir Cell Mol Biol 32:490–497

Eversole LR, Reichart PA, Ficarra G, Schmidt-Westhausen A, Romagnoli P, Pimpinelli N (1997) Oral keratinocyte immune responses in HIV-associated candidiasis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 84:372–380

Eyerich K, Foerster S, Rombold S, Seidl HP, Behrendt H, Hofmann H, Ring J, Traidl-Hoffmann C (2008) Patients with chronic mucocutaneous candidiasis exhibit reduced production of Th17-associated cytokines IL-17 and IL-22. J Invest Dermatol 128:2640–2645

Ferwerda B, Kibiki GS, Netea MG, Dolmans WM, van der Ven AJ (2007) The Toll-like receptor 4 Asp299Gly variant and tuberculosis susceptibility in HIV-infected patients in Tanzania. AIDS 21:1375–1377

Ferwerda B, Ferwerda G, Plantinga TS, Willment JA, van Spriel AB, Venselaar H, Elbers CC, Johnson MD, Cambi A, Huysamen C, Jacobs L, Jansen T, Verheijen K, Masthoff L, Morre SA, Vriend G, Williams DL, Perfect JR, Joosten LA, Wijmenga C, van der Meer JW, Adema GJ, Kullberg BJ, Brown GD, Netea MG (2009) Human dectin-1 deficiency and mucocutaneous fungal infections. N Engl J Med 361:1760–1767

Fidel PL (2002) Distinct protective host defenses against oral and vaginal candidiasis. Med Mycol 40:359–375

Fidel PL Jr, Ginsburg KA, Cutright JL, Wolf NA, Leaman D, Dunlap K, Sobel JD (1997) Vaginal-associated immunity in women with recurrent vulvovaginal candidiasis: evidence for vaginal Th1-type responses following intravaginal challenge with Candida antigen. J Infect Dis 176:728–739

Filewod NC, Pistolic J, Hancock RE (2009) Low concentrations of LL-37 alter IL-8 production by keratinocytes and bronchial epithelial cells in response to proinflammatory stimuli. FEMS Immunol Med Microbiol 56:233–240

Filler SG, Yeaman MR, Sheppard D (2005) Tumor necrosis factor inhibition and invasive fungal infections. Clin Infect Dis 41(Suppl 3):S208–S212

Franchi L, Munoz-Planillo R, Reimer T, Eigenbrod T, Nunez G (2010) Inflammasomes as microbial sensors. Eur J Immunol 40:611–615

Gaffen SL (2009) Structure and signalling in the IL-17 receptor family. Nat Rev Immunol 9:556–567

Gantner BN, Simmons RM, Canavera SJ, Akira S, Underhill DM (2003) Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. J Exp Med 197:1107–1117

Gauglitz GG, Callenberg H, Weindl G, Korting HC (2012) Host defence against *Candida albicans* and the role of pattern-recognition receptors. Acta Derm Venereol 92:291–298

Gazi U, Rosas M, Singh S, Heinsbroek S, Haq I, Johnson S, Brown GD, Williams DL, Taylor PR, Martinez-Pomares L (2011) Fungal recognition enhances mannose receptor shedding through dectin-1 engagement. J Biol Chem 286:7822–7829

Glocker EO, Hennigs A, Nabavi M, Schaffer AA, Woellner C, Salzer U, Pfeifer D, Veelken H, Warnatz K, Tahami F, Jamal S, Manguiat A, Rezaei N, Amirzargar AA, Plebani A, Hannesschlager N, Gross O, Ruland J, Grimbacher B (2009) A homozygous CARD9 mutation in a family with susceptibility to fungal infections. N Engl J Med 361:1727–1735

Godaly G, Bergsten G, Hang L, Fischer H, Frendéus B, Lundstedt AC, Samuelsson M, Samuelsson P, Svanborg C (2001) Neutrophil recruitment, chemokine receptors, and resistance to mucosal infection. J Leukoc Biol 69:899–906

Gross O, Poeck H, Bscheider M, Dostert C, Hannesschlager N, Endres S, Hartmann G, Tardivel A, Schweighoffer E, Tybulewicz V, Mocsai A, Tschopp J, Ruland J (2009) Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. Nature 459:433–436

Hardison SE, Brown GD (2012) C-type lectin receptors orchestrate antifungal immunity. Nat Immunol 13:817–822

Herre J, Gordon S, Brown GD (2004) Dectin-1 and its role in the recognition of beta-glucans by macrophages. Mol Immunol 40:869–876

Hise AG, Tomalka J, Ganesan S, Patel K, Hall BA, Brown GD, Fitzgerald KA (2009) An essential role for the NLRP3 inflammasome in host defense against the human fungal pathogen *Candida albicans*. Cell Host Microbe 5:487–497

Huang W, Na L, Fidel PL, Schwarzenberger P (2004) Requirement of interleukin-17A for systemic anti-Candida albicans host defense in mice. J Infect Dis 190:624–631

Hube B, Naglik J (2001) *Candida albicans* proteinases: resolving the mystery of a gene family. Microbiology 147:1997–2005

Janeway CA, Medzhitov R (2002) Innate immune recognition. Annu Rev Immunol 20:197–216

Joly S, Ma N, Sadler JJ, Soll DR, Cassel SL, Sutterwala FS (2009) Cutting edge: *Candida albicans* hyphae formation triggers activation of the Nlrp3 inflammasome. J Immunol 183:3578–3581

Kisand K, Boe Wolff AS, Podkrajsek KT, Tserel L, Link M, Kisand KV, Ersvaer E, Perheentupa J, Erichsen MM, Bratanic N, Meloni A, Cetani F, Perniola R, Ergun-Longmire B, Maclaren N, Krohn KJ, Pura M, Schalke B, Strobel P, Leite MI, Battelino T, Husebye ES, Peterson P, Willcox N, Meager A (2010) Chronic mucocutaneous candidiasis in APECED or thymoma patients correlates with autoimmunity to Th17-associated cytokines. J Exp Med 207:299–308

Kumar H, Kumagai Y, Tsuchida T, Koenig PA, Satoh T, Guo Z, Jang MH, Saitoh T, Akira S, Kawai T (2009) Involvement of the NLRP3 inflammasome in innate and humoral adaptive immune responses to fungal beta-glucan. J Immunol 183:8061–8067

Kummer JA, Broekhuizen R, Everett H, Agostini L, Kuijk L, Martinon F, van Bruggen R, Tschopp J (2007) Inflammasome components NALP 1 and 3 show distinct but separate expression profiles in human tissues suggesting a site-specific role in the inflammatory response. J Histochem Cytochem 55:443–452

Lamagni TL, Evans BG, Shigematsu M, Johnson EM (2001) Emerging trends in the epidemiology of invasive mycoses in England and Wales (1990–9). Epidemiol Infect 126:397–414

Laube DM, Dongari-Bagtzoglou A, Kashleva H, Eskdale J, Gallagher G, Diamond G (2008) Differential regulation of innate immune response genes in gingival epithelial cells stimulated with Aggregatibacter actinomycetemcomitans. J Periodont Res 43:116–123

Lee HM, Shin DM, Choi DK, Lee ZW, Kim KH, Yuk JM, Kim CD, Lee JH, Jo EK (2009a) Innate immune responses to Mycobacterium ulcerans via Toll-like receptors and dectin-1 in human keratinocytes. Cell Microbiol 11:678–692

Lee HM, Yuk JM, Shin DM, Jo EK (2009b) Dectin-1 is inducible and plays an essential role for mycobacteria-induced innate immune responses in airway epithelial cells. J Clin Immunol 29:795–805

Leibundgut-Landmann S, Gross O, Robinson MJ, Osorio F, Slack EC, Tsoni SV, Schweighoffer E, Tybulewicz V, Brown GD, Ruland J, Reis e Sousa C (2007) Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. Nat Immunol 8:630–638

Li L, Dongari-Bagtzoglou A (2009) Epithelial GM-CSF induction by Candida glabrata. J Dent Res 88:746–751

Li L, Redding S, Dongari-Bagtzoglou A (2007) Candida glabrata: an emerging oral opportunistic pathogen. J Dent Res 86:204–215

López-García B, Lee PH, Yamasaki K, Gallo RL (2005) Anti-fungal activity of cathelicidins and their potential role in *Candida albicans* skin infection. J Invest Dermatol 125:108–115

Ma CS, Chew GY, Simpson N, Priyadarshi A, Wong M, Grimbacher B, Fulcher DA, Tangye SG, Cook MC (2008) Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. J Exp Med 205:1551–1557

Mahanonda R, Pichyangkul S (2007) Toll-like receptors and their role in periodontal health and disease. Periodontol 2000 43:41–55

Martinon F, Mayor A, Tschopp J (2009) The inflammasomes: guardians of the body. Annu Rev Immunol 27:229–265

Milner JD, Brenchley JM, Laurence A, Freeman AF, Hill BJ, Elias KM, Kanno Y, Spalding C, Elloumi HZ,

Paulson ML, Davis J, Hsu A, Asher AI, O'Shea J, Holland SM, Paul WE, Douek DC (2008) Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. Nature 452:773–776

Mostefaoui Y, Claveau I, Rouabhia M (2004) In vitro analyses of tissue structure and interleukin-1beta expression and production by human oral mucosa in response to *Candida albicans* infections. Cytokine 25:162–171

Moyes DL, Naglik JR (2011) Mucosal immunity and *Candida albicans* infection. Clin Dev Immunol 2011:346307

Moyes DL, Runglall M, Murciano C, Shen C, Nayar D, Thavaraj S, Kohli A, Islam A, Mora-Montes H, Challacombe SJ, Naglik JR (2010) A biphasic innate immune MAPK response discriminates between the yeast and hyphal forms of *Candida albicans* in epithelial cells. Cell Host Microbe 8:225–235

Moyes DL, Murciano C, Runglall M, Islam A, Thavaraj S, Naglik JR (2011) *Candida albicans* yeast and hyphae are discriminated by MAPK signaling in vaginal epithelial cells. PLoS One 6:e26580

Moyes DL, Murciano C, Runglall M, Kohli A, Islam A, Naglik JR (2012) Activation of MAPK/c-Fos induced responses in oral epithelial cells is specific to *Candida albicans* and Candida dubliniensis hyphae. Med Microbiol Immunol 201:93–101

Murciano C, Moyes DL, Runglall M, Islam A, Mille C, Fradin C, Poulain D, Gow NA, Naglik JR (2011) *Candida albicans* cell wall glycosylation may be indirectly required for activation of epithelial cell proinflammatory responses. Infect Immun 79:4902–4911

Naglik JR, Moyes D (2011) Epithelial cell innate response to *Candida albicans*. Adv Dent Res 23:50–55

Naglik JR, Moyes D, Makwana J, Kanzaria P, Tsichlaki E, Weindl G, Tappuni AR, Rodgers CA, Woodman AJ, Challacombe SJ, Schaller M, Hube B (2008) Quantitative expression of the *Candida albicans* secreted aspartyl proteinase gene family in human oral and vaginal candidiasis. Microbiology 154:3266–3280

Naglik JR, Moyes DL, Wachtler B, Hube B (2011) *Candida albicans* interactions with epithelial cells and mucosal immunity. Microbes Infect 13:963–976

Netea MG, van Tits LJ, Curfs JH, Amiot F, Meis JF, van der Meer JW, Kullberg BJ (1999) Increased susceptibility of TNF-alpha lymphotoxin-alpha double knockout mice to systemic candidiasis through impaired recruitment of neutrophils and phagocytosis of *Candida albicans*. J Immunol 163:1498–1505

Netea MG, Sutmuller R, Hermann C, van der Graaf CA, Van der Meer JW, van Krieken JH, Hartung T, Adema G, Kullberg BJ (2004) Toll-like receptor 2 suppresses immunity against *Candida albicans* through induction of IL-10 and regulatory T cells. J Immunol 172:3712–3718

Netea MG, Gow NA, Munro CA, Bates S, Collins C, Ferwerda G, Hobson RP, Bertram G, Hughes HB, Jansen T, Jacobs L, Buurman ET, Gijzen K, Williams DL, Torensma R, McKinnon A, MacCallum DM, Odds FC, Van der Meer JW, Brown AJ, Kullberg BJ (2006) Immune sensing of *Candida albicans* requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. J Clin Invest 116:1642–1650

Netea MG, Brown GD, Kullberg BJ, Gow NA (2008) An integrated model of the recognition of *Candida albicans* by the innate immune system. Nat Rev Microbiol 6:67–78

Osorio F, LeibundGut-Landmann S, Lochner M, Lahl K, Sparwasser T, Eberl G, Reis e Sousa C (2008) DC activated via dectin-1 convert Treg into IL-17 producers. Eur J Immunol 38:3274–3281

Pandiyan P, Conti HR, Zheng L, Peterson AC, Mathern DR, Hernandez-Santos N, Edgerton M, Gaffen SL, Lenardo MJ (2011) CD4(+)CD25(+)Foxp3(+) regulatory T cells promote Th17 cells in vitro and enhance host resistance in mouse *Candida albicans* Th17 cell infection model. Immunity 34:422–434

Papadopoulos AI, Ferwerda B, Antoniadou A, Sakka V, Galani L, Kavatha D, Panagopoulos P, Poulakou G, Kanellakopoulou K, van der Meer JW, Giamarellos-Bourboulis EJ, Netea MG (2010) Association of Toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms with increased infection risk in patients with advanced HIV-1 infection. Clin Infect Dis 51:242–247

Pivarcsi A, Bodai L, Réthi B, Kenderessy-Szabó A, Koreck A, Széll M, Beer Z, Bata-Csörgoo Z, Magócsi M, Rajnavölgyi E, Dobozy A, Kemény L (2003) Expression and function of Toll-like receptors 2 and 4 in human keratinocytes. Int Immunol 15:721–730

Plantinga TS, Hamza OJ, Willment JA, Ferwerda B, van de Geer NM, Verweij PE, Matee MI, Banahan K, O'Neill LA, Kullberg BJ, Brown GD, van der Ven AJ, Netea MG (2010) Genetic variation of innate immune genes in HIV-infected African patients with or without oropharyngeal candidiasis. J Acquir Immune Defic Syndr 55:87–94

Plantinga TS, Johnson MD, Scott WK, Joosten LA, van der Meer JW, Perfect JR, Kullberg BJ, Netea MG (2012) Human genetic susceptibility to Candida infections. Med Mycol 50:785–794

Reaves TA, Chin AC, Parkos CA (2005) Neutrophil transepithelial migration: role of Toll-like receptors in mucosal inflammation. Mem Inst Oswaldo Cruz 100(Suppl 1):191–198

Reid DM, Montoya M, Taylor PR, Borrow P, Gordon S, Brown GD, Wong SY (2004) Expression of the beta-glucan receptor, dectin-1, on murine leukocytes in

situ correlates with its function in pathogen recognition and reveals potential roles in leukocyte interactions. J Leukoc Biol 76:86–94

Rice PJ, Adams EL, Ozment-Skelton T, Gonzalez AJ, Goldman MP, Lockhart BE, Barker LA, Breuel KF, Deponti WK, Kalbfleisch JH, Ensley HE, Brown GD, Gordon S, Williams DL (2005) Oral delivery and gastrointestinal absorption of soluble glucans stimulate increased resistance to infectious challenge. J Pharmacol Exp Ther 314:1079–1086

Roeder A, Kirschning CJ, Rupec RA, Schaller M, Weindl G, Korting HC (2004) Toll-like receptors as key mediators in innate antifungal immunity. Med Mycol 42:485–498

Romani L (1999) Immunity to *Candida albicans*: Th1, Th2 cells and beyond. Curr Opin Microbiol 2:363–367

Rouabhia M, Ross G, Page N, Chakir J (2002) Interleukin-18 and gamma interferon production by oral epithelial cells in response to exposure to *Candida albicans* or lipopolysaccharide stimulation. Infect Immun 70:7073–7080

Ruhnke M (2006) Epidemiology of *Candida albicans* infections and role of non-Candida-albicans yeasts. Curr Drug Targets 7:495–504

Rupniak HT, Rowlatt C, Lane EB, Steele JG, Trejdosiewicz LK, Laskiewicz B, Povey S, Hill BT (1985) Characteristics of four new human cell lines derived from squamous cell carcinomas of the head and neck. J Natl Cancer Inst 75:621–635

Ryan KR, Hong M, Arkwright PD, Gennery AR, Costigan C, Dominguez M, Denning D, McConnell V, Cant AJ, Abinun M, Spickett GP, Lilic D (2008) Impaired dendritic cell maturation and cytokine production in patients with chronic mucocutanous candidiasis with or without APECED. Clin Exp Immunol 154:406–414

Samaranayake LP, Keung Leung W, Jin L (2009) Oral mucosal fungal infections. Periodontol 2000 49:39–59

Savolainen J, Rantala A, Nermes M, Lehtonen L, Viander M (1996) Enhanced IgE response to *Candida albicans* in postoperative invasive candidiasis. Clin Exp Allergy 26:452–460

Schaller M, Weindl G (2009) Models of oral and vaginal candidiasis based on in vitro reconstituted human epithelia for the study of host-pathogen interactions. Methods Mol Biol 470:327–345

Schaller M, Schäfer W, Korting HC, Hube B (1998) Differential expression of secreted aspartyl proteinases in a model of human oral candidosis and in patient samples from the oral cavity. Mol Microbiol 29:605–615

Schaller M, Korting HC, Schäfer W, Bastert J, Chen W, Hube B (1999) Secreted aspartic proteinase (Sap) activity contributes to tissue damage in a model of human oral candidosis. Mol Microbiol 34:169–180

Schaller M, Mailhammer R, Grassl G, Sander CA, Hube B, Korting HC (2002) Infection of human oral epithelia with Candida species induces cytokine expression correlated to the degree of virulence. J Invest Dermatol 118:652–657

Schaller M, Boeld U, Oberbauer S, Hamm G, Hube B, Korting HC (2004) Polymorphonuclear leukocytes (PMNs) induce protective Th1-type cytokine epithelial responses in an in vitro model of oral candidosis. Microbiology 150:2807–2813

Schaller M, Borelli C, Korting HC, Hube B (2005) Hydrolytic enzymes as virulence factors of *Candida albicans*. Mycoses 48:365–377

Schneider JJ, Unholzer A, Schaller M, Schäfer-Korting M, Korting HC (2005) Human defensins. J Mol Med 83:587–595

Sheikh F, Baurin VV, Lewis-Antes A, Shah NK, Smirnov SV, Anantha S, Dickensheets H, Dumoutier L, Renauld JC, Zdanov A, Donnelly RP, Kotenko SV (2004) Cutting edge: IL-26 signals through a novel receptor complex composed of IL-20 receptor 1 and IL-10 receptor 2. J Immunol 172:2006–2010

Steubesand N, Kiehne K, Brunke G, Pahl R, Reiss K, Herzig KH, Schubert S, Schreiber S, Folsch UR, Rosenstiel P, Arlt A (2009) The expression of the beta-defensins hBD-2 and hBD-3 is differentially regulated by NF-kappaB and MAPK/AP-1 pathways in an in vitro model of Candida esophagitis. BMC Immunol 10:36

Strober W (2004) Epithelial cells pay a Toll for protection. Nat Med 10:898–900

Sugawara Y, Uehara A, Fujimoto Y, Kusumoto S, Fukase K, Shibata K, Sugawara S, Sasano T, Takada H (2006) Toll-like receptors, NOD1, and NOD2 in oral epithelial cells. J Dent Res 85:524–529

Sutmuller RP, den Brok MH, Kramer M, Bennink EJ, Toonen LW, Kullberg BJ, Joosten LA, Akira S, Netea MG, Adema GJ (2006) Toll-like receptor 2 controls expansion and function of regulatory T cells. J Clin Invest 116:485–494

Szolnoky G, Bata-Csorgo Z, Kenderessy AS, Kiss M, Pivarcsi A, Novak Z, Nagy Newman K, Michel G, Ruzicka T, Marodi L, Dobozy A, Kemeny L (2001) A mannose-binding receptor is expressed on human keratinocytes and mediates killing of *Candida albicans*. J Invest Dermatol 117:205–213

Takeda K, Kaisho T, Akira S (2003) Toll-like receptors. Annu Rev Immunol 21:335–376

Tardif F, Goulet JP, Zakrazewski A, Chauvin P, Rouabhia M (2004) Involvement of interleukin-18 in the inflammatory response against oropharyngeal candidiasis. Med Sci Monit 10:BR239–BR249

Taylor PR, Brown GD, Reid DM, Willment JA, Martinez-Pomares L, Gordon S, Wong SY (2002) The beta-glucan receptor, dectin-1, is predominantly expressed on the surface of cells of the

monocyte/macrophage and neutrophil lineages. J Immunol 169:3876–3882

Taylor PR, Gordon S, Martinez-Pomares L (2005a) The mannose receptor: linking homeostasis and immunity through sugar recognition. Trends Immunol 26:104–110

Taylor PR, Martinez-Pomares L, Stacey M, Lin H, Brown GD, Gordon S (2005b) Macrophage receptors and immune recognition. Annu Rev Immunol 23:901–944

Taylor PR, Tsoni SV, Willment JA, Dennehy KM, Rosas M, Findon H, Haynes K, Steele C, Botto M, Gordon S, Brown GD (2007) Dectin-1 is required for beta-glucan recognition and control of fungal infection. Nat Immunol 8:31–38

Tomalka J, Ganesan S, Azodi E, Patel K, Majmudar P, Hall BA, Fitzgerald KA, Hise AG (2011) A novel role for the NLRC4 inflammasome in mucosal defenses against the fungal pathogen *Candida albicans*. PLoS Pathog 7:e1002379

Uehara A, Takada H (2008) Synergism between TLRs and NOD1/2 in oral epithelial cells. J Dent Res 87:682–686

Uehara A, Sugawara Y, Kurata S, Fujimoto Y, Fukase K, Kusumoto S, Satta Y, Sasano T, Sugawara S, Takada H (2005) Chemically synthesized pathogen-associated molecular patterns increase the expression of peptidoglycan recognition proteins via Toll-like receptors, NOD1 and NOD2 in human oral epithelial cells. Cell Microbiol 7:675–686

Uehara A, Fujimoto Y, Fukase K, Takada H (2007) Various human epithelial cells express functional Toll-like receptors, NOD1 and NOD2 to produce anti-microbial peptides, but not proinflammatory cytokines. Mol Immunol 44:3100–3111

Underhill DM, Rossnagle E, Lowell CA, Simmons RM (2005) Dectin-1 activates Syk tyrosine kinase in a dynamic subset of macrophages for reactive oxygen production. Blood 106:2543–2550

van de Veerdonk FL, Joosten LA, Devesa I, Mora-Montes HM, Kanneganti TD, Dinarello CA, van der Meer JW, Gow NA, Kullberg BJ, Netea MG (2009a) Bypassing pathogen-induced inflammasome activation for the regulation of interleukin-1beta production by the fungal pathogen *Candida albicans*. J Infect Dis 199:1087–1096

van de Veerdonk FL, Marijnissen RJ, Kullberg BJ, Koenen HJ, Cheng SC, Joosten I, van den Berg WB, Williams DL, van der Meer JW, Joosten LA, Netea MG (2009b) The macrophage mannose receptor induces IL-17 in response to *Candida albicans*. Cell Host Microbe 5:329–340

van de Veerdonk FL, Joosten LA, Shaw PJ, Smeekens SP, Malireddi RK, van der Meer JW, Kullberg BJ, Netea MG, Kanneganti TD (2011) The inflammasome drives protective Th1 and Th17 cellular responses in disseminated candidiasis. Eur J Immunol 41:2260–2268

van der Graaf CA, Netea MG, Franke B, Girardin SE, Van der Meer JW, Kullberg BJ (2006) Nucleotide oligomerization domain 2 (Nod2) is not involved in the pattern recognition of *Candida albicans*. Clin Vaccine Immunol 13:423–425

Vignali DA, Collison L, Workman C (2008) How regulatory T cells work. Nat Rev Immunol 8:523–532

Vylkova S, Li XS, Berner JC, Edgerton M (2006) Distinct antifungal mechanisms: beta-defensins require *Candida albicans* Ssa1 protein, while Trk1p mediates activity of cysteine-free cationic peptides. Antimicrob Agents Chemother 50:324–331

Wagener J, Mailänder-Sanchez D, Schaller M (2012a) Immune responses to *Candida albicans* in models of in vitro reconstituted human oral epithelium. Methods Mol Biol 845:333–344

Wagener J, Weindl G, de Groot PW, de Boer AD, Kaesler S, Thavaraj S, Bader O, Mailänder-Sanchez D, Borelli C, Weig M, Biedermann T, Naglik JR, Korting HC, Schaller M (2012b) Glycosylation of *Candida albicans* cell wall proteins is critical for induction of innate immune responses and apoptosis of epithelial cells. PLoS One 7:e50518

Weindl G, Naglik JR, Kaesler S, Biedermann T, Hube B, Korting HC, Schaller M (2007) Human epithelial cells establish direct antifungal defense through TLR4-mediated signaling. J Clin Invest 117:3664–3672

Weindl G, Wagener J, Schaller M (2010) Epithelial cells and innate antifungal defense. J Dent Res 89:666–675

Weindl G, Wagener J, Schaller M (2011) Interaction of the mucosal barrier with accessory immune cells during fungal infection. Int J Med Microbiol 301:431–435

Willment JA, Brown GD (2008) C-type lectin receptors in antifungal immunity. Trends Microbiol 16:27–32

Wilson D, Thewes S, Zakikhany K, Fradin C, Albrecht A, Almeida R, Brunke S, Grosse K, Martin R, Mayer F, Leonhardt I, Schild L, Seider K, Skibbe M, Slesiona S, Waechtler B, Jacobsen I, Hube B (2009) Identifying infection-associated genes of *Candida albicans* in the postgenomic era. FEMS Yeast Res 9:688–700

Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R (2004) IL-22 increases the innate immunity of tissues. Immunity 21:241–254

Xie MH, Aggarwal S, Ho WH, Foster J, Zhang Z, Stinson J, Wood WI, Goddard AD, Gurney AL (2000) Interleukin (IL)-22, a novel human cytokine that signals through the interferon receptor-related proteins CRF2-4 and IL-22R. J Biol Chem 275:31335–31339

Yamashiro S, Kamohara H, Wang JM, Yang D, Gong WH, Yoshimura T (2001) Phenotypic and

functional change of cytokine-activated neutrophils: inflammatory neutrophils are heterogeneous and enhance adaptive immune responses. J Leukoc Biol 69:698–704

Zelante T, De Luca A, Bonifazi P, Montagnoli C, Bozza S, Moretti S, Belladonna ML, Vacca C, Conte C, Mosci P, Bistoni F, Puccetti P, Kastelein RA, Kopf M, Romani L (2007) IL-23 and the Th17 pathway promote inflammation and impair antifungal immune resistance. Eur J Immunol 37:2695–2706

Zelensky AN, Gready JE (2005) The C-type lectin-like domain superfamily. FEBS J 272:6179–6217

Zhou M, Yang B, Ma R, Wu C (2008) Memory Th-17 cells specific for C. albicans are persistent in human peripheral blood. Immunol Lett 118:72–81

Clinical Presentations and Epidemiology of Fungal Infections

10 Invasive Aspergillosis in the Intensive Care Unit

Wouter Meersseman[1]

CONTENTS

I. Is Invasive Aspergillosis a Problem in the Intensive Care Unit?

Autopsy studies have revealed the emergence of *Aspergillus* species as major pathogens, as well as the **expansion of the spectrum of patients at risk for invasive aspergillosis (IA)**. In a non-selected patient population at an academic hospital, the prevalence of invasive fungal infection increased from 2.2 % to 5.1 % over a 12-year period, largely in association with an increase in the rate of *Aspergillus* infection (Groll et al. 1996). However, estimates about the incidence of IA among critically ill patients are sparse and variable. For various reasons, figures about the true incidence of IA are difficult to generate. First, with cultures that are positive for *Aspergillus* species, discrimination between colonization and infection remains challenging. Second, very few institutions perform postmortem examinations routinely, although in most cases this is the only way to prove the definite nature of the diagnosis (Roosen et al. 2000; Dimopoulos et al. 2004; Esteban and Fernandez-Segoviano 2003). Third, characteristic radiological signs of IA are usually absent in the non-neutropenic intensive care unit (ICU) patient. Confounding variables such as atelectasis, bacterial ventilator-associated pneumonia or pleural effusions obscure the typical halo sign. Moreover, the halo sign is seen most often in neutropenic patients, which represent only a minority of cases in the ICU. Finally, to date, the diagnostic utility of recently available non-culture-based microbiological tools, including immunological methods for the detection of fungal antigens and the polymerase chain reaction (PCR) for the detection of *Aspergillus*-specific DNA, has not been properly validated in the nonhematology ICU population. In addition, the European Organization for Research and Treatment of Cancer/Mycosis Study Group (EORTC/MSG) guidelines were not designed for patient categories other than patients with cancer and patients who have undergone bone marrow transplantation. The revised definitions apply to immunocompromised patients but not necessarily to critically ill patients in the ICU who, nonetheless, may develop possible or probable IA (de Pauw et al. 2008).

In our medical ICU, we observed a high incidence of IA in two separate, retrospective, **autopsy-controlled** studies. In the larger study,

[1]Department of General Internal Medicine, Gasthuisberg University Hospital, Herestraat 49, Leuven 3000, Belgium; e-mail: wouter.meersseman@uz.leuven.be

Human Fungal Pathogens, 2nd Edition
The Mycota XII
O. Kurzai (Ed.)

127 (6.9 %) of 1,850 hospitalized patients had microbiologic or histopathologic evidence of aspergillosis during their ICU stay, including 89 cases (70 %) in which there was not an underlying hematological malignancy. The observed mortality rate of 80 % was much higher than the mortality rate predicted on the basis of the Simplified Acute Physiology Score II (48 %) (Meersseman et al. 2004). An earlier study sought unsuspected causes of death in the same medical ICU and revealed that, among 100 autopsies, there were 15 cases of IA, of which 5 were missed before death (Roosen et al. 2000). These data are in line with previous autopsy findings, suggesting that invasive fungal infections are among the most commonly missed diagnoses in ICU patients (Silfvast et al. 2003; Mort and Yeston 1999; Combes et al. 2004; Bulpa et al. 2001; Garnacho-Montero et al. 2005; Vandewoude et al. 2006). In a recent study that examined the etiology of patients with septic shock, the prevalence of IA was 0.3 % (Kumar et al. 2006). Valles et al. (2003) reported 13 (19 %) of 67 episodes of IA with pathologic and/or microbiologic evidence of aspergillosis in a cohort of patients with severe hospital-acquired pneumonia who had been admitted to the ICU. During a 6-year period, Cornillet et al. (2006) found that a mean number of 15 patients per year received a diagnosis of IA; approximately one-half of these patients were in the ICU. These intercenter differences can be explained by differences in underlying patient characteristics, case mixes, and autopsy policies.

II. How to Deal with the ICU Patient Who Has One or More *Aspergillus*-Positive Endotracheal Aspirates?

A European multicenter ($n=30$) observational study included critically ill patients with one or more *Aspergillus*-positive endotracheal aspirates (Blot et al. 2012). Among 115 histopathology-controlled patients, 79 had proven aspergillosis. A clinical algorithm based upon quantitative culture results judged 86 of 115 cases to have putative aspergillosis. The diagnosis was confirmed in 72 and rejected in 14 patients. The algorithm judged 29 patients to have *Aspergillus* colonizaton. This was confirmed in 22 and rejected in 7 patients. In a total cohort of 524 patients, 15 % had proven disease and 6 % had probable disease. According to the algorithm, the remainder of the patients were judged to be colonizers (46.9 %) or having putative disease ("possible" cases without histological confirmation) (38 %).

III. Who Is at Risk of Developing Aspergillosis in the ICU?

Over the past two decades, IA has emerged as a life-threatening fungal infection in patients with hematological diseases (Denning 1996). Although many infected patients will eventually be admitted to the ICU for advanced supportive care, it seems that IA has also gained a foothold in less severely immunocompromised ICU patients. So, can a threshold of immunosuppression needed for the development of IA be defined? We grouped the risk factors for IA in the ICU into three categories: high, intermediate, and low (Table 10.1).

Various factors, including the prolonged use of antibiotics and the use of central venous catheters and/or mechanical ventilation, adversely affect the defense systems of previously healthy individuals (Stevens et al. 2000). Although these factors are present in most ICU patients, many of these patients do not develop IA. One of the intriguing hypotheses for immunosuppression in the apparently immunocompetent patient with multiple-organ dysfunction relates to the **biphasic response to sepsis** (Hartemink et al. 2003). The initial hyperinflammatory phase is followed by relative immunoparalysis (Kox et al. 2000). This latter process is characterized by neutrophil deactivation, and it may put the patient at risk of developing opportunistic infections such as IA. Additional epidemiological studies are warranted to better delineate this phase of **immunoparalysis**.

Patients in the ICU (medical and surgical) are often treated with **steroids**. Recent work concluded that the mortality rate is reduced if

Table 10.1 Risk of invasive aspergillosis among patients admitted to the intensive care unit (medical, mixed, or surgical)

High-risk category	Neutropenia (neutrophil count $<500/mm^3$)
	Allogeneic bone marrow transplantation
	Hematological malignancy
Intermediate-risk category	Prolonged treatment with corticosteroids before admission to the ICU
	Autologous bone marrow transplantation
	Chronic obstructive pulmonary disease stage III or IV
	Liver cirrhosis Child C
	Solid-organ cancer
	ARDS treated with >80 mg methylprednisolone per day (for at least 3 days)
	Lung transplantation
	Systemic diseases such Wegener's granulomatosis and microscopic polyangiitis
	HIV infection
Low-risk category	Severe burns
	Prolonged stay in the ICU (>21 days)
	Chronic obstructive pulmonary disease stage II
	Other solid-organ transplant recipients (heart, kidney, or liver)
	Post-cardiac surgery status
	Malnutrition (BMI <18)
	Steroid treatment with a duration of less than 3 days

patients with septic shock who have adrenal dysfunction receive hydrocortisone for a 7-day period (Annane et al. 2002). However, in vitro pharmacological concentrations of hydrocortisone accelerate the growth of *Aspergillus* species (Lionakis and Kontoyiannis 2003). Clearly, high steroid intake diminishes both lines of cellular defense against IA (i.e., macrophages and neutrophils). This has been demonstrated in hematopoietic stem cell transplant recipients who received prolonged courses of steroids for the treatment of graft-versus-host disease (O'Donnell et al. 1994; Martino et al. 2002). Palmer et al. (1991) reported that the threshold steroid concentration varies according to the type of patient, and they emphasized that underlying lung disease is a risk factor for IA even when low doses of steroids are administered. Cases of IA have even been reported in association with inhaled steroids (Leav et al. 2000). Additional studies are needed to investigate whether administration of the 7-day course of hydrocortisone (200 mg/day) to patients with septic shock puts them at risk of developing IA, knowing that recognition of fungal infection may be delayed because the anti-inflammatory properties of steroids blunt the signs of infection (Graham and Tucker 1984). Recently, during the H1N influenza outbreak of 2009, 23 % of our patients with proven H1N1 developed probable or proven aspergillosis during the course of their illness. Almost all of them were treated with corticosteroids for the underlying acute respiratory distress syndrome (ARDS) caused by H1N1 (Wauters et al. 2012).

Two at-risk groups not included in the EORTC/MSG definitions stand out with regard to IA: patients with **chronic obstructive pulmonary disease** (COPD) and patients with cirrhosis. Patients with COPD have an increasingly recognized risk of developing IA and, in some institutions, cases of IA among patients with COPD outnumber those cases in "classic" patients. Bulpa et al. (2001) analyzed a group of 16 patients with COPD who had proven or probable IA and who required ICU admission. All patients were receiving steroid treatment. The outcome was invariably poor. This is in accordance with the findings of Rello et al. (1998), who described another 8 patients with COPD and IA, among whom the outcome was universally fatal.

Hepatic failure is generally not recognized as a risk factor for IA. A literature review revealed that 5 of 14 previously reported cases of IA in seemingly immunocompetent hosts were associated with liver disease (Ascah et al. 1984).

Our study revealed three fatal cases of IA (Meersseman et al. 2004). Patients with cirrhosis experience depressed phagocytosis, which may increase their risk for severe infections (Bailey et al. 1976).

It is expected that new risk categories of IA will arise as new immunosuppressive agents, such as alemtuzumab and etanercept (a TNF-α blocker), are made available (Martin et al. 2006; Warris et al. 2001).

IV. Do Patients Acquire Aspergillosis in the ICU?

There are numerous sources of *Aspergillus* species for patients in the ICU (Patterson 2005). Some studies suggest that fungal colonization of the lungs is present before entry into the hospital (Lass-Flörl et al. 1999). It is believed that the primary ecological niche is decomposing material (Segal and Walsh 2006). However, aerosolized spores may become a potential source of infection through improperly cleaned ventilation systems, water systems, or even computer consoles (Warris and Verweij 2005). The use of high-efficiency particulate air filtration reduces the risk of IA but does not reduce it to zero, probably in part because patients may be colonized before admission to the ICU, and partly because of breaks in airflow (Munoz et al. 2004). Pittet et al. (1996) described two patients who developed fatal IA in the ICU. In retrospect, high concentrations of airborne *Aspergillus* spores could be found in close proximity to an air filter change in the ICU. An outbreak in a Spanish post-cardiac surgery unit was reported in 2012. The demonstration of matches between air and clinical genotypes reinforces the role of environmental air in the acquisition of aspergillosis. High-efficiency particulate air filters were not able to prevent the outbreak (Pelaez et al. 2012). Environmental monitoring of *Aspergillus* spores in the air is mandatory but, to our knowledge, a study of ventilators as a source of infection has not been undertaken. Of note, the development of IA depends on an interplay between the inoculating dose, the ability of the host to resist infection, and the virulence of the organism.

In addition to the airborne route, contaminated water has been implicated as a source of infection (Anaissie et al. 2002; Warris et al. 2002).

In the retrospective study performed in our unit, 63 of 102 patients (62 %) with a culture positive for *Aspergillus* species had received the positive culture result within 1 week after admission to the ICU. Almost all patients were undergoing mechanical ventilation, and the mean duration of ICU stay was 20days. Of the patients with proven cases, 18 of 26 (69 %) with an underlying hematological malignancy and 11 of 30 (37 %) without a malignancy had clinical evidence of IA at the time of admission to the ICU (Meersseman et al. 2004). However, there is no consensus about the incubation period and estimates range from 2 days to 3 months. Moreover, culture results and clinical evidence alone are not reliable predictors for invasive disease. The concept that the increasing fungal burden associated with specific ICU treatments for diseases other than IA (e.g., steroid therapy for septic shock) parallels the progression from subclinical to clinical aspergillosis needs to be explored using more secretion specimens as a modality for surveillance is an interesting topic for research in the ICU.

V. Disease Manifestations in the ICU

There are **several manifestations of IA disease in the ICU**. There are three types of pulmonary pathogen–host interactions (Soubani and Chandrasekar 2002). The most frequent interaction is colonization of the airways; this can be present in patients with defective mucociliary clearance and structural changes in the bronchial wall (Hope et al. 2005a). These changes are present in almost every patient who is undergoing mechanical ventilation, making them particularly susceptible to colonization. IA will not develop in these patients unless a critical level of immunodeficiency has been reached.

The second type of interaction is "allergic" in nature and is beyond the scope of this review. The most relevant form of interaction for ICU physicians is the invasive disease that develops in persons with impaired immunity. The lungs and sinuses are implicated in >90 % of these cases (Patterson et al. 2000). The aggressive angioinvasive form of IA is frequently encountered in neutropenic patients, whereas cavitating infiltrates are observed most frequently in patients who are receiving steroids, patients with COPD, patients with cirrhosis, solid-organ transplant recipients, etc. (Denning et al. 2003). In lung transplant recipients, anastomotic infections are the most frequently occurring presentations (Nathan et al. 2000; Mehrad et al. 2001).

Other, rarer, presentations include endocarditis, wound infection, mediastinitis (after cardiac surgery), infection of vascular grafts, and osteomyelitis; these are occasionally a problem in immunocompromised patients or during epidemic outbreaks. A detailed description of all disease entities is beyond the scope of this article and was recently reviewed elsewhere (Pasqualotto and Denning 2006; Nunley et al. 2002). Infection of the central nervous system is frequently an ominous sign and may arise from hematogenous seeding (for which the lung is the most common primary site), from spread of the pathogen from the sinuses, or after neurosurgery (Lin et al. 2001; Marr et al. 2002).

The pathogenesis of IA in patients with steroid-associated immunosuppression differs greatly from that in neutropenic patients. Data demonstrate that pathologic lesions are often widespread and that death is related to a high fungal burden in neutropenic animals, whereas the pathogenesis in non-neutropenic, steroid-treated animals is driven by an adverse inflammatory host response that is frequently confined to the lungs, with a low fungal burden in the lung parenchyma and other organs (Balloy et al. 2005; Chamilos et al. 2006).

Clinical signs are usually nonspecific and do not necessarily differ from those for other causes of nosocomial pneumonia. In addition, critically ill patients with prolonged stays in the ICU often develop pulmonary infiltrates, atelectasis, and/or acute respiratory distress syndrome (ARDS), whereas patients with prior lung disease (e.g., COPD) may present with preexisting cavities noted by conventional chest radiography.

VI. Are the Available Diagnostic Tools Applicable to Patients in the ICU?

Making a timely diagnosis of IA in the ICU population is probably even more challenging than establishing an early diagnosis in patients with hematologic disease. Basically, this is because the **index of suspicion** is lower in the ICU population because most patients do not belong to one of the well-established risk groups. Moreover, the diagnostic tools were developed in hematology patients. In general, a diagnosis is made on the basis of a combination of compatible clinical findings, abnormal radiologic findings, and microbiologic confirmation or on the basis of histologic proof of tissue invasion by the fungus (Hope et al. 2005).

Over the past few years, lung computed tomography (CT) has become one of the most important diagnostic tools. Diagnostic signs of angioinvasive pulmonary mycosis – not only that due to *Aspergillus* species, but occasionally that due to Mucorales species – include single or multiple small nodules with the halo sign. It should be recognized that the utility of this sign has been evaluated almost exclusively in neutropenic patients (Caillot et al. 1997). In other groups, including ICU patients, similar CT findings are frequently absent, and if the signs are present, they are far less specific. Many ICU patients have nonspecific interfering radiologic abnormalities associated with atelectasis or ARDS (Figs. 10.1, 10.2, 10.3, and 10.4).

A positive result from a culture of a respiratory specimen or positive findings from a direct microscopic examination is present in only about half of patients with IA (Mennink-Kersten et al. 2004). The predictive value of a positive culture result depends largely on whether the patient is immunocompromised and ranges from 20 % to 80 % (Perfect et al. 2001). Given the ubiquitous nature of *Aspergillus* spores, differentiation of colonization from infection remains problematic. Two studies have examined the significance of isolation of *Aspergillus* species in ICU patients and have confirmed the

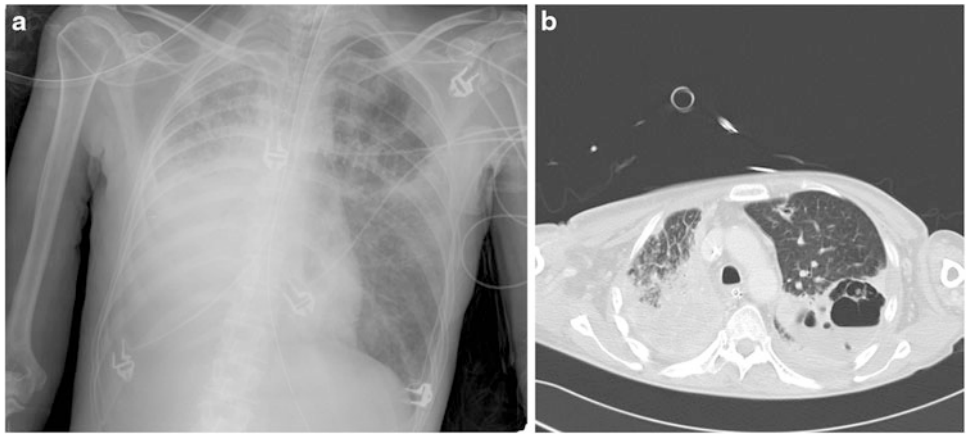


Fig. 10.1. (**a**) Chest X-ray and (**b**) CT scan of a 42-year-old lady with adenocarcinoma of the right lung with adjacent malignant pleural effusion who was receiving chemotherapy (cisplatinum and gemcitabine) and who was admitted to the intensive care unit because of fever and new pulmonary infiltrates. No neutropenia. A large right-sided pleural effusion and a cavity in the upper zone of the left lung were seen in the chest X-ray. CT scan confirmed a complex cavitary lesion in the left lung and a pleural effusion around the right lung. Bronchoalveolar lavage (BAL) culture was positive for *Haemophilus influenzae* and negative for fungi. The results of a serum galactomannan test were negative but revealed a value of 6.2 ng/ml in the BAL fluid specimen. Despite administration of voriconazole, the patient died. Autopsy revealed invasive aspergillosis that was confined to the left lung. Residual malignant lesion was present in the right pleura and lung parenchyma

poor positive predictive values (Tarrand et al. 2003). However, although culture and microscopic examination of respiratory tract samples are performed on a regular basis in most ICUs (once or twice weekly, as a means of surveillance), it is not an appropriate guide for clinical practice.

Serologic testing techniques based on the detection of circulating fungal cell wall components, such as **galactomannan** (GM) or β-D-glucan, and detection of circulating fungal DNA by PCR techniques hold promise for patients with hematologic malignancy, but they have not been systematically studied for the diagnosis of IA in the ICU. GM and β-D-glucan are polysaccharide fungal cell wall components that are released during tissue invasion and that can be detected in specimens of body fluids (e.g., serum and bronchoalveolar lavage fluid) obtained from patients with IA (Klont et al. 2004). Studies of neutropenic patients have revealed high rates of sensitivity (67–100 %) and specificity (86–99 %) (Pfeiffer et al. 2006; Maertens et al. 2002). However, in a retrospective observational study of a medical ICU population, serum GM was elevated in only 53 % of patients with IA (Meersseman et al. 2004). Detection of serum GM is probably not a sensitive marker for IA (especially in non-neutropenic patients), as demonstrated in lung and liver transplant recipients (Husain et al. 2004; Kwak et al. 2004). Viable fungi can endure in the lung tissue (with encapsulation by an inflammatory process), whereas circulating markers can remain undetectable because of clearance by circulating neutrophils. Bronchoalveolar lavage (BAL) fluid could be a better specimen for GM detection. In a prospective study performed at our unit, 110 patients (10 % of total population) underwent BAL for culture and GM detection, serum GM measurement, and CT scan. Only 22 % of the patients were neutropenic. Sensitivity and specificity of BAL GM detection with a cut-off value of 0.5 was 88 % and 87 %, respectively. This is in contrast with a sensitivity of only 42 % for serum GM (taken on the same day as BAL GM and using the same cut-off value) (Meersseman et al. 2008). The use of **β-D-glucan detection** in the ICU is hampered by false-positive results (associated with the use of albumin, wound gauze, hemodialysis, and bacterial infections) (Pazos et al. 2005; Digby et al. 2003; Ostrosky-Zeichner et al. 2005). GM

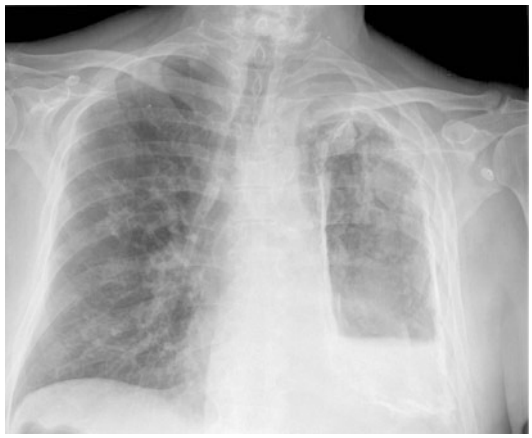


Fig. 10.2. Chest radiograph of 72-year-old patient referred for pneumectomy. At the time of referral, he had major hemoptysis. He was known to have suffered from tuberculosis 20 years earlier. Chest X-ray preoperatively shows a destroyed left lung, an air-fluid level with widespread pleural calcifications. Culture from a tracheal aspirate revealed *Aspergillus fumigatus*. GM value of a BAL specimen was 9.8 ng/ml. Serum galactomannan was below detection limit. No reactivation of tuberculosis was found. Patient was treated with voriconazole. Pneumectomy was uneventful. Biopsy of necrotic tissue per-operatively showed dichotomous branching hyphae, which turned out to be *Aspergillus fumigatus* upon culture

detection yields fewer false-positive results, although the use of β-lactam antibiotics posed a problem in the past. Although some residual GM might still be present in piperacillin/tazobactam, the preparations seem no longer responsible for false-positive GM results (Mikulska et al. 2012). Plasma-lyte, a frequently uses resuscitation fluid, might also cause false positivity of the GM assay (Petraitiene et al. 2011).

Thus far, no prospective data on PCR detection are available for ICU patients.

Critical care physicians need a helpful tool to guide clinical practice. A pre-emptive based approach using GM-detection in BAL might lead to earlier treatment of aspergillosis in the ICU.

VII. Antifungals for the Treatment of Aspergillosis in the ICU

Amphotericin B has been the mainstay of the treatment of IA for a long time. However, this formulation is renowned for being associated with serious adverse effects (e.g., nephrotoxicity, hypokalemia, and fever). These events often result in the use of suboptimal dosing regimens. Fortunately, over the past few years, lipid-based formulations of amphotericin B and new antifungal drugs with more favorable tolerability and safety profiles (including voriconazole, posaconazole, and the echinocandins) have become available as alternatives.

Ten years ago, voriconazole (a derivative of fluconazole) became the new standard of care for treating IA. A significantly better outcome (response rate, 52.8 % versus. 30.6 %) was demonstrated in a randomized study that compared initial treatment with voriconazole versus conventional amphotericin B (Herbrecht et al. 2002). Posaconazole is a new, oral, broad-spectrum triazole that is effective against several fungi that are resistant to most other antifungals; it is well tolerated and holds promise as a prophylactic agent in neutropenic patients (Cornely et al. 2007). Caspofungin, micafungin, and anidulafungin belong to a new class of antifungal drugs, the echinocandins, which act by inhibiting the synthesis of β-(1,3)- D-glucan in the fungal cell wall. Echinocandins display activity against *Aspergillus* species, as demonstrated in several studies of salvage therapy, but convincing data on their use as first-line treatment are still lacking (Denning 2003). The latter criticism also applies to first-line treatment with lipid-based formulations of amphotericin B (Sastry et al. 2005).

However, most patients who were recruited in these first- and second-line treatment studies were experiencing an underlying hematological disorder or were transplant recipients. These studies usually exclude patients with baseline characteristics that are commonly seen in ICU patients, including patients with liver function abnormalities, coagulation disorders, or renal dysfunction and patients in need of advanced cardiovascular or pulmonary support, including mechanical ventilation. Non-neutropenic ICU patients and patients who are not transplant recipients largely tend to be underrepresented in all major trials; given the impact of these co-morbidities, lower response rates can be anticipated.

In addition, many aspects of antifungal therapy that are relevant to the ICU population have not been sufficiently addressed in clinical

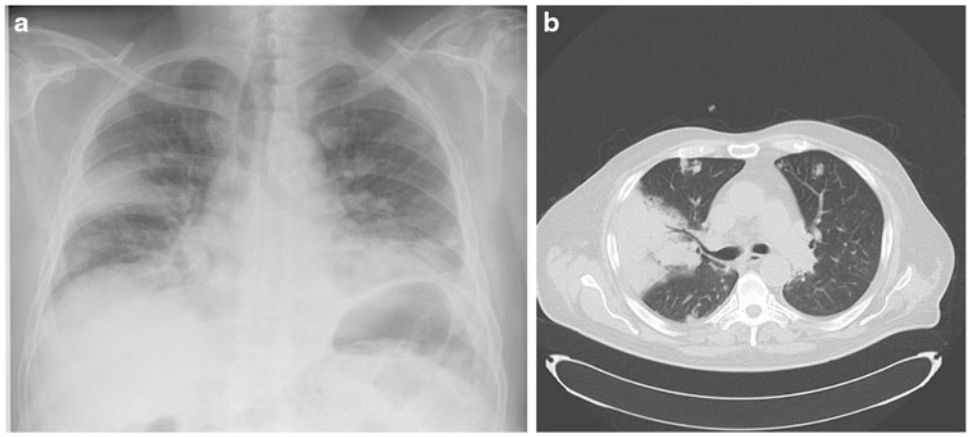


Fig. 10.3. (**a**) Chest radiograph and (**b**) CT of a 65-year-old man who was diagnosed as having Wegener's granulomatosis 1 month prior to presentation. Patient had renal involvement without pulmonary involvement initially. He was treated with high-dose steroids and cyclophosphamide. One month later, he developed fever, hemoptysis, and acute respiratory failure. He required mechanical ventilation. Chest X-ray shows bilateral patchy infiltrates with a large wedge-shaped lesion on the right side. CT scan showed a large triangled infiltrate in the right lung suggestive of pulmonary infarction. Widespread nodules are seen throughout both lungs. Bronchial culture for fungi remained negative, although BAL GM and serum GM values were both raised (8.4 and 3.2 ng/ml respectively). Patient died soon after admission to the ICU following ventricular fibrillation. Autopsy revealed disseminated aspergillosis (lung, thyroid gland, heart, adrenals, and spleen)

studies, including the **pharmacokinetic profile of antifungals in patients with underlying renal, hepatic, and/or cardiac dysfunction**; the dose–response relationship; the best route of administration (oral, enteral, or parenteral); the monitoring of drug-related toxicities (e.g., how to monitor voriconazole-induced visual disturbances in sedated patients); and, especially, drug interactions with frequently used "ICU drugs". Studies measuring levels of voriconazole in ICU patients are eagerly awaited. The echinocandins have not been studied as first-line therapy but offer the advantage of being free of nephrotoxicity; dose adjustments are not required in the event of renal failure or in patients who are undergoing continuous hemofiltration. In addition, few clinically significant drug–drug interactions have been reported.

VIII. Future Directions

In an era of increased availability of new immunosuppressive drugs and better intensive care, with prolonged patient survival, we can expect a continuing increase in the incidence of IA. The occurrence of IA in the ICU usually entails a poor prognosis, despite major recent improvements in the diagnosis and treatment of IA in patients with hematologic diseases. Multicenter studies are warranted to explore the exact incidence of IA in the ICU and to better delineate the difference between hospital-acquired, ICU-acquired, and community-acquired aspergillosis. Evaluating the value of galactomannan, β-D-glucan, and PCR analyses in non-neutropenic, critically ill patients with different sample types (and, especially, with respiratory samples) is urgently needed, as is a better delineation of the patient population at risk for IA in the broad group of critically ill patients. Finally, antifungal pharmacokinetics, pharmacodynamics, and interactions with other drugs need to be explored more thoroughly. Meanwhile, all new diagnostic techniques and therapeutic measures must be validated against postmortem findings, because only proven cases of IA offer the most valuable information.

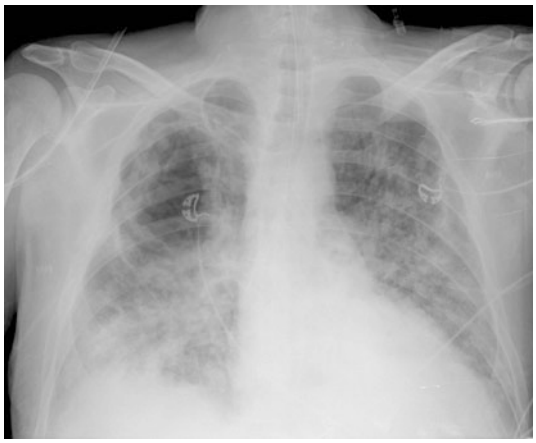


Fig. 10.4. Chest radiograph (day 22 post-ICU admission) of a 52-year-old man with Child C liver cirrhosis. He was originally admitted for a spontaneous bacterial peritonitis. Blood cultures revealed *Escherichia coli*. He developed multiple organ failure. On day 20 post-admission to the ICU, he was still mechanically ventilated and developed new-onset fever and progressively increased oxygen requirements. Bronchial culture revealed *Pseudomonas aeruginosa*. Despite meropenem, he deteriorated. A chest X-ray showed bilateral infiltrates with the possibility of a cavity in the right lung. A CT scan was not feasible. A new bronchial culture obtained on day 23 revealed *Aspergillus fumigatus*. BAL GM was 3.1 ng/ml and serum GM was 0.7 ng/ml. Anidulafungin was started on day 25. No voriconazole IV was given because of severe renal failure. Voriconazole via nasogastric tube was unsuccessful because of large residual volumes in the stomach. Patient died on day 30 post-admission. Autopsy showed a necrotizing right-sided pneumonia due to *Aspergillus fumigatus*

Acknowledgments Potential conflicts of interest: W.M. has been a member of the speakers' bureau for Pfizer and Merck Sharp and Dome.

References

Anaissie EJ, Stratton SL, Dignani MC et al (2002) Pathogenic *Aspergillus* species recovered from a hospital water system: a 3-year prospective study. Clin Infect Dis 34:780–789

Annane D, Sebille V, Charpentier C et al (2002) Effect of treatment with low doses of hydrocortisone fludrocortisone on mortality in patients with septic shock. JAMA 288:862–871

Ascah KJ, Hyland RH, Hutcheon MA et al (1984) Invasive aspergillosis in a "healthy" patient. Can Med Assoc J 131:332–335

Bailey RJ, Woolf IL, Cullens H, Williams R (1976) Metabolic inhibition of polymorphonuclear leucocytes in fulminant hepatic failure. Lancet 1:1162–1163

Balloy V, Huerre M, Latgé JP, Chignard M (2005) Differences in patterns of infection inflammation for corticosteroid treatment chemotherapy in experimental invasive pulmonary aspergillosis. Infect Immun 73:494–503

Blot SI, Taccone FS, Van den Abeele AM et al (2012) A clinical algorithm to diagnose invasive aspergillosis in critically ill patients. Am J Resp Crit Care Med 186:56–64

Bulpa PA, Dive AM, Garrino MG et al (2001) Chronic obstructive pulmonary disease patients with invasive pulmonary aspergillosis: benefits of intensive care? Intensive Care Med 27:59–67

Caillot D, Casasnovas O, Bernard A et al (1997) Improved management of invasive pulmonary aspergillosis in neutropenic patients using early thoracic computed tomographic scan surgery. J Clin Oncol 15:139–147

Chamilos G, Luna M, Lewis R et al (2006) Invasive fungal infections in patients with hematological malignancies in a tertiary care center: an autopsy study over a 15-year period (1989–2003). Haematologica 91:986–989

Combes A, Mokhtari M, Couvelard A et al (2004) Clinical autopsy diagnoses in the intensive care unit: a prospective study. Arch Intern Med 164:389–392

Cornely O, Maertens J, Winston DJ et al (2007) Posaconazole vs. fluconazole or itraconazole in patients with neutropenia. N Engl J Med 356:348–359

Cornillet A, Camus C, Nimubona S et al (2006) Comparison of epidemiological, clinical biological features of invasive aspergillosis in neutropenic nonneutropenic patients: a 6-year survey. Clin Infect Dis 43:577–584

de Pauw B, Walsh TJ, Donnelly JP et al on behalf of the Invasive Fungal Infections Cooperative Group of the European Organization for Research Treatment of Cancer Mycoses Study Group of the National Institute of Allergy Infectious Diseases (2008) Revised definitions of invasive fungal disease from the EORTC/MSG consensus group. Clin Infect Dis 46(12):1813–1821

Denning DW (1996) Therapeutic outcome in invasive aspergillosis. Clin Infect Dis 23:608–615

Denning DW (2003) Echinocandin antifungal drugs. Lancet 362:1142–1151

Denning DW, Riniotis K, Dobrashian R, Sambatakou H (2003) Chronic cavitary fibrosing pulmonary pleural aspergillosis: case series, proposed nomenclature change, review. Clin Infect Dis 37(Suppl 3): S265–S280

Digby J, Kalbfleisch J, Glenn A, Larsen A, Browder W, Williams D (2003) Serum glucan levels are not specific for presence of fungal infections in intensive care unit patients. Clin Diagn Lab Immunol 10:882–885

Dimopoulos G, Piagnerelli M, Berré J, Salmon I, Vincent JL (2004) Post mortem examination in the intensive care unit: still useful? Intensive Care Med 30:2080–2085

Esteban A, Fernandez-Segoviano P (2003) Is autopsy dead in the ICU? Intensive Care Med 29:522–525

Garnacho-Montero J, Amaya-Villar R, Ortiz-Leyba C et al (2005) Isolation of *Aspergillus* spp. from the respiratory tract in critically ill patients: risk factors, clinical presentation outcome. Crit Care 9:R191–R199

Graham BS, Tucker WS Jr (1984) Opportunistic infections in endogenous Cushing's syndrome. Ann Intern Med 101:334–338

Groll AH, Shah PM, Mentzel C, Schneider M, Just-Nuebling G, Huebner K (1996) Trends in the post-mortem epidemiology of invasive fungal infections at a university hospital. J Infect 33:23–32

Hartemink KJ, Paul MA, Spijkstra JJ, Girbes A, Polderman KH (2003) Immunoparalysis as a cause for invasive aspergillosis? Intensive Care Med 29:2068–2071

Herbrecht R, Denning DW, Patterson TF et al (2002) Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. N Engl J Med 347:408–415

Hope WW, Walsh TJ, Denning DW (2005a) Laboratory diagnosis of invasive aspergillosis. Lancet Infect Dis 5:609–622

Hope WW, Walsh TJ, Denning DW (2005b) The invasive saprophytic syndromes due to *Aspergillus* spp. Med Mycol 43(Suppl 1):S207–S238

Husain S, Kwak E, Obman A et al (2004) Prospective assessment of Platelia *Aspergillus* galactomannan antigen for the diagnosis of invasive aspergillosis in lung transplant recipients. Am J Transplant 4:796–802

Klont R, Messink-Kersten M, Verwije PE (2004) Utility of *Aspergillus* antigen detection in specimens other than serum specimens. Clin Infect Dis 39:1467–1474

Kox WJ, Volk T, Kox SN et al (2000) Immunomodulatory therapies in sepsis. Intensive Care Med 26(Suppl 1):S124–S128

Kumar A, Roberts D, Wood KE et al (2006) Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock. Crit Care Med 34:1589–1596

Kwak E, Husain S, Obman A et al (2004) Efficacy of galactomannan antigen in the Platelia *Aspergillus* enzyme immunoassay for diagnosis of invasive aspergillosis in liver transplant recipients. J Clin Microbiol 42:435–438

Lass-Flörl C, Salzer G, Schmid T, Rabl W, Ulmer H, Dierichi M (1999) Pulmonary *Aspergillus* colonization in humans: its impact on management of critically ill patients. Br J Haematol 104:745–747

Leav BA, Fanburg B, Hadley S (2000) Invasive pulmonary aspergillosis associated with high-dose inhaled fluticasone. N Engl J Med 343:586

Lin SJ, Schranz J, Teutsch M (2001) Aspergillosis case-fatality rate: systematic review of the literature. Clin Infect Dis 32:358–366

Lionakis MS, Kontoyiannis DP (2003) Glucocorticoids invasive fungal infections. Lancet 362:1828–1838

Maertens J, Van Eldere J, Verhaegen J, Verbeken Verschakelen J, Boogaerts M (2002) Use of circulating galactomannan screening for early diagnosis of invasive aspergillosis in allogeneic stem cell transplant recipients. J Infect Dis 186:1297–1306

Marr KA, Patterson T, Denning DW (2002) Aspergillosis: pathogenesis, clinical manifestations therapy. Infect Dis Clin North Am 16:875–894

Martin SI, Marty FM, Fiumara K, Treon SP, Gribben JG, Baden LR (2006) Infectious complications associated with alemtuzumab use for lymphoproliferative disorders. Clin Infect Dis 43:16–24

Martino R, Subira M, Rovira M et al (2002) Invasive fungal infections after allogeneic peripheral blood stem cell transplantation: incidence risk factors in 395 patients. Br J Haematol 116:475–482

Meersseman W, Vandecasteele SJ, Wilmer A, Verbeken E, Peetermans WE, Van Wijngaerden E (2004) Invasive aspergillosis in critically ill patients without malignancy. Am J Respir Crit Care Med 170:621–625

Meersseman W, Lagrou K, Maertens J et al (2008) Galactomannan in broncho-alveolar lavage fluid: a tool for diagnosing aspergillosis in ICU patients. Am J Resp Crit Care 1:27–34

Mehrad B, Paciocco G, Martinez FJ, Ojo TC, Ianettoni MD, Lynch JP (2001) Spectrum of *Aspergillus* infection in lung transplant recipients: case series review of the literature. Chest 119:169–175

Mennink-Kersten MA, Donelly JP, Verweij PE (2004) Detection of circulating galactomannan for the diagnosis management of invasive aspergillosis. Lancet Infect Dis 4:349–357

Mikulska M, Furfaro E, Del Bono V et al (2012) Piperacillin/tazobactam seem to be no longer responsible for false positive results of the galactomannan assay. J Antimicrob Chemother 67:1746–1748

Mort TC, Yeston NS (1999) The relationship of pre mortem diagnoses post mortem findings in a surgical intensive care unit. Crit Care Med 27:299–303

Munoz P, Guinea J, Pelaez T, Duran C, Blanco JL, Bouza E (2004) Nosocomial invasive aspergillosis in a heart transplant recipient acquired during a break in the HEPA air filtration system. Transpl Infect Dis 6:50–54

Nathan SD, Shorr AF, Schmidt ME, Burton NA (2000) Aspergillus endobronchial abnormalities in lung transplant recipients. Chest 118:403–407

Nunley DR, Gal AA, Vega JD, Perlino C, Smith P, Lawrence CE (2002) Saprophytic fungal infections complications involving the bronchial anastomosis following human lung transplantation. Chest 122:1185–1191

O'Donnell MR, Schmidt GM, Tegtmeier BR et al (1994) Prediction of systemic fungal infection in allogeneic marrow recipients: impact of amphotericin prophylaxis in high-risk patients. J Clin Oncol 12:827–834

Ostrosky-Zeichner L, Alexander BD, Kett DH et al (2005) Multicenter clinical evaluation of the (1→3) β-D-glucan assay as an aid to diagnosis of fungal infections in humans. Clin Infect Dis 41:654–659

Palmer LB, Greenberg HE, Schiff MJ (1991) Corticosteroid treatment as a risk factor for invasive aspergillosis in patients with lung disease. Thorax 46:15–20

Pasqualotto AC, Denning DW (2006) Post-operative aspergillosis. Clin Microbiol Infect 12:1060–1076

Patterson TF (2005) Advances challenges in management of invasive mycoses. Lancet 366:1013–1025

Patterson TF, Kirkpatrick WR, White M et al (2000) Invasive aspergillosis: disease spectrum, treatment practices, outcome. Medicine (Baltimore) 79:250–260

Pazos C, Ponton J, Del Palacio A (2005) Contribution of 1,3 beta-D glucan chromogenic assay to diagnosis therapeutic monitoring of invasive aspergillosis in neutropenic adult patients: a comparison with serial screening for circulating galactomannan. J Clin Microbiol 43:299–305

Pelaez T, Munoz P, Guinea J et al (2012) Outbreak of invasive aspergillosis after major heart surgery caused by spores in the air of the intensive care unit. Clin Infect Dis 54:24–31

Perfect JR, Cox GM, Lee JY et al (2001) The impact of culture isolation of *Aspergillus* species: a hospital-based survey of aspergillosis. Clin Infect Dis 33:1824–1833

Petraitiene R, Petraitis V, Witt JR et al (2011) Galactomannan-antigenemia after infusion of gluconate-containing plasma-lyte. J Clin Microbiol 48:4330–4332

Pfeiffer C, Fine J, Safdar N (2006) Diagnosis of invasive aspergillosis using a galactomannan assay: a meta-analysis. Clin Infect Dis 42:1417–1427

Pittet D, Huguenin T, Dharan S et al (1996) Unusual cause of lethal pulmonary aspergillosis in patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 154:541–544

Rello J, Esandi ME, Mariscal D, Gallego M, Domingo C, Valles J (1998) Invasive pulmonary aspergillosis in patients with chronic obstructive pulmonary disease: report of eight cases review. Clin Infect Dis 26:1473–1475

Roosen J, Frans E, Wilmer A, Knockaert D, Bobbaers H (2000) Comparison of premortem clinical diagnoses in critically ill patients subsequent autopsy findings. Mayo Clin Proc 75:562–567

Sastry P, Parikh P, Kulkarni P, Bhagwart R, Gadade H (2005) Use of liposomal amphotericin B in bone marrow transplant. J Postgrad Med 51:S49–S52

Segal BH, Walsh TJ (2006) Current approaches to diagnosis treatment of invasive aspergillosis. Am J Respir Crit Care Med 173:707–717

Silfvast T, Takkunen O, Kolho E, Andersson L, Rosenberg P (2003) Characteristics of discrepancies between clinical autopsy diagnoses in the intensive care unit: a 5-year review. Intensive Care Med 29:321–324

Soubani AO, Chandrasekar PH (2002) The clinical spectrum of pulmonary aspergillosis. Chest 121:1988–1999

Stevens DA, Kan VL, Judson MA (2000) Practice guidelines for diseases caused by *Aspergillus*. Clin Infect Dis 30:696–709

Tarrand JJ, Lichterfeld M, Warraich I et al (2003) Diagnosis of invasive septate mold infections: a correlation of microbiological culture histologic or cytologic examination. Am J Clin Pathol 119:854–858

Valles J, Mesalles E, Marsical D et al (2003) A 7-year prospective study of severe-hospital acquired pneumonia requiring ICU admission. Intensive Care Med 29:1981–1988

Vandewoude KH, Blot SI, Depuydt P et al (2006) Clinical relevance of *Aspergillus* isolation from respiratory tract samples in critically ill patients. Crit Care 10:R31

Warris A, Verweij PE (2005) Clinical implications of environmental sources for *Aspergillus*. Med Mycol 43(Suppl 1):S59–S65

Warris A, Bjorneklett A, Gaustad P et al (2001) Invasive aspergillosis associated with infliximab therapy. N Engl J Med 344:1099–1100

Warris A, Voss A, Abrahamsen TG, Verwije PE (2002) Contamination of hospital water with *Aspergillus fumigatus* other molds. Clin Infect Dis 34:1159–1160

Wauters J, Baar I, Meersseman P et al (2012) Invasive pulmonary aspergillosis is a frequent complication of critically ill H1N1 patients: a retrospective study. Intensive Care Med 38:1761–1768

11 Molecular Epidemiology of *Pneumocystis* Outbreaks

Philippe M. Hauser[1], Joseph A. Kovacs[2]

CONTENTS

I. Introduction

Pneumocystis jirovecii is a fungus that causes severe pneumonia in immunocompromised humans. Recognized initially among presumably malnourished infants, and subsequently among immunosuppressed cancer patients, *Pneumocystis jirovecii* pneumonia (PCP) assumed medical prominence with the advent of the HIV epidemic. With the development and broad utilization of combination antiretroviral therapy, the disease has become sporadic in HIV-infected persons, primarily occurring among those who are unaware of their status, as well as in solid organ transplantation recipients and patients with hemato-oncologic or autoimmune diseases. Notably, multiple **nosocomial outbreaks** of PCP among renal transplant recipients (RTRs) have been reported, especially over the past 15 years. These outbreaks suggest inter-human transmission of the pathogen or its acquisition from a common source. Because a detailed review of studies of PCP outbreaks has recently been published (de Boer et al. 2011), the present chapter focuses on new insights from more recent publications. In addition, we comment on the limitations of the molecular typing methods available for investigation of PCP outbreaks.

[1]Institute of Microbiology, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland; e-mail: Philippe.Hauser@chuv.ch
[2]Critical Care Medicine Department, NIH Clinical Center, National Institutes of Health, Bethesda, MD, USA

II. Biology

P. jirovecii specifically infects humans, whereas other *Pneumocystis* species infect other mammals (Thomas and Limper 2007; Cushion and Stringer 2010). *P. jirovecii* grows extracellularly in the alveoli of human lungs. In the absence of a long-term in vitro culture system, the life cycle remains hypothetical and is postulated to include asexual (trophic cell) and sexual (ascus, or cyst) forms. Although PCR techniques are being increasingly utilized for diagnosis, the gold standard still remains the detection of organisms within respiratory specimens using various staining methods, such as methenamine silver and direct immunofluorescence (examples are shown in Fig. 11.1). A number of biological features of *Pneumocystis* species suggest that they are obligate parasites without a free-living form: (i) organisms can incorporate host cholesterol (Furlong et al. 1997), (ii) they possess a system of surface antigen variation (Stringer and Keely 2001), (iii) they appear to have co-evolved with their hosts (Demanche et al. 2001), and (iv) they lack most of the enzymes that are dedicated to the biosyntheses of amino acids in fungi (Table 11.1) (Hauser et al. 2010; Cissé et al. 2012). Moreover, the

Human Fungal Pathogens, 2nd Edition
The Mycota XII
O. Kurzai (Ed.)

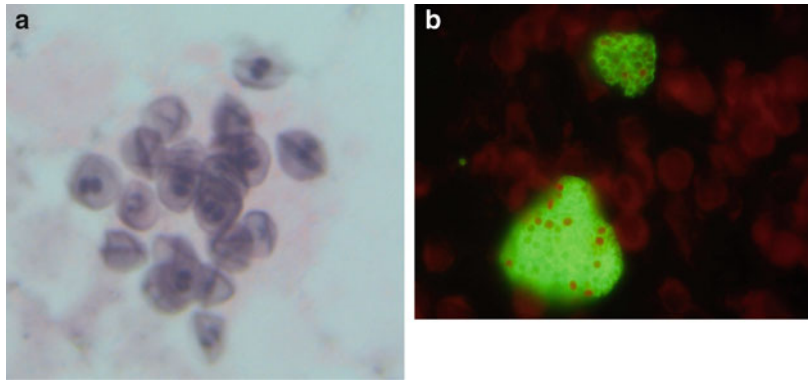


Fig. 11.1 (**a**) *P. jirovecii* asci visualized in a patient's bronchoalveolar lavage using Grocott's methenamine silver staining as described by Churukian and Schenk (1977). The *parentheses-like structure* in each ascus resembles opercula observed in other asci. Ascomycetous operculum is thought to be involved in the dehiscence (release of endospores) upon contact with humidity, suggesting that asci might be the airborne *P. jirovecii* infectious forms (picture from Institute of Microbiology, Centre Hospitalier Universitaire Vaudois). (**b**) *P. jirovecii* organisms visualized in a patient's induced sputum using direct immunofluorescence kit BioRad MONOFLUO Pneumocystis (Picture from Critical Care Medicine Department, NIH Clinical Center, National Institutes of Health)

P. jirovecii genome lacks known virulence factors and has a relatively small size and reduced GC content (Cissé et al. 2012). Together with epidemiological studies, which have failed to identify a free-living form of the fungus, these features suggest that *P. jirovecii* is an obligate parasite that has developed specialized systems for infecting humans but that causes clinically significant disease only in immunocompromised hosts.

III. Epidemiology

Immunity against *P. jirovecii* develops during the **first years of life**, presumably after a primary infection that occurs prior to 2 years of age (Pifer et al. 1978; Vargas et al. 2001). Thus, carriage of the fungus as a latent infection and reactivation upon immunosuppression was the first hypothesis for the development of PCP later in life. However, a number of recent observations have suggested that infection de novo from exogenous sources is responsible for the majority of episodes: (i) the occurrence of PCP outbreaks caused by a single genotype (de Boer et al. 2011), (ii) the elimination of *Pneumocystis* after experimental infection in the rat model (Vargas et al. 1995), and (iii) the presence of mutations within the dihydropteroate synthase gene in patients who never received sulfa drugs (Huang et al. 2000; Nahimana et al. 2003a). The latter observation provides especially strong evidence supporting **de novo infection** because these mutations (i) are associated with failure of sulfa prophylaxis, (ii) are absent in *P. jirovecii* isolated before the use of sulfa drugs for treating or preventing PCP (Meshnick 1999), and (iii) have been shown to be selected by sulfonamide use within single patients (Nahimana et al. 2003b).

The occurrence of infection de novo raises questions about the source of the pathogen and mode(s) of transmission. Obligate parasitism implies that the reservoir and source of infection are probably only humans.

A number of experiments in animal models have helped in understanding the epidemiology of *P. jirovecii* in humans. Studies on rats demonstrated that air is the route of transmission for de novo infection (Hughes 1982; Hughes et al. 1983). Horizontal transfer of the fungus can occur from animals with PCP to susceptible animals (Powles et al. 1992; Vogel et al. 1993; Ceré et al. 1997), but also from immunocompetent murine carriers of the fungus following a single day of exposure (Dumoulin et al. 2000; Chabé et al. 2004).

Table 11.1 Number of enzymes dedicated to the biosyntheses of amino acids in *P. jirovecii, P. carinii,* and *S. pombe*

	Number of dedicated enzymes			
Amino acid	In *S. pombe* (reference)[a]	In *S. pombe*	In *P. jirovecii*	In *P. carinii*
Ala	1	1	1	0
Asp	1	1	1	1
Asn	1[b]	1	0	0
Arg	3	3	0	0
Cys	2	1	0	0
Glu	1[b]	1	1	1
Gln	1	1	1	0
Gly	1[b]	1	1	0
His	6	6	0	0
Ile[c]	4	4	1	0
Leu[d]	3	3	1	0
Lys from aspartate	0	0	0	0
Lys from pyruvate	7	3	0	0
Met	3	2	0	0
Phe	2	1	0	0
Pro	1[b]	1	0	0
Ser	3	3	1	0
Thr from glycine	1	1	0	0
Thr from homoserine	2	2	0	0
Trp	5	3	0	0
Tyr	2	1	0	0
Val[c]	4	4	1	0
Total	54	44	9	2

Taken from Table S5 of Cissé et al. (2012)

[a]The reference gene numbers of *S. pombe* are those obtained from KEGG; data from Hauser et al. (2010)

[b]Universally conserved (Payne and Loomis 2006)

[c]The four enzymes are the same for Ile and Val syntheses

[d]One of the enzymes is also involved in Ile and Val syntheses

Carriage of *P. jirovecii* without overt PCP by immunocompromised (Nevez et al. 1999; Hauser et al. 2000) and immunocompetent (Vargas et al. 2000; Miller et al. 2001; Tipirneni et al. 2009) humans has been documented. The genotypes observed in carriers (Hauser et al. 2000) and in infants, presumably experiencing primary infection (Totet et al. 2003, 2004), were not different from those in PCP patients, suggesting that these could be sources of the pathogen. However, one study of larger cohorts found that infants may harbor different genotypes from AIDS patients (Beard et al. 2005). This study suffers from the fact that the infant and adult populations were from different geographical areas, which may have had different predominant genotypes. The dispersion of *Pneumocystis* organisms in the air around PCP patients (Choukri et al. 2010; Bartlett et al. 1997), as well as around infected rats (Choukri et al. 2011) has been examined by PCR techniques. Significant fungal burdens were detected in the air at 1 m from the source, then decreased with distance but were detected up to 8 m away. For rats, air burdens correlated with lung burdens. The same *P. jirovecii* genotypes were present in the lungs of PCP patients and in air particles collected in their room (Bartlett et al. 1997; Damiani et al. 2012). Together, these observations suggest that inter-human transmission from infected humans through the air is the main route of dissemination of *P. jirovecii*. The possible categories of humans that may act as a source include patients with active or developing PCP, infants experiencing primary infection, and colonized immunocompromised

patients, but also, possibly, transient carriers such as elderly people (Vargas et al. 2010), pregnant women (Vargas et al. 2003), and healthcare workers (Miller et al. 2001; Tipirneni et al. 2009). Nevertheless, the actual human source(s) of infection for sporadic cases and their relative importance remain to be elucidated. We speculate that infants experiencing primary infection might play a major role because, being immunologically naive, multiplication of the fungus might be more productive than in other categories of colonized humans.

IV. Molecular Methods for Investigating PCP Outbreaks

In order to test the hypothesis that inter-human transmission of *P. jirovecii* is involved in outbreaks, molecular typing methods are crucial because they can document identical *P. jirovecii* genotypes in patients between whom transmission is suspected. Typing methods for *P. jirovecii* rely on the natural genetic heterogeneity among isolates. In the absence of a culture method, current typing methods utilize **PCR amplification of variable regions of the *P. jirovecii* genome**, followed by the detection of polymorphisms by DNA sequencing, single-strand conformation polymorphism (SSCP) analysis, or restriction fragment length polymorphism (RFLP) analysis. Initially, one to two genetic regions were sequenced, including a variable region of the mitochondrial 26S rRNA gene (hereafter called mt26S) (Keely et al. 1995) or the internal transcribed spacers number 1 and 2 of the nuclear rRNA operon (ITS1 and ITS2; ITSs sequencing) (Lee et al. 1993; Lu et al. 1994; Tsolaki et al. 1996). Subsequently, a multilocus approach was developed that utilized SSCP to analyze four genomic regions (Hauser et al. 1997, 2001a; Nahimana et al. 2000), including ITS1, mt26S, the intron of the nuclear 26S rRNA gene (26S), and the β-tubulin intron 6 region (b-tub). As DNA sequencing prices decreased, SSCP was replaced by direct sequencing, i.e., without cloning PCR products (multilocus sequence typing, MLST) (Schmoldt et al. 2008; Gianella et al. 2010; Pliquett et al. 2012). RFLP analysis of a PCR product from a variant region of the gene family encoding the major surface glycoprotein (RFLP-MSG) was shown to be discriminative and useful for investigating outbreaks (Ripamonti et al. 2009; Sassi et al. 2012). The polymorphisms in all markers that were used for typing appeared to be **stable over months** (Keely et al. 1995; Hauser et al. 1997, 2001a; Hauser 2004), which is a prerequisite for microbe-based epidemiological studies (Hauser et al. 1998a). The index of discrimination power is the average probability that two unrelated specimens randomly chosen will be assigned to different types by the method. It has been determined only for the SSCP method and was estimated to be 0.93 (Hauser et al. 2001a), which can be considered as satisfactory because the minimal desired level for a typing system to be used as a single method is 0.95 (Struelens and ESGEM 1996). SSCP and ITSs sequencing identified, respectively, 43 (Hauser 2004) and more than 60 different *P. jirovecii* genotypes (Lu and Lee 2008). The same markers have been analyzed by SSCP and MLST, but the latter is more discriminative because it detects all polymorphisms.

The interpretation of data using the current typing methods for *P. jirovecii* must take into account two important caveats. First, **co-infection** of a single patient with two or more *P. jirovecii* genotypes has been reported using multiple typing methods (Tsolaki et al. 1996; Hauser et al. 1998a; Nahimana et al. 2000; Ma et al. 2002; Ripamonti et al. 2009), which obviously complicates epidemiological studies (Hauser et al. 1998b). The proportion of co-infected patients varies considerably, ranging from a few percent using direct sequencing to ~70% using SSCP. SSCP can detect a co-infecting type if it represents at least 11% of the population (Nahimana et al. 2000). Cloning of PCR products followed by DNA sequencing can also be used to detect co-infections; the minimal proportion detected will depend on the number of clones that are analyzed. Direct sequencing of PCR products without cloning would not be able to detect low-abundance co-infecting types. Geographical variation or other parameters probably account for some

Table 11.2 Numbers of ITS types corresponding to *P. jirovecii* genotypes found in various samples of PCP patients by Helweg-Larsen et al. (2001a)

Patient	Respiratory specimen[a]	Autopsy lungs	In common between respiratory specimen and autopsy lungs
I	1	1	1
II	1	3	1
III	3	10	1

[a]Patients I and II, bronchoalveolar lavage; patient III, oral wash

of the reported differences in the proportion of co-infected patients. Identifying all co-infecting genotypes present in clinical specimens of infected humans is probably necessary to fully understand *P. jirovecii* epidemiology, and may also be useful for analysis of outbreaks, because some genotypes might be more transmissible than others. Nonetheless, at present most investigations of outbreaks use methods that detect only the most abundant genotype(s). Accordingly, recent outbreaks have often been investigated by MLST without cloning to detect co-infections, and this method was able to provide evidence that a single *P. jirovecii* genotype accounted for all or most of the PCP cases in a given outbreak (Schmoldt et al. 2008; Gianella et al. 2010; Phipps et al. 2011; Pliquett et al. 2012). ITSs sequencing with or without cloning PCR products also reached this conclusion (Yazaki et al. 2009; Le Gal et al. 2012).

The second caveat in interpreting data from typing methods is that the *P. jirovecii* **genotypes present in respiratory samples may not include all those present in the patient's lungs**. Indeed, analysis of lungs from three autopsied individuals in one study demonstrated a compartmentalization of different co-infecting *P. jirovecii* genotypes in the lungs of two patients. Importantly, some genotypes identified in specific regions of the lungs were absent in the corresponding respiratory specimen (see Table 11.2) (Helweg-Larsen et al. 2001a). As previously stressed by Hughes (2007), this raises questions about studies that concluded that recurrent PCP episodes represent de novo infection, because genotype(s) already present in the lungs may have been detected only during the second and not the first episode. On the other hand, this apparently has not caused problems for investigations of outbreaks since the same *P. jirovecii* genotype was observed in many instances (see following section).

As far as ITSs typing is concerned, amplification by PCR has been reported to create an artifactual diversity of genotypes, i.e., 37% of them were chimeras (Beser et al. 2007). A substantial decrease in the proportion of these artifacts was obtained by increasing elongation time, primer concentration, and annealing temperature, as well as by decreasing the number of cycles and using a proofreading polymerase (Beser et al. 2007). Nevertheless, two recent studies successfully detected the same ITSs genotype in outbreak PCP cases, apparently without using the advised modifications to decrease chimera formation (Yazaki et al. 2009; Le Gal et al. 2012). Infection with a single genotype may account for this because there would be no opportunity for recombination.

V. Molecular Epidemiology of *Pneumocystis* Outbreaks

In a recent publication, de Boer et al. (2011) reviewed studies of 16 nosocomial PCP outbreaks among RTRs, six of which used molecular typing to characterize the genotype. A transmission map describing timings of outpatient visits and hospitalizations was generally provided in these studies to assess potential occurrence of infectious encounters (an example is shown in Fig. 11.2). Molecular typing revealed in five out of six instances that all or the vast majority of the outbreak cases with suspected infectious contact(s) harbored the same *P. jirovecii* genotype, strongly suggesting that **inter-human transmission** of *P. jirovecii* had occurred. Importantly, all outbreaks shared the three following features, which probably contributed to the outbreaks: (i) cases received no or inadequate anti-PCP

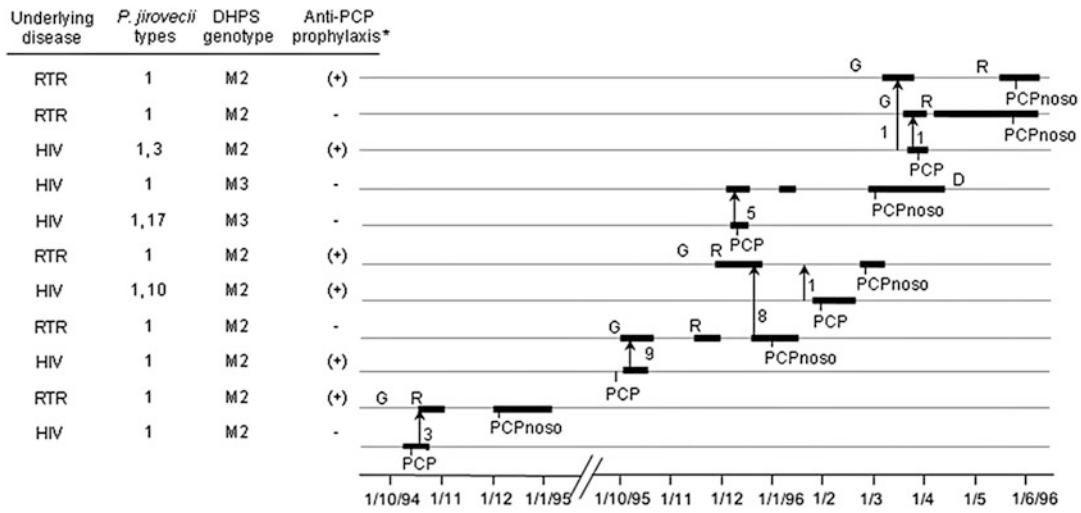


Fig. 11.2 Potential encounters compatible with nosocomial inter-human transmission of *P. jirovecii* in one building of a hospital. *Thicker parts of solid lines* represent periods of hospitalization. Each encounter or consecutive encounters are figured by an *arrow* pointing to the direction of the presumed transmission; the number of encounters is indicated close to each *arrow*. *Anti-PCP prophylaxis with Fansidar was suboptimal (25 mg pyrimethamine and 500 mg sulfadoxine once or twice every 2 weeks). *D* death, *G* graft, *R* rejection episode, *RTR* renal transplant recipient, *PCPnoso* nosocomial case, *DHPS* dihydropteroate synthase, *M2* amino acid change within the putative sulfa-binding site at position 57 (Pro to Ser) [Taken from Fig. 4 of Rabodonirina et al. (2004)]

prophylaxis, (ii) contact could occur between potential source patients with clinical or incubating PCP and susceptible patients (in outpatient waiting rooms or during a simultaneous hospitalization), and (iii) no isolation procedure of PCP patients was utilized. However, being retrospective, these studies could not firmly exclude that cases acquired the fungus from asymptomatic colonized health-care worker(s), from an unidentified environmental source, or by indirect transmission from an index patient through nosocomial carriers.

Since the review of de Boer et al. (2011), eight additional PCP outbreaks have been reported in seven studies, nearly all in RTRs. One study provided evidence of transmission only on the basis of possible infectious encounters (Mori et al. 2010). Of note, the latter is the first among outpatients with rheumatoid arthritis. In addition to possible infectious encounters, the other six studies reported that a single molecular *P. jirovecii* genotype accounted for most or all of the RTR outbreak cases (Phipps et al. 2011; Thomas et al. 2011; Wynckel et al. 2011; Brunot et al. 2012; Pliquett et al. 2012; Le Gal et al. 2012). In all eight outbreaks, patients were not receiving anti-PCP prophylaxis at the time of suspected exposure, and there was no policy to isolate PCP patients. Thus, these recent studies provide further evidence that inter-human transmission is involved in such outbreaks, but they also could not exclude a common source or indirect transmission.

These studies highlight at least three new issues that could help explain PCP outbreaks:

(i) For the first time, three out of ten potential **source patients were identified as carriers of *P. jirovecii*** without clinical symptoms of PCP (Le Gal et al. 2012). This is important because carriers are not routinely identified within hospitals and they may be even more numerous than PCP patients, and as such they may constitute an important nosocomial source of *P. jirovecii*. The risk of transmission by such individuals needs to be determined, potentially by measuring dissemination of the fungus in the air as previously described for PCP patients (Choukri et al. 2010). If such individuals are an important source of infection, approaches to minimizing such transmission would need to be evaluated.

(ii) The new studies identified new clinical risk factors for PCP that may have played a role in the outbreaks. Alteration in immunosuppressive therapy, graft rejection, cytomegalovirus infection, and older age were previously identified (de Boer et al. 2011). A cohort analysis including 14 cases and 324 control patients identified two new independent risk factors: underlying pulmonary disease and transplanted organ dysfunction (Phipps et al. 2011). Lower CD4 lymphocyte counts at 3 months after transplantation (Brunot et al. 2012) or just before PCP diagnosis (Struijk et al. 2011) were also identified as risk factors. Although they need confirmation because they were identified in single studies, these findings may be important for preventing outbreaks by better identifying patients who would benefit from PCP prophylaxis.

(iii) Two different *P. jirovecii* genotypes were involved in two outbreaks that occurred at a relatively close geographical distance of 50 km (Liverpool and Manchester) (Thomas et al. 2011). This raises new issues for the analysis of the different outbreak-associated *P. jirovecii* genotypes, which we present in the following section.

VI. *P. jirovecii* Outbreak Genotypes

An important finding concerning *P. jirovecii* genotypes involved in outbreaks has been reported recently (Sassi et al. 2012). Genotypes linked to two PCP outbreaks that occurred contemporaneously in two European cities at a distance of 300 km (Zurich and Munich) were found to be identical using both MLST and RFLP-MSG. Subsequently, the same genotype was identified using MLST in a third contemporaneous outbreak at a distance of 400 km from the two previous ones (Frankfurt) (Hauser et al. 2013). However, this genotype was different from that involved in a contemporaneous Japanese outbreak (Nagoya) (Sassi et al. 2012), as well as from that observed in another European outbreak that occurred about 12 years earlier at a distance of 400–800 km (Lyon) (Hauser et al. 2013).

Two epidemiological factors may have played a role in the variation of the genotype involved in the different outbreaks: the **period of time** and the **geographical location** (Hauser et al. 2013). However, the presence of the same genotype in three contemporaneous and geographically close outbreaks is particularly intriguing. As previously noted (Sassi et al. 2012; de Boer 2012), two hypotheses could explain this latter finding: (i) the **genotype was predominant** in an area covering the three European locations and infected index patients of the three outbreaks, or (ii) the genotype carried **uncharacterized pathogenic factor (s)** and some unidentified contact (e.g., a patient or colonized individual) existed between the three outbreaks. Because the few sporadic contemporaneous cases analyzed in the three outbreaks (six, three, and two in Munich, Zurich, and Frankfurt, respectively) were infected by other genotypes, the outbreak genotype was considered not to be predominant, but was hypothesized to be more pathogenic in RTRs (Sassi et al. 2012).

On the other hand, by analysis of a large number of isolates, the Lyon outbreak genotype was demonstrated to be the predominant one at the time in the affected hospital as well as in the local area (Rabodonirina et al. 2004), and one of the most prevalent in several European locations (Hauser et al. 2001a). Nevertheless, the Lyon genotype may have been not only predominant but also more transmissible. Indeed, the potential infectious encounters during the outbreak also involved other genotypes, but these apparently were not transmitted (Rabodonirina et al. 2004). The **increased transmissibility or pathogenicity** of the Lyon genotype might have been due to unidentified specific factors, but also potentially to the presence of one or two amino acid changes within the putative sulfa-binding site of *P. jirovecii* dihydropteroate synthase (Pro to Ser at position 57, mutation M2; Thr to Ala and Pro to Ser at positions 55 and 57, respectively, double mutation M3). All but one potential donor in the encounters harbored M2, whereas M3 was present in the remaining donor

(Fig. 11.2). Epidemiological studies have shown that these mutations confer some level of resistance to sulfonamides (Kazanjian et al. 1998; Helweg-Larsen et al. 1999; Nahimana et al. 2003a), and the results were confirmed by functional complementation in *Escherichia coli* (Iliades et al. 2005) and *Saccharomyces cerevisiae* (Meneau et al. 2004). Consequently, the Lyon outbreak genotype may have been selected among the different genotypes encountered because several patients were receiving suboptimal prophylaxis (Fig. 11.2). Thus, the observations in Lyon suggest that the two hypotheses, preponderance and increased pathogenicity of the outbreak genotypes, may not be mutually exclusive.

In order to further investigate this issue, we analyzed all outbreak genotypes that could be compared with each other because they shared at least one sequenced marker (see Table 11.3). The results of Wynckel et al. (2011) could not be included because ITS types are not given. Although only one marker could be compared, the ITS1 allele confirmed that the Japanese genotype was different from all others. The ITS1 and mt26S alleles showed that the two genotypes involved in the Manchester and Liverpool outbreaks (Thomas et al. 2011) were also different from all others. The ITS1 allele of the Brest genotype (Le Gal et al. 2012) was the same as that in the three nearly contemporaneous European outbreaks, but the discrimination power of a single marker is not sufficient to draw any conclusions. As revealed by three markers, the Australian outbreak (Phipps et al. 2011) may have harbored the same genotype as that of Lyon. Our analysis demonstrates a great diversity of the outbreak genotypes. Notably, the two contemporaneous English outbreaks involved different genotypes although they occurred at a close geographic distance, which contrasts with the three European outbreaks involving a single genotype. *P. jirovecii* genotypes from nine (Phipps et al. 2011) and two (Pliquett et al. 2012) unlinked control patients were different from the outbreak genotype, favoring the pathogenicity hypothesis. On the other hand, the Brest outbreak genotype was also predominant among a larger cohort of 22 controls, favoring the preponderance hypothesis. The latter observation also suggests that **analysis of numerous controls**, as performed only in Brest and Lyon (Table 11.3), may be necessary to confidently determine genotype frequencies.

VII. Biological and Epidemiological Considerations

P. jirovecii has most probably evolved for millions of years as a **colonizer of human lungs**. Its pathogenic manifestation as acute PCP may have become frequent only recently in history, especially with the advent of HIV-associated immunodeficiency and the increase in the number of immunosuppressed humans due to the progress of medicine. Consequently, *P. jirovecii* is probably **not a primary pathogen** like, for example, pathogenic dimorphic fungi, and thus is not expected to harbor virulence factors that have been selected to cause disease. Its potential virulence factors could be considered "accidental", i.e., selected for improved survival in colonized humans. *Pneumocystis* has also developed a sophisticated system for **antigenic variation** that presumably provides a mechanism for evading host immune responses, possibly T cell responses (Bishop et al. 2012). Thus, the biology of *P. jirovecii* suggests that selection of factors conferring increased pathogenicity is unexpected, which is in favor of the hypothesis of the predominance of the outbreak genotypes. Nevertheless, events that convert non-pathogens to pathogens cannot be excluded.

Studies that reported the association of certain *P. jirovecii* genotypes with various phenotypes may support the hypothesis of pathogenic factors in outbreak genotypes. Some genotypes were reported to be associated with milder disease (Miller and Wakefield 1999), failure of prophylaxis (Hauser et al. 2001b), failure of PCP treatment (Matos et al. 2003), reduced severity of PCP (van Hal et al. 2009), or increased mortality rate (Rabodonirina et al. 2013). The same genotype was associated with related phenotypes by two independent laboratories (Miller and Wakefield 1999; Hauser et al. 2001b), which strengthens the conclusions drawn. However, in the absence of culture, these phenotypes could not be confirmed and, moreover, other studies failed to confirm some of these associations (Helweg-Larsen et al. 2001b; Valerio et al. 2007). The recent release of the *P. jirovecii* genome sequence (Cissé et al. 2012) should facilitate studies addressing such issues.

Table 11.3 Features of outbreaks involving genotypes that are comparable by the sequence of at least one marker

Country	City	Period	No. cases genotyped/ no. cases	No. controls genotyped	Initial typing method	Alleles ITS1/mt26S/ b-tub/26S[a]	References
France	Lyon	1994–1996	9/10	151	SSCP	B1/8/1/1	Rabodonirina et al. (2004) Hauser et al. (2013)
Japan	Nagoya	2004–2005	8/27	0	ITSs sequencing[b]	A2	Yazaki et al. (2009) Sassi et al. (2012)
Germany	Frankfurt	2005–2008	4/29	2	MLST	B/7/1/4	Pliquett et al. (2012) Hauser et al. (2013)
Germany	Munich	2005–2008	16/16	6	MLST	B/7/1/4	Schmoldt et al. (2008) Sassi et al. (2012)
Switzerland	Zurich	2005–2008	7/7	3	MLST	B/7/1/4	Gianella et al. (2010) Sassi et al. (2012)
England	Manchester	2008–2010	7/11	0	MLST[c]	B2/8	Thomas et al. (2011)
England	Liverpool	2008–2010	10/21	0	MLST[c]	B1/3	Thomas et al. (2011)
France	Brest	2008–2010	14/18	22	ITSs sequencing[d]	B	Le Gal et al. (2012)
Australia	Sydney	2010	11/11	9	MLST[e]+ITS2+DHPS sequencing	B1/8/1	Phipps et al. (2011)

[a]Alleles labels are according to Hauser et al. (1997)
[b]Without cloning PCR products
[c]b-tub and 26S were not analyzed
[d]With cloning PCR products and sequencing four to ten clones
[e]26S was not analyzed

VIII. Conclusions and Perspectives

The data summarized above strongly support the hypothesis that inter-human transmission of *P. jirovecii* is important in spreading infection in PCP outbreaks. It is noteworthy that outbreaks appeared to occur in settings in which there was no or inadequate use of prophylaxis, contact occurred between susceptible and infected (and infectious) patients, and PCP patients were not placed in respiratory isolation. Thus, **host susceptibility** and **clinical management** of PCP patients may play major roles in the outbreaks. How can such outbreaks be prevented in the future? One approach that has been validated in clinical trials is the appropriate use of **PCP prophylaxis** for at-risk populations. In most of the outbreaks reported to date, widespread implementation of prophylactic measures led to elimination of additional cases. Preventing transmission by other mechanisms, such as isolation of PCP patients, is logical but has not been validated. It is possible that by the time PCP is diagnosed, transmission to susceptible patients has already occurred, given that progressive infection may be present for weeks before diagnosis. Alternatively, attempts to screen all potential carriers of *Pneumocystis*, including asymptomatic patients as well as medical personnel, does not seem practical, feasible, or cost effective.

The data available to date are insufficient to determine if outbreak genotypes are causing disease because they are the preponderant local genotype, or because they have increased pathogenicity. It is possible that both factors play a role because they are not mutually exclusive, although our current understanding of the biology of the fungus tends to favor the preponderance hypothesis. One approach to addressing this issue would be to **sequence the entire genome of multiple genotypes** and look for genetic differences that may contribute to pathogenicity. As sequencing techniques are becoming more efficient and cost effective, this may be feasible in the near future.

To better understand PCP outbreaks, the dynamics of populations of *P. jirovecii* genotypes need to be better understood. Genotypes should ideally be determined for each outbreak in PCP patients and colonized individuals within hospitals, as well as, if possible, in their relatives, including infants as they may represent an important reservoir. The use of a standardized typing method, e.g. MLST, would have the advantage of allowing interlaboratory comparison of different isolates. Parallel DHPS genotyping would allow investigation of the importance of sulfa drug resistance.

The optimal utilization of prophylaxis in RTRs needs to be defined. Currently, anti-PCP prophylaxis is given (if administered) for varying periods at different centers, generally for 3–6 months after transplantation. Given the recent outbreaks, it seems prudent to administer prophylaxis for at least 6 months. However, cases can occur after prophylaxis has been discontinued, with a report up to 15 years after transplantation (Le Gal et al. 2012). As clinical risk factors for development of PCP are identified, prophylaxis can potentially be administered after 6 months to patients with these risk factors, such as in cases of graft rejection.

The **occurrence of even two potentially nosocomial cases of PCP** should raise concerns and be evaluated as a potential outbreak. *P. jirovecii* genotyping can determine if in fact an outbreak is occurring and if additional measures to minimize transmission need to be implemented.

Acknowledgments Work in PMH's laboratory is supported by the Swiss National Science Foundation grants 310030-124998 and 310030-146135. Supported in part by the Intramural Research Program of the NIH Clinical Center, National Institutes of Health, Bethesda, MD, USA.

References

Bartlett MS, Vermund SH, Jacobs R, Durant PJ, Shaw MM, Smith JW, Tang X, Lu JJ, Li B, Jin S, Lee CH (1997) Detection of *Pneumocystis carinii* DNA in air samples: likely environmental risk to susceptible persons. J Clin Microbiol 35:2511–2513

Beard CB, Fox MR, Lawrence GG, Guarner J, Hanzlick RL, Huang L, del Rio C, Rimland D, Duchin JS, Colley DG (2005) Genetic differences in *Pneumocystis* isolates recovered from immunocompetent infants and from adults with AIDS: epidemiological Implications. J Infect Dis 192:1815–1818

Beser J, Hagblom P, Fernandez V (2007) Frequent in vitro recombination in internal transcribed

spacers 1 and 2 during genotyping of *Pneumocystis jirovecii*. J Clin Microbiol 45:881–886
Bishop LR, Helman D, Kovacs JA (2012) Discordant antibody and cellular responses to *Pneumocystis* major surface glycoprotein variants in mice. BMC Immunol 13:39
Brunot V, Pernin V, Chartier C, Garrigue V, Vetromile F, Szwarc I, Delmas S, Portalès P, Basset D, Mourad G (2012) An epidemic of *Pneumocystis jiroveci* pneumonia in a renal transplantation center: role of T-cell lymphopenia. Transplant Proc 44:281–2820
Ceré N, Polack B, Chanteloup NK, Coudert P (1997) Natural transmission of *Pneumocystis carinii* in nonimmunosuppressed animals: early contagiousness of experimentally infected rabbits (*Oryctolagus cuniculus*). J Clin Microbiol 35:2670–2672
Chabé M, Dei-Cas E, Creusy C, Fleurisse L, Respaldiza N, Camus D, Durand-Joly I (2004) Immunocompetent hosts as a reservoir of *Pneumocystis* organisms: histological and rt-PCR data demonstrate active replication. Eur J Clin Microbiol Infect Dis 23:89–97
Choukri F, Menotti J, Sarfati C, Lucet JC, Nevez G, Garin YJ, Derouin F, Totet A (2010) Quantification and spread of *Pneumocystis jirovecii* in the surrounding air of patients with *Pneumocystis* pneumonia. Clin Infect Dis 51:259–265
Choukri F, el Aliouat M, Menotti J, Totet A, Gantois N, Garin YJ, Bergeron V, Dei-Cas E, Derouin F (2011) Dynamics of *Pneumocystis carinii* air shedding during experimental pneumocystosis. J Infect Dis 203:1333–1336
Churukian CJ, Schenk EA (1977) Rapid Grocott's methenamine silver nitrate method for fungi and *Pneumocystis carinii*. Am J Clin Pathol 68:427–428
Cissé OH, Pagni M, Hauser PM (2012) De novo assembly of the *Pneumocystis jirovecii* genome from a single bronchoalveolar lavage fluid specimen from a patient. MBio 4:e00428–12. doi:10.1128/mBio.00428-12
Cushion MT, Stringer JR (2010) Stealth and opportunism: alternative lifestyles of species in the fungal genus *Pneumocystis*. Annu Rev Microbiol 64:431–452
Damiani C, Choukri F, Le Gal S, Menotti J, Sarfati C, Nevez G, Derouin F, Totet A (2012) Possible nosocomial transmission of *Pneumocystis jirovecii*. Emerg Infect Dis 18:877–878
de Boer MG (2012) Linking *Pneumocystis* epidemiology, transmission, and virulence. Clin Infect Dis 54:1445–1447
de Boer MGJ, de Fijter JW, Kroon FP (2011) Outbreaks and clustering of *Pneumocystis* pneumonia in kidney transplant recipients: a systematic review. Med Mycol 49:673–680
Demanche C, Berthelemy M, Petit T, Polack B, Wakefield AE, Dei-Cas E, Guillot J (2001) Phylogeny of *Pneumocystis carinii* from 18 primate species confirms host specificity and suggests coevolution. J Clin Microbiol 39:2126–2133
Dumoulin A, Mazars E, Seguy N, Gargallo-Viola D, Vargas S, Cailliez JC, Aliouat EM, Wakefield AE, Dei-Cas E (2000) Transmission of *Pneumocystis carinii* disease from immunocompetent contacts of infected hosts to susceptible hosts. Eur J Clin Microbiol Infect Dis 19:671–678
Furlong ST, Koziel H, Bartlett MS, McLaughlin GL, Shaw MM, Jack RM (1997) Lipid transfer from human epithelial cells to *Pneumocystis carinii in vitro*. J Infect Dis 175:661–668
Gianella S, Haeberli L, Joos B, Ledergerber B, Wuthrich RP, Weber R, Kuster H, Hauser PM, Fehr T, Mueller NJ (2010) Molecular evidence of interhuman transmission in an outbreak of *Pneumocystis jirovecii* pneumonia among renal transplant recipients. Transpl Infect Dis 12:1–10
Hauser PM (2004) The development of a typing method for an uncultivable microorganism: the example of *Pneumocystis jirovecii*. Infect Genet Evol 4:199–203
Hauser PM, Francioli P, Bille J, Telenti A, Blanc DS (1997) Typing of *Pneumocystis carinii* f. sp. *hominis* by single-strand conformation polymorphism of four genomic regions. J Clin Microbiol 35:3086–3091
Hauser PM, Blanc DS, Bille J, Francioli P (1998a) Typing methods to approach *Pneumocystis carinii* genetic heterogeneity. FEMS Immunol Med Microbiol 22:27–35
Hauser PM, Blanc DS, Telenti A, Nahimana A, Bille J, Francioli P (1998b) Potential coinfections complicate typing of *Pneumocystis carinii* sp.f. *hominis*. J Clin Microbiol 36:311
Hauser PM, Blanc DS, Bille J, Nahimana A, Francioli P (2000) Carriage of *Pneumocystis carinii* by immunosuppressed patients and molecular typing of the organisms. AIDS 14:461–463
Hauser PM, Blanc DS, Sudre P, Senggen Manoloff E, Nahimana A, Bille J, Weber R, Francioli P, Study group (2001a) Genetic diversity of *Pneumocystis carinii* in HIV-positive and -negative patients as revealed by PCR-SSCP typing. AIDS 15:461–466
Hauser PM, Sudre P, Nahimana A, Francioli P, Study group (2001b) Prophylaxis failure is associated with a specific *Pneumocystis carinii* genotype. Clin Infect Dis 33:1080–1082
Hauser PM, Burdet FX, Cissé OH, Keller L, Taffé P, Sanglard D, Pagni M (2010) Comparative genomics suggests that the fungal pathogen *Pneumocystis* is an obligate parasite scavenging amino acids from its host's lungs. PLoS One 5:e15152. doi:10.1371/journal.pone.0015152
Hauser PM, Rabodonirina M, Nevez G (2013) *Pneumocystis jirovecii* genotypes involved in PCP outbreaks among renal transplant recipients. Clin Infect Dis 56:165–166
Helweg-Larsen J, Benfield TL, Eugen-Olsen J, Lundgren JD, Lundgren B (1999) Effects of mutations in *Pneumocystis carinii* dihydropteroate synthase gene on outcome of AIDS-associated *P. carinii* pneumonia. Lancet 354:1347–1351
Helweg-Larsen J, Lundgren B, Lundgren JD (2001a) Heterogeneity and compartmentalization of *Pneumocystis carinii* f. sp. *hominis* genotypes in autopsy lungs. J Clin Microbiol 39:3789–3792
Helweg-Larsen J, Lee CH, Jin S, Hsueh JY, Benfield TL, Hansen J, Lundgren JD, Lundgren B (2001b) Clinical

correlation of variations in the internal transcribed spacer regions of rRNA genes in *Pneumocystis carinii* f.sp. *hominis*. AIDS 15:451–459

Huang L, Beard CB, Creasman J, Levy D, Duchin JS, Lee S, Pieniazek N, Carter JL, del Rio C, Rimland D, Navin TR (2000) Sulfa or sulfone prophylaxis and geographic region predict mutations in the *Pneumocystis carinii* dihydropteroate synthase gene. J Infect Dis 182:1192–1198

Hughes WT (2007) Transmission of *Pneumocystis* species among renal transplant recipients. Clin Infect Dis 44:1150–1151

Hughes WT (1982) Natural mode of acquisition for de novo infection with *Pneumocystis carinii*. J Infect Dis 145:842–848

Hughes WT, Bartley DL, Smith BM (1983) A natural source of infection due to *Pneumocystis carinii*. J Infect Dis 147:595

Iliades P, Meshnick SR, Macreadie IG (2005) Analysis of *Pneumocystis jirovecii* DHPS alleles implicated in sulfamethoxazole resistance using an *Escherichia coli* model system. Microb Drug Resist 11:1–8

Kazanjian P, Locke AB, Hossler PA, Lane BR, Bartlett MS, Smith JW, Cannon M, Meshnick SR (1998) *Pneumocystis carinii* mutations associated with sulfa and sulfone prophylaxis failures in AIDS patients. AIDS 12:873–878

Keely SP, Stringer JR, Baughman RP, Linke MJ, Walzer PD, Smulian AG (1995) Genetic variation among *Pneumocystis carinii hominis* isolates in recurrent pneumocystosis. J Infect Dis 172:595–598

Le Gal S, Damiani C, Rouillé A, Grall A, Tréguer L, Virmaux M, Moalic E, Quinio D, Moal MC, Berthou C, Saliou P, Le Meur Y, Totet A, Nevez G (2012) A cluster of *Pneumocystis* infections among renal transplant recipients: molecular evidence of colonized patients as potential infectious sources of *Pneumocystis jirovecii*. Clin Infect Dis 54:e62–e71. doi:10.1093/cid/cir996

Lee CH, Lu JJ, Bartlett MS, Durkin MM, Liu TH, Wang J, Jiang B, Smith JW (1993) Nucleotide sequence variation in *Pneumocystis carinii* strains that infect humans. J Clin Microbiol 31:754–757

Lu JJ, Lee CH (2008) *Pneumocystis* pneumonia. J Formos Med Assoc 107:830–842

Lu JJ, Bartlett MS, Shaw MM, Queener SF, Smith JW, Ortiz-Rivera M, Leibowitz MJ, Lee CH (1994) Typing of *Pneumocystis carinii* strains that infect humans based on nucleotide sequence variations of internal transcribed spacers of rRNA genes. J Clin Microbiol 32:2904–2912

Ma L, Kutty G, Jia Q et al (2002) Analysis of variation in tandem repeats in the intron of the major surface glycoprotein expression site of the human form of *Pneumocystis carinii*. J Infect Dis 186:1647–1654

Matos O, Lee CH, Jin S, Li B, Costa MC, Gonçalves L, Antunes F (2003) *Pneumocystis jiroveci* in Portuguese immunocompromised patients: association of specific ITS genotypes with treatment failure, bad clinical outcome and childhood. Infect Genet Evol 3:281–285

Meneau I, Sanglard D, Bille J, Hauser PM (2004) *Pneumocystis jiroveci* dihydropteroate synthase polymorphisms confer resistance to sulfadoxine and sulfanilamide in *Saccharomyces cerevisiae*. Antimicrob Agents Chemother 48:2610–2606

Meshnick SR (1999) Drug-resistant *Pneumocystis carinii*. Lancet 354:1318–1319

Miller RF, Wakefield AE (1999) *Pneumocystis carinii* genotypes and severity of pneumonia. Lancet 353:2039–2040

Miller RF, Ambrose HE, Wakefield AE (2001) *Pneumocystis carinii* f. sp. *hominis* DNA in immunocompetent health care workers in contact with patients with *P. carinii* pneumonia. J Clin Microbiol 39:3877–3882

Mori S, Cho I, Sugimoto M (2010) A cluster of *Pneumocystis jirovecii* infection among outpatients with rheumatoid arthritis. J Rheumatol 37:1547–1548

Nahimana A, Blanc DS, Francioli P, Bille J, Hauser PM (2000) Typing of *Pneumocystis carinii* f. sp. *hominis* by PCR-SSCP to indicate a high frequency of co-infections. J Med Microbiol 49:753–758

Nahimana A, Rabodonirina M, Zanetti G, Meneau I, Francioli P, Bille J, Hauser PM (2003a) Association between a specific *Pneumocystis jiroveci* dihydropteroate synthase mutation and failure of pyrimethamine/sulfadoxine prophylaxis in human immunodeficiency virus-positive and -negative patients. J Infect Dis 188:1017–1023

Nahimana A, Rabodonirina M, Helweg-Larsen J, Meneau I, Francioli P, Bille J, Hauser PM (2003b) Sulfa resistance and dihydropteroate synthase mutants in recurrent *Pneumocystis carinii* pneumonia. Emerg Infect Dis 9:864–867

Nevez G, Raccurt C, Jounieaux V, Dei-Cas E, Mazars E (1999) Pneumocystosis versus pulmonary *Pneumocystis carinii* colonization in HIV-negative and HIV-positive patients. AIDS 13:535–536

Payne SH, Loomis WF (2006) Retention and loss of amino acid biosynthetic pathways based on analysis of whole-genome sequences. Eukaryot Cell 5:272–276

Phipps LM, Chen SC, Kable K, Halliday CL, Firacative C, Meyer W, Wong G, Nankivell BJ (2011) Nosocomial *Pneumocystis jirovecii* pneumonia: lessons from a cluster in kidney transplant recipients. Transplantation 92:1327–1334

Pifer LL, Hughes WT, Stagno S, Woods D (1978) *Pneumocystis carinii* infection: evidence for high prevalence in normal and immunosuppressed children. Pediatrics 61:35–41

Pliquett RU, Asbe-Vollkopf A, Hauser PM, Presti LL, Hunfeld KP, Berger A, Scheuermann EH, Jung O, Geiger H, Hauser IA (2012) A *Pneumocystis jirovecii* pneumonia outbreak in a single kidney-transplant center: role of cytomegalovirus co-infection. Eur J Clin Microbiol Infect Dis 31:2429–2437

Powles MA, McFadden DC, Pittarelli LA, Schmatz DM (1992) Mouse model for *Pneumocystis carinii* pneumonia that uses natural transmission to initiate infection. Infect Immun 60:1397–1400

Rabodonirina M, Vanhems P, Couray-Targe S, Gillibert RP, Ganne C, Nizard N, Colin C, Fabry J, Touraine

JL, van Melle G, Nahimana A, Francioli P, Hauser PM (2004) Molecular evidence of interhuman transmission of *Pneumocystis* pneumonia among renal transplant recipients hospitalized with HIV-infected patients. Emerg Infect Dis 10:1766–1773

Rabodonirina M, Vaillant L, Taffé P, Nahimana A, Gillibert R-P, Vanhems P, Hauser PM (2013) *Pneumocystis jirovecii* genotype associated with increased death rate of HIV-infected patients with pneumonia. Emerg Infect Dis 19:21–28

Ripamonti C, Orenstein A, Kutty G, Huang L, Schuhegger R, Sing A, Fantoni G, Atzori C, Vinton C, Huber C, Conville PS, Kovacs JA (2009) Restriction fragment length polymorphism typing demonstrates substantial diversity among *Pneumocystis jirovecii* isolates. J Infect Dis 200:1616–1622

Sassi M, Ripamonti C, Mueller NJ, Yazaki H, Kutty G, Ma L, Huber C, Gogineni E, Oka S, Goto N, Fehr T, Gianella S, Konrad R, Sing A, Kovacs JA (2012) Outbreaks of *Pneumocystis* pneumonia in 2 renal transplant centers linked to a single strain of *Pneumocystis*: implications for transmission and virulence. Clin Infect Dis 54:1437–1444

Schmoldt S, Schuhegger R, Wendler T, Huber I, Söllner H, Hogardt M, Arbogast H, Heesemann J, Bader L, Sing A (2008) Molecular evidence of nosocomial *Pneumocystis jirovecii* transmission among 16 patients after kidney transplantation. J Clin Microbiol 46:966–971

Stringer JR, Keely SP (2001) Genetics of surface antigen expression in *Pneumocystis carinii*. Infect Immun 69:627–639

Struelens MJ, Members of the European Study Group on Epidemiological Markers (ESGEM) (1996) Consensus guidelines for appropriate use and evaluation of microbial epidemiologic typing systems. Clin Microbiol Infect 2:2–11

Struijk GH, Gijsen AF, Yong SL, Zwinderman AH, Geerlings SE, Lettinga KD, van Donselaar-van der Pant KA, ten Berge IJ, Bemelman FJ (2011) Risk of *Pneumocystis jiroveci* pneumonia in patients long after renal transplantation. Nephrol Dial Transplant 26:3391–3398

Thomas CF Jr, Limper AH (2007) Current insights into the biology and pathogenesis of *Pneumocystis* pneumonia. Nat Rev Microbiol 5:298–308

Thomas S, Vivancos R, Corless C, Wood G, Beeching NJ, Beadsworth MB (2011) Increasing frequency of *Pneumocystis jirovecii* pneumonia in renal transplant recipients in the United Kingdom: clonal variability, clusters, and geographic location. Clin Infect Dis 53:307–308

Tipirneni R, Daly KR, Jarlsberg LG, Koch JV, Swartzman A, Roth BM, Walzer PD, Huang L (2009) Healthcare worker occupation and immune response to *Pneumocystis jirovecii*. Emerg Infect Dis 15:1590–1597

Totet A, Respaldiza N, Pautard JC, Raccurt C, Nevez G (2003) *Pneumocystis jiroveci* genotypes and primary infection. Clin Infect Dis 36:1340–1342

Totet A, Duwat H, Magois E, Jounieaux V, Roux P, Raccurt C, Nevez G (2004) Similar genotypes of *Pneumocystis jirovecii* in different forms of *Pneumocystis* infection. Microbiology 150:1173–1178

Tsolaki AG, Miller RF, Underwood AP, Banerji S, Wakefield AE (1996) Genetic diversity at the internal transcribed spacer regions of the rRNA operon among isolates of *Pneumocystis carinii* from AIDS patients with recurrent pneumonia. J Infect Dis 174:141–156

Valerio A, Tronconi E, Mazza F, Fantoni G, Atzori C, Tartarone F, Duca P, Cargnel A (2007) Genotyping of *Pneumocystis jiroveci* pneumonia in Italian AIDS patients. Clinical outcome is influenced by dihydropteroate synthase and not by internal transcribed spacer genotype. J Acquir Immune Defic Syndr 45:521–528

van Hal SJ, Gilgado F, Doyle T, Barratt J, Stark D, Meyer W, Harkness J (2009) Clinical significance and phylogenetic relationship of novel Australian *Pneumocystis jirovecii* genotypes. J Clin Microbiol 47:1818–1823

Vargas SL, Hughes WT, Wakefield AE, Oz HS (1995) Limited persistence in and subsequent elimination of *Pneumocystis carinii* from the lungs after *P. carinii* pneumonia. J Infect Dis 172:506–510

Vargas SL, Ponce CA, Gigliotti F, Ulloa AV, Prieto S, Muñoz MP, Hughes WT (2000) Transmission of *Pneumocystis carinii* DNA from a patient with *P. carinii* pneumonia to immunocompetent contact health care workers. J Clin Microbiol 38:1536–1538

Vargas SL, Hughes WT, Santolaya ME, Ulloa AV, Ponce CA, Cabrera CE, Cumsille F, Gigliotti F (2001) Search for primary infection by *Pneumocystis carinii* in a cohort of normal, healthy infants. Clin Infect Dis 32:855–861

Vargas SL, Ponce CA, Sanchez CA, Ulloa AV, Bustamante R, Juarez G (2003) Pregnancy and asymptomatic carriage of *Pneumocystis jiroveci*. Emerg Infect Dis 9:605–606

Vargas SL, Pizarro P, López-Vieyra M, Neira-Avilés P, Bustamante R, Ponce CA (2010) *Pneumocystis* colonization in older adults and diagnostic yield of single versus paired noninvasive respiratory sampling. Clin Infect Dis 50:e19–e21. doi:10.1086/649869

Vogel P, Miller CJ, Lowenstine LL, Lackner AA (1993) Evidence of horizontal transmission of *Pneumocystis carinii* pneumonia in simian immunodeficiency virus-infected rhesus macaques. J Infect Dis 168:836–843

Wynckel A, Toubas D, Noël N, Toupance O, Rieu P (2011) Outbreak of *Pneumocystis* pneumonia occurring in late post-transplantation period. Nephrol Dial Transplant 26:2417

Yazaki H, Goto N, Uchida K, Kobayashi T, Gatanaga H, Oka S (2009) Outbreak of *Pneumocystis jiroveci* pneumonia in renal transplant recipients: *P. jirovecii* is contagious to the susceptible host. Transplantation 88:380–385

12 Infections Caused by Mucorales

Caroline B. Moore[1,2], Malcolm D. Richardson[1,2]

CONTENTS

[1]Mycology Reference Centre, University Hospital of South Manchester, Manchester, UK; e-mail: caroline.moore@manchester.ac.uk
[2]Manchester Academic Health Science Centre, University of Manchester, Manchester, UK; e-mail: caroline.moore@manchester.ac.uk

I. Introduction

The term mucormycosis is now used to refer to infections caused by moulds belonging to the order Mucorales (Richardson and Warnock 2012). Traditionally, this order was assigned to the phylum Zygomycota together with the order Entomophthorales, and the different forms of disease caused by the two groups of organisms were often referred to as zygomycosis. However, following molecular analysis, the phylum Zygomycota is no longer accepted due to its **polyphyletic nature**. The subphylum Mucoromycotina has been proposed to accommodate the Mucorales and the subphylum Entomophthoromycotina has been created for the Entomophthorales.

A European Confederation of Medical Mycology (ECMM) Working Group on Zygomycosis was set up in 2004, and subsequently joined the International Society for Human and Animal Mycology (ISHAM) in 2009. This group has produced a number of influential publications during this time, and the reader is referred to one such recent publication, a special issue of *Persoonia* dedicated to the biodiversity, phylogeny and ecology of zygomycetous fungi (Voigt and de Hoog 2013).

Fungi of the order Mucorales can cause **rhinocerebral, pulmonary, gastrointestinal,**

Human Fungal Pathogens, 2nd Edition
The Mycota XII
O. Kurzai (Ed.)

cutaneous or disseminated disease in predisposed individuals, the different clinical forms often being associated with particular underlying disorders (Richardson and Warnock 2012). Mucormycosis is the second most frequent mould infection seen in immunocompromised individuals. These infections are worldwide in distribution.

II. Taxonomy

The biological classification of the kingdom Fungi is evolving, primarily due to the advent of molecular phylogenetic analysis. The order Mucorales is known to be phylogenetically ancient, belonging to a section of the lower fungi, and historically was placed in the phylum Zygomycota. More recently, species within the order have been analysed using comparative sequencing of a number of genes. These studies have established that species within the phylum descended from different evolutionary origins (polyphyletic) and, thus the phylum name can no longer be recognised. The taxa have been reclassified into the phylum Glomeromycota and four new subphyla *incertae sedis* (of uncertain placement), until the taxonomy of these fungi is further resolved (Hibbett et al. 2007; Brandt and Warnock 2011; Vitale et al. 2012).

At present, the order Mucorales has been placed in one such new subphylum, Mucoromycotina, and comprises six families: **Cunninghamellaceae, Lichtheimiaceae, Mucoraceae, Saksenaeaceae, Thamnidiaceae** and **Syncephalastraceae** (Gomes et al. 2011; Garcia-Hermoso et al. 2011). Species from all six families have been implicated in human infection (Table 12.1) (Ribes et al. 2000; Gomes et al. 2011). Clinically, the previously common term zygomycosis is no longer accurate, and **the umbrella term mucormycosis should be used** to describe infections caused by these moulds.

Human pathogenic species principally belong to the genera *Rhizopus*, *Mucor* and *Lichtheimia* (Table 12.1). *Rhizopus* species predominate, accounting for around 50% of cases, with *R. oryzae* (syn: *R. arrhizus*) being the most common clinical isolate reported, followed by *R. microsporus* var. *rhizopodiformis*. *Mucor* and *Lichtheimia* account for a further 20–30% of reported cases of mucormycosis (Gomes et al. 2011; Reiss et al. 2012).

Other genera, such as *Rhizomucor*, *Cunninghamella*, *Apophysomyces* and *Saksenaea* cause human infection to a much lesser extent. *Cokeromyces recurvatus* and *Syncephalastrum racemosum* have been described as the causative agent in only a small percentage of cases. However, the possibility of incorrect speciation in reported cases cannot be ignored, especially because of the complexities of identification, together with the changing nomenclature of these fungi. Additionally, cases of the more unusual species may be underestimated due to incomplete or incorrect identification (Gomes et al. 2011). Regardless of causative species, the disease process and diagnostic strategies are similar.

III. Habitat and Sources of Mucorales

The species isolated from cases of human and animal infection are **thermotolerant**, and many are ubiquitous in the soil and on decomposing organic matter. These moulds are found in the indoor and outdoor air, on food items and in dust. The environmental microbiology literature provides a limited insight into how common the Mucorales are in the environment and provides few clues about which ecological niches these fungi are found in (Richardson 2009). In particular, examination of numerous air sampling surveys in indoor and outdoor environments might indicate the level of exposure or help explain the apparent seasonality of mucormycosis. Similar analyses of other environments might reveal specific point sources of fungal communities. These and other environmental issues have been reviewed previously (Richardson 2009) but are summarised here.

A. Indoor and Outdoor Environments

Sporangiospores released by the Mucorales range from 3 to 11 μm in diameter, are **easily aerosolised and readily dispersed** throughout the environment. This is the major mode of transmission. Spores can also be carried by

Table 12.1 Taxonomy of human pathogenenic Mucormycetes of the order Mucorales

Order	Family	Genus	Species
Mucorales	Syncephalastraceae	*Syncephalastrum*	*Syncephalastrum racemosum*
	Saksenaeaceae	*Apophysomyces*	*Apophysomyces elegans* complex
			Apophysomyces trapeziformis
		Saksenaea	*Saksenaea vasiformis* complex[a]
			Saksenaea erythrospora[b]
	Cunninghamellaceae	*Cunninghamella*	*Cunninghamella bertholletiae*
	Mucoraceae	*Absidia*	*Absidia coerulea*[c]
		Actinomucor	*Actinomucor elegans*
		Mucor	*Mucor circinelloides* (*Rhizomucor variabilis*)
			Mucor hiemalis
			Mucor indicus
			Mucor racemosus[c]
			Mucor ramosissimus
		Rhizomucor	*Rhizomucor pusillus*
		Rhizopus	*Rhizopus azygosporus*
			Rhizopus microsporus
			Rhizopus oryzae (*Rhizopus arrhizus*)
			Rhizopus schipperae
			Rhizopus stolonifer[c]
	Thamnidiaceae	*Cokeromyces*	*Cokeromyces recurvatus*
	Lichtheimiaceae	*Lichtheimia* (former *Absidia*)	*Lichtheimia corymbifera* (*Mycocladus corymbifer*, *Absidia corymbifera*)
			Lichtheimia ramosa (*Absidia ramosa*)

Adapted from Gomes et al. (2011)

[a]*S. vasiformis* is a complex of species but two new species also reside within this family, *Saksenaea oblongispora* and *S. erythrospora* (Alvarez et al. 2010)

[b]Data from Alvarez et al. (2010)

[c]Implication in human infection is not confirmed

insects, especially flies. However, there are very little data concerning the levels of mucormycete sporangiospores in outdoor and indoor air, especially in geographical areas where mucormycosis is particularly prevalent. The numbers of airborne mucormycete sporangiospores appear to depend on the climatic conditions that favour growth and dispersal.

A survey of indoor and outdoor air in and around 17 homes in Cincinnati, Ohio using mould-specific quantitative PCR failed to detect any pathogenic Mucorales (Meklin et al. 2007). During a 2-year air sampling survey in Barcelona, Spain, the following genera were found in decreasing order: *Aureobasidum, Rhizopus, Mucor, Arthinium, Phoma, Fusarium, Trichoderma* and *Botrytis* (Calvo et al. 1980). A 1-year aeromycological study was conducted in the area of Zagreb, in order to establish seasonal variations in the composition and concentration of aeromycota (Klaric and Pepeljnjak 2006). Sampling was carried out at three locations at regular intervals. Airborne fungi peaked during spring and summer (110–284 cfu/m^3), whereas lower levels were detected in autumn and winter at each of the sampling sites (6–128 cfu/m^3). In contrast to *Cladosporium, Pencillium* and *Alternaria*, very low levels of *Mucor* and *Rhizopus* were found.

In what appears to be the largest study of airborne indoor and outdoor fungal species and concentrations to date, Shelton and colleagues (2002) examined 12,026 fungal air samples (9,619 indoor samples and 2,407 outdoor samples) from 1,717 buildings located across the USA; these samples were collected during indoor air quality investigations performed from 1996 to 1998. For all buildings, both indoor and outdoor air samples were collected. The culturable airborne fungal concentrations in indoor air were lower than those in outdoor air. The fungal levels were highest in autumn and summer and lowest in the winter and spring. Geographically, the highest fungal levels were found in the Southwest, Far West and Southeast. The most common culturable airborne fungi, both indoors and outdoors and

in all seasons and regions, were *Cladosporium, Penicillium*, non-sporulating fungi and *Aspergillus*. *Stachybotrys chartarum* was identified in the indoor air in 6% of the buildings studied and in the outdoor air of 1% of the buildings studied. *Mucor* (not specified), *Rhizopus* (not specified) and *Cunninghamella* were detected in indoor air more than outdoor air, although this varied from one geographical area of the USA to another. However, agents of mucormycosis were not included in the category of "common fungal types".

A comparison of populations of mould species in homes in the UK and USA using mould-specific quantitative PCR **did not detect any appreciable level of Mucorales** in dust samples (Vesper et al. 2005). Mucormycetes do not appear to be common in buildings (Hyvärinen et al. 2002), suggesting that the various building materials used in house construction do not support the growth of these fungi, compared to the wide profile of deuteromycetes found on damp substrates. A significant proportion of private residences, offices and work places are known to be damp; estimates range from 20 % to 50 %. Numerous publications have reported the moisture levels required for growth of fungi on construction, finishing or furnishing materials. *Rhizopus* species have a high moisture requirement for growth and are classified as hydrophilic. Members of the Mucorales are non-cellulolytic microorganisms and do not have any enzymatic activity, even against the most susceptible forms of cellulose, and therefore are not very likely to be found on building materials. **All of these observations suggest that house residents are not generally exposed to Mucormycetes in their home environment, apart from mould-contaminated food items such as bread and fruit.** This notion is supported by the absence of Mucormycetes from indoor air sampling surveys (Shelton et al. 2002).

B. Soil and Composting Vegetation

The simplistic view is that the agents of mucormycosis are found in the soil, in composting vegetation, on rotting fruit, in dust, during heavy excavation and construction, and in air-conditioning filters. The elevated temperatures found in composting vegetation are **selective for thermophilic species**, such as some species of *Lichtheimia, Mucor, Rhizopus* and *Rhizomucor*. In general, these fungi are unable to utilise cellulose and lignin. They are characterised by rapid germination. Some species are found growing on dung.

At present, there are approximately 3,300 species of currently known soil fungi. Very few studies have identified Mucorales at particular sites in areas where cases of mucormycosis have been reported. For many years there has been the view that most soil fungi were cosmopolitan and that species at a particular site were only selected by various soil parameters. It has also been the view that most fungal species would potentially spend part of their life in the soil. This view has now been modified considerably as very many plant-parasitic species are never isolated from the soil. This is particularly pertinent when considering the Mucorales.

In general, composting is considered to be an aerobic process, suggesting high biological activity. It has been clearly demonstrated that the rise in temperature and the decomposition of composting plant materials is brought about by thermophilic microorganisms, including fungi (Ingold 1978). Temperature and changes in the available food supply probably exert the greatest influence in determining the species of organism comprising the population in a compost stack at any one time. Fungi, including *Aspergillus fumigatus* and Mucormycetes play an important role in the decomposition of cellulose, lignin and other more resistant materials, despite being confined primarily to the outer layers and becoming active only during the latter part of the composting period (Ingold 1978).

Many of the opportunistic Mucorales are **typical inhabitants of natural composts, tropical soils and other heated materials**. Indoor sites particularly associated with these fungi therefore may be those where humid organic material is exposed to heat, most notably within poorly maintained heating ducts and attached humidifier structures, in soils of potted plants, especially those placed in warm locations, and in indoor composts (James et al. 2006).

Rhizopus oryzae and *Lichtheimia corymbifera* (*Absidia corymbifera*) were found in potted

plant soil in a haematology unit where a leukaemia patient developed invasive mucormycosis (Ribes et al. 2000). They were also abundantly represented in bird dung accumulating in an aviary (Ribes et al. 2000). In hospitals, wooden materials such as tongue depressors have repeatedly been a source of problematic *Rhizopus* inoculum (Ribes et al. 2000). *Rhizopus oryzae* has been isolated from grains, onions, various nuts and stored seed potatoes (Ribes et al. 2000).

A number of studies mention soil contamination of areas of soft-tissue damage followed by mucormycosis, apparently in immunocompetent patients (Vainrub et al. 1988). **Traumatic inoculation of spores can lead to extensive necrotic cutaneous infections**. This form of disease is most often seen in patients with burns and other forms of local trauma, such as traumatic implantation of sporangiospores in dirt, needle-stick injuries, illicit drug injection sites, tattooing, insect bites and stings. Disruption of the cutaneous barrier seems to be a prerequisite for acquiring cutaneous mucormycosis, with region-specific Mucormycetes being isolated from soil at the site of trauma as well as from the patient.

An illustrative case is where a patient who, whilst attempting to extinguish presumably burning clothes, rolled in moist soil that was found to be contaminated with *Apophysomyces elegans* (Cooter et al. 1990). *Apophysomyces elegans* was first isolated from soil samples collected from a mango orchard in northern India (Misra et al. 1979). In diabetic or immunosuppressed patients, cutaneous lesions may arise at an insulin injection site or a catheter insertion site. Cutaneous mucormycosis is an aggressive disease. It can lead to necrotising fasciitis or to widespread disseminated infection.

Biological soil crusts are the community of organisms living at the surface of desert soils. Major components are cyanobacteria, green algae, microfungi, mosses, liverworts and lichens. Mucormycetes do not appear to survive in this environment. Species of Mucormycetes have been cultured from geothermal soils in Yellowstone National Park, including species of *Absidia* and *Cunninghamella*, especially in close proximity to the perennial plant *Dichanthelium lanuginosum*, the hot springs panic grass (Redman et al. 1999).

C. Natural Disasters

Natural disasters have also revealed specific environmental niches of Mucormycetes. **This was illustrated particularly by the Asian tsunami**. Most of the tsunami survivors who experienced near-drowning events remained in unclean and traumatic conditions without receiving any immediate medical care for several hours. Patients lay for several hours or days in warm and stagnant water; normally poorly virulent environmental bacteria, fungi and amoebae found the ideal conditions to colonise open wounds and bone fractures and disseminate to other body sites. Many survivors presented with necrotising fasciitis, which is a soft-tissue infection associated with infarction of the dermis and subcutaneous tissue. The cause of this condition is usually polymicrobial but cutaneous mucormycosis is recognised as a cause of necrotising fasciitis since agents of mucormycosis exhibit vasculotropism, which is important in the pathophysiology of this condition, with the endpoint being ischaemic necrosis of the affected tissue (Snell and Tavakoli 2007).

The following case is illustrative (Snell and Tavakoli 2007). A survivor of the Asian tsunami with soft-tissue injuries and bilateral displaced fractures of her inferior and superior pubic rami was found to be infected with *Apophysomyces elegans*. Liposomal amphotericin was commenced and a total course of 42 days was maintained. After surgical débridement, topical negative pressure dressings, nursing care and antifungal therapy, split skin grafting was utilised to cover the resulting defect, with good result. It was assumed that the affected areas had become infected with the fungus in contaminated water or soil as a result of destruction of the urban infrastructure by the tsunami.

Tornados and hurricanes have been the cause of cutaneous infection by Mucorales species. Neblett Fanfair and colleagues (2012) describe a large cluster of cases of mucormycosis, with 13 *Apophysomyces trapeziformis* infections in persons injured during a tornado in Joplin, USA. Morbidity and mortality among the case patients were substantial; the **primary risk factors for infection were penetrating trauma**. These findings suggest that clinicians should consider environmental fungi as potential agents

of soft-tissue infections in injured patients after natural disasters.

D. Other Sources of Mucormycetes

Even though many previous reviews on the epidemiology of mucormycosis highlight the growth and isolation of the Mucorales on intact and rotting fruit, bread and other food items (Ribes et al. 2000), there are very few systematic surveys. It is apparent from the recent literature at least that the Mucorales do not constitute a major part of the mycoflora of food-handling facilities.

IV. Exposure Pathways

A. Exposure Through Colonisation of Nasal Mucus

Because of their rapid growth and prolific spore production, inhalation of sporangiospores must be a daily occurrence (Sugar 2005). It is known that the sporangiospores of some Mucorales in indoor environments have a very low settling rate and remain airborne for a long time. Airborne fungal spores are almost ubiquitous and can be found on all human surfaces in contact with air, especially on upper and lower airway mucosa. **Some 15,000–170,000 spores are inhaled over a 24 h period by an adult person, depending on environmental factors**. Regional and climatic factors contribute to exposure. Fungal spores impacted on the mucus of airway mucosa are cleared by mucociliary transport. Therefore, it is surprising that members of the Mucorales are very rarely found in nasal mucus. Nasal mucus is produced by the nasal mucosa, and mucus lining the airways (trachea, bronchus, bronchioles) is produced by specialised airway epithelial cells (goblet cells) and submucosal glands. Small particles such as dust, particulate pollutants and allergens as well as infectious agents such as bacteria become caught in the viscous nasal or airway mucus.

Do mycological studies of the biodiversity of the nasal mucus help? The nose is a very efficient air sampler, and analysis of the nasal mucosa should reflect the air spora of the environment where a patient has been residing and working. In one study conducted in Austria, nasal mucus samples from patients suffering from chronic rhinosinusitis and from healthy persons were monitored over 28 months (Buzina et al. 2003). Mucus samples were obtained by flushing the noses of patients with saline or by endoscopic sinus surgery. Fungi from mucus were cultured and identification was performed microscopically and by polymerase chain reaction (PCR) with subsequent sequencing of the ribosomal internal transcribed spacer region. Altogether, 619 strains of fungi were cultivated from 233 subjects. Eighty-one species were identified, with a maximum of nine different species per person. The most prevalent isolates belonged to the genera *Penicillium, Aspergillus, Cladosporium, Alternaria* and *Aureobasidium*. Interestingly, Mucormycetes were found in only two subjects: in one, *Rhizopus oryzae*, and in another *Cunninghamella bertholletiae*.

Mucorales are found occasionally in water-damaged buildings, as demonstrated by air sampling and analysis of settled dust by quantitative PCR. However, inhalation of sporangiospores in dust has been linked to outbreaks of rhinocerebral or pulmonary mucormycosis linked to excavation, construction or contaminated air-conditioning filters. Some reports suggest that the lower airways are colonised with agents of mucormycosis (Connolly et al. 1999). **Nevertheless, in patients with appropriate underlying conditions, the isolation of Mucorales species, even from non-sterile samples, is generally regarded as potentially significant** (Torres-Narbona et al. 2008).

B. Exposure Through the Gastrointestinal Tract

Ingestion of contaminated food materials, such as fermented milk, fermented porridge, herbal/homeopathic remedies, and the use of contaminated tongue depressors are recognised exposure risks for gastrointestinal mucormycosis. Gastrointestinal mucormycosis is a rare condition that has usually been encountered in **malnourished infants or children**. Persistent ingestion of non-nutritional substances, gastric

ulcers, severe systemic illness, age extremes and systemic immunosuppression are additional predisposing conditions for gastrointestinal tract mucormycosis.

C. Contaminated Medical Devices

Whereas most infections with Mucormycetes are community acquired, nosocomial acquisition, both sporadic cases and pseudo-outbreaks, have been linked with **contaminated bandages, needles and tongue depressors** used to construct splints for intravenous and arterial cannulation sites in preterm infants (Ribes et al. 2000). Percutaneous routes of exposure are very important in causing infection by Mucormycetes. Traumatic implantation of spores in dirt has been seen in a number of patients. Needle-stick exposures have been implicated in mucormycotic infections occurring at the site of medicine injection, catheter insertion sites, injection sites for illicit drug use and tattooing. Insect bites or stings have also been implicated in disease transmission in cases of cutaneous and subcutaneous mucormycosis. The development of wound mucormycosis has been seen with a variety of adhesive products used in the hospital setting.

D. Exposure in Hospital Environments

Hospital-acquired mucormycosis, both sporadic cases and pseudo-outbreaks, has been reported but is rare. Fifteen hospital **outbreaks or clusters of infection** are described in the English literature between 1966 and 2010, from the USA, Canada, China and Europe. These included cutaneous, pulmonary, rhinocerebral, gastrointestinal and disseminated infections. *Rhizopus* was identified as the cause in the majority of cases, although *Rhizomucor*, *Lichtheimia*, *Cunninghamella* and *Mucor* have also been implicated (Antoniadou 2009; Gomes et al. 2011).

The most common route of entry was via contact with contaminated objects, and sources included non-sterile bandages and adhesive dressings, and wooden tongue depressors used to prepare oral medications administered via nasogastric tubes, as well as to construct splints for intravenous and arterial cannulation sites in preterm infants. Less frequently, inhalation was the route of exposure, and cases have been linked to construction or renovation work, contaminated ventilation systems and water damage to a linen store and patient's shower room. Corn-starch used in the manufacturing of allopurinol tablets and commercial ready-to-eat food was implicated in an outbreak of intestinal mucormycosis (Antoniadou 2009; Richardson 2009; Gomes et al. 2011).

V. Epidemiology

A. Modes of Transmission

Mucorales are thermotolerant fungi, generally reported in the literature to enjoy a **global distribution**, being common inhabitants of soil, found on plants and in decomposing organic matter. They are also found in the indoor environment, in dust and on foodstuffs. Such fungi are common plant pathogens and contaminants of grain, fruit and bread (Richardson 2009). Mucorales infections are also reported worldwide.

Mucorales fungi have efficient dispersion mechanisms, via sporangiospore release from sporangia. Each sporangium can contain **between hundreds and hundreds of thousands of spores**, depending on species, and when maturation is reached, the sporangium wall dissolves, discharging the spores into the environment (Fig. 12.1). Most species produce aerial growth only when the surrounding air is extremely damp. In such species, a splash dispersal mechanism is used, whereby water passes into the spore-mass through the columella as the sporangium wall dissolves, resulting in a "sporangial drop". Other species can form sporangiospores in dry air, producing powdery spores that are readily aerosolised and dispersed by low-speed winds. The foraging of small animals also aids the dissemination of spores (Richardson 2009).

Such dispersal mechanisms suggest that humans must be exposed to these fungal spores on a regular basis, both via inhalation and topical exposure. Inhalation from environmental sources is regarded as the main portal of entry into the body, and certainly the nasal sinuses and lungs are the commonest initial

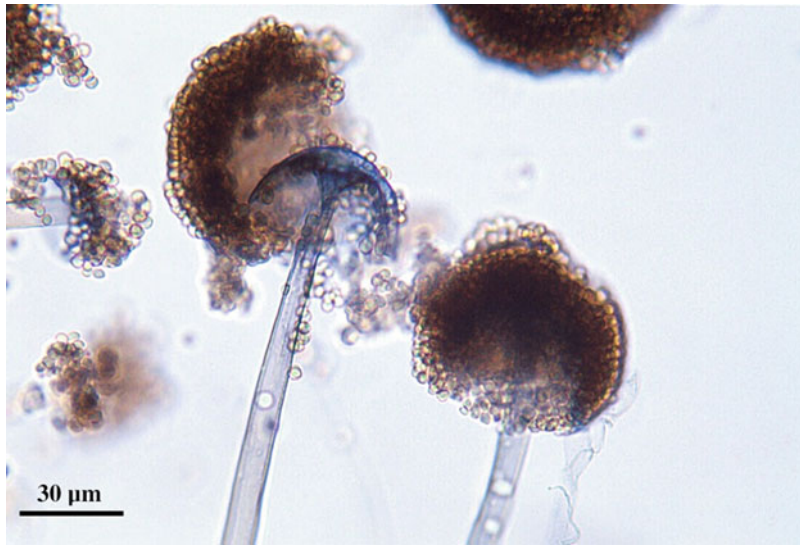


Fig. 12.1. Microscopic morphology of *Rhizopus oryzae*

sites of infection. However, despite such repeated contact, the frequency of human disease is relatively low and directly correlates with the immune status of the host. Studies suggest that those without properly functioning phagocytes are at increased risk of disease (Ibrahim et al. 2012; Roilides et al. 2012). **Neutropenic individuals are therefore the predominant sufferers of mucormycosis.** Less commonly, infection can also occur following cutaneous or percutaneous injuries. Intact skin is an effective anatomical barrier to infection; conversely, spores can be directly inoculated into abraded skin, often following trauma, or into occluded skin or mucosa as a result of contaminated dressings. Needlestick exposures after medicinal or illicit drug injections, catheter insertion, or tattoos have been reported. Ingestion of fungal elements from contaminated foodstuffs is a rare cause of gastrointestinal disease (Ribes et al. 2000; Richardson and Warnock 2012; Liao et al. 2013).

B. Prevalence of Infection

Mucormycosis remains the second most common mould infection in immunocompromised individuals, after aspergillosis, although it is still regarded as relatively rare (Neofytos et al. 2013). Roden and colleagues reviewed the English language literature from 1885 to the 1990s, discovering 929 cases of confirmed mucormycosis (Roden et al. 2005). **Estimates of prevalence in the USA, based on a population-based surveillance study during 1992 and 1993, indicated about 1.7 cases/million population, or approximately 500 cases per year** (Rees et al. 1998). Some European and North American studies suggest an increase in such infections during the last decade, especially in cancer centres (Marr et al. 2002; Lass-Flörl 2009a; Waness et al. 2009), whereas other surveys do not support this (Torres-Narbona et al. 2008; Prasad et al. 2012).

Spanish laboratories report an isolation rate of 6 per 100,000 samples, with no significant increase over a decade (Torres-Narbona et al. 2008). Analysis of paediatric infections between 2003 and 2010 in the USA found no evidence of an increase in mucormycosis (Prasad et al. 2012). In contrast, in France, population-based surveillance, conducted between 1997 and 2006, showed an increase in incidence from 0.7 to 1.2 cases per million population per annum. Most of the increase was accounted for by infections among haematopoietic stem cell transplant (HSCT) recipients or persons with haematological malignancies (Richardson and Warnock 2012). In the USA, an increase in the frequency of mucormycosis has been reported since the mid-1990s, and is now identified in up to 6.8% of autopsies (Brown 2005).

A contrast in worldwide epidemiology appears to have arisen. While mucormycosis remains relatively rare in developed countries, primarily affecting haematology patients, **in developing countries, especially India, the incidence of Mucorales disease is rapidly increasing and is predominantly related to an increase in individuals with uncontrolled diabetes** (Meis and Chakrabarti 2009). This association has also been reported in Japan and Taiwan (Slavin and Chakrabarti 2012).

Reasons suggested to explain this observed global rise in cases of mucormycosis include changes in transplantation procedures, an increased use of immunosuppressive drugs, improved diagnostic ability, increased incidence of diabetes mellitus, and the introduction of antifungal agents, including voriconazole and caspofungin, with no Mucorales activity (Brown 2005; Cuenca-Estrella et al. 2009; Waness et al. 2009).

Five main clinical manifestations are encountered, varying in frequency: rhino-orbital-cerebral (craniofacial; 39%), pulmonary (24%), cutaneous (19%), gastrointestinal and disseminated disease (Roden et al. 2005). **These different clinical forms are associated with particular underlying disorders** (see Section V, D). Risk of multi-organ dissemination varies, depending on the underlying disease and immune status of the host; rates in the literature range from 3% to >50%.Dissemination has been reported in patients with cerebral (48% of cases), cutaneous (39%) and pulmonary (20%) disease (Garcia-Hermoso et al. 2011; Sun and Singh 2011; Richardson and Warnock 2012). Other presentations include renal, cardiac and bone disease (Gomes et al. 2011; Liao et al. 2013).

C. Is Mucormycosis a Seasonal Disease?

Mucormycosis has not previously been regarded to have a seasonal pattern. Seasonal variations in the atmospheric concentration of fungal spores for some moulds in several geographical locations have been reported. In the USA, the highest levels were found in the autumn and summer and lowest in the winter and spring but agents of mucormycosis were not commonly found (Shelton et al. 2002). Seasonal peaks of *Aspergillus* conidia and other moulds have been reported from Europe and the USA (Ribes et al. 2000) but little data exist on the seasonal frequency of the Mucorales or the seasonal prevalence of mucormycosis. Only one study by Talmi and colleagues (2002) reported dates of onset of symptoms in 19 patients in Israel with rhino-orbital and rhino-orbito-cerebral mucormycosis and those appeared to vary seasonally but did not establish statistical significance. A more recent study from the Lebanon suggested a clustering of onset of mucormycosis at the end of a dry, warm period that lasted from May to October (Al-Ajam et al. 2006).

D. Risk Factors

Mucormycosis predominantly affects immunocompromised patients. The main predisposing factors, pathogenic mechanisms and clinical presentations are summarised in Table 12.2. Ultimately, **host factors play the most important role** in the development of Mucorales disease. Patients most at risk are those with prolonged or profound neutropenia, poorly controlled diabetes with or without ketoacidosis, other metabolic acidosis, neonatal prematurity and extensive skin lesions following trauma or burns. Malnutrition, long-term corticosteroid use, illicit intravenous drug use, iron overload with or without desferrioxamine iron chelation therapy and use of broad-spectrum antifungal prophylactic agents are also described as risk factors (Lass-Flörl 2009a; Petrikkos and Drogari-Apiranthitou 2011; Richardson and Warnock 2012; Liao et al. 2013). Other possible predisposing factors include those with HIV infection, chronic infections such as tuberculosis, septicaemia with multi-organ failure, use of antibiotic therapy, chronic renal failure and sarcoidosis (Waness et al. 2009; Garcia-Hermoso et al. 2011).

Certain underlying conditions appear to be more commonly associated with particular clinical forms of disease. Generally, patients with haematological malignancies and solid organ transplant (SOT) recipients are most likely to develop pulmonary disease. In SOT patients,

Table 12.2 Predisposing conditions for mucormycosis, pathogenic mechanisms and clinical presentation

Predisposing conditions for mucormycosis	Pathogenic mechanism(s)	Clinical presentation forms (in order of frequency)
Haematological malignancies Haematopoietic stem cell transplantation	Prolonged neutropenia	1. Pulmonary, rhinocerebral 2. Cutaneous 3. Sino-orbital
Diabetic ketoacidosis Uncontrolled diabetes mellitus	Impairment of neutrophil activation (functional neutropenia)/Fe usage by Mucormycetes for growth	1. Rhinocerebral 2. Sino-orbital 3. Pulmonary 4. Cutaneous
Prolonged treatment with corticosteroids Autoimmune disease	Defects in macrophages and neutrophils, corticosteroid induced diabetes, hypocomplementaemia	1. Disseminated 2. Renal 3. Cutaneous 4. Rhinocerebral 5. Gastrointestinal
Solid organ transplantation (SOT)/graft-versus-host disease	Cellular immune suppression, corticosteroid induced diabetes	1. Pulmonary 2. Sinus 3. Cutaneous 4. Rhinocerebral 5. Disseminated
HIV infection/intravenous illicit drug use	Injection of spores contained in drugs	1. Cerebral 2. Cutaneous 3. Renal, heart 4. Rhinocerebral 5. Disseminated
Iron overload	Fe usage by Mucormycetes for growth	1. Disseminated 2. Pulmonary
Iron/aluminium chelation therapy with desferrioxamine (DFO)	Fe-DFO action as siderophore	3. Rhinocerebral 4. Cerebral 5. Cutaneous 6. Gastrointestinal
Skin or soft-tissue breakdown Burn/trauma/surgical wound/insect bite	Direct cutaneous inoculation with high number of spores	1. Cutaneous 2. Pulmonary 3. Sino-orbital 4. Rhinocerebral 5. Gastrointestinal
Prolonged use of broad-spectrum antifungal agents (voriconazole, itraconazole and caspofungin)	Breakthrough infection due to resistance to these agents	1. Sino-pulmonary
Miscellaneous		
Neonatal prematurity	Ingestion of spores	1. Gastrointestinal 2. Cutaneous
Malnourishment	Ingestion of spores	3. Pulmonary 4. Sino-orbital
Prolonged use of broad-spectrum antimicrobial agents	Replacement of normal bacterial biota	5. Rhinocerebral

Taken (with minor adaptations) from Petrikkos and Drogari-Apiranthitou (2011)

dissemination occurs preferentially to cutaneous and soft tissues, rather than the brain. Diabetic individuals are at most risk of sinusitis, sino-orbital or rhinocerebral disease, whereas those with iron overload or treated with desferrioxamine are more likely to develop disseminated disease. Gastrointestinal infection is most probable to occur in low-birthweight premature infants, in malnourished individuals or as a result of peritoneal dialysis. Injection drug users are most likely to develop cerebral infection, often without sinus involvement. Of note, mucormycosis in HIV-infected individuals but without intravenous drug abuse is very rare.

Immunocompetent individuals can develop cutaneous infection, largely after penetrating trauma, burns or road traffic accidents (Meis and Chakrabarti 2009; Petrikkos and Drogari- Apiranthitou 2011; Garcia-Hermoso et al. 2011; Sun and Singh 2011; Richardson and Warnock 2012).

1. Haematological Malignancies, Haematopoietic Stem Cell and Solid Organ Transplantation

Patients with acute leukaemia and recipients of allogeneic haematopoietic stem cells are at high risk of developing mucormycosis, especially pulmonary infection. **Most cases occur later (more than 3 months) after transplantation**, when patients have developed graft-versus-host disease (GVHD), with resulting treatment with systemic steroids. The incidence of mucormycosis in bone marrow transplant patients is <0.25% in the first 6 months following transplant, and accounts for 8% of invasive fungal infections in these patients (Marr et al. 2002; Kontoyiannis et al. 2010; Garcia-Hermoso et al. 2011). SOT patients, particularly kidney recipients, are at risk of developing Mucorales infection, with mucormycosis representing 2% of invasive fungal infections in this setting. Liver transplantation carries a higher risk of dissemination, compared to other SOT recipients, with infection occurring earlier after transplantation (Garcia-Hermoso et al. 2011).

Voriconazole was introduced in 2002, and is extensively used in patients with high-risk malignancies or following haematopoietic stem cell transplant (HSCT), for prophylaxis and treatment of aspergillosis. However, the drug has no activity against Mucorales (Alastruey-Izquierdo et al. 2009; Petrikkos and Drogari-Apiranthitou 2011). Whole genome sequencing has revealed **duplication of the ergosterol biosynthetic pathway** in *R. oryzae*, and this may contribute to this lack of susceptibility (Ma et al. 2009). Furthermore, fruit fly and murine models have shown an increased virulence in *R. oryzae* strains pre-exposed to voriconazole (Lamaris et al. 2009). Prophylactic use of voriconazole, and less commonly caspofungin, has corresponded to an increase in mucormycosis in many institutions worldwide, and has been suggested as a causal factor (Meis and Chakrabarti 2009; Cuenca-Estrella et al. 2009; Richardson and Warnock 2012). Indeed, published reports detail voriconazole breakthrough infections in at least 23 cases (Reiss et al. 2012). Breakthrough mucormycosis in individuals whilst on caspofungin therapy has also been described (Ramos et al. 2009; Pang et al. 2012).

Although several factors may contribute to an increase in mucormycosis, it is clear that the use of broader-spectrum antifungal agents for prophylaxis has the potential to lead to the selection of organisms, such as the Mucorales, that are intrinsically resistant to available antifungal agents, and moreover, such use may selectively enhance Mucorales virulence (Lamaris et al. 2009; Richardson and Warnock 2012).

2. Diabetes Mellitus

Diabetes mellitus provides a significant risk factor for developing mucormycosis; around **17–36% of Mucorales infections in Europe are associated with this condition and around 74% of cases in India.** Type 2 diabetes represents the major threat, and indeed mucormycosis can present as the diabetes-defining illness (Roden et al. 2005; Rüping et al. 2010; Rammaert et al. 2012). Although diabetes is increasing worldwide, rates of diabetes-associated Mucorales disease across Europe are largely falling, which has been attributed to the increased use of statins (Petrikkos and Drogari-Apiranthitou 2011). Fulminant rhino-orbital-cerebral disease represents the primary site of infection (Rammaert et al. 2012). Impairment of macrophage and neutrophil function has been recognised as the key factor responsible for this increased risk. Ketoacidosis can further increase the risk, although most infections are seen in poorly controlled diabetics without ketoacidosis (Rammaert et al. 2012).

Iron has an important role in the pathogenesis of infections caused by the Mucorales, with **fungal growth being promoted in the presence of increased iron uptake**. The availability of serum iron is increased in the presence of an

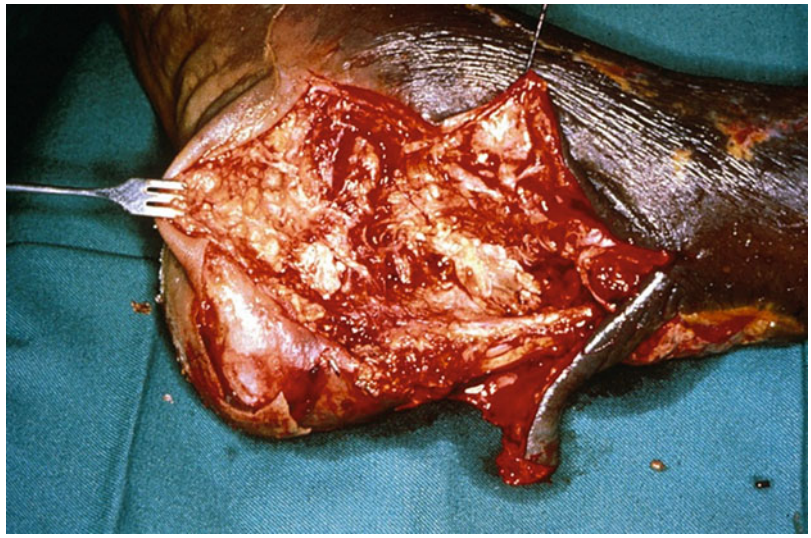


Fig. 12.2. Subcutaneous mucormycosis caused by *Apophysomyces elegans* following extensive burns

acidic pH, and this could help to account for the development of mucormycosis in patients with diabetic ketoacidosis (Richardson and Warnock 2012). Uncontrolled diabetes mellitus is the main cause of acidosis in the majority of patients with mucormycosis; however, the disease is also manifest in patients with persistent metabolic acidosis due to other causes, such as renal disease (Waness et al. 2009). **Desferrioxamine is an iron chelator that mobilises iron from the host and makes it available to the infecting fungus.** The use of this drug in patients with iron overload or on dialysis has been associated with the development of more severe, disseminated, and often lethal mucormycosis (Richardson and Warnock 2012).

3. Infection of the Immunocompetent Host

Immunocompetent individuals are at risk of developing cutaneous mucormycosis, most commonly after experiencing **burns (Fig. 12.2) or penetrating trauma**, including motorcycle or other road traffic accidents (Richardson 2009). Insect bites, scorpion stings or bird pecks have also been implicated in disease transmission in cases of cutaneous disease (Lechevalier et al. 2008; Wilson 2008; Richardson 2009; Pourahmad et al. 2013). Subcutaneous Mucorales infection of a tattoo site in an otherwise healthy individual has been reported (Fig. 12.3) (Parker et al. 1986). Mucoraceous infections have also been observed after combat injuries (Hospenthal et al. 2011; Evriviades et al. 2011). Natural disasters, including tsunamis and tornados, have given rise to numerous cases of mucormycosis after traumatic implantation of spores from dirt or contaminated water into sustained wounds (Richardson 2009; Slavin and Chakrabarti 2012; Neblett Fanfair et al. 2012).

E. Mortality Rates

Without appropriate and timely treatment, the **survival rate of mucormycosis is extremely low**, around 3%, depending on underlying illness and clinical manifestation of Mucorales disease (Petrikkos and Drogari-Apiranthitou 2011). Regardless of treatment, prognosis can still be poor. Overall, the survival rate of patients with mucormycosis is around 50%, but varies greatly depending on the form of the disease (Meis and Chakrabarti 2009). Mortality ranges from 35% among individuals with no underlying condition, 44% among patients with diabetes and 66% in patients with malignancies. The higher survival rate among diabetics than among those with underlying malignancies has been attributed to the relative ease with which the underlying ketoacidosis can be reversed. Mortality rates also vary

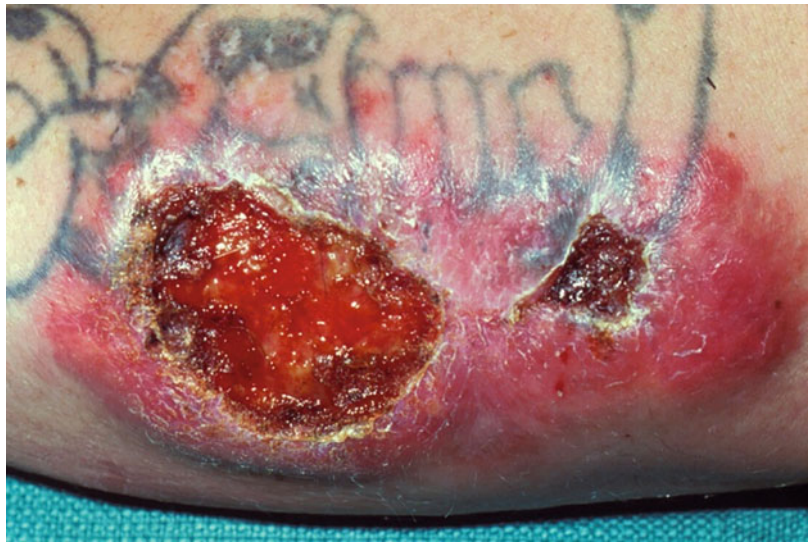


Fig. 12.3. An ulcerated, erythematous plaque on a tattooed area of the left forearm of an otherwise healthy 25-year-old male. The lesion had begun 15 months previously as a papule following a scratch. *Saksenaea vasiformis* was isolated

according to the site of infection, ranging from 62% among those with rhinocerebral disease, 76% in those with pulmonary disease, 85% among those with gastrointestinal disease and 96% in those with disseminated disease. **Pulmonary mucormycosis has a high mortality rate** because it is difficult to diagnose, frequently occurs in neutropenic patients and because surgical intervention is seldom possible (Roden et al. 2005; Meis and Chakrabarti 2009; Richardson and Warnock 2012).

Breakthrough infections after voriconazole therapy carry a high degree of mortality, reportedly 73% in one series of patients with haematological malignancies (Trifilio et al. 2007). Other poor prognostic indicators include disseminated disease, renal failure and infection with *Cunninghamella* species (Roden et al. 2005; Lass-Flörl 2009a). Furthermore, improved outcomes are observed when surgery is combined with medical therapy, rather than medical treatment alone (Petrikkos and Drogari-Apiranthitou 2011).

F. Prevention of Disease

Since mortality rates are so high, prevention of mucormycosis is paramount and centres around two main control measures: **reduction of environmental exposure to the fungus and prompt correction of underlying risk factors** (Petrikkos and Drogari-Apiranthitou 2011; Richardson and Warnock 2012).

In the non-immunocompromised individual, preventive measures should focus on correcting any underlying risk factors that could lead to the development of mucormycosis. Patient education in the control of diabetes mellitus, the use of iron chelators other than desferrioxamine, and limiting the use of aluminium-containing buffers in dialysis are among the best preventive measures that can be taken. Individuals, in particular those with diabetes, should avoid and report to their health care provider any kind of trauma, especially if the incident resulted in contact with contaminated water or soil (Gomes et al. 2011; Richardson and Warnock 2012).

The first steps in the prevention of infection in the immunocompromised patient, should consist of measures to **reduce or eliminate obvious sources of environmental exposure**, such as removing plants and flower arrangements from rooms where these individuals are being treated. Food items, such as old bread and fruit that are often contaminated with mucoraceous moulds, should not be offered to immunocompromised patients.

Inhalation of spores is thought to be the commonest mode of infection with mucormycosis in immunocompromised patients. The most effective, and expensive, method of protecting these individuals from nosocomial infection is to confine them to hospital rooms provided with **HEPA-filtered air**. Although this reduces the risk of disease to an insignificant level, infection can still occur if patients are colonised before their admission to hospital, or are moved from the protected environment to other parts of the hospital for essential procedures to be performed. Immunocompromised patients should not be nursed in units with ongoing, adjacent construction work, but if this cannot be avoided, measures should be instituted to minimise the entry of dust and contaminated air (Richardson and Warnock 2012).

Regardless of immune status of the host, care should be taken to use only sterile dressings for wounds and aseptic techniques for percutaneous procedures (Gomes et al. 2011). On hospital discharge, patients most at risk should also be given guidance on measures to limit potential environmental exposure, such as ensuring good ventilation and dust control in housing, avoidance of building works and close contact with soil, such as gardening (Gomes et al. 2011; Richardson and Warnock 2012).

There is currently no effective antifungal prophylaxis available for prevention of mucormycosis.

VI. Clinical Manifestations

There are five major clinical forms of mucormycosis (Table 12.2), of which rhinocerebral and pulmonary infections are by far the most common (Richardson and Warnock 2012). Other manifestations include cutaneous, gastrointestinal and disseminated disease. Like the aetiological agents of aspergillosis, the causal organisms of mucormycosis have a predilection for **vascular invasion causing thrombosis, infarction and necrosis** of the surrounding tissue. The clinical hallmark of mucormycosis is the rapid onset of necrosis and fever. In most cases, progress is rapid and death follows unless aggressive treatment is initiated.

A. Rhinocerebral Mucormycosis

The terms rhinocerebral and craniofacial mucormycosis are used to describe an infection that begins in the paranasal sinuses and then spreads to involve the orbit, face, palate and/or brain (Teixeira et al. 2013). The clinical presentation is similar to that of acute invasive *Aspergillus* rhinosinusitis. This is a **rapidly progressive disease** that is most commonly seen in diabetics with uncontrolled ketoacidosis, as well as in neutropenic cancer patients and HSCT recipients. Immunocompetent individuals are seldom affected. It is the commonest clinical form of mucormycosis, accounting for 39% of all cases of the disease, and 66% of cases in diabetics. If left untreated, the infection is often fatal within a week of onset.

The presenting symptoms include fever, unilateral facial swelling, unilateral headache, nasal or sinus congestion or pain, and a blood-tinged nasal discharge. Necrotic black ulcerations on the hard palate or nasal turbinate are a characteristic diagnostic sign. As the infection spreads into the orbit, periorbital or perinasal swelling occurs and progresses to disfiguring destruction of facial tissue. Ptosis, proptosis, ophthalmoplegia and loss of vision can occur. Drainage of black pus from the eye is a useful diagnostic sign. From the orbit, infection may spread into the brain leading to frontal lobe necrosis and abscess formation.

Imaging studies are helpful in assessing the extent of bone and soft-tissue involvement, but are more useful in guiding invasive procedures to obtain biopsy specimens than in establishing a diagnosis. Computed tomographic (CT) scans often reveal involvement of several sinuses (in particular ethmoid and sphenoid), but with a clear unilateral predilection, no air-fluid levels, thickening of sinus linings, and destruction of surrounding bone. Magnetic resonance imaging (MRI) is superior to CT scanning for detecting the extension of infection to the adjacent soft tissue of the orbit and brain, and may be preferred for the diabetic patient in whom CT contrast agents may be contraindicated.

B. Pulmonary Mucormycosis

This is the second most common clinical form of mucormycosis. It is most commonly seen in **neutropenic patients** with underlying haematological malignancies who are undergoing remission induction treatment. It has also been reported in allogeneic HSCT recipients, SOT recipients and those receiving desferrioxamine treatment. Mucormycosis may develop in the lungs as a result of aspiration of infectious material, following inhalation, or from haematogenous or lymphatic spread during dissemination. In about 50% of reported cases, the infection remains confined to the lung; in the remainder it spreads into adjacent tissues or becomes more widely disseminated. If left untreated, the infection is usually fatal within 2–3 weeks.

The **clinical presentation** is non-specific. Patients often present with an unremitting fever (greater than 38 °C) that fails to respond to broad-spectrum antibacterial treatment. Non-productive cough is a common presenting symptom. Haemoptysis and pleuritic chest pain are uncommon, but when present are helpful in suggesting a fungal infection. However, there are no characteristic symptoms or clinical signs to distinguish mucormycosis from aspergillosis. The **radiological signs** are also non-specific, but infiltrates and nodules are more frequent than consolidation or cavitation. Pleural effusion is uncommon. As with pulmonary aspergillosis, high-resolution CT scanning is more sensitive than plain chest radiographs for early diagnosis of infection and is the best method for determining the extent of the disease. The halo and air crescent signs, which are recognised radiological signs of aspergillosis, have also been reported in patients with mucormycosis. In patients with pulmonary mucormycosis, the presence of an air crescent sign is associated with an increased risk of massive haemoptysis. Mucormycosis has been reported to have a predilection for the upper lobes; however, any part of the lung may be involved, and bilateral disease is not uncommon.

C. Cutaneous Mucormycosis

Although inhalation is the usual route of infection in patients with mucormycosis, traumatic inoculation of spores can lead to extensive necrotic cutaneous infections. This form of disease is most often seen in patients with burns and severe soft-tissue trauma. Cutaneous mucormycosis has sometimes developed as a result of the application of contaminated surgical dressings, tape, adhesive bandages and splints. In diabetic or immunosuppressed patients, cutaneous lesions may arise at an insulin injection site or a catheter insertion site. **Cutaneous mucormycosis is an aggressive disease, even in the face of surgical débridement and antifungal treatment**. In 44% of cases, it leads to extensive local necrotising fasciitis or widespread disseminated infection.

The initial signs include cutaneous erythema and subcutaneous swelling. The margins of the lesion become raised and indurated, and the central region becomes necrotic and evolves into an ulcer covered with a black eschar. The lesions are indistinguishable from those caused by *Aspergillus* species, and can resemble ecthyma gangrenosum. The lesions are painful, and the patient can be febrile. The development of severe underlying necrosis and infarction in a burn should suggest the diagnosis.

Much less commonly, cutaneous mucormycosis develops following haematogenous dissemination of the fungus in immunosuppressed patients. The lesions begin as an erythematous, indurated painful cellulitis, then evolve into ulcers covered with a black eschar.

D. Gastrointestinal Mucormycosis

This is a rare condition that has usually been encountered in malnourished individuals and low-birthweight infants, in whom it may present as necrotising enterocolitis. All segments of the gastrointestinal tract can be involved, but lesions are **most common in the stomach, colon and ileum**. The disease is seldom diagnosed during life.

The symptoms are varied and depend on the site affected. Fever, abdominal pain, distention, vomiting and haematemesis are typical. Complications include gastric or intestinal perforation, perirenal abscesses and renal infarction. Intestinal mucormycosis is a fulminant illness ending in death within several weeks due to bowel infarction, sepsis or haemorrhagic shock.

E. Disseminated Mucormycosis

This may follow any of the four forms of mucormycosis described so far, but it is most frequently associated with pulmonary disease. Less commonly, dissemination occurs from the gastrointestinal tract, burns or other cutaneous lesions. The commonest site of spread is the **brain**, but metastatic necrotic lesions have also been found in the **spleen, heart and other organs**. Disseminated mucormycosis is seldom diagnosed during life, but occasionally patients develop metastatic cutaneous lesions, which permit an earlier diagnosis.

F. Other Forms of Mucormycosis

Isolated mucormycotic brain lesions without rhino-orbital involvement have been reported in leukaemic patients and injection drug users. The lesions often lead to focal neurological signs. The diagnosis is difficult, but should be considered in a neutropenic patient who becomes lethargic, confused or obtunded. CT and MRI scans are useful in locating the lesions, but the findings are non-specific. Other rare forms of mucormycosis include endocarditis, osteomyelitis and pyelonephritis. Isolated peritonitis has been associated with peritoneal dialysis.

G. Differential Diagnosis

Rhinocerebral mucormycosis is a dramatic and distinctive condition, but it can be confused with cavernous sinus thrombosis, bacterial orbital cellulitis and other forms of acute invasive fungal sinusitis, such as aspergillosis. The clinical manifestations of pulmonary mucormycosis cannot be distinguished from Gram-negative bacterial pneumonia, aspergillosis or hyalohyphomycosis.

VII. Laboratory Diagnosis

Early and reliable diagnosis of infections caused by fungi of the order Mucorales is crucial. These agents of disease are aggressive and rapidly invade tissue; thus infections often prove fatal. Furthermore, Mucorales fungi are frequently resistant to widely used antifungal agents, including voriconazole and caspofungin (Alastruey-Izquierdo et al. 2009), and breakthrough infections have occurred during prophylaxis (Reiss et al. 2012; Pang et al. 2012). Prompt initiation of appropriate antifungal therapy may increase survival rates and improve morbidity (Lass-Flörl et al. 2007; Walsh et al. 2012).

A. Microscopic Investigations

Microscopic investigation of clinical specimens can be performed using direct, cytopathological or histopathological techniques. Microscopy can be performed rapidly and provides a same-day diagnosis. This allows optimal therapy to be commenced at the soonest, and other strategies, including surgery, to be evaluated. Appropriate clinical specimens depend on the site of infection (Table 12.3). Biopsied tissue is the preferred specimen type; however, it may not be appropriate to obtain tissue during thrombocytopenia and from critically ill patients, thus preventing histopathological examination.

1. Direct and Cytopathological Examination

Microscopic evidence of Mucorales fungi in clinical specimens is of increased significance compared to their isolation in culture, especially from necrotic wound material and upper respiratory tract secretions. Furthermore, **positive microscopy must be regarded as significant even if the culture result is negative** (Lass-Flörl 2009b; Richardson and Warnock 2012).

Gram stain is generally the most commonly used stain in laboratories, and may be used to detect fungal hyphae. Generally, hyphae stain Gram-negative; however, the **staining pattern may be irregular** (Brandt and Warnock 2011; Walsh et al. 2012). Potassium hydroxide (KOH) preparations are traditionally used to examine clinical material for fungal elements (Fig. 12.4), although observation of hyphae in KOH wet

Table 12.3 Specimen types for main manifestations of mucormycosis

Clinical manifestation	Appropriate clinical specimens
Rhino-orbital-cerebral (craniofacial)	Sinus aspirates or scrapings Nasal discharge, scrapings or tissue
With evidence of CNS involvement	Brain biopsy (Cerebrospinal fluid)
Pulmonary	Biopsies of lesions – guided by transbronchial or percutaneous computerised tomography Bronchoalveolar lavage fluid (Sputum)
Cutaneous	Skin biopsy or scrapings
Gastrointestinal	Biopsy tissue of the affected area
Disseminated	Biopsy tissue of the affected organ

Adapted from Lass-Flörl (2009b)
Specimen types in parentheses are alternatives but not optimal

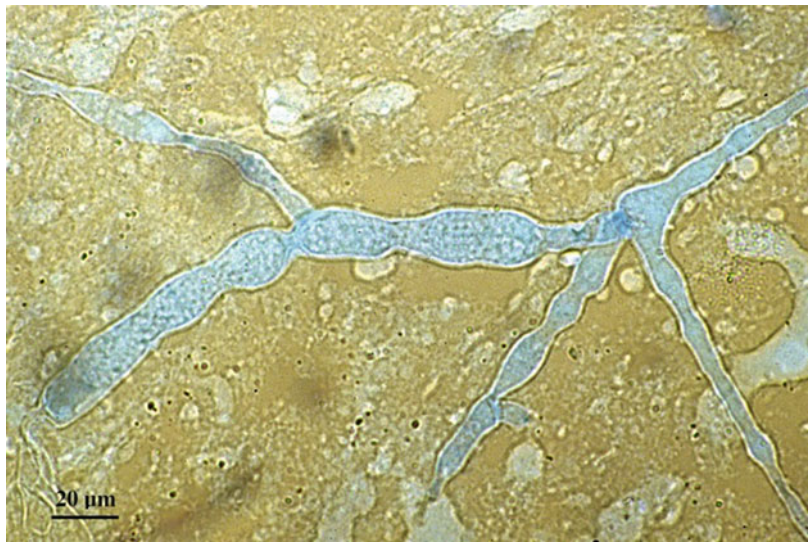


Fig. 12.4. Direct mount in potassium hydroxide of aspirated material from a case of subcutaneous mucormycosis, showing distinctive thin-walled hyphae with focal bulbous dilations and irregular branching

mounts can be difficult. Sensitivity can be improved by the addition of a fluorescent whitener, such as Blankophor or Calcofluor white, which binds to chitin in the fungal cell wall and is visualised using fluorescence microscopy. Calcofluor white staining has been demonstrated to aid differentiation between septate and non-septate hyphae in lung biopsied tissue (Lass-Flörl et al. 2007). **Fluorescent brighteners may also be applied to previously Gram-stained slides if necessary.** Giemsa stain is not appropriate for Mucorales hyphae due to poor uptake of stain (Shea 2011).

Typically, these fungi are characterised by broad (6–16 µm), non-septate (or occasionally sparsely septate) thin-walled hyaline hyphae showing wide-angled branching of 90° or more (Fig. 12.4). Due to lack of support from septal walls, hyphae can collapse and fold into irregular shapes, often appearing as ribbon-like structures, with bulbous areas. These fragile hyphae can easily become fragmented during collection and processing of clinical material. Difficulties in detecting these fragments in preparations may result in a falsely negative report. Thus, multiple specimens should be

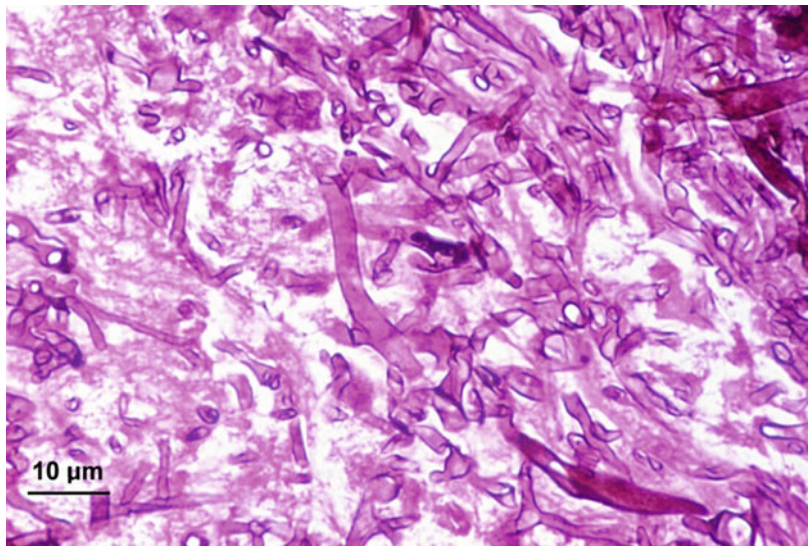


Fig. 12.5. Stained section (H&E) of tissue from an enlarging necrotic ulcer from a patient's leg, showing broad, infrequently septate, thin-walled hyphae with focal bulbous dilations and irregular, non-dichotomous, often right-angled, branching. *Saksenaea vasiformis* was isolated

taken in an attempt to increase the probability of obtaining a positive result and clinicians should be guided in handling of the specimen (Lass-Flörl 2009b; Brandt and Warnock 2011; Walsh et al. 2012).

Due to differing susceptibilities, it is crucially important to **distinguish between Mucorales fungi and other hyaline moulds**, including the more commonly encountered *Aspergillus* species. These non-mucoraceous moulds characteristically have thinner, septate hyphae displaying acute-angled branching. Nevertheless, care should be taken when patients have received prior antifungal therapy as hyphal appearance can be atypical, leading to difficulties in differentiation of filamentous fungi.

2. Histopathological Examination

Because Mucorales species may represent contamination in culture, direct demonstration of organisms in tissue provides conclusive evidence of invasive disease. However, as with direct microscopy, such detection may prove problematic. Specialised fungal stains, such as periodic acid-Schiff (PAS) or Gomori methenamine silver (GMS) stains are preferable to the standard haematoxylin and eosin (H&E) stain (Fig. 12.5). Hyphal appearance is generally similar to that seen in direct preparations, with fragmentation and distortion also occurring (Fig. 12.5). Furthermore, hyphal cross-sections, of varying diameter, will be evident in tissue sections, resulting in a vacuolated appearance and might be misinterpreted as yeast cells (Lass-Flörl 2009b; Walsh et al. 2012; Shea 2011).

An acute inflammatory response is commonly observed, with neutrophil infiltration. Foci of granulomatous inflammation may also be present. Angioinvasion is typically seen, which results in thrombosis and tissue infarction. Penetration of the perineural space has also been reported in biopsies containing nerves (Frater et al. 2001; Garcia-Hermoso et al. 2011). Immunohistochemistry using commercially available monoclonal antibodies has been reported to increase diagnostic specificity in mucormycosis (Jensen et al. 1997). Regardless of staining methods, **microscopy alone is generally insufficient to enable identification of any Mucorales species**; cultures are required. Therefore, all specimens should also be sent to the clinical microbiology laboratory with a request for culture.

B. Microbiology

Obtaining a positive culture is helpful, both for identification of the causative agent and also to allow in vitro susceptibility testing to be performed. Studies have suggested that susceptibility profiles vary between different Mucorales species and between geographic areas (Rüping et al. 2010; Gomes et al. 2011). However, not all samples are optimal for recovery of fungus. Blood cultures are almost always negative despite the predilection for invading blood vessels seen with Mucorales. With central nervous system involvement, cultures of cerebrospinal fluid are of rare diagnostic value, and in pulmonary disease, sputum cultures lack sensitivity. Isolation of mucoraceous fungi in patients with definitive invasive disease is around 75%, **although higher yields may be obtained when tissue samples are not ground** (Torres-Narbona et al. 2008).

Culture results must be interpreted in conjunction with microscopy and clinical data because positive cultures may be a reflection of patient colonisation or environmental contamination, especially for material from necrotic lesions and upper respiratory tract fluids (Torres-Narbona et al. 2008; Petrikkos and Drogari-Apiranthitou 2011). Importantly, if the patient is diabetic or immunosuppressed, a fungal isolate should never be disregarded (Richardson and Warnock 2012).

All clinical material should be transferred to the laboratory for culture as quickly as possible, without refrigeration, to maximise potential fungal yield (Denning et al. 2003). **Cultures of tissue samples should be prepared with as little manipulation as is necessary.** Since mucoraceous hyphae lack septal walls, they are easily damaged, resulting in cytoplasm leakage and a decrease in viability. Tissue samples should be carefully teased apart into small pieces, without grinding, and placed onto regular mycological media, such as Sabouraud dextrose agar, at 30 °C and 37 °C for a minimum of 5 days. An incubation temperature of 37 °C enhances recovery of Mucorales hyphae from deep sites of infection (Kontoyiannis et al. 2007). Mucorales species grow well on most microbiological media, with the exception of cycloheximide-containing agar. Most Mucorales are inhibited by cycloheximide (actidione) and such media should be avoided. Some species, such as *Apophysomyces elegans* and *S. vasiformis*, require specialist media to induce sporulation. If necessary, sterile bread without preservatives can be employed as a growth medium for Mucorales from clinical samples (Petrikkos and Drogari-Apiranthitou 2011). Many species are thermotolerant and can grow at temperatures above 40 °C; indeed maximal growth temperatures can be helpful in the identification process.

Mucorales species grow very rapidly in vitro, up to 20 mm per day. Lack of septal walls results in faster streaming of cytoplasm contents, allowing easier transport within the hyphae. Furthermore, sequencing studies suggest that *R. oryzae* has undergone an ancestral whole-genome duplication event; doubling of genes responsible for metabolism could help explain this fast growth rate (Ma et al. 2009; Reiss et al. 2012). Clinical cultures are positive generally within 24–48 h, producing **profusely sporing floccose (woolly), cottony white, grey or brownish colonies (Fig. 12.6), and microscopically are characterised by wide (6–15 μm) non-septate (or almost so) hyphae showing ≥90° branching.** Asexual reproductive structures are also produced and, depending on species, may include branched or unbranched sporangiophores, sporangia, columellae and sporangiospores (Fig. 12.1). Other structures such as root-like rhizoids may also be seen (Fig. 12.7). Phenotypic speciation of these fungi relies on such macroscopic and microscopic characteristics, can be time-consuming and requires expertise. Carbon assimilation profiling using commercial kits has been reported to be a useful technique in identifying these moulds (Schwarz et al. 2007). Molecular methods have also been reported for genotypic identification, providing the potential advantages of speed and accuracy. However, no standardised method has been published. Nonetheless, recommendations of the International Society for Human and Animal Mycology Working Group suggest that the use of **ribosomal DNA sequencing of the ITS region (see also chapter "Diagnostics of Fungal**

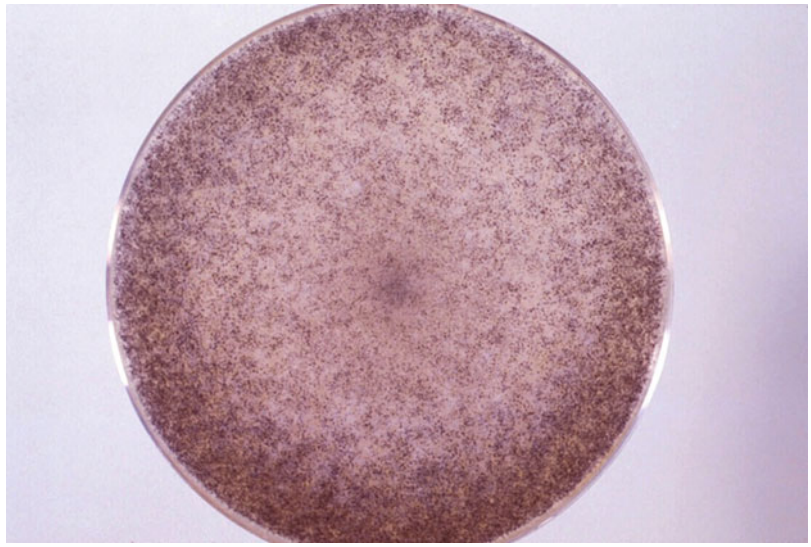


Fig. 12.6. *Rhizopus oryzae* on Sabouraud dextrose agar. Colonies are very fast growing at 25 °C, about 5–8 mm high, with some tendency to collapse. They are white cottony at first becoming brownish grey to blackish-grey depending on the amount of sporulation

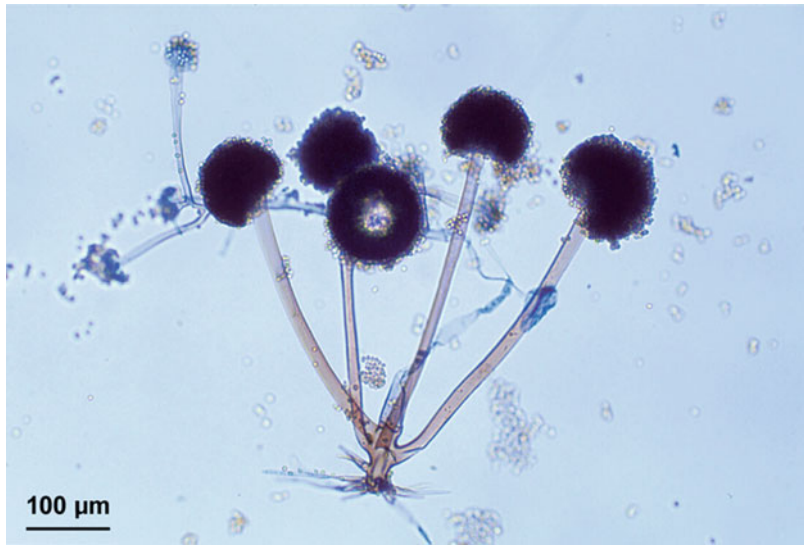


Fig. 12.7. Microscopic morphology of *Rhizopus oryzae*

Infections" by Willinger et al.) provides reliable identification of most medically important species of Mucorales, and would be a suitable choice for identification of an unknown organism with no a priori knowledge (Dannaoui 2009). However, discrimination between closely related species may not be possible with ITS sequencing, and heterogeneity of the ITS sequence has been noted for members of the order Mucorales. Sequencing of other gene targets, such as the D1/D2 region of the 28S rDNA or a conserved region of the Mucormycetes cytochrome, may also be valuable (Balajee et al. 2009; Gomes et al. 2011).

C. Serological Methods of Detection

Over the years, several authors have endeavoured to develop methods for sero-diagnosis of mucormycosis. These have included immunodiffusion and ELISA techniques, with varying levels of sensitivity and specificity (Kaufman et al. 1989; Sankapal and Abraham 2011). None have proved clinically useful and, currently, no specific antigen or antibody detection methods are commercially available (Ribes et al. 2000; Garcia-Hermoso et al. 2011).

Testing for β-glucan as a biomarker in mucormycosis is futile because most of these fungi do not possess (1,3)-β-D-glucan in their cell wall. However, a negative result may help to rule out invasive aspergillosis, the most likely differential diagnosis (Skiada et al. 2013).

D. Molecular Methods of Detection and Identification

Molecular procedures have been developed for the detection of mucoraceous fungi directly from clinical specimens (Bernal-Martínez et al. 2013). Such methods have the potential to offer greater speed and enhanced sensitivity, especially because these fragile organisms can be difficult to grow and to induce sporulation. Discrimination between morphologically similar species may also be faster and easier. PCR-based techniques have been the most widely described, although no standardised or commercial methods are currently available. Ideally, tissue specimens should be fresh or frozen because formalin will damage DNA (Gomes et al. 2011). Nevertheless, formalin-fixed paraffin-embedded tissues may be the only samples available and reported methods can be utilised, although yields are likely to be lower (Garcia-Hermoso et al. 2011).

The advantage of increased sensitivity of molecular detection can also be problematic; false-positive results can occur as a result of environmental contamination, especially when the clinical specimen is not collected aseptically or contains material from necrotic lesions and upper respiratory tract fluids (Gomes et al. 2011). Different regions of DNA, including ribosomal DNA genes, have been used for molecular detection of Mucorales fungi. Assays have often used the small-subunit (18S) or ITS rDNA gene region as targets because of the conserved nature and high copy number of these regions (Gomes et al. 2011). However, in both murine models and clinical samples, histopathology results had demonstrably better sensitivity compared with panfungal PCR followed by ITS1 sequencing (Garcia-Hermoso et al. 2011). Other targets, such as the 28S rRNA gene and a conserved region of the Mucormycetes cytochrome, have been shown to be specific for Mucorales species (Gomes et al. 2011). A recent study demonstrated the utility of specific quantitative PCR assays using hydrolysis probes targeting several species of Mucorales, with positive serum results obtained between 3 and 68 days prior to diagnosis by means of histopathology and/or positive culture. Such tests could be useful in screening high-risk patients (Millon et al. 2013).

Currently, molecular diagnosis of mucormycosis is limited to the research laboratory; further work is required to optimise methods, particularly to improve the sensitivity with fixed-tissue specimens, before routine clinical use is achieved (Gomes et al. 2011; Garcia-Hermoso et al. 2011; Walsh et al. 2012).

References

Al-Ajam MR, Bizri AR, Mokbbat J et al (2006) Mucormycosis in the Eastern Mediterranean: a seasonal disease. Epidemiol Infect 134:341–346

Alastruey-Izquierdo A, Castelli MV, Cuesta I et al (2009) In vitro activity of antifungals against Zygomycetes. Clin Microbiol Infect 15(Suppl 5):71–76

Alvarez E, Garcia-Hermoso D, Sutton DA et al (2010) Molecular phylogeny and proposal of two new species of the emerging pathogenic fungus *Saksenaea*. J Clin Microbiol 48:4410–4416

Antoniadou A (2009) Outbreaks of zygomycosis in hospitals. Clin Microbiol Infect 15(Suppl 5):55–59

Balajee SA, Borman AM, Brandt ME et al (2009) Sequence-based identification of *Aspergillus*, *Fusarium*, and Mucorales species in the clinical mycology laboratory: where are we and where should we go from here. J Clin Microbiol 47:877–884

Bernal-Martínez L, Buitrago MJ, Castelli MV et al (2013) Development of a single tube multiplex real-time PCR to detect the most clinically relevant Mucormycetes species. Clin Microbiol Infect 19: E1–E7

Brandt ME, Warnock DW (2011) Taxonomy and classification of fungi. In: Versalovic J, Carroll KC, Funke G et al (eds) Manual of clinical microbiology, vol 2, 10th edn. ASM Press, Washington, DC, pp 1749–1755

Brown J (2005) Zygomycosis: an emerging fungal infection. Am J Health Syst Pharm 62:2593–2596

Buzina W, Braun H, Freudenschuss K et al (2003) Fungal biodiversity – as found in nasal mucus. Med Mycol 41:149–161

Calvo MA, Guarro J, Suarez G et al (1980) Air-borne fungi in the air of Barcelona (Spain). IV. Various isolated genera. Mycopathologia 71:119–123

Connolly JE, McAdams HP, Erasmus JJ et al (1999) Opportunistic fungal pneumonia. J Thorac Imaging 14:51–62

Cooter RD, Lim IS, Ellis DH et al (1990) Burn wound zygomycosis caused by *Apophysomyces elegans*. J Clin Microbiol 28:2151–2153

Cuenca-Estrella M, Bernal-Martinez L, Isla G et al (2009) Incidence of zygomycosis in transplant recipients. Clin Microbiol Infect 15(Suppl 5):37–40

Dannaoui E (2009) Molecular tools for identification of Zygomycetes and the diagnosis of zygomycosis. Clin Microbiol Infect 15(Suppl 5):66–70

Denning DW, Kibbler CC, Barnes RA (2003) British Society for Medical Mycology proposed standards of care for patients with invasive fungal infections. Lancet Infect Dis 3:230–240

Evriviades D, Jeffery S, Cubison T et al (2011) Shaping the military wound: issues surrounding the reconstruction of injured servicemen at the Royal Centre for Defence Medicine. Philos Trans R Soc Lond B Biol Sci 366:219–230

Frater JL, Hall GS, Procop GW (2001) Histologic features of zygomycosis: emphasis on perineural invasion and fungal morphology. Arch Pathol Lab Med 125:375–378

Garcia-Hermoso D, Dannaoui E, Lortholory O et al (2011) Agents of systemic and subcutaneous mucormycosis and entomophthoromycosis. In: Versalovic J, Carroll KC, Funke G et al (eds) Manual of clinical microbiology, vol 2, 10th edn. ASM Press, Washington, DC, pp 1880–1901

Gomes MZ, Lewis RE, Kontoyiannis DP (2011) Mucormycosis caused by unusual mucomycetes, non-*Rhizopus*, -*Mucor*, and –*Lichtheimia* species. Clin Microbiol Rev 24:411–445

Hibbett DS, Binder M, Bischoff JF et al (2007) A higher-level phylogenetic classification of the fungi. Mycol Res 111:509–547

Hospenthal DR, Chung KK, Lairet K et al (2011) *Saksenaea erythrospora* infection following combat trauma. J Clin Microbiol 49:3707–3709

Hyvärinen A, Meklin T, Vepsäläinen A et al (2002) Fungi and actinobacteria in moisture-damaged building materials – concentrations and diversity. Int Biodeterior Biodegrad 49:27–37

Ibrahim AS, Spellberg B, Walsh TJ et al (2012) Pathogenesis of mucormycosis. Clin Infect Dis 54(Suppl 1): S16–S22

Ingold CT (1978) The biology of *Mucor* and its allies, vol 88, Studies in biology. Edward Arnold, London

James TY, Kauff F, Schoch CL et al (2006) Reconstructing the early evolution of Fungi using a six-gene phylogeny. Nature 443:818–822

Jensen HE, Salonen J, Ekfors TO (1997) The use of histochemistry to improve sensitivity and specificity in the diagnosis of systemic mycoses in patients with haematological malignancies. J Pathol 181:100–105

Kaufman L, Turner LF, McLaughlin DW (1989) Indirect enzyme-linked immunosorbent assay for zygomycosis. J Clin Microbiol 27:1979–1982

Klaric MS, Pepeljnjak S (2006) A year-round aeromycological study in Zagreb area, Croatia. Ann Agric Environ Med 13:55–64

Kontoyiannis DP, Chamilos G, Hassan SA et al (2007) Increased culture recovery of Zygomycetes under physiologic temperature conditions. Am J Clin Pathol 127:208–212

Kontoyiannis DP, Marr KA, Park BJ et al (2010) Prospective surveillance for invasive fungal infections in hematopoietic stem cell transplant recipients, 2001–2006: overview of the Transplant-Associated Infection Surveillance Network (TRANSNET) Database. Clin Infect Dis 50:1091–1100

Lamaris GA, Ben-Ami R, Lewis RE et al (2009) Increased virulence of Zygomycetes organisms following exposure to voriconazole: a study involving fly and murine models of zygomycosis. J Infect Dis 199:1399–1406

Lass-Flörl C (2009a) The changing face of epidemiology of invasive fungal disease in Europe. Mycoses 52:197–205

Lass-Flörl C (2009b) Zygomycosis: conventional laboratory diagnosis. Clin Microbiol Infect 15(Suppl 5):60–65

Lass-Flörl C, Resch G, Nachbaur D et al (2007) The value of computed tomography-guided percutaneous lung biopsy for diagnosis of invasive fungal infection in immunocompromised patients. Clin Infect Dis 45:e101–e104

Lechevalier P, Hermoso DG, Carol A et al (2008) Molecular diagnosis of *Saksenaea vasiformis* cutaneous infection after scorpion sting in an immunocompetent adolescent. J Clin Microbiol 46:3169–3172

Liao Y et al (2013) Epidemiology of opportunitistic invasive fungal infections in China: review of the literature. Chin Med J 126:361–368

Ma LJ, Ibrahim AS, Skory C et al (2009) Genomic analysis of the basal lineage fungus *Rhizopus oryzae* reveals a whole-genome duplication. PLoS Genet 5:e1000549. doi:10.1371/journal.pgen.1000549, Epub 2009 Jul 3

Marr KA, Carter RA, Crippa F et al (2002) Epidemiology and outcome of mould infections in hematopoietic stem cell transplant recipients. Clin Infect Dis 34:909–917

Meis JF, Chakrabarti A (2009) Changing epidemiology of an emerging infection: zygomycosis. Clin Microbiol Infect 15(Suppl 5):10–14

Meklin T, Reponen T, McKinstry C et al (2007) Comparison of mold concentrations quantified by MSQPCR in indoor and outdoor air sampled simultaneously. Sci Total Environ 382:130–134

Millon L, Larosa F, Lepiller Q et al (2013) Quantitative polymerase chain reaction detection of circulating DNA in serum for early diagnosis of mucormycosis in immunocompromised patients. Clin Infect Dis 56:e95–e101

Misra PC, Srivastava KJ, Lata K et al (1979) *Apophysomyces*, a new genus of the Mucorales. Mycotaxon 8:377–382

Neblett Fanfair R, Benedict K, Bos J et al (2012) Necrotizing cutaneous mucormycosis after a tornado in Joplin, Missouri, in 2011. N Engl J Med 367:2214–2225

Neofytos D, Treadway S, Ostrander D et al (2013) Epidemiology, outcomes, and mortality predictors of invasive mold infections among transplant recipients: a 10-year, single-centre experience. Trans Infect Dis. doi:10.1111/tid.12060

Pang KA, Godet C, Fekkar A et al (2012) Breakthrough invasive mould infections in patients treated with caspofungin. J Infect 64:424–429

Parker C, Kaminski G, Hill D (1986) Zygomycosis in a tattoo, caused by *Saksenaea vasiformis*. Aust J Dermatol 27:107–111

Petrikkos G, Drogari-Apiranthitou M (2011) Zygomycosis in immunocompromised non-haematology patients. Mediterr J Hematol Infect Dis 2011: e2011012

Pourahmad M, Sepidkar A, Farokhnia MH et al (2013) Mucormycosis after scorpion sting: case report. Mycoses. doi:10.1111/myc.12066 [Epub ahead of print]

Prasad PA, Vaughan AM, Zaoutis TE (2012) Trends in zygomycosis in children. Mycoses 55:352–356

Rammaert B et al (2012) Diabetes and mucormycosis: a complex interplay. Diabetes Metab 38:193–204

Ramos A, Cuervas-Mons V, Noblejas A et al (2009) Breakthrough rhinocerebral mucormycosis in a liver transplant patient receiving caspofungin. Transplant Proc 41:1972–1975

Redman RS, Litvintseva A, Sheehan KB et al (1999) Fungi from geothermal soils in Yellowstone National Park. Appl Environ Microbiol 65:5193–5197

Rees JR, Pinner RW, Hajjeh RA et al (1998) The epidemiological features of invasive mycotic infections in the San Francisco Bay area, 1992–1993: results of population-based laboratory active surveillance. Clin Infect Dis 27:1138–1147

Reiss E, Shadomy HJ, Lyon GM (2012) Fundamental medical mycology. Wiley-Blackwell, Hoboken

Ribes JA, Vanover-Sams CL, Baker DJ (2000) Zygomycetes in human disease. Clin Microbiol Rev 13:236–301

Richardson M (2009) The ecology of the Zygomycetes and its impact on environmental exposure. Clin Microbiol Infect 15(suppl 5):2–9

Richardson MD, Warnock DW (2012) Fungal infection: diagnosis and management, 4th edn. Wiley-Blackwell, Chichester

Roden MM, Zaoutis TE, Buchanan WL et al (2005) Epidemiology and outcome of zygomycosis: a review of 929 reported cases. Clin Infect Dis 41:634–653

Roilides E, Kontoyiannis DP, Walsh TJ (2012) Host defenses against zygomycetes. Clin Infect Dis 54 (Suppl 1):S61–S66

Rüping MJ, Heinz WJ, Kindo AJ et al (2010) Forty-one recent cases of invasive zygomycosis from a global clinical registry. J Antimicrob Chemother 65:296–302

Sankapal SR, Abraham J (2011) Antibody response in central nervous system to the antigenic preparation of *Mucor* and *Aspergillus*. Jundishapur J Microbiol 4:223–228

Schwarz P et al (2007) Carbon assimilation profiles as a tool for identification of zygomycetes. J Clin Microbiol 45:1433–1439

Shea Y (2011) General approaches for direct detection of fungi. In: Versalovic J, Carroll KC, Funke G et al (eds) Manual of clinical microbiology, vol 2, 10th edn. ASM Press, Washington, DC, pp 1776–1792

Shelton BG, Kirkland KH, Flanders WD et al (2002) Profiles of airborne fungi in buildings and outdoor environments in the United States. Appl Environ Microbiol 68:1743–1753

Skiada A, Lanternier F, Groll AH et al (2013) Third European Conference on Infections in Leukemia, Diagnosis and treatment of mucormycosis in patients with hematological malignancies: guidelines from the 3rd European Conference on Infections in Leukemia (ECIL 3). Haematologica 98: 492–504

Slavin MA, Chakrabarti A (2012) Opportunistic fungal infections in the Asia-Pacific area. Med Mycol 50:18–25

Snell BJ, Tavakoli K (2007) Necrotising fasciitis caused by *Apophysomyces elegans* complicating soft-tissue and pelvic injuries in a tsunami survivor from Thailand. Plast Reconstr Surg 119:448–449

Sugar A (2005) Agents of mucomycosis and related species. In: Mandell GL, Bennett JE, Dolin R (eds) Principles and practice of infectious diseases, 6th edn. Elsevier, New York

Sun HY, Singh N (2011) Mucormycosis: its contemporary face and management strategies. Lancet Infect Dis 11:301–311

Talmi YP, Goldschmied-Reouven A, Bakon M et al (2002) Rhino-orbital and rhino-orbito-cerebral mucormycosis. Otolaryngol Head Neck Surg 127:22–31

Teixeira CA, Medeiros PB, Leushner P et al (2013) Rhinocerebral mucormycosis: literature review apropos of a rare entity. BMJ Case Rep. doi:10.1136/bcr-2013-008552

Torres-Narbona M, Guinea J, Martinez-Alarcón J et al (2008) Workload and clinical significance of the isolation of zygomycetes in a tertiary general hospital. Med Mycol 46:225–230

Trifilio S et al (2007) Breakthrough fungal infections after allogeneic hematopoietic stem cell transplantation in patients on prophylactic voriconazole. Bone Marrow Transplant 40:451–456

Vainrub B, Macareno A, Mandel S et al (1988) Wound zygomycosis (mucormycosis) in otherwise healthy adults. Am J Med 84:546–548

Vesper SJ, Wymer LJ, Meklin T et al (2005) Comparison of populations of mould species in homes in the UK and USA using mould-specific quantitative PCR. Lett Appl Microbiol 41:367–373

Vitale RG, de Hoog GS, Schwarz P et al (2012) Antifungal susceptibility and phylogeny of opportunistic members of the order Mucorales. J Clin Microbiol 50:66–75

Voigt K, de Hoog GS (eds) (2013) The zygomycetes in a phylogenetic perspective. Persoonia – Molecular phylogeny and evolution of fungi, vol 30. Nationaal Herbarium Nederland, Lieden, pp 1–125

Walsh TJ, Gamaletsou MN, McGinnis MR et al (2012) Early clinical and laboratory diagnosis of invasive pulmonary, extrapulmonary, and disseminated mucormycosis (zygomycosis). Clin Infect Dis 54 (Suppl 1):S55–S60

Waness A, Dawsari GA, Jahdali HA (2009) The rise of an opportunistic infection called "invasive zygomycosis". J Glob Infect Dis 1:131–138

Wilson PA (2008) Zygomycosis due to *Saksenaea vasiformis* caused by a magpie peck. Med J Aust 189:521

13 Diagnostics of Fungal Infections

Birgit Willinger[1], Daniela Kienzl[2], Oliver Kurzai[3]

CONTENTS

[1]Division of Clinical Microbiology, Department of Laboratory Medicine, Medical University of Vienna, Währinger Gürtel 18-20/5P, Vienna A-1090, Austria; e-mail: birgit.willinger@meduniwien.ac.at

[2]University Clinic for Radiodiagnostics, Medical University of Vienna, Währinger Gürtel 18-20, Vienna A-1090, Austria

[3]Septomics Research Center, Friedrich-Schiller-Universität and Leibniz-Institut für Naturstoff-Forschung und Infektionsbiologie – Hans-Knöll-Institut, Jena, Albert-Einstein-Strasse 10, Jena 07745, Germany

I. Introduction

Whereas invasive fungal infections were once a rare event, a constant increase has been observed for decades and fungi nowadays represent a major threat for hospitalized and immunocompromised patients all over the world (Leone et al. 2003; Lehrnbecher et al. 2010; Miceli et al. 2011; Martin 2012). This development is mainly related to the constant increase in patients with compromised immunity. The rise of human immunodeficiency virus (HIV), introduction of increasingly invasive therapeutic measures in intensive care medicine, and iatrogenic immunosuppression associated with solid organ transplants and bone marrow/stem cell transplantation has generated new – and constantly growing – risk collectives for invasive fungal infections. Newly arising forms of immunosuppression have also significantly changed epidemiology of fungal infections. The worldwide spread of HIV infection has resulted in a dramatic increase in opportunistic mycoses like *Cryptococcus* meningitis or *Pneumocystis* pneumonia. In fact, the latter was originally a disease entity known to affect malnourished or premature infants and has now become a classical infection in patients with defects in T cell responses, including AIDS patients (Vanek and Jirovec 1952). The increasing number of fungal infections is not solely due to

Human Fungal Pathogens, 2nd Edition
The Mycota XII
O. Kurzai (Ed.)

an increase in well-known pathogenic fungi like *Candida albicans, Aspergillus fumigatus, Cryptococcus neoformans*, and *Pneumocystis jirovecii* (formerly known as *P. carinii*), although these species are still responsible for the majority of cases. In contrast, descriptions of infections caused by fungi that were previously considered apathogenic or of species that have never been observed before are now found in the literature in increasing numbers (Chakrabarti and Slavin 2011). The identification of these "novel" pathogens presents major problems for the diagnostic laboratories. In addition, the diagnosis of even the most common invasive fungal infections like candidemia and aspergillosis by conventional methods is fairly insensitive and often only available at late (sometimes too late) phases of the infection. This is especially true for invasive aspergillosis (IA), a disease entity for which the need for early specific therapy has unequivocally been shown. This chapter summarizes current diagnostic procedures in medical mycology, including classical culture- and morphology-based methods as well as novel molecular tools. These latter including protocols targeted at molecular species identification and molecular approaches to the diagnosis of fungal infections directly from patient samples and mass spectrometry. In a recent editorial in *Science*, it has been estimated, that "billions of people each year" are infected by fungi, with the majority being "relatively minor infections" (Brown et al. 2012).

However, exact numbers for invasive fungal infections are scarce and although studies have suggested a dramatic increase in the 1980s, incidence rates available have mostly been estimated for defined risk collectives in single centers (Wilson et al. 2002). Based on the analysis of large patient cohort data from hospital discharge surveys, Wilson et al. calculated incidences from candidiasis, aspergillosis, cryptococcosis, and histoplasmosis (Wilson et al. 2002). Based on these data, the authors estimated incidence rates of approximately 228 per million US population for candidiasis, 34 per million for aspergillosis and 30 per million for cryptococcosis. Accordingly, candidiasis accounts for 80% of all invasive fungal infections in the USA. Whereas, as expected, HIV-patients accounted for most cases of invasive cryptococcosis (79%) and cancer patients accounted for 42% of cases of IA, most patients hospitalized for invasive fungal infections were neither HIV-positive nor transplant or cancer patients (56%). Several studies from the USA and European countries have estimated the incidence of candidemia to be between 20 and 110 per million; however, other analyses reported numbers of up to 240 per million (Pfaller and Diekema 2005), which is in line with the above-mentioned study by Wilson et al. (2002).

II. Clinical Considerations in Diagnosing Fungal Infections

With the notable exception of endemic mycoses due to the dimorphic fungal pathogens and infection by *Cryptococcus gattii* (Byrnes et al. 2011), systemic fungal infection mainly occurs in immunocompromised patients. Consequently, the rapid evolvement of clinical importance is tightly linked to a growing number of immunocompromised patients. Major developments in the last 100 years that have contributed to this are solid organ transplantations, bone marrow or hematopoietic stem cell transplantation (BMT/HSCT), and modern intensive care therapy (see chapter "Invasive Aspergillosis in the Intensive Care Unit" by Meersseman). In addition to this, the HIV pandemia has resulted in a dramatic increase in immunocompromised patients in some parts of the world that are also predisposed to fungal infections. Whereas the type and degree of immunosuppression may not always be well defined (e.g., in surgical intensive care unit patients), it seems clear that a fully functional human immune system is largely protective against invasive mycoses. Knowledge about the specific immune status of a patient on the other hand allows predictions about the risk of fungal infections that may aid in both diagnosis and clinical management. Rather than providing an exhaustive review of known risk factors for all kinds of fungal infections, some defined clinical situations will be highlighted in the following section to underline basic concepts in protective immunity against fungal infection.

A. Fungal Infections in Chronic Granulomatous Disease

Several congenital immune defects are associated with an increased risk of invasive fungal infections. Among the most important conditions is chronic granulomatous disease (CGD), which is characterized by **dramatically reduced or absent formation of reactive oxygen intermediates due to a defect in the enzyme complex NADPH-oxidase**, responsible for generating superoxide in neutrophilic granulocytes. Invasive aspergillosis (IA) is one of the most common infections in these patients and frequently results in dissemination, with *Aspergillus* species being one of the most common causative agents of brain abscesses. These infections tend to have a high case fatality rate and IA is responsible for more than one third of all fatalities in CGD patients (Winkelstein et al. 2000; van den Berg et al. 2009; Antachopoulos 2010). In contrast to IA, the incidence of invasive candidiasis is only slightly elevated in CGD patients. Whereas this may indicate a higher functional redundancy of immune effector mechanisms against *C. albicans*, this finding may also be related to functional integrity of the gastrointestinal epithelial barrier, which prevents translocation of *C. albicans* from its natural reservoir (Koh et al. 2008). Recently, it has been shown that the genetic defect underlying CGD also prevents the formation of neutrophil extracellular traps (NETs) (Bianchi et al. 2009). Successful treatment of invasive infection due to *Aspergillus nidulans* was achieved by gene therapy and speculated to be related to restoration of NET formation in the patients' neutrophils (Bianchi et al. 2009), which may indicate that the role of NADPH-oxidase goes beyond the direct effects of reactive oxygen intermediates. CGD patients are also at risk of other mold infections, including many genera that are known to cause infection in humans (Antachopoulos 2010).

B. Fungal Infections in HIV/AIDS

The discovery of AIDS and its causative agent HIV resulted from descriptions of unusual occurrences of several opportunistic infections in young adult men. Among those infections was *Pneumocystis carinii* (now *Pneumocystis jirovecii*) pneumonia (PcP). PcP is still one of the most important AIDS-defining diseases in Western Europe and the USA. Before the HIV/AIDS epidemic, PcP was rather uncommon and had, in fact, been recognized initially as a pulmonal infection in malnourished low-birthweight infants (Vanek and Jirovec 1952). After the emergence of AIDS, PcP became the leading AIDS-defining diagnosis in the USA and in Europe (Huang and Hecht 2000). In addition to PcP, invasive cryptococcosis is of major importance in HIV-infected patients (Warkentien and Crum-Cianflone 2010) and has developed into one of the most important HIV-related opportunistic infections. Especially in Africa, cases of cryptococcal meningitis may outnumber cases of bacterial meningitis in countries with a high AIDS prevalence, and it has been estimated that each year one million cases of cryptococcal meningitis occur worldwide (Park et al. 2009). However, due to the introduction of highly active combination antiretroviral therapy (HAART), numbers of cryptococcosis have declined substantially in North America and Western Europe (Park et al. 2009). With regard to infections by *Candida* spp., oral candidiasis (thrush) is very common in HIV-infected patients. More importantly, esophageal candidiasis is one of the major AIDS-defining illnesses. Oropharyngeal candidiasis may be one of the first clinical signs of HIV infection, occurring in 50–95% of all HIV-infected patients (Rabeneck et al. 1993; Fidel 2006). In contrast, systemic candidiasis is much rarer in HIV.

C. Fungal Infections in Hematopoietic Stem Cell Transplantation

Since its beginnings, transplantation of bone marrow cells or peripheral blood hematopoietic stem cells (HSCT) has developed into a curative approach for several diseases. Congenital immune deficiencies, benign hematological disorders, autoimmune diseases, and hematological malignancies are the major fields of

application of HSCT (Jenq and van den Brink 2010). In the latter case, rather than just "replacing" a patient's own hematopoietic system, HSCT can also be considered one of the most effective immune therapies, as the newly transplanted immune system has been shown to attack remaining tumor cells in what has been termed the graft-versus-tumor (GVT) effect (Jenq and van den Brink 2010). Several factors contribute to immunosuppression in HSCT patients and increased risk of fungal (and other) infections, including the underlying disease, the conditioning treatment leading to more or less complete eradication of the patients own hematopoietic system, and (later in the course) the immunomodulatory treatment required for treating graft-versus-host disease (GvHD), a major complication of allogeneic stem cell transplantation. Consequently, several risk factors for developing invasive fungal infection in HSCT recipients, including the intensity of total body irradiation, the degree of donor human leukocyte antigen (HLA) mismatch, time to engraftment, corticosteroid therapy, and many others have been described. Fungal infections have been as high as 20% in allogeneic HSCT recipients and could increase to 40% in patients with severe GvHD (Jenq and van den Brink 2010). Initially, systemic candidiasis was the major fungal infection in allogeneic HSCT patients, with an incidence of about 11% and an associated mortality of 39% (Goodrich et al. 1991; Marr 2008). Both *Candida* sepsis (acute invasive candidiasis) and chronic disseminated candidiasis (typically with hepatosplenic involvement) were observed in HSCT patients. However, invasive *Candida* infections have decreased considerably following the implementation of azole prophylaxis, which has proven to be highly effective in preventing these infections (Marr 2008). The introduction of new conditioning regimens with less myeloablation and less damage to mucosal barriers has further decreased the incidence of invasive candidiasis (Fukuda et al. 2003). During the 1990s, invasive infections caused by *Aspergillus fumigatus* were noted in HSCT recipients and found to be associated with very high mortality rates of 80–90% (Marr 2008). Since then and despite the availability of new and more effective treatment regimens (Herbrecht et al. 2002) and antifungal prophylaxis regimens covering *A. fumigatus* (Cornely et al. 2007), **IA continues to be a major infectious complication in allogenic HSCT**. In recent years, increasing frequencies of other mold infections have been observed in many centers. Of particular importance are infections caused by zygomycetes (Vehreschild et al. 2013), but other fungal genera including *Fusarium* and *Scedosporium* have also been described as emerging fungal pathogens. Therefore, the epidemiology of fungal infections in allogenic HSCT patients has been constantly changing during the clinical development of clinical protocols and will most likely continue to do so, demanding epidemiological awareness and high-quality mycological diagnostics to be available at HSCT centers.

D. Fungal Infections in Solid Organ Transplant Recipients

The risk of infectious complications in solid organ transplant recipients is increased by the immunosuppressive therapy and by the potential introduction of pathogens through the transplanted organs. In addition, the underlying organ failure and the surgical intervention itself can add to the individual risk of infection (Grim and Clark 2011). Early infectious complications (generally defined as occurring up to day 30 post transplant) are often related to surgery. Maximal immunosuppression usually occurs after that period and up to 6 months after transplantation (Grim and Clark 2011). In most solid organ transplants, with the notable exception of lung transplantation, invasive candidiasis is the most common fungal infection. It is most common in transplants affecting the integrity of the gastrointestinal tract, including liver, small bowel, and enterically drained pancreas transplant recipients. In these patients antifungal prophylaxis is generally recommended. In lung transplant recipients, IA is the most common fungal infection, most probably due to the constant exposure of the transplanted organ to the ubiquitous spores of this pathogen. In addition, impaired ciliary clearance and decreased cough reflex may add to the risk of

aerogenic IA (Grim and Clark 2011). In recent years, other mold infections have increasingly been observed in these patients, including zygomycosis (Lanternier et al. 2012). Finally, infections with *P. jirovecii* have been frequently diagnosed in solid organ transplants, including several outbreaks in kidney transplant recipients. The underlying reasons for the observed increase in outbreaks of PcP in these patients remains unclear. As a result, trimethoprim-sulfamethoxazole prophylaxis has become a widely accepted practice incorporated into many kidney transplantation guidelines (de Boer et al. 2011). Outbreak situations of PcP, with up to 28 cases in a given setting, may require the use of molecular typing methods for unequivocal documentation of epidemiological coherence. These approaches are described in chapter "Molecular Epidemiology of Pneumocystis Outbreaks" by Hauser and Kovacs. Invasive cryptococcosis plays a minor role in solid organ transplant recipients (Osawa et al. 2010).

III. Diagnostic Criteria for Invasive Fungal Infections

The unequivocal diagnosis of invasive fungal infections (IFI) is still a major problem in the clinical setting. Patients at risk of IFI in general are also prone to a number of other infections as well as several syndromes or pathophysiologies that may cause **related or even identical symptoms**. It has been conceived that this diagnostic uncertainty leads to over-diagnosis and over-treatment of fungal infections in at-risk cohorts (Kibbler 2005) due to empirical treatment of suspected cases. Recognizing the practical problems arising from this situation – and most importantly a notable discrepancy between cases of IFI included in therapeutic studies (mostly fulfilling quite rigorous criteria for diagnosis of infection) and the average patient in clinical routine – the Invasive Fungal Infections Cooperative Group of the European Organization for Research and Treatment of Cancer (EORTC) and the Mycoses Study Group (MSG) of the National Institute of Allergy and Infectious Diseases established a **consensus effort** to standardize the definitions of IFI. This standardization was (and still is) explicitly targeted towards clinical research and notably was never designed to guide clinical decisions on therapeutic intervention – although the definitions may of course aid in these decisions (Ascioglu et al. 2002).

In the first version of the EORTC/MSG criteria, three levels of diagnostic certainty were defined: "proven", "probable," and "possible." Patients classified as "possible" according to the initial version of the definitions were considered to present enough information to warrant some form of empirical treatment but, at the same time, inclusion of these patients into clinical trials for antifungal drugs was discouraged due to the low specificity of the diagnosis (Ascioglu et al. 2002). Assignment to a level of diagnostic accuracy was based on a combination of "host factors," "clinical manifestations" (with two levels of evidence, termed major and minor, within this category), and "mycological results" (Ascioglu et al. 2002). Importantly, usage of the definitions was restricted to patients with cancer and to HSCT recipients, thus addressing two major populations at risk of IFI but clearly also excluding several other risk cohorts, e.g., patients in an intensive care unit (ICU).

In 2008, the EORTC/MSG consensus group published a revised version of these definitions (De Pauw et al. 2008). In the meantime, the original definitions had formed the basis for several large scale clinical trials of antifungal drug efficacy and several other studies including the formulation of clinical practice guidelines (Herbrecht et al. 2002; Walsh et al. 2002a, b; Cornely et al. 2007; Ullmann et al. 2007; De Pauw et al. 2008; O'Connell and Walsh 2008) and evidently gained considerable acceptance worldwide. In the revised definitions, now targeted towards diagnosis of invasive fungal disease (IFD) rather than IFI, only minor changes were introduced in the "proven" level, whereas the definitions of probable and possible cases were modified significantly. In the revised version, probable cases required the presence of host factor + clinical feature + mycological criterion, whereas possible cases were defined by the presence of host factor + clinical feature but absence of mycological evidence for IFD (De Pauw et al. 2008). The impact of the revision published in 2008 on classification of patients should not be underestimated: In a study analyzing 589 high-risk patients, as many as **81% of**

"possible" and 75% of "probable" cases according to the initial definition had to be regrouped as "non-classifiable" (Tsitsikas et al. 2012), indicating that the overall specificity was increased by the criteria modification. This was mainly due to the elimination of minor clinical criteria and the emphasis placed on specific computed tomography (CT) findings (Tsitsikas et al. 2012). One major problem of the revised definitions is the fact that patients with host-factor and mycological criteria but without defined clinical symptoms are grouped as non-classifiable. In addition, application of the EORTC/MSG criteria is still restricted to immunocompromised patients and therefore does not allow a general application, especially when regarding intensive care patients, which have become a major at-risk cohort (Martin et al. 2003; Meersseman and Van Wijngaerden 2007; De Pauw et al. 2008). Furthermore, it has to be emphasized, that the EORTC/MSG definitions are still intended for use in clinical and epidemiological research and are not meant to guide clinical diagnosis in individual cases (De Pauw et al. 2008). Despite this and beside facilitating our ability to test the efficacy of therapeutic regimens and strategies (De Pauw et al. 2008), these definitions also set the standards for evaluating diagnostic tools and provide a commonly accepted reference in the absence of a reliable gold standard for unequivocal diagnosis of invasive fungal disease.

IV. Radiology in the Diagnosis of Fungal Infections

Early detection of fungal infections is very important because the morbidity and mortality rates of the disease are high, especially in immunocompromised patients (Krowka et al. 1985; Soubani et al. 1996). The radiological diagnosis of intrapulmonary fungal infections, in particular, is sometimes challenging due to the multitude of either infectious or malignant differential diagnoses. Although the diagnostic value for the detection of fungal infections on chest radiographs (CXR) is poor (Korones et al. 1997), in cases of clinical suspicion of a pulmonary fungal infection CXR should be the first-line modality. In more severe cases, computer tomography (CT) needs to be performed, even if the CXR is normal. In contrast to radiography, **CT can give a picture of the whole extent of disease** and, in some cases, point to specific pathogens involved in the disease (Wah et al. 2003). Particularly in younger patients, CT should be performed at a low dose (LDCT). Magnetic resonance imaging (MRI) is an imaging method especially suited for detecting visceral mycotic lesions. In these cases, MRI is more sensitive in detecting, as well as in defining, the stage of the disease than is contrast-enhanced CT (Semelka et al. 1992). The most common pathogens of pulmonary fungal infections in immunocompromised patients are *Aspergillus* species, *Cryptococcus neoformans,* and *Candida* species. Pulmonary *Aspergillus* infections in immunocompromised patients can be grouped into **angio-invasive aspergillosis** (AIA) and the less common **broncho-invasive aspergillosis** (BIA). CT findings of AIA include a halo sign (defined as a ground glass opacity surrounding the circumference of a nodule or mass), infarct-shaped consolidations, and an internal, low-attenuation cavity or air-crescent sign (Figs. 13.1, 13.2, and 13.3). Typical CT patterns of BIA are small airway lesions, peribronchial consolidations, and/or bronchiectasis. In a late manifestation of AIA, an air-crescent sign can be observed, which is caused by an intracavitary fungus mass of AIA that moves when the patient changes position (Aquino et al. 1994; Shibuya et al. 2004). The air-crescent sign can also appear in saprophytic aspergillosis in immunocompetent and immunosuppressed patients when pre-formed cavities are colonized with *Aspergillus* spp. (Fred and Gardiner 2009). Pulmonary cryptococcosis is an emerging disease in neutropenic patients. The most common radiological features in pulmonary infection with cryptococcosis are poorly defined nodules (commonly between 7 and 20 mm) or patchy air space consolidations. Cavities, ground glass opacities, the halo sign, mediastinal lymphadenopathy, and pleural effusion are rarely seen in pulmonary manifestations (Chang et al. 2006). Common CT findings in patients with *Candida* infections

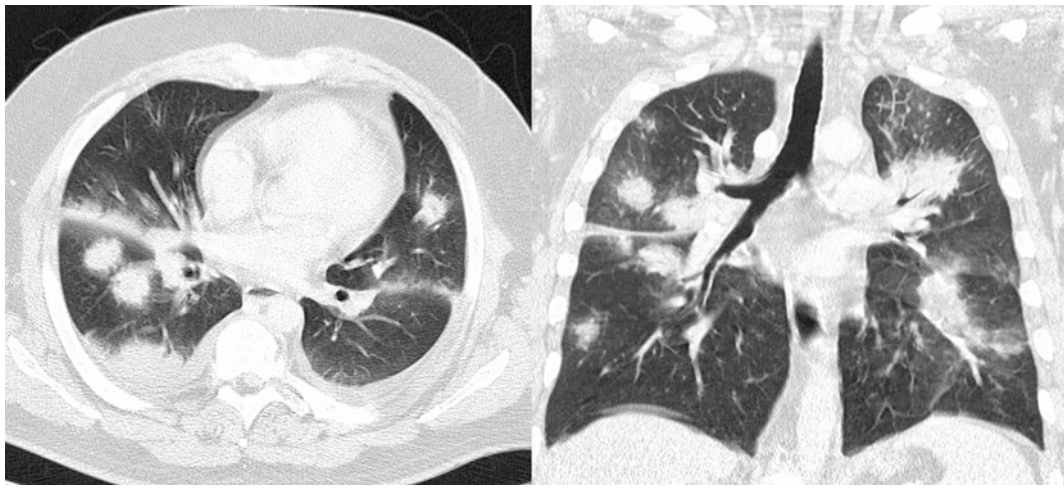


Fig. 13.1. Angioinvasive aspergillosis in a 35-year-old man with acute lymphatic leukemia. CT scan shows bilateral peribronchovascular nodular lung lesions with surrounding halo of ground-glass attenuation representing adjacent hemorrhage

include random bilateral nodules with a diameter between 3 and 30 mm. Consolidations and centrilobular nodules are rarely seen (Althoff Souza et al. 2006). In hematogenous fungal disseminations that affect the central nervous system and the hepatosplenic system, microabscesses can be observed by using MR imaging rather than contrast-enhanced CT (Semelka et al. 1992; Anttila et al. 1996). However, MRI is sometimes not available and the examination can last about 30–40 min, which, in many cases, is not feasible for the critically ill patient. Contrast-enhanced CT and ultrasound (US) are the routinely performed first-line modalities in the radiological assessment of hepatosplenic fungal abscesses.

V. Classical Laboratory Diagnosis

A. Pre-analytical Considerations in Diagnosing Fungal Infections

As with other microbial infections, the diagnosis of fungal infections depends upon a combination of clinical observation and the application of various laboratory techniques. Superficial fungal infections are often associated with suspicious lesions, which guide clinical diagnosis. In contrast, invasive fungal infections rarely show specific signs. As a consequence, the selection, collection, possible storage, and transportation of diagnostic material are of utmost importance in order to establish the diagnosis. During transportation, fungi must remain viable in order to be recovered on culture, and specimens should not be allowed to desiccate. Transportation to the laboratory should occur at room temperature. Specimens should be delivered within 2 h after sampling but should not arrive at the laboratory later than 24 h after collection. Specimen collections from the central nervous system should be processed as soon as possible. When immediate processing is not possible, the specimen should be held at ambient temperature (CLSI 2012).

To establish or confirm diagnosis, it is essential that the laboratory is provided with an adequate specimen. For the diagnosis of invasive fungal infections, specimens originating from sterile locations are preferred. Blood cultures should be performed whenever deep fungal infections are suspected. All *Candida* species or other organisms such as *Histoplasma* spp. have been shown to be reliably detected by the current available **blood culture** systems. For aspergillosis and mucormycosis, blood cultures are of limited utility. However, disseminated

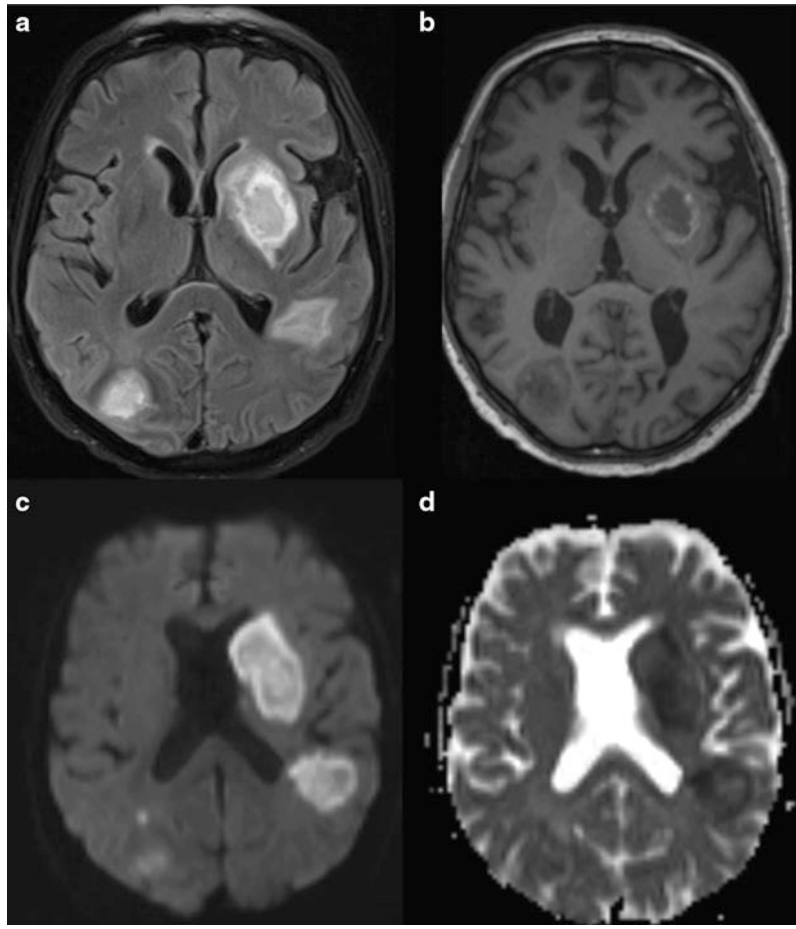


Fig. 13.2. Invasive aspergillosis of the brain in a 70-year-old male patient with lung carcinoma and chemotherapy. (**a**) Axial T2 tirm MR image shows bihemispheric hyperintense lesions. (**b**) Unenhanced axial T1 weighted MR image shows bihemispheric hypointense lesions with a rim of high signal intensity representing blood products. (**c**) Diffusion-weighted images shows high signal intensity in all lesions. (**d**) Apparent diffusion coefficient (ADC) map shows low signal intensity in the center of the lesions, a finding indicative of restricted diffusion

infections caused by *Fusarium* spp. and *Scedosporium* spp. are associated with the recovery of the pathogen in blood cultures in approximately 50% of all cases (Arendrup et al. 2012).

In case of suspected meningitis, 3–5 ml of **cerebrospinal fluid** (CSF) should be collected in sterile, screw-capped tubes. Immediate transport of all collected fluid to the laboratory at room temperature is mandatory (CLSI 2012). CSF is best for detection of *Cryptococcus* meningitis (Denning et al. 2003), but is less sensitive for the detection of central nervous system aspergillosis or candidiasis (Arendrup et al. 2012).

Tissue biopsies are of high diagnostic value. However, for some fungi (e.g. Mucorales), homogenization of the biopsy material reduces the culture yield and is it preferable to inoculate culture plates with slices of minimally manipulated tissue (Arendrup et al. 2012).

The confirmation of many fungal infections is based on samples obtained from lower respiratory tract specimens. Sputum specimens are acceptable, but increasing the number of sputum samples examined increases the sensitivity of detection, with three samples providing optimum yield in invasive aspergillosis.

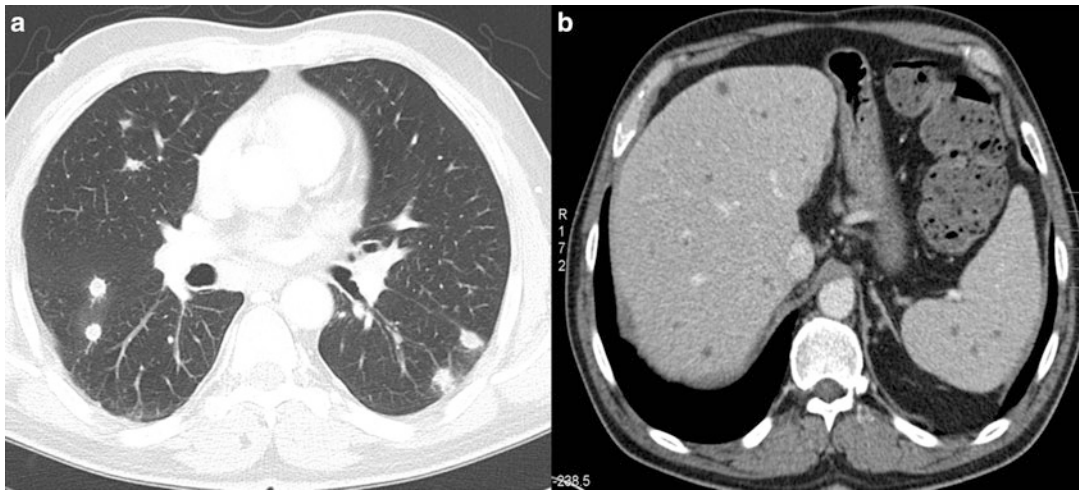


Fig. 13.3. Systemic candidiasis in a 40-year-old male patient with chronic myeloic leukemia. (**a**) Axial CT scan shows multiple bilateral intrapulmonary nodules smaller than 1 cm. (**b**) Axial contrast-enhanced CT shows countless hypoattenuating lesions in the liver and the spleen representing *Candida* microabscesses

Bronchoalveolar lavage (BAL) fluid provides a more representative sample from the lower respiratory tract and allows CT scan abnormalities to be directly sampled. The presence of *Candida* spp. in BAL fluid, similarly, does not correlate with invasive lung infection and should be interpreted as of low significance (Meersseman et al. 2009; Arendrup et al. 2012).

B. Currently Available Diagnostic Methods

Currently available laboratory methods for diagnosing invasive fungal infections include microscopic detection, isolation of the fungus, serologic detection of antibodies and antigen, and histopathologic evidence of invasion (Alexander 2002). For definite diagnosis of proven invasive fungal infections, histological and cultural evidence from biopsies, resection material, or other specimens obtained from normally sterile body sites is required (De Pauw et al. 2008). However, it is not always possible to fulfill these criteria and a combination of different methods is necessary to detect and identify the fungal organism causing the disease.

1. Histopathology

Histopathology is the cornerstone for diagnosis and identification of fungal pathogens. However, as with culture methods, it may not be possible to obtain a tissue sample from critically ill patients. In addition, identifying the specific pathogen based solely on morphological characteristics can be difficult or impossible because several different organisms may have similar histopathological characteristics, e.g., *Fusarium* spp. and other filamentous fungi are indistinguishable from *Aspergillus* in tissue biopsies (Alexander and Pfaller 2006). Because *Aspergillus* is far more commonly encountered than the other pathogens mentioned, a pathologist often may describe an organism as *Aspergillus* or *Aspergillus*-like on the basis of morphological features alone. This can hinder diagnosis and may entail inappropriate therapy (Chandrasekar 2010). Molecular tools can aid in the identification of fungal pathogens from histopathology samples (see Sect. 6).

2. Microscopy

Direct microscopy is most useful in the diagnosis of superficial and subcutaneous fungal

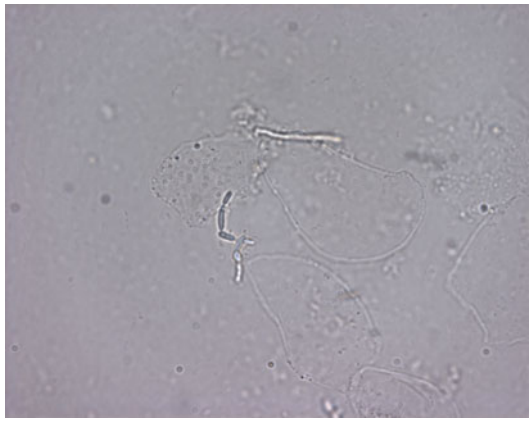


Fig. 13.4 KOH preparation demonstrating pseudohyphae of *Candida* spp.

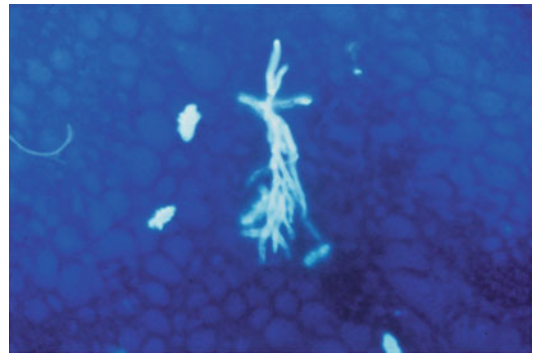


Fig. 13.5 Microscopy with an optical brightener showing dichotomously branched hyphae indicative of *Aspergillus*

infections. Recognition of fungal elements can provide a reliable and rapid indication of the mycosis involved. Various methods can be used: unstained wet-mount preparations can be examined by light-field, dark-field, or phase contrast illumination (Richardson and Warnock 2003).

The most common direct microscopic procedure relies on the use of 10–20% potassium hydroxide (KOH; Fig. 13.4), which degrades the proteinaceous components of specimens while leaving the fungal cell wall intact, thus allowing their visualization (Lease and Alexander 2011).

The visibility of fungi within clinical specimens can be further enhanced by the addition of calcofluor white or blankophores (Willinger 2006). These are fluorophores, which are members of a group of compounds known as fluorescent brighteners, optical brighteners, or "whitening agents" and bind to β-1-3 and β-1-4 polysaccharides, such as found in cellulose and chitin. When excited with ultraviolet or violet radiation, these substances will fluoresce with an intense bluish/white color (Harrington and Hageage 2003). The high intensity of the elicited fluorescence allows rapid and reliable microscopic screening (Fig. 13.5). Optical brightener methods have been shown to be more sensitive than KOH wet mount.

This has also been shown in a study of respiratory samples (mostly BAL) from transplant recipients and neutropenic patients, indicating a sensitivity of 88% and a specificity of 99% for the detection of *Aspergillus*-like elements by blankophor in comparison with a 76% sensitivity for culture (Andreas et al. 2000; Vyzantiadis et al. 2012). The procedure is also suitable for disclosing fungi in Gram-stained microscopical mounts. Filamentous fungi like aspergilli, which stain poorly by the Gram procedure, may be unveiled on Gram-stained microscopic mounts after removal of immersion oil by subsequent blankophor staining (Ruchel and Schaffrinski 1999). In addition, optical brightener methods have been shown to be more sensitive than KOH wet mount in a number of specimen types (Chander et al. 1993). Furthermore, calcofluor or blankophor can be combined with 10–20% KOH, although it is better to pre-soften the tissue with KOH before adding the fluorescent stain (Vyzantiadis et al. 2012).

Because optical brighteners provide a rapid and sensitive method for the detection of most fungi, their use is encouraged for respiratory samples, pus, tissue samples, and fluids from sterile sites when a fluorescence microscope is available.

Similarly, lactophenol cotton blue is easy to handle and often used for the detection and identification of fungi (Fig. 13.6). Other stains are frequently used in direct microscopy, such as the India ink wet mount, which is useful for visualization of encapsulated fungi, particularly *Cryptococcus neoformans*. Although a negative direct examination cannot rule out fungal disease, visualization of fungal elements in specimens can often secure initial information helpful in the selection of empirical antifungal therapy (Lease and Alexander 2011).

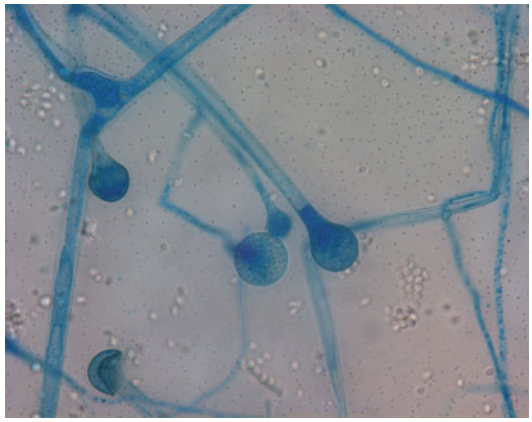


Fig. 13.6. Staining of *Lichtheimia corymbifera* using lactophenol cotton blue

Because yeast and molds can stain variably with the Gram stain, a more specific fungal stain is recommended (CLSI 2012). Microscopy may help to discern whether an infection is caused by yeast or molds (Table 13.1). The presence of pseudohyphae and optionally blastoconidia indicates the presence of yeast. Molds are most commonly seen as hyaline hyphomycetes, generally characterized by parallel cell walls, septation (cross-wall formation in hyphae), lack of pigmentation, and progressive dichotomous branching as in *Aspergillus*, *Fusarium*, or *Scedosporium* species (Vyzantiadis et al. 2012). *Aspergillus* septate hyphae have a diameter of 3–6 μm and each branch is approximately equal in width to the originating stem. Branching at 45° angles is characteristic. Because many other filamentous fungi present with a similar appearance in clinical samples on direct microscopy, one can usually only say that the infection is due to one of the agents of **hyalohyphomycosis**, although the balance of probability is that most infections will be due to *Aspergillus* (Patterson 2003).

In addition, it is important to look for septate and non-septate hyphae, thus allowing to distinguish between *Aspergillus* spp. and members of the Mucorales. **Mucoraceous molds** have large (3–25 μm in diameter, average 12 μm) ribbon-like, multinucleated hyphal cells with nonparallel walls and infrequent septa. The branching is not dichotomous, irregular, and sometimes at right angles. Hyphae can appear distorted with swollen cells, or compressed, twisted, and folded (Vyzantiadis et al. 2012). Another group of molds causing tissue invasion with a distinctive appearance are the agents of phaeohyphomycosis, such as *Alternaria* and *Curvularia*. These fungi have melanin in their cell walls and appear as pigmented, septate hyphae. These are the agents of phaeohyphomycosis (Revankar 2007). The detection of fungal hyphae and/or arthrospores in skin, nail, or hair samples may indicate the presence of dermatophytes but give no special hint as to the species involved.

3. Antigen and Antibody Detection

Except for **cryptococcosis**, where antigen testing can reliably prove infection, serological tests mainly provide supplemental information for the diagnosis of invasive fungal infections. Antibody tests are often used in the diagnosis of endemic mycoses, which may be difficult to detect by traditional methods. In some cases, antibody tests are a supplemental test in the diagnosis of invasive candidosis, but have two major drawbacks: detection of anti-*Candida* antibodies fails to discriminate between disseminated and superficial infections, and antibodies are often present in colonized but uninfected patients. In order to diagnose invasive candidosis, a rise in antibody titers has to be observed in serial serum samples. However, because immunocompromised patients do not reliably produce antibodies it is rendered nearly impossible to diagnose invasive candidosis in these patients solely by antibody detection (Willinger 2006). *Aspergillus* antibodies are only infrequently detectable in immunocompromised patients, but are often helpful in patients with aspergilloma or allergic bronchopulmonary aspergillosis and in patients with cystic fibrosis (Kappe and Rimek 2010). Another approach to the diagnosis of fungal infections is the detection of antigens in blood or other body fluids, whether it is used as a single test or in combination with antibody detection.

Table 13.1. Characteristic morphology of fungi in clinical specimens by direct microscopic examination (selection of certain fungi)

Fungal group and representatives	Microscopic morphology in clinical specimens
Yeasts	
Candida spp. (except *C. glabrata*)	Single budding of moderately variable size 2.5–7.5 μm in size Pseudohyphae and true hyphae may be present in certain species
C. glabrata	Blastoconidia of uniform size (2.5–4 μm) No production of hyphae or pseudohyphae
Cryptococcus neoformans	Spherical budding yeasts of variable size 2–15 μm in diameter Capsule may be present or absent No hyphae or pseudohyphae
Pneumocystis jirovecii	Intracystic bodies that resemble parentheses facing one another are pathognomonic 4–6 μm in size Seen on histological stains Cysts often found in clusters No budding Best visualized in histological sections using special stains Trophozoites seen on staining with giemsa or immunofluorescent stains
Hyaline hyphomycetes	
Aspergillus *Acremonium* *Fusarium* *Paecilomyces* *Scedosporium* *Scopulariopsis* *Trichoderma*	Hyaline, septate, dichotomously branching hyphae Uniform width (3–6 μm) *Aspergillus* may produce conidial heads in specimens from cavitary pulmonary lesions Conidiation may be visualized on histopathological examination of some of the non-*Aspergillus* hyphomycetes
Mucorales	
Cunninghamella *Lichtheimia* (formerly *Absidia*) *Mucor* *Rhizomucor* *Rhizopus* *Saksenaea*	Broad, thin-walled, non-septated hyphae 6–25 μm wide Nonparallel sides and random branches
Dematiaceous fungi	
Alternaria *Bipolaris* *Curvularia* *Cladophialophora*	Pigmented (brown, tan, or black) Septate hyphae 2–6 μm wide
Dimorphic fungi	
Blastomyces dermatitidis	Large, spherical, thick-walled, budding yeast cells 8–15 (max. 40) μm diameter Typically broad-based junction between mother and daughter cells Cells may appear multinucleate
Coccidioides immitis	Spherical, thick-walled spherules Vary in size (20–30 μm) Mature spherules contain endospores Released endospores may be mistaken for yeast Hyphae and arthroconidia may be found in cavitary lesions
Histoplasma capsulatum	Small budding yeasts 2–5 μm in size
Paracoccidoides brasiliensis	Large, multiple-budding yeasts Variable size 2–30 μm in diameter Large cells are surrounded by smaller buds around the periphery ("mariner's wheel appearance")

(a) Invasive Candidosis

Mannan is a *Candida* cell wall constituent and can be detected in the serum of patients with invasive candidosis (IC), but it is quickly degraded and cleared from the bloodstream. Furthermore, antimannan antibodies are found in patients either colonized or infected by *Candida* spp. However, it has been shown in patients developing IC that mannan and antimannan antibody titers complement each other (Peman and Zaragoza 2012). In fact, when these tests are used together, their sensitivity and specificity for diagnosing IC improves significantly (Marchetti et al. 2012). In practice, the two more commonly used tests are Platelia Candida Ag® and Platelia Candida Ab® assays (Bio-Rad Laboratories, Marnes-la-Coquette, France). It has been shown that detection of both antigen and antibody is superior to evaluation of each one separately as it presents a sensitivity of 83% and a specificity of 86% in IC; consequently, its use might be helpful in these patients (Mikulska et al. 2010). Nevertheless, the results of mannan antigen and antibody detection varies depending on the *Candida* species implicated; the tests perform best for *C. albicans, C. glabrata*, and *C. tropicalis* and worst for *C. parapsilosis* and *C. krusei*. Another important benefit of this combined antigen–antibody detection is the ability to make an earlier diagnosis than with blood cultures, in some cases. Serial determinations may be necessary. These assays can help to detect the infection early because they can be positive 6 days, on average, prior to blood cultures. The method also shows very high negative predictive value (>85%) and can be used to rule out infection (Cuenca-Estrella et al. 2012). However convincing this may be, many experts feel that these assays do not have sufficient sensitivity or specificity to influence their clinical decision-making (Kullberg et al. 2011).

Another option is the Cand-Tec latex agglutination test (Ramco Laboratories, Houston, TX, USA), which detects an antigen, not specifically defined. Though the sensitivity and specificity of this test vary among reports (Yeo and Wong 2002), it is still widely used. However, false-positive reactions due to the rheumatoid factor and renal insufficiency (Ruchel 1993) have been observed and it is generally recommended to use better-defined assays.

An indirect immunofluorescence assay to detect *Candida albicans* germ tube-specific antibodies (CAGTA) is commercially available as *C. albicans* IFA IgG® (Vircell Laboratories, Granada, Spain) and has shown an overall sensitivity of 77–89% and a specificity of 91–100% thus being useful in the diagnosis of IC in critically and noncritically ill patients (Quindos et al. 2004). In addition, Peman et al. (2011) found a high rate of positive results when using this test. The presence of this biomarker was the only protective factor independently associated with mortality, and positivity was not modified by either colonization or previous antifungal treatment. However, there are no recommendations to use this biomarker yet.

(b) Cryptococcosis

The detection of cryptococcal capsular polysaccharide is one of the most valuable rapid serodiagnostic tests for fungi performed on a routine basis. The cryptococcal antigen can be detected either by the latex agglutination test or by enzyme-linked immunosorbent assay (ELISA). The test has gained widespread appeal and is suited to prove disseminated cryptococcosis (De Pauw et al. 2008). False-positive reactions have been reported in patients with disseminated trichosporonosis, *Capnocytophaga canimorsus* septicemia, malignancy, and positive rheumatoid factor. Another assay format is the Premier Cryptococcal Antigen enzyme immunoassay (Meridian Diagnostics, Inc., Cincinnati, OH, USA) that utilizes a polyclonal capture system and a monoclonal detection system. The Premier EIA was reported to be as sensitive as the latex agglutination system for the detection of capsular polysaccharide in serum and cerebrospinal fluid. In addition, it does not react with rheumatoid factor and gives fewer false-positive results (Yeo and Wong 2002).

(c) Invasive Aspergillosis

The most commonly used, commercially available antigen test for *Aspergillus* detection is the double-sandwich ELISA test Platelia Aspergillus® (Bio-Rad Laboratories, Marnes, France), which is validated for use in serum and bronchoalveolar lavage (BAL) fluid and

Table 13.2. Characteristics of the galactomannan antigenemia test

Parameter	Description
Specimen	Serum, BAL
Criteria for positivity	Two consecutive serum specimens with GMI $\geq$0.5 Always repeat the test before implementing therapy for IA
Population	Prolonged neutropenia, allogeneic SCT, ICU patients, COPD patients

GMI galactomannan index, *SCT* stem cell transplantation

Table 13.3. Causes for false-positivity or cross-reactivity in the galactomannan antigenemia test

Patient groups with false-positive results	False-positivity caused by galactomannan contamination	Cross-reactivity caused by similar cell wall galactomannan
Infants with intestinal colonization by *Bifidobacterium* spp.	Piperacillin-tazobactam Amoxicillin-clavulanate	*Penicillium* spp. *Alternaria* spp.
Chronic graft- versus-host disease	Other beta-lactam antibiotics Plasmalyte (sodium gluconate)	*Paecilomyces* spp. *Cryptococcus* spp.
People following diets rich in soy protein and other foods	Other intravenous hydration or nutrition fluids containing sodium gluconate Echinocandins Chronic graft- versus-host disease Diets rich in soy protein and other foods	*Histoplasma capsulatum* *Geotrichum* *Neosartoria* *Lichtheimia ramosa*

has been evaluated in oncohematologic patients and in receptors of hematopoietic precursors with prolonged neutropenia (Table 13.2). This test has also been evaluated in non-hematological ICU patients and patients suffering from chronic obstructive pulmonary disease (COPD) (Meersseman et al. 2008; Guinea et al. 2010; Hage et al. 2011; He et al. 2011; He et al. 2012). The Platelia Aspergillus® detects **galactomannan** (GM), which represents a heteropolysaccharide cell wall component of the *Aspergillus* cell wall and is released by *Aspergillus* species during hyphal growth. To optimize its diagnostic value in patients at risk of infection, it is recommended to perform two GM determinations a week, mainly in oncohematologic patients and in hematopoietic stem cell transplant recipients with prolonged neutropenia (Peman and Zaragoza 2012). Circulating GM may be detected at a median of 5–8 days before clinical signs and symptoms of IA become evident. Furthermore, its concentration corresponds to the fungal tissue burden, and may therefore be used to monitor the patient's response to antifungal treatment (Maertens et al. 2009).

Pfeiffer et al. (2006) presented a meta-analysis of the diagnostic value of GM detection with the Platelia Aspergillus® assay, showing that for proven and probable cases of IA, the pooled (adult and pediatric) sensitivity was only 61%, whereas the overall specificity was 93%. In general, the negative predictive value (NPV) and the specificity are excellent (>95%), suggesting that the assay can be used to rule out the diagnosis of IA. However, false positive and negative results of GM have been described in certain patient groups (Table 13.3) by various authors (Wheat and Walsh 2008; Hage et al. 2011; Peman and Zaragoza 2012). Solely testing for antigenemia does not replace other tests for IA. To maximize sensitivity, testing should precede empiric antifungal therapy and positive results should be confirmed on a new specimen (Wheat 2003). Generally lower sensitivity rates have been obtained from pediatric patients than adult patients in studies evaluating the utility of GM antigenemia test in early diagnosis

of IA (Oz and Kiraz 2011). When using this test it has to be considered that results may be falsely positive or negative and have to be correlated with clinical and laboratory findings.

Antigen has been detected in body fluids other than sera. The detection of antigen in BAL fluid was described in various studies, indicating the presence of pulmonary aspergillosis. A meta-analysis evaluating the accuracy of BAL galactomannan determined the sensitivity to be 90% and specificity 94% (Guo et al. 2010). A positive result increased the probability of having IA by about sixfold, whereas a negative result decreased the probability to 1%. Whereas the sensitivity remained unchanged, at about 85%, the specificity increased from 89% to 94% by using a cut-off of 1.0 instead of 0.5. BAL was judged to be more useful than serum, by comparison with results of another meta-analysis (Pfeiffer et al. 2006).

Hage et al. (2011) state when using BAL, positive results caused by airway colonization may complicate the use of the test for diagnosis. As shown, results may be false-positive in patients colonized with *Aspergillus*, *Penicillium*, and *Paecilomyces* (Clancy et al. 2007; Husain et al. 2007). When combined results of those studies were analyzed, sensitivity was unchanged but specificity increased using a cut-off of 1.0 (Muller et al. 2002). Results in colonized specimens were at least 1.0 in 8 out of 12 cases (Husain et al. 2008). However, BAL galactomannan was negative in hematology patients who were colonized with *Aspergillus* species (Bergeron et al. 2010). As colonization is undesirable in solid organ transplant or hematology patients at high risk of IA, results attributed to colonization should not be disregarded, but rather prompt additional investigation to exclude invasive disease or to assess the effectiveness of antifungal prophylaxis or therapy, and follow-up evaluation for subsequent invasive disease (Hage et al. 2011). All in all, GM detection in BAL fluid seems to be useful in establishing or excluding the diagnosis of IA in ICU patients (Hage et al. 2011).

In addition, the test might allow the detection of GM in other specimens such as cerebrospinal fluid, urine, and homogenates/extracts of tissue specimens. Few data exist on the performance of the galactomannan assay for homogenized tissue specimens, nevertheless the data are promising (Lackner and Lass-Flörl 2013).

Monitoring for antigen clearance or rebound may provide useful information for assessing the effectiveness of therapy. Declining levels in patients responding to therapy and rising concentrations in those with fatal outcomes have been observed by several authors (Bretagne et al. 1997; Maertens et al. 2001). Thus, failure of antigenemia to decline may suggest treatment failure and support consideration of modifying the therapy.

Studies on patients who have undergone solid organ transplantation report sensitivity ranging from 67% to 100% and specificity from 40% to 98% (Clancy et al. 2007; Husain et al. 2007; Pasqualotto et al. 2010). Detection of antigen in BAL was more sensitive than detection of antigen in serum or isolation of *Aspergillus* from the BAL or lung biopsy, as highlighted in an evaluation of proven cases in hematology patients (Maertens et al. 2010). In that study, the sensitivity was 100% for BAL antigen, 55% for serum antigen, 62% for culture, and 74% for microscopy. In organ transplant recipients, sensitivity was 82% for antigen detection compared to 73% for culture and/or microscopy (Husain et al. 2008).

Recently Thornton et al. (2012) described a new promising diagnostic method for the detection of *Aspergillus* in patients suffering from hematological malignancies. The technology is based on the detection of *Aspergillus*-specific JF5 by MabJF5 monoclonal antibodies. The JF5 is an extracellular glycoprotein that is exclusively secreted during active growth of the fungus and represents a surrogate marker of *Aspergillus* infection. Evaluating exclusively the activity of *Aspergillus* rather than the antigen presents a major advantage compared with the GM test and, at the same time, limits the risk of cross-reactivity or a false-positive result due to contaminated materials because the JF5 test only detects germinating conidia that represent a potential infectious risk. The technique has been incorporated into an immuno-chromatographic lateral-flow device ('point of care' diagnostic tool), which is easy to use. The test aims to detect invasive pulmonary aspergillosis using blood or/and serum samples from patients in only 15 min. However, further studies are needed to prove the test's performance in a clinical setting (Lackner and Lass-Flörl 2013).

4. (1,3)-β-D-Glucan as a Marker for Invasive Fungal Infection

(1,3)-β-D-Glucan (BDG) is a component of the cell walls of many fungal organisms; its presence can be detected in sera when observing activation of the factor G of the horseshoe crab coagulation cascade (Bellanger et al. 2011). The presence of BDG in serum has been shown to be a reliable marker of invasive fungal infection (Karageorgopoulos et al. 2011), and it has recently been added to the EORTC/MSG guidelines as a biological criterion for invasive fungal diseases other than zygomycosis and cryptococcosis (De Pauw et al. 2008). It has also been proposed as a noninvasive marker for pneumocystosis (Bellanger et al. 2011). However, one has to be aware that this test does not detect cryptococcosis and fungal colonization or superficial infections.

The contribution of BDG to IFI screening has mainly been investigated for patients with hematological malignancies. More recently, studies have reported the contribution of the BDG assay for detecting invasive candidiasis in patients in surgical intensive care units (Mohr et al. 2011; Ostrosky-Zeichner et al. 2011).

Karageorgopoulos et al. (2011) recently presented a meta-analysis including 2979 patients (594 with proven or probable IFIs) showing a pooled sensitivity of 76.8% and a specificity of 85.3%. However, marked statistical heterogeneity was noted. BDG has good diagnostic accuracy for distinguishing proven or probable IFIs from no IFIs and can be useful in clinical practice, if implemented in the proper setting and interpreted after consideration of its limitations. In addition, the detection of BDG precedes the appearance of symptoms, radiologic signs, and the empirical antifungal treatment in some patients (Peman and Zaragoza 2012).

5. Culturing Techniques

Culture remains one of the key methods for diagnosing fungal infection. Though often slow, sometimes insensitive, and sometimes confusing with respect to contamination, culture may yield the specific etiological agent and may allow susceptibility testing to be performed. Proper collection and transportation of the specimen is essential. In particular, sterile materials are important for diagnosis of IFI.

Blood cultures (BC) are the first-line test and currently considered the "gold standard" in the event of any suspected case of systemic mycosis (Ostrosky-Zeichner 2012). Several commercial blood culture systems are available.

Lysis centrifugation has been considered the "gold standard" for recovering pathogenic yeasts, as well as thermally dimorphic fungi from blood (Willinger 2006). In particular, dimorphic molds can be most reliably and rapidly recovered in the lysis centrifugation system. Lysis centrifugation involves incorporation of a tube containing an anticoagulant and a lytic agent and exploits the possibility that white blood cell lysis releases viable fungal cells. However, it is time consuming and prone to contamination (Berenguer et al. 1993). When older BC systems are used as comparators, lysis centrifugation procedures show higher efficacy, whereas current automated systems have improved the sensitivity of blood cultures for detecting microorganisms (Cuenca-Estrella et al. 2012). In comparison to current automated blood culture systems, in recent studies lysis centrifugation does not appear more sensitive for the majority of invasive fungi (Morrell et al. 1996; Arendrup et al. 2010).

Of the automated systems, there are two that are widely used: the Bactec System (BD Diagnostic System, Sparks, MD) and the BacT/Alert System (bioMérieux, Marcy l'Etoile, France). Studies have documented that these systems match the performance of lysis centrifugation methods for the detection of yeast. The Bactec system proposes a specifically formulated medium for the isolation of fungi, called Mycosis IC/F medium. This medium is a brain–heart broth enriched with sucrose, chloramphenicol, and tobramycin to inhibit bacterial growth, and a lysis agent, saponin. The incubation period recommended by the manufacturers for Bactec Mycosis IC/F and BacT/Alert FA vials is 14 and 5 days, respectively. In various studies, the vast majority of the *Candida* species were detected in 5 days (Meyer et al. 2004; Ericson et al. 2012). The main reason for 14 days of incubation for Bactec Mycosis IC/F vials is to detect the growth of filamentous fungi, which may take longer.

In 2012, recommendations concerning diagnostic procedures for detection of *Candida* diseases were published by the ESCMID Fungal Infection Study Group (Cuenca-Estrella et al. 2012). Concerning candidemia, the number of BC recommended in a single session is 3 (2–4), with a total volume varying according to the age of the patient: 40–60 ml for adults, 2–4 ml for children under 2 kg, 6 ml between 2 and 12 kg, and 20 ml between 12 and 36 kg. The timing for obtaining the BC is one right after the other from different sites, and venipuncture remains the technique of choice. A BC set comprises 60 ml blood for adults obtained in a single session within a 30-min period and divided into 10-ml aliquots among three aerobic and three anaerobic bottles. The frequency recommended is daily when candidemia is suspected, and the incubation period must be at least 5 days.

When these recommendations have been followed, the sensitivity of BC to detect *Candida* is 50–75% although lower sensitivity rates in neutropenic patients and those undergoing antifungal treatment have been reported (Arendrup et al. 2008, 2011). **It should be noted that fungal pathology in direct Gram stain from positive BC flasks can provide a reasonably reliable basis for discrimination between *C. albicans* and non-*albicans* species** (Harrington et al. 2007).

Despite the advances in blood culture technology, the recovery of fungi from blood remains an insensitive marker for IFI. Filamentous fungi will be detected to a much lesser extent than yeasts. For IA, BCs represent a limited diagnostic tool because *Aspergillus fungaemia* is rarely encountered, even in the setting of disseminated disease (Duthie and Denning 1995; Denning 1998; Girmenia et al. 2001). Mainly, *A. terreus* has been described as being detected by blood cultures, other *Aspergillus* species very rarely. However, recently it has been described (Rosa et al. 2011) that BACTEC automated systems may allow detection of *A. fumigatus*, *A. flavus*, and *A. terreus*. In this study, BACTEC Plus Aerobic/F vials seemed to be advantageous for *Aspergillus* detection in blood samples collected from patients under antifungal treatment.

Culture is highly sensitive (98%) in patients with *Cryptococcus* meningitis (Denning et al. 2003). However, in central nervous system aspergillosis or candidiasis, cerebrospinal fluid (CSF) cultures are less sensitive (Arendrup et al. 2012). For *Candida* meningitis, 80% of CSF cultures have been described as positive (Sanchez-Portocarrero et al. 2000). All yeasts and molds obtained from sterile sites, including blood and continuous ambulatory peritoneal dialysis (CAPD) fluids, and intravenous-line tips should be identified to species level. This is also valid for bronchoscopically gained specimens. Treatment of clinically obvious or severe cases should not be delayed for culture results, although treatment may need to be altered according to the species. The presence or absence of fungal elements on microscopy is not always predictive of positive culture results, and if a clinician is faced with unexpectedly negative results, investigations should be repeated while alternative diagnoses are considered (Moriarty et al. 2012).

However, the interpretation of results obtained from a culture of the respiratory tract specimens is difficult because of the possible transient colonization by yeasts, on the one hand, and the ubiquitous nature of airborne conidia and the risk of accidental contamination with molds, on the other hand. It has been observed that the cultivation of *Aspergillus* spp. from normally sterile sites and the presence of *Aspergillus* in respiratory samples from immunocompromised children at risk of IA are highly indicative of infection (Muller et al. 2002). Detection of *Aspergillus* species in BAL fluid is highly suspicious for invasive disease in febrile granulocytopenic patients with new pulmonal infiltrates. However, the absence of hyphal elements or a negative culture does not exclude the diagnosis.

6. Identification of Cultured Fungi

Yeasts are identified by their **assimilation pattern** and their **microscopic morphology**, and molds by their **macroscopic and microscopic morphology**. Due to the slow growth of some species/isolates this can take days to weeks. As for *Candida* spp., the use of chromogenic media and test kits allows rapid identification and can accelerate the otherwise slow identification of yeasts. Identification of the most com-

mon yeasts can be performed immediately when visible growth is observed (Fig. 13.7). Chromagar Candida (Becton Dickinson, Franklin Lakes, NJ, USA) and Candida ID Agar (bioMérieux, Marcy l'Etoile, France) have been shown to allow easier differentiation of *Candida* species in mixed yeast populations than the traditional Sabouraud Glucose agar. Moreover, more rapid identification of *Candida* species can be achieved on these media than with conventional test kits. These media reduce the need for subculture and further biochemical tests and considerably simplify and shorten the identification procedure, mainly for *C. albicans*, *C. tropicalis*, and *C. krusei* (Willinger and Manafi 1999; Fricker-Hidalgo et al. 2001; Willinger et al. 2001). However, *C. glabrata* the second most common *Candida* species cannot be identified reliably on chromogenic media.

Identifying filamentous fungi can be much more cumbersome. Generally, macroscopic and microscopic morphology is the key to identification (de Hoog et al. 2009). Macroscopic examination of the colonies can reveal important characteristics concerning color, texture, exudates, pigments, specific structures, growth rate and growth zones, and aerial (the hyphae projecting above the medium surface) and submerged or vegetative mycelium (the portion that penetrates the medium in order to secure the colony and absorb nutrients). The color of the reverse of the colony must be recorded along with any pigment that diffuses into the medium. In addition, microscopic elements have to be evaluated for identification. The production of characteristic conidia and hyphae enables the identification of a large number of molds, at least to the genus level (Vyzantiadis et al. 2012). Standard references including identification algorithms are available (de Hoog et al. 2009) but, despite this, morphology-based identification of molds requires a high degree of experience.

Commercially available biochemical test systems identify most of the commonly isolated species of yeast accurately but may result in no identification or misidentification of more unusual isolates (Freydiere et al. 2001). Furthermore, samples for these tests must be incubated for 1–3 days before results are obtained. Identification of molds may also be cumbersome and tedious because it is mainly based on morphology. As an alternative to the conventional identification schemes, proteomic profiling by mass spectral analysis was recently evaluated for use in species differentiation of a variety of microorganisms. Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF) is emerging as a rapid and accurate tool for identifying pathogens, not only for bacteria but also for molds and yeast species. This technology generates characteristic mass spectral fingerprints that are unique signatures for each microorganism and are thus ideal for an accurate microbial identification at the genus and species levels and have the potential to be used for strain typing and identification (Stevenson et al. 2010; Croxatto et al. 2012). Furthermore, molecular tools can aid in the identification of cultured fungi (see below)

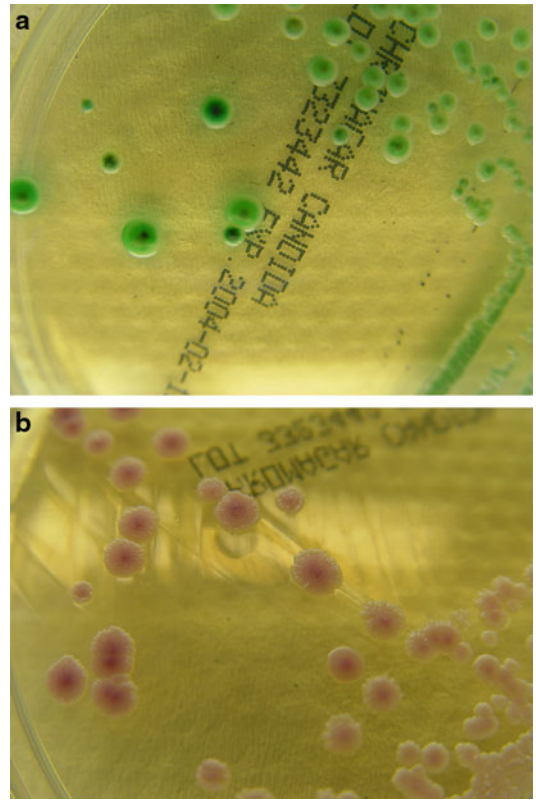


Fig. 13.7. Pigmentation of various *Candida* species on ChromagarCandida® : (**a**) *C. albicans* and (**b**) *C. parapsilosis*)

7. Mass-Spectrometry-Based Identification of Fungal Pathogens

This technique probably presents the major paradigm change of the past decades in the way in which species are determined in microbiology because it allows the identification of microorganisms within a few minutes. Currently, there are four commercial systems available: the MALDI Biotyper (Bruker Daltonics, Bremen, Germany), the AXIMA@SARAMIS database (AnagnosTec, Potsdam, Germany and Shimadzu, Duisburg, Germany), and the Andromas (Andromas, Paris, France) and VITEK MS systems (bioMérieux, Marcy l'Etoile, France). Among fungi, ascomycetous and basidiomycetous yeasts including *Candida*, *Pichia*, and *Cryptococcus* genera are most easily processed and analyzed (Bader 2013).

Recent studies showed that far more than 90% of clinical *Candida* isolates from different species could be correctly identified by MALDI-TOF MS (Marklein et al. 2009; van Veen et al. 2010). Furthermore, closely related yeast species that cannot be discriminated with common biochemical methods, such as *Candida ortho-/meta-/parapsilosis*, *Candida glabrata/bracarensis/nivariensis*, *Candida albicans/dubliniensis*, *Candida haemulonii* group I and II complexes, or the phenotypically similar species *Candida palmioleophila*, *Candida famata*, and *Candida guilliermondii*, can be resolved without difficulty by MALDI-TOF MS (Bader 2013).

This technique has also been applied directly on positive blood cultures without the need for prior culturing and has thus reducing the time required for microbiological diagnosis. The reliability of this technique has not only been evaluated for bacteria but also for yeasts directly from blood culture bottles. Spanu et al. (2012) demonstrated that the identification results were concordant with those of the conventional culture-based method for 95.9% of *Candida albicans* and 86.5% of non-*albicans Candida* species. Results were available in 30 min, suggesting that this approach is a reliable, time-saving tool for routine identification of *Candida* species causing bloodstream infection.

Far less data are available for the differentiation of molds like *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp., and dermatophytes (Wieser et al. 2012). As MALDI-TOF MS identification can only be performed from cultured fungi, the various growth forms of molds, such as mycelium and conidia, complicate the analysis due to differences in protein composition. Also, in strongly pigmented isolates such as those from *Fusarium* spp. or *A. niger*, the conidial melanin pigment inhibits the ionization of the analyte (Dong et al. 2009; Buskirk et al. 2011). This can be overcome by growth in liquid cultures suppressing pigment formation or by pre-analytical washing steps. However, adjustments and optimizations are needed to enhance the performance of MALDI-TOF MS-based identification systems for routine diagnostics of molds (Bader 2013).

MALDI-TOF MS also has the potential to be a useful and rapid identification method for dermatophytes. The identification of dermatophytes is currently based on morphological criteria, is time consuming, and is hindered by intraspecies morphological variability and the atypical morphology of some clinical isolates. A more rapid method seems to be important for improving and shortening the period of identification. In recent studies using different systems, a high level of identification was obtained for the most important clinical fungal dermatophytes species, *Trichophyton rubrum*, *T. interdigitale*, *T. tonsurans*, and *Arthroderma benhamiae*, thus demonstrating that MALDI-TOF MS might also represent a fast and very specific method for species identification of dermatophytes (Erhard et al. 2008; de Respinis et al. 2012; Nenoff et al. 2013).

C. Antifungal Susceptibility Testing

In vitro antifungal susceptibility testing (AST) is often used to select agents with likely activity for a given infection, but perhaps its most important use is in identifying agents that will not work, i.e., to detect resistance. Currently, there are **two independent standards** for broth microdilution (BMD) susceptibility testing of

Candida and filamentous fungi: the Clinical and Laboratory Standards Institute (CLSI) methods and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methods (Lass-Flörl et al. 2010; Pfaller 2012). Both of these methods use BMD, although there are some differences in inoculum size and minimum inhibitory concentration (MIC) endpoint determination, results obtained when testing azoles and echinocandins against *Candida* and azoles against *Aspergillus* species are in close agreement (Pfaller et al. 2011).

Data gathered by these standardized tests are useful (in conjunction with other forms of data) for calculating clinical breakpoints and epidemiologic cut-off values (ECOFFs). Clinical breakpoints should be selected to optimize detection of non-wild-type strains of pathogens and should be species-specific and not divide wild-type distributions of important target species. ECOFFs are the most sensitive means of identifying strains with acquired resistance mechanisms (Pfaller 2012). The wild-type distribution for a species is defined as the distribution of the MIC for isolates that exhibit no acquired or mutational resistance to the drug in question. This also means that a non-wild-type isolate possesses an acquired or mutational resistance mechanism. The upper limit of the wild-type population is defined as the ECOFF. Breakpoints should never divide the wild-type population, as this will lead to a random susceptibility classification (Rodriguez-Tudela et al. 2010). Both reference test methods offer breakpoints and interpretative data for *Candida* spp. and *Aspergillus*. The CLSI breakpoints for fluconazole, voriconazole, and the echinocandins have recently been revised for *Candida* species (Pfaller and Diekema 2012). Although the CLSI documents only provide guidelines for *Candida* spp., some investigators have applied the CLSI breakpoints to *Cryptococcus* spp. and correlations have been demonstrated between higher MICs and treatment failures (Jenkins and Schuetz 2012). For *Aspergillus* species, breakpoints do not yet exist, but susceptibility results can be interpreted as wild type or higher than wild type using ECOFFs (Arendrup et al. 2012).

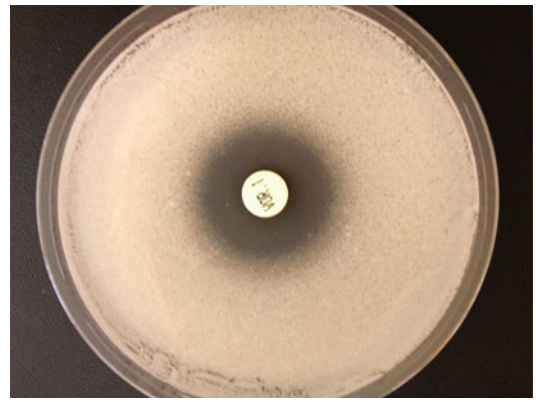


Fig. 13.8. Disk diffusion test (RPMI Agar)

Because the BMD testing method is difficult to perform in daily practice in clinical microbiology laboratories, other testing approaches have been investigated. Disk diffusion antifungal susceptibility testing is a simple and cost-effective method for both yeasts and molds (Fig. 13.8). The CLSI has developed agar-based, disk diffusion testing for yeasts (CLSI 2004, 2009) and molds (CLSI 2010a, b). Disk diffusion testing has been standardized for fluconazole and voriconazole, as well as for echinocandins versus *Candida* species. Also, interpretative breakpoints have been provided for azoles, caspofungin, and micafungin.

There are also commercially available test kits for MIC determination that exhibit potential advantages in terms of ease of use, flexibility, standardization, and rapidity of results. **Etest** (bioMérieux, Marcy l' Etoile, France) is very often used and directly quantifies antifungal susceptibility in terms of discrete MIC values (Fig. 13.9). RPMI-based agars are recommended for use (Pfaller et al. 2000). Also, it is recommended that Mueller-Hinton agar is supplemented with 2% glucose and 0.5 μg/ml methylene blue, which appears to enhance the formation of inhibition ellipses with clear edges and less growth within the ellipse (Pfaller et al. 2004). The method is reliable and reproducible, is suitable for yeast and molds, and has been shown to correlate well with the CLSI methodology (Szekely et al. 1999). A clear benefit of utilizing Etest is in assessing the susceptibility to amphotericin B, as this method gives much broader MIC ranges than BMD. Etest is also highly suitable for determining the activity of echinocandins against yeasts because it produces easy-to-read, sharp zones of inhibition. However, for echinocandins the so-called paradoxical effect has been

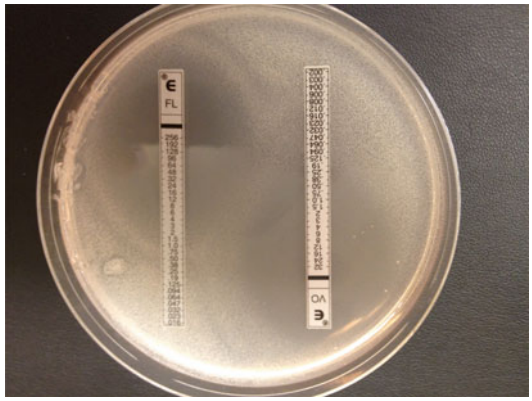


Fig. 13.9. *Candida albicans* and Etest (fluconazole, voriconazole), RPMI-medium

observed for *Candida* and *Aspergillus* in vitro. The paradoxical effect refers to an attenuation of echinocandin activity at higher concentrations despite an inhibitory effect at lower drug levels. It appears to be species-related and varies with the echinocandin. The effect has been noted most often for caspofungin and is not related to FKS1 mutations or upregulation of echinocandin sensitivity of the glucan synthase complex in the presence of drug. The clinical relevance of this in vitro effect is uncertain (Wiederhold 2009; Chen et al. 2011).Another test system is the **VITEK 2**, a fully automated commercial system (bioMérieux, Marcy l'Etoile, France) intended for antifungal susceptibility testing of yeasts. The system reliably detects resistance among *Candida* spp. and demonstrates excellent quantitative, qualitative, and categorical agreement with the reference method in various studies (Pfaller et al. 2004, 2007; Posteraro et al. 2009). However, correct discrimination between fluconazole-susceptible and intermediate *C. glabrata* isolates is not possible because the revised species-specific susceptibility breakpoint is not included in the VITEK 2 detection range (MIC of $\leq$0.250 to $\geq$4 mg/l). Astvad et al. (2013) demonstrated that the VITEK 2 allowed correct categorization of all wild-type isolates as susceptible for caspofungin. However, despite an acceptable categorical agreement, it failed to reliably classify isolates harboring *fks* hot spot mutations as intermediate or resistant, which was in part due to the fact that the detection range did not span the susceptibility breakpoint for *C. glabrata*. Furthermore, EUCAST breakpoints for Caspofungin have not been established, thus the VITEK 2 cannot be used with echinocandins as an alternative for the EUCAST reference method. **Sensititre YeastOne** (Trek Diagnostic Systems, Sun Prairie, WI, USA) is a colorimetric antifungal susceptibility testing MIC plate that exhibits high agreement with the CLSI BMD method (Pfaller et al. 2008).

Susceptibility testing is indicated to provide a basis for selection of appropriate antifungal treatment in individual patient cases and for epidemiological reasons in order to continuously follow susceptibility patterns and thereby detect any emergence of resistance at an early stage. Recommendations for AST are displayed in Table 13.4. However, for individual patient care the isolate should be identified to species level to predict the susceptibility pattern. Important examples of fungi that have low susceptibility to antifungal agents include: *C. krusei*, which is intrinsically resistant to fluconazole and less susceptible to amphotericin B than other *Candida* spp.; *Aspergillus* spp., *Scedosporium apiospermum*, *Trichosporon* spp., and *Scopulariopsis* spp., which are resistant to amphotericin B; zygomycetes, which are resistant to all licensed azoles; and *C. glabrata*, which is frequently less susceptible to fluconazole than other *Candida* spp. For better illustration, Table 13.5 shows the susceptibility pattern of the most common *Candida* spp. In cases where the susceptibility pattern cannot be reliably predicted on the basis of species identification alone, antifungal susceptibility testing should be performed (Arendrup et al. 2012).

VI. Molecular Tools in the Diagnosis of Fungal Pathogens

Molecular species identification has become increasingly important in medical mycology. In the past, this method was mainly applied to fungal isolates that did not form fruiting bodies in culture or to rare isolates that could not be identified by classical phenotype-based methods. However, there is a constantly increasing demand for fast diagnosis in the clinical setting and molecular tools allow rapid species (or at least genus) identification from minute amounts of culture without the need for prolonged subculture to induce fruiting body formation. Only recently have these advantages have been challenged by MS-based identification (see above). Furthermore, molecular

Table 13.4. Antifungal susceptibility testing: when and how to test

When to test?
Routine antifungal testing of fluconazole and an echinocandin against *C. glabrata* from deep sites
In invasive fungal infections
In invasive and mucosal infections failing therapy
For *Candida* species from sterile sites
For isolates considered clinically relevant particularly in patients exposed to antifungals
How to test?
Identify the isolate to species level
Perform routine susceptibility testing for fluconazole and, according to the local epidemiology, include other azoles
No testing of isolates with a high rate of intrinsic resistance
C. lusitaniae and amphotericin
C. krusei and fluconazole, flucytosine
C. guilliermondii and echinocandins
A. terreus and amphotericin B

As described by Lass-Flörl et al. (2010), Arendrup et al. (2012), Pfaller and Diekema (2012) – modified

Table 13.5. General susceptibility patterns of *Candida* species

Candida spp.	AmB	Flucytosin	FLU	ITRA	VOR	POS	EC
C. albicans	S	S	S	S	S	S	S
C. tropicalis	S	S	S	S	S	S	S
C. parapsilosis	S	S	S	S	S	S	S to R
C. glabrata	S to I	S	S-DD to R	S-DD to R	S-DD to R	S-DD to R	S
C. krusei	S to I	I to R	R	S-DD to R	S	S	S
C. lusitaniae	S to R	S	S	S	S	S	S

As described by Pappas et al. (2009) – modified

AmB amphotericin B, *FLU* fluconazole, *ITRA* itraconazole, *VOR* voriconazole, *POS* posaconazol, *EC* echinocandins, *S* susceptible, *SDD* susceptible dose-dependent, *I* intermediate, *R* resistant

species identification (as with MS-based species identification) requires a far lesser expertise of the personnel and – if reliable databases are used for species identification – is likely to generate more reliable results than phenotype-based identification. **Consequently, modern medical mycology has incorporated molecular tools for standard diagnostic procedures.** It is likely that these tools will continue to find their niches in medical mycology even in view of the growing applicability of MS-based species identification. The reason for this is that even with new, as-yet unrecognized fungal pathogens that are not available within identification databases, molecular data can assign an unidentified pathogen to a group of fungi much better than MS patterns.

A. Identification of Cultured Fungal Pathogens

Several technologies have been used in the past for species identification of fungal pathogens. These include restriction-fragment-length polymorphism, random amplified polymorphic DNA, and hybridization-based tools including microarrays and others (Balajee et al. 2007). However, with **sequence-based** tools having become affordable and technically accessible for many medical mycology laboratories, these tools have certainly evolved to be of major importance in the medical mycology laboratory. These techniques can also identify rare species as long as the database is sufficiently large and can suggest related species in the case of novel fungal pathogens (Kurzai et al. 2003).

DNA extraction and PCR amplification from cultured fungi are normally unproblematic and several protocols for extraction of fungal DNA have been described in the literature. A simple protocol, which generally results in sufficient amounts and quality of DNA, is included in the *Atlas of Clinical Fungi* (de Hoog et al. 2009). Some available protocols have much lower yields, mostly due to suboptimal procedures for breaking the fungal cell wall (e.g., protocols based on repeated freezing/thawing), but due to sufficient amounts of DNA in the culture material they can still be useful for extracting fungal DNA. Conventional PCR amplification with 25–35 cycles is sufficient to amplify the target region for sequencing. Several **target regions** have been described in older literature, including parts of the ribosomal gene clusters like the D1/D2 domain of the large subunit (LSU). Sequencing of the LSU in the British reference laboratory between 2004 and 2006 allowed an identification of all clinical isolates that could not be identified by conventional methods (Linton et al. 2007). The most useful target regions for species identification in general are the **internal transcribed spacer regions** (ITS1/ITS2) (Guarro et al. 1999; Balajee et al. 2007).

These regions are intercalated between the more conserved sequences encoding for the ribosomal RNAs (rRNAs), which can be used as a starting point for PCR amplification using "universal" primers, and accumulate mutations with higher frequency than the "functional" rRNAs. The typical fungal ribosomal gene cluster encodes for the 18S rRNA followed by the first internal spacer (ITS1), the 5,8S rRNA, ITS2, and the 28S rRNA gene, which is similar to the arrangements found in other eukaryotic cells (Iwen et al. 2002).

It has been shown that ITS sequencing is regularly sufficient to identify unknown fungal isolates (Rakeman et al. 2005). However, it must be kept in mind that the variability of the ITS regions may differ between fungal species. In fact, ITS sequencing has been used for subspecies strain typing in some species (e.g., for *Pneumocystis jirovecii*), whereas other species have been found to have highly conserved ITS sequences (Iwen et al. 2002). Disadvantages of ribosomal target sequences include a lack of discriminative power in some clinical situations (e.g., discrimination of *Aspergillus* species complexes) (Balajee and Marr 2006) or the presence of heterologous copies in some genera (e.g., some *Fusarium* spp.) that may interfere with amplification and/or sequencing (O'Donnell and Cigelnik 1997). In these cases, other target sequences like elongation factor 1 (EF-1), the RNA-polymerase subunit RPB2, rodlet protein RodA, or β-tubulin can be useful (O'Donnell et al. 1998; Balajee et al. 2007). It has been stated that the success of a sequencing strategy in a clinical microbiology laboratory lies in: (i) the choice of locus, (ii) the amenability of the region to PCR amplification and sequencing, (iii) the reliability of interpretation of the results, and (iv) the availability of a sequence database for comparison (Balajee et al. 2007). The last step of sequence-based species identification relies on sequence comparisons using BLAST tools (most commonly BLAST for comparison of nucleotide sequences) and is strongly dependent on the quality of the database. In most cases, GenBank, a non-curated open-source database is used for initial BLAST analysis. Several studies have clearly demonstrated, that GenBank includes wrong and wrongly assigned sequences, low quality sequences, and other traps that may interfere with a correct species identification (de Hoog and Horre 2002; Nilsson et al. 2006).

The study by Nilsson et al. (2006) suggested that many entries are to some degree incorrect (20%!) and lack reasonable and up-to-date annotation. This problem is further aggravated by constant changes in fungal taxonomy, which are normally not reflected by the database, and the fungal specificity of several names reflecting an identical organism (names for teleomorph, anamorph, and synanamorph). Because sequences deposited in GenBank cannot easily be corrected – it requires action by the initial depositor – erroneous database entries do accumulate over time.

Thus, although GenBank is undoubtedly a highly important tool, a rigorous evaluation of all molecular species identification results is mandatory and a reference laboratory should always be involved in cases of remaining doubt. Evaluation can be achieved by selectively

looking for homology with type strain-derived sequences from known sources and/or authors. In addition, the presence of a number of homologous sequences from the same species but from different sources is usually an encouraging sign. Finally, all molecular results should be carefully evaluated together with the morphological characteristics of the respective isolate and current taxonomic resources. In fact, other web-based repositories can be used for this purpose, including for example the homepage of the Centraalbureau voor Schimmelcultures (CBS), DoctorFungus, or MycoBank (Crous et al. 2004; Robert et al. 2005). When these limitations are kept in mind, species identification based on ITS sequencing is usually reliable with homologies >98%, although there is no clear cut-off for unequivocal species identification (Nilsson et al. 2008). Some commercial databases have become available but are so far not used broadly (Balajee et al. 2007).

B. Molecular Tools for Diagnosing Invasive Aspergillosis

Although a vast variety of PCR protocols for detecting *Aspergillus* species in specimens now exist, no standard protocol has been defined and included in the internationally accepted criteria for the diagnosis of IA (EORTC/MSG) (Löffler and Kurzai 2011). Reasons for this are the missing evaluation of the clinical usefulness and the lack of standardization. Because *Aspergillus* spores are ubiquitously present, it is a great challenge to avoid false-positive results due to contamination, especially in respiratory samples. Therefore, the focus has changed to blood or serum samples with lower risk of contamination. However, recently, the analysis of respiratory samples has been reconsidered for PCR diagnostics of IA.

In general, **fungal DNA concentration in blood or serum samples is very low**. Therefore, a sample volume of at least 3 ml needs to be used for DNA isolation to gain a sufficient amount of *Aspergillus* DNA for PCR analysis, as recommended by the European Aspergillus PCR Initiative (EAPCRI). Due to its inhibitory effect on DNA-degrading enzymes, EDTA should be the anticoagulant of choice. Also, EDTA does not interfere with PCR as other anticoagulants may do. Choosing the right DNA isolation protocol has great impact on the diagnostic quality of *Aspergillus* PCR (White et al. 2010; White et al. 2011). Critical steps in DNA isolation that influence the efficacy of DNA extraction are the lysis of erythrocytes and leucocytes, fungal cell wall disruption, and the elution volume. Omitting lysis of either blood cells or fungal cell wall is associated with a decreased analytic sensitivity. As recommended by EAPCRI, fungal cell wall disruption following blood cell lysis should not be performed with protocols based on enzymatic digestion because they are time consuming, costly, and bear the risk of fungal DNA-contaminated enzyme preparations. For subsequent DNA purification and DNA extraction, commercially available kits can be used (Loeffler et al. 2002). Controls are essential to detect possible fungal DNA contamination of buffers, which has been reported (Loeffler et al. 1999). The optimum elution volume is 60 µl. Neither an increase above 100 µl nor an arbitrary reduction of the elution volume is useful because they lead to a significant decrease in detection rate and in DNA concentration, respectively (White et al. 2010).

The impact of fungal DNA amplification on the diagnostic quality is not as high as the previously described DNA extraction. To ensure enhanced sensitivity of the PCR, a target sequence with multiple genomic copies, high cycle numbers, and amplification with real-time PCR should be preferred. The target sequences most commonly used are within the ribosomal gene clusters 5,8S rRNA, 18S rRNA, and 26 rRNA, with the internal spacers ITS-1 and ITS-2 in between, as mentioned earlier. The genome of *A. fumigatus* contains 38–91 copies of this gene cluster, although this may vary strongly between different strains (Herrera et al. 2009). Mitochondrial DNA sequences also exist in high copy numbers and can also be used (Costa et al. 2002). Combining both target sequences could gain additional sensitivity (Millon et al. 2011).

Several commercial assay are available. MycAssay™ Aspergillus from Myconostica (Manchester, UK) detects *Aspergillus* DNA from lower respiratory tract specimens and serum by real-time PCR. An appropriate fungal DNA extraction and purification kit

(MycXtra®) is offered as well. Another diagnostic assay based on real-time PCR analysis is the SeptiFast kit offered by Roche Diagnostics (Mannheim, Germany). Although originally designed for sepsis diagnostics, it was the first PCR system on the market to detect *A. fumigatus* (Steinmann et al. 2009). SeptiFast not only detects *A. fumigatus*, but also five different *Candida* species. It appears that, for *Candida* spp. and *A. fumigatus*, the SeptiFast methodology is more sensitive than conventional BC (Westh et al. 2009). ViraCor-IBT Laboratories (Lee's Summit, MO, USA) provides a laboratory service for detection of *Aspergillus* spp. that is also based on real-time PCR. Molzym (Bremen, Germany) offers a variety of kits depending on the type of specimen (e.g., SepsiTest for whole blood samples) to detect more than 345 different pathogens including *C. albicans, C. tropicalis, C. parapsilosis, C. krusei, C. glabrata, Cryptococcus neoformans*, and *A. fumigatus* by DNA sequencing. In the case of SepsiTest, broad range primers hybridize within conserved sequences on rRNA genes. SIRS Lab (Jena, Germany) created the DNA detection system VYOO that allows multiplex PCR and detection of 34 bacteria and 7 fungal pathogens (*C. albicans, C. dubliniensis, C. tropicalis, C. parapsilosis, C. krusei, C. glabrata*, and *A. fumigatus*) as well as 5 antibiotic resistance genes in one step by hybridization of microbial DNA onto microarrays. Prior to DNA detection, pathogen DNA is selectively purified using a novel protocol designed by SIRS LAB (Bruns et al. 2009; Sachse et al. 2009). Pathogen DNA can be enriched by affinity chromatography due to selective binding of a specific protein to non-methylated CpG dinucleotide motifs mainly found in microbes. However, this method is not feasible for samples from neutropenic patients (Springer et al. 2011).

C. Other Molecular Tools for Diagnosing Fungal Infection from Clinical Specimens

A vast variety of PCR protocols ranging from specific protocols for selected pathogens to pan-fungal PCRs have been described in the literature and we have restricted ourselves here to describing protocols for diagnosis of IA because these are clearly the best-evaluated molecular tools in medical mycology. However, with the changing epidemiology of fungal infections and the constant emergence of rare pathogens, new studies on molecular tools for aiding diagnosis of invasive mycoses are certainly warranted. The example of *Aspergillus* PCR can serve as a **role-model** and will most likely open the way to standardization and defined quality control, which are urgently required for diagnostic tools in the twenty-first century.

References

Alexander B, Pfaller M (2006) Contemporary tools for the diagnosis and management of invasive mycoses. Clin Infect Dis 43:S15–S27

Alexander BD (2002) Diagnosis of fungal infection: new technologies for the mycology laboratory. Transpl Infect Dis 4(Suppl 3):32–37

Althoff Souza C, Muller NL et al (2006) Pulmonary invasive aspergillosis and candidiasis in immunocompromised patients: a comparative study of the high-resolution CT findings. J Thorac Imaging 21 (3):184–189

Andreas S, Heindl S et al (2000) Diagnosis of pulmonary aspergillosis using optical brighteners. Eur Respir J 15(2):407–411

Antachopoulos C (2010) Invasive fungal infections in congenital immunodeficiencies. Clin Microbiol Infect 16(9):1335–1342

Anttila VJ, Lamminen AE et al (1996) Magnetic resonance imaging is superior to computed tomography and ultrasonography in imaging infectious liver foci in acute leukaemia. Eur J Haematol 56(1–2):82–87

Aquino SL, Kee ST et al (1994) Pulmonary aspergillosis: imaging findings with pathologic correlation. AJR Am J Roentgenol 163(4):811–815

Arendrup MC, Bergmann OJ et al (2010) Detection of candidaemia in patients with and without underlying haematological disease. Clin Microbiol Infect 16(7):855–862

Arendrup MC, Bille J et al (2012) ECIL-3 classical diagnostic procedures for the diagnosis of invasive fungal diseases in patients with leukaemia. Bone Marrow Transplant 47(8):1030–1045

Arendrup MC, Bruun B et al (2011) National surveillance of fungemia in Denmark (2004 to 2009). J Clin Microbiol 49(1):325–334

Arendrup MC, Fuursted K et al (2008) Semi-national surveillance of fungaemia in Denmark 2004–2006: increasing incidence of fungaemia and numbers of

isolates with reduced azole susceptibility. Clin Microbiol Infect 14(5):487–494
Ascioglu S, Rex JH et al (2002) Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. Clin Infect Dis 34(1):7–14
Astvad KM, Perlin DS et al (2013) Evaluation of caspofungin susceptibility testing by the new Vitek 2 AST-YS06 yeast card using a unique collection of FKS wild-type and hot spot mutant isolates, including the five most common candida species. Antimicrob Agents Chemother 57 (1):177–182
Bader O (2013) MALDI-TOF-MS-based species identification and typing approaches in medical mycology. Proteomics 13:788–799
Balajee SA, Marr KA (2006) Phenotypic and genotypic identification of human pathogenic aspergilli. Future Microbiol 1(4):435–445
Balajee SA, Sigler L et al (2007) DNA and the classical way: identification of medically important molds in the 21st century. Med Mycol 45(6):475–490
Bellanger AP, Grenouillet F et al (2011) Retrospective assessment of beta-D-(1,3)-glucan for presumptive diagnosis of fungal infections. APMIS 119 (4–5):280–286
Berenguer J, Buck M et al (1993) Lysis-centrifugation blood cultures in the detection of tissue-proven invasive candidiasis. Disseminated versus single-organ infection. Diagn Microbiol Infect Dis 17(2):103–109
Bergeron A, Belle A et al (2010) Contribution of galactomannan antigen detection in BAL to the diagnosis of invasive pulmonary aspergillosis in patients with hematologic malignancies. Chest 137(2):410–415
Bianchi M, Hakkim A et al (2009) Restoration of NET formation by gene therapy in CGD controls aspergillosis. Blood 114(13):2619–2622
Bretagne S, Marmorat-Khuong A et al (1997) Serum *Aspergillus* galactomannan antigen testing by sandwich ELISA: practical use in neutropenic patients. J Infect 35(1):7–15
Brown GD, Denning DW et al (2012) Tackling human fungal infections. Science 336(6082):647
Bruns T, Sachse S et al (2009) Identification of bacterial DNA in neutrocytic and non-neutrocytic cirrhotic ascites by means of a multiplex polymerase chain reaction. Liver Int 29(8):1206–1214
Buskirk AD, Hettick JM et al (2011) Fungal pigments inhibit the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis of darkly pigmented fungi. Anal Biochem 411 (1):122–128
Byrnes EJ 3rd, Bartlett KH et al (2011) *Cryptococcus gattii*: an emerging fungal pathogen infecting humans and animals. Microbes Infect 13 (11):895–907
Chakrabarti A, Slavin MA (2011) Endemic fungal infections in the Asia-Pacific region. Med Mycol 49 (4):337–344
Chander J, Chakrabarti A et al (1993) Evaluation of calcofluor staining in the diagnosis of fungal corneal ulcer. Mycoses 36(7–8):243–245
Chandrasekar P (2010) Diagnostic challenges and recent advances in the early management of invasive fungal infections. Eur J Haematol 84(4): 281–290
Chang WC, Tzao C et al (2006) Pulmonary cryptococcosis: comparison of clinical and radiographic characteristics in immunocompetent and immunocompromised patients. Chest 129(2):333–340
Chen SC, Slavin MA et al (2011) Echinocandin antifungal drugs in fungal infections: a comparison. Drugs 71(1):11–41
Clancy CJ, Jaber RA et al (2007) Bronchoalveolar lavage galactomannan in diagnosis of invasive pulmonary aspergillosis among solid-organ transplant recipients. J Clin Microbiol 45(6):1759–1765
Clinical and Laboratory Standards Institute (2004) Method for antifungal disk diffusion susceptibility testing of yeasts: approved guideline. CLSI Document M44-A2. Clinical and Laboratory Standards Institute, Wayne
Clinical and Laboratory Standards Institute (2009) Zone diameter interpretative standards, corresponding minimal inhibitory concentration (MIC) interpretative breakpoints, and quality control limits for anti- fungal disk diffusion susceptibility testing of yeasts; informational supplement, 3rd ed. CLSI document M44-S3. Clinical and Laboratory Standards Institute, Villanova
Clinical and Laboratory Standards Institute (2010a) Reference method for antifungal disk diffusion testing of non-dermatophyte filamentous fungi; approved guideline. CLSI document M51-A. Clinical and Laboratory Standards Institute, Villanova
Clinical and Laboratory Standards Institute (2010b) Performance standards for antifungal disk diffusion testing of non-dermatophyte filamentous fungi: informational supplement. CLSI document M51-S1. Clinical and Laboratory Standards Institute, Villanova
Clinical and Laboratory Standards Institute (2012) Principles and procedures for detection of fungi in clinical specimens—direct examination and culture; approved guideline. CLSI document M54-A. Clinical and Laboratory Standards Institute, Villanova
Cornely OA, Maertens J et al (2007) Posaconazole vs. fluconazole or itraconazole prophylaxis in patients with neutropenia. N Engl J Med 356(4):348–359
Costa C, Costa JM et al (2002) Real-time PCR coupled with automated DNA extraction and detection of galactomannan antigen in serum by enzyme-linked immunosorbent assay for diagnosis of invasive aspergillosis. J Clin Microbiol 40(6):2224–2227

Crous PW, Gams W, Stalpers JA, Robert V, Stegehuis G (2004) MycoBank: an online initiative to launch mycology into the 21st century. Stud Mycol 50:19–22

Croxatto A, Prod'hom G et al (2012) Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. FEMS Microbiol Rev 36 (2):380–407

Cuenca-Estrella M, Verweij PE et al (2012) ESCMID guideline for the diagnosis and management of *Candida* diseases 2012: diagnostic procedures. Clin Microbiol Infect 18(Suppl 7):9–18

de Boer D, Delnoij D et al (2011) The discriminative power of patient experience surveys. BMC Health Serv Res 11:332

de Hoog GS, Horre R (2002) Molecular taxonomy of the alternaria and *Ulocladium* species from humans and their identification in the routine laboratory. Mycoses 45(8):259–276

de Hoog S, Guarro J et al (2009) Atlas of clinical fungi: electronic version 3.1. ASM, Washington

De Pauw B, Walsh TJ et al (2008) Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/ Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. Clin Infect Dis 46(12): 1813–1821

de Respinis S, Tonolla M et al (2012) Identification of dermatophytes by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Med Mycol

Denning DW (1998) Invasive aspergillosis. Clin Infect Dis 26(4):781–803, quiz 804785

Denning DW, Kibbler CC et al (2003) British Society for Medical Mycology proposed standards of care for patients with invasive fungal infections. Lancet Infect Dis 3(4):230–240

Dong H, Kemptner J et al (2009) Development of a MALDI two-layer volume sample preparation technique for analysis of colored conidia spores of *Fusarium* by MALDI linear TOF mass spectrometry. Anal Bioanal Chem 395(5):1373–1383

Duthie R, Denning DW (1995) *Aspergillus fungemia*: report of two cases and review. Clin Infect Dis 20 (3):598–605

Erhard M, Hipler UC et al (2008) Identification of dermatophyte species causing onychomycosis and tinea pedis by MALDI-TOF mass spectrometry. Exp Dermatol 17(4):356–361

Ericson EL, Klingspor L et al (2012) Clinical comparison of the Bactec Mycosis IC/F, BacT/Alert FA, and BacT/Alert FN blood culture vials for the detection of candidemia. Diagn Microbiol Infect Dis 73(2): 153–156

Fidel PL Jr (2006) *Candida*-host interactions in HIV disease: relationships in oropharyngeal candidiasis. Adv Dent Res 19(1):80–84

Fred HL, Gardiner CL (2009) The air crescent sign: causes and characteristics. Tex Heart Inst J 36(3): 264–265

Freydiere AM, Guinet R et al (2001) Yeast identification in the clinical microbiology laboratory: phenotypical methods. Med Mycol 39(1):9–33

Fricker-Hidalgo H, Orenga S et al (2001) Evaluation of *Candida* ID, a new chromogenic medium for fungal isolation and preliminary identification of some yeast species. J Clin Microbiol 39(4):1647–1649

Fukuda T, Boeckh M et al (2003) Risks and outcomes of invasive fungal infections in recipients of allogeneic hematopoietic stem cell transplants after nonmyeloablative conditioning. Blood 102(3):827–833

Girmenia C, Nucci M et al (2001) Clinical significance of *Aspergillus fungaemia* in patients with haematological malignancies and invasive aspergillosis. Br J Haematol 114(1):93–98

Goodrich JM, Reed EC et al (1991) Clinical features and analysis of risk factors for invasive candidal infection after marrow transplantation. J Infect Dis 164 (4):731–740

Grim SA, Clark NM (2011) Management of infectious complications in solid-organ transplant recipients. Clin Pharmacol Ther 90(2):333–342

Guarro J, GeneJ J et al (1999) Developments in fungal taxonomy. Clin Microbiol Rev 12(3):454–500

Guinea J, Torres-Narbona M et al (2010) Pulmonary aspergillosis in patients with chronic obstructive pulmonary disease: incidence, risk factors, and outcome. Clin Microbiol Infect 16(7):870–877

Guo YL, Chen YQ et al (2010) Accuracy of BAL galactomannan in diagnosing invasive aspergillosis: a bivariate metaanalysis and systematic review. Chest 138(4):817–824

Hage CA, Knox KS et al (2011) Antigen detection in bronchoalveolar lavage fluid for diagnosis of fungal pneumonia. Curr Opin Pulm Med 17(3):167–171

Harrington A, McCourtney K et al (2007) Differentiation of *Candida albicans* from non-albicans yeast directly from blood cultures by Gram stain morphology. Eur J Clin Microbiol Infect Dis 26(5):325–329

Harrington BJ, Hageage GJ (2003) Calcofluor white: a review of its uses and application in clinical mycology and parasitology. Lab Med 34:361–367

He H, Ding L et al (2011) Value of consecutive galactomannan determinations for the diagnosis and prognosis of invasive pulmonary aspergillosis in critically ill chronic obstructive pulmonary disease. Med Mycol 49(4):345–351

He H, Ding L et al (2012) Role of galactomannan determinations in bronchoalveolar lavage fluid samples from critically ill patients with chronic obstructive pulmonary disease for the diagnosis of invasive pulmonary aspergillosis: a prospective study. Crit Care 16(4):R138

Herbrecht R, Denning DW et al (2002) Voriconazole versus amphotericin B for primary therapy of

invasive aspergillosis. N Engl J Med 347 (6):408–415
Herrera ML, Vallor AC et al (2009) Strain-dependent variation in 18S ribosomal DNA Copy numbers in *Aspergillus fumigatus*. J Clin Microbiol 47(5): 1325–1332
Huang L, Hecht FM (2000) Why does *Pneumocystis carinii* pneumonia still occur? AIDS 14 (16):2611–2612
Husain S, Clancy CJ et al (2008) Performance characteristics of the platelia *Aspergillus* enzyme immunoassay for detection of *Aspergillus* galactomannan antigen in bronchoalveolar lavage fluid. Clin Vaccine Immunol 15(12):1760–1763
Husain S, Paterson DL et al (2007) *Aspergillus* galactomannan antigen in the bronchoalveolar lavage fluid for the diagnosis of invasive aspergillosis in lung transplant recipients. Transplantation 83 (10):1330–1336
Iwen PC, Hinrichs SH et al (2002) Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. Med Mycol 40(1):87–109
Jenkins SG, Schuetz AN (2012) Current concepts in laboratory testing to guide antimicrobial therapy. Mayo Clin Proc 87(3):290–308
Jenq RR, van den Brink MR (2010) Allogeneic haematopoietic stem cell transplantation: individualized stem cell and immune therapy of cancer. Nat Rev Cancer 10(3):213–221
Kappe R, Rimek D (2010) Mycoserology–did we move on? *Aspergillus*. Mycoses 53(Suppl 1):26–29
Karageorgopoulos DE, Vouloumanou EK et al (2011) Beta-D-glucan assay for the diagnosis of invasive fungal infections: a meta-analysis. Clin Infect Dis 52(6):750–770
Kibbler C (2005) Defining invasive fungal infections in neutropenic or stem cell transplant patients. J Antimicrob Chemother 56(Suppl 1):i12–i16
Koh AY, Kohler JR et al (2008) Mucosal damage and neutropenia are required for *Candida albicans* dissemination. PLoS Pathog 4(2):e35
Korones DN, Hussong MR et al (1997) Routine chest radiography of children with cancer hospitalized for fever and neutropenia: is it really necessary? Cancer 80(6):1160–1164
Krowka MJ, Rosenow EC 3rd et al (1985) Pulmonary complications of bone marrow transplantation. Chest 87(2):237–246
Kullberg BJ, Verweij PE et al (2011) European expert opinion on the management of invasive candidiasis in adults. Clin Microbiol Infect 17(Suppl 5):1–12
Kurzai O, Keith P et al (2003) Postmortem isolation of *Pseudotaeniolina globosa* from a patient with aortic aneurysm. Mycoses 46(3–4):141–144
Lackner M, Lass-Flörl C (2013) Up-date on diagnostic strategies of invasive aspergillosis. Curr Pharm Des 19:3595–3614
Lanternier F, Sun HY et al (2012) Mucormycosis in organ and stem cell transplant recipients. Clin Infect Dis 54(11):1629–1636
Lass-Flörl C, Perkhofer S et al (2010) In vitro susceptibility testing in fungi: a global perspective on a variety of methods. Mycoses 53(1):1–11
Lease ED, Alexander BD (2011) Fungal diagnostics in pneumonia. Semin Respir Crit Care Med 32 (6):663–672
Lehrnbecher T, Frank C et al (2010) Trends in the postmortem epidemiology of invasive fungal infections at a university hospital. J Infect 61 (3):259–265
Leone M, Albanese J et al (2003) Long-term epidemiological survey of *Candida* species: comparison of isolates found in an intensive care unit and in conventional wards. J Hosp Infect 55(3):169–174
Linton CJ, Borman AM et al (2007) Molecular identification of unusual pathogenic yeast isolates by large ribosomal subunit gene sequencing: 2 years of experience at the United Kingdom mycology reference laboratory. J Clin Microbiol 45 (4):1152–1158
Loeffler J, Hebart H et al (1999) Contaminations occurring in fungal PCR assays. J Clin Microbiol 37 (4):1200–1202
Loeffler J, Schmidt K et al (2002) Automated extraction of genomic DNA from medically important yeast species and filamentous fungi by using the MagNA Pure LC system. J Clin Microbiol 40 (6):2240–2243
Löffler J, Kurzai O (2011) Diagnostic utility of DNA from Aspergillus in whole blood specimens. Curr Fungal Infect Rep 5:179–1815
Maertens J, Buve K et al (2009) Galactomannan serves as a surrogate endpoint for outcome of pulmonary invasive aspergillosis in neutropenic hematology patients. Cancer 115(2):355–362
Maertens J, Verhaegen J et al (2001) Screening for circulating galactomannan as a noninvasive diagnostic tool for invasive aspergillosis in prolonged neutropenic patients and stem cell transplantation recipients: a prospective validation. Blood 97(6): 1604–1610
Maertens JA, Madero L et al (2010) A randomized, double-blind, multicenter study of caspofungin versus liposomal amphotericin B for empiric antifungal therapy in pediatric patients with persistent fever and neutropenia. Pediatr Infect Dis J 29(5): 415–420
Marchetti O, Lamoth F et al (2012) ECIL recommendations for the use of biological markers for the diagnosis of invasive fungal diseases in leukemic patients and hematopoietic SCT recipients. Bone Marrow Transplant 47(6):846–854
Marklein G, Josten M et al (2009) Matrix-assisted laser desorption ionization-time of flight mass spectrometry for fast and reliable identification of clin-

ical yeast isolates. J Clin Microbiol 47(9): 2912–2917
Marr KA (2008) Fungal infections in hematopoietic stem cell transplant recipients. Med Mycol 46(4):293–302
Martin GS (2012) Sepsis, severe sepsis and septic shock: changes in incidence, pathogens and outcomes. Expert Rev Anti Infect Ther 10(6):701–706
Martin GS, Mannino DM et al (2003) The epidemiology of sepsis in the United States from 1979 through 2000. N Engl J Med 348(16):1546–1554
Meersseman W, Lagrou K et al (2008) Galactomannan in bronchoalveolar lavage fluid: a tool for diagnosing aspergillosis in intensive care unit patients. Am J Respir Crit Care Med 177(1):27–34
Meersseman W, Lagrou K et al (2009) Significance of the isolation of *Candida* species from airway samples in critically ill patients: a prospective, autopsy study. Intensive Care Med 35(9):1526–1531
Meersseman W, Van Wijngaerden E (2007) Invasive aspergillosis in the ICU: an emerging disease. Intensive Care Med 33(10):1679–1681
Meyer MH, Letscher-Bru V et al (2004) Comparison of Mycosis IC/F and plus Aerobic/F media for diagnosis of fungemia by the bactec 9240 system. J Clin Microbiol 42(2):773–777
Miceli MH, Diaz JA et al (2011) Emerging opportunistic yeast infections. Lancet Infect Dis 11(2):142–151
Mikulska M, Calandra T et al (2010) The use of mannan antigen and anti-mannan antibodies in the diagnosis of invasive candidiasis: recommendations from the third European conference on infections in leukemia. Crit Care 14(6):R222
Millon L, Grenouillet F et al (2011) Ribosomal and mitochondrial DNA target for real-time PCR diagnosis of invasive aspergillosis. J Clin Microbiol 49 (3):1058–1063
Mohr JF, Sims C et al (2011) Prospective survey of (1–>3)-beta-D-glucan and its relationship to invasive candidiasis in the surgical intensive care unit setting. J Clin Microbiol 49(1):58–61
Moriarty B, Hay R et al (2012) The diagnosis and management of tinea. BMJ 345:e4380
Morrell RM Jr, Wasilauskas BL et al (1996) Performance of fungal blood cultures by using the Isolator collection system: is it cost-effective? J Clin Microbiol 34(12):3040–3043
Muller FM, Trusen A et al (2002) Clinical manifestations and diagnosis of invasive aspergillosis in immunocompromised children. Eur J Pediatr 161 (11):563–574
Nenoff P, Erhard M et al (2013) MALDI-TOF mass spectrometry – a rapid method for the identification of dermatophyte species. Med Mycol 51(1): 17–24
Nilsson RH, Kristiansson E et al (2008) Intraspecific ITS variability in the kingdom fungi as expressed in the international sequence databases and its implications for molecular species identification. Evol Bioinform Online 4:193–201
Nilsson RH, Ryberg M et al (2006) Taxonomic reliability of DNA sequences in public sequence databases: a fungal perspective. PLoS One 1:e59
O'Connell S, Walsh G (2008) Application relevant studies of fungal beta-galactosidases with potential application in the alleviation of lactose intolerance. Appl Biochem Biotechnol 149(2):129–138
O'Donnell K, Cigelnik E (1997) Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Mol Phylogenet Evol 7(1):103–116
O'Donnell K, Kistler HC et al (1998) Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. Proc Natl Acad Sci USA 95(5):2044–2049
Osawa R, Alexander BD et al (2010) Geographic differences in disease expression of cryptococcosis in solid organ transplant recipients in the United States. Ann Transplant 15(4):77–83
Ostrosky-Zeichner L (2012) Invasive mycoses: diagnostic challenges. Am J Med 125(1 Suppl):S14–S24
Ostrosky-Zeichner L, Kullberg BJ et al (2011) Early treatment of candidemia in adults: a review. Med Mycol 49(2):113–120
Oz Y, Kiraz N (2011) Diagnostic methods for fungal infections in pediatric patients: microbiological, serological and molecular methods. Expert Rev Anti Infect Ther 9(3):289–298
Pappas PG, Silveira FP et al (2009) *Candida* in solid organ transplant recipients. Am J Transplant 9 (Suppl 4):S173–S179
Park BJ, Wannemuehler KA et al (2009) Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. AIDS 23(4):525–530
Pasqualotto AC, Xavier MO et al (2010) Diagnosis of invasive aspergillosis in lung transplant recipients by detection of galactomannan in the bronchoalveolar lavage fluid. Transplantation 90 (3):306–311
Patterson TF (2003) Aspergillosis. In: Dismukes WE, Pappas PG, Sobel JD (eds) Clinical mycology. Oxford University Press, New York, pp 221–240
Peman J, Zaragoza R (2012) Combined use of nonculture-based lab techniques in the diagnosis and management of critically ill patients with invasive fungal infections. Expert Rev Anti Infect Ther 10(11):1321–1330
Pemán J, Zaragoza R, Quindós G, Alkorta M, Cuétara MS, Camarena JJ, Ramírez P, Giménez MJ, Martín-Mazuelos E, Linares-Sicilia MJ, Pontón J; study group *Candida albicans* Germ Tube Antibody Detection in Critically Ill Patients (2011) Clinical factors associated with a *Candida albicans* Germ Tube Antibody positive test in Intensive Care Unit

patients. BMC Infect Dis 11:60. doi:10.1186/1471-2334-11-60

Pfaller M, Boyken L et al (2011) Comparison of the broth microdilution methods of the European Committee on Antimicrobial Susceptibility Testing and the Clinical and Laboratory Standards Institute for testing itraconazole, posaconazole, and voriconazole against *Aspergillus* isolates. J Clin Microbiol 49(3):1110–1112

Pfaller MA (2012) Antifungal drug resistance: mechanisms, epidemiology, and consequences for treatment. Am J Med 125(1 Suppl):S3–S13

Pfaller MA, Boyken L et al (2004) Evaluation of the etest method using Mueller-Hinton agar with glucose and methylene blue for determining amphotericin B MICs for 4,936 clinical isolates of *Candida* species. J Clin Microbiol 42(11):4977–4979

Pfaller MA, Chaturvedi V et al (2008) Clinical evaluation of the Sensititre YeastOne colorimetric antifungal panel for antifungal susceptibility testing of the echinocandins anidulafungin, caspofungin, and micafungin. J Clin Microbiol 46(7):2155–2159

Pfaller MA, Diekema DJ (2005) Unusual fungal and pseudofungal infections of humans. J Clin Microbiol 43(4):1495–1504

Pfaller MA, Diekema DJ (2012) Progress in antifungal susceptibility testing of *Candida* spp. by use of Clinical and Laboratory Standards Institute broth microdilution methods, 2010 to 2012. J Clin Microbiol 50(9):2846–2856

Pfaller MA, Diekema DJ et al (2007) Multicenter comparison of the VITEK 2 antifungal susceptibility test with the CLSI broth microdilution reference method for testing amphotericin B, flucytosine, and voriconazole against *Candida* spp. J Clin Microbiol 45(11):3522–3528

Pfaller MA, Messer SA et al (2000) In vitro susceptibility testing of filamentous fungi: comparison of Etest and reference microdilution methods for determining itraconazole MICs. J Clin Microbiol 38(9):3359–3361

Pfeiffer CD, Fine JP et al (2006) Diagnosis of invasive aspergillosis using a galactomannan assay: a meta-analysis. Clin Infect Dis 42(10):1417–1427

Posteraro B, Martucci R et al (2009) Reliability of the Vitek 2 yeast susceptibility test for detection of in vitro resistance to fluconazole and voriconazole in clinical isolates of *Candida albicans* and *Candida glabrata*. J Clin Microbiol 47(6):1927–1930

Quindos G, Moragues MD et al (2004) Is there a role for antibody testing in the diagnosis of invasive candidiasis? Rev Iberoam Micol 21(1):10–14

Rabeneck L, Crane MM et al (1993) A simple clinical staging system that predicts progression to AIDS using CD4 count, oral thrush, and night sweats. J Gen Intern Med 8(1):5–9

Rakeman JL, Bui U et al (2005) Multilocus DNA sequence comparisons rapidly identify pathogenic molds. J Clin Microbiol 43(7):3324–3333

Revankar SG (2007) Dematiaceous fungi. Mycoses 50 (2):91–101

Richardson MD, Warnock DW (2003) Fungal infection: diagnosis and management. Blackwell, Massachusetts

Robert V, Stegehuis G et al (2005) The MycoBank engine and related databases. http://de.mycobank.org

Rodriguez-Tudela JL, Arendrup MC et al (2010) EUCAST breakpoints for antifungals. Drug News Perspect 23(2):93–97

Rosa C, Araujo R et al (2011) Detection of *Aspergillus* species in BACTEC blood cultures. J Med Microbiol 60(Pt 10):1467–1471

Ruchel R (1993) Diagnosis of invasive mycoses in severely immunosuppressed patients. Ann Hematol 67(1):1–11

Ruchel R, Schaffrinski M (1999) Versatile fluorescent staining of fungi in clinical specimens by using the optical brightener Blankophor. J Clin Microbiol 37 (8):2694–2696

Sachse S, Straube E et al (2009) Truncated human cytidylate-phosphate-deoxyguanylate-binding protein for improved nucleic acid amplification technique-based detection of bacterial species in human samples. J Clin Microbiol 47(4):1050–1057

Sanchez-Portocarrero J, Perez-Cecilia E et al (2000) The central nervous system and infection by *Candida* species. Diagn Microbiol Infect Dis 37 (3):169–179

Semelka RC, Shoenut JP et al (1992) Detection of acute and treated lesions of hepatosplenic candidiasis: comparison of dynamic contrast-enhanced CT and MR imaging. J Magn Reson Imaging 2(3): 341–345

Shibuya K, Ando T et al (2004) Pathophysiology of pulmonary aspergillosis. J Infect Chemother 10 (3):138–145

Soubani AO, Miller KB et al (1996) Pulmonary complications of bone marrow transplantation. Chest 109 (4):1066–1077

Spanu T, Posteraro B et al (2012) Direct maldi-tof mass spectrometry assay of blood culture broths for rapid identification of *Candida* species causing bloodstream infections: an observational study in two large microbiology laboratories. J Clin Microbiol 50(1):176–179

Springer J, Loeffler J et al (2011) Pathogen-specific DNA enrichment does not increase sensitivity of PCR for diagnosis of invasive aspergillosis in neutropenic patients. J Clin Microbiol 49(4):1267–1273

Steinmann J, Buer J et al (2009) Invasive aspergillosis in two liver transplant recipients: diagnosis by SeptiFast. Transpl Infect Dis 11(2):175–178

Stevenson LG, Drake SK et al (2010) Evaluation of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of clinically important yeast species. J Clin Microbiol 48(10):3482–3486

Szekely A, Johnson EM et al (1999) Comparison of E-test and broth microdilution methods for antifungal drug susceptibility testing of molds. J Clin Microbiol 37(5):1480–1483

Thornton C, Johnson G et al (2012) Detection of invasive pulmonary aspergillosis in haematological malignancy patients by using lateral-flow technology. J Vis Exp 61:3721

Tsitsikas DA, Morin A et al (2012) Impact of the revised (2008) EORTC/MSG definitions for invasive fungal disease on the rates of diagnosis of invasive aspergillosis. Med Mycol 50(5):538–542

Ullmann AJ, Lipton JH et al (2007) Posaconazole or fluconazole for prophylaxis in severe graft-versus-host disease. N Engl J Med 356(4):335–347

van den Berg JM, van Koppen E et al (2009) Chronic granulomatous disease: the European experience. PLoS One 4(4):e5234

van Veen SQ, Claas EC et al (2010) High-throughput identification of bacteria and yeast by matrix-assisted laser desorption ionization-time of flight mass spectrometry in conventional medical microbiology laboratories. J Clin Microbiol 48(3):900–907

Vanek J, Jirovec O (1952) Parasitic pneumonia. Interstitial plasma cell pneumonia of premature, caused by *Pneumocystis carinii*. Zentralbl Bakteriol Parasitenkd Infektionskr Hyg 158(1–2):120–127

Vehreschild JJ, Birtel A et al (2013) Mucormycosis treated with posaconazole: review of 96 case reports. Crit Rev Microbiol 39:310–324

Vyzantiadis TA, Johnson EM et al (2012) From the patient to the clinical mycology laboratory: how can we optimise microscopy and culture methods for mould identification? J Clin Pathol 65(6):475–483

Wah TM, Moss HA et al (2003) Pulmonary complications following bone marrow transplantation. Br J Radiol 76(906):373–379

Walsh TJ, Lutsar I et al (2002a) Voriconazole in the treatment of aspergillosis, scedosporiosis and other invasive fungal infections in children. Pediatr Infect Dis J 21(3):240–248

Walsh TJ, Pappas P et al (2002b) Voriconazole compared with liposomal amphotericin B for empirical antifungal therapy in patients with neutropenia and persistent fever. N Engl J Med 346(4):225–234

Warkentien T, Crum-Cianflone NF (2010) An update on *Cryptococcus* among HIV-infected patients. Int J STD AIDS 21(10):679–684

Westh H, Lisby G et al (2009) Multiplex real-time PCR and blood culture for identification of bloodstream pathogens in patients with suspected sepsis. Clin Microbiol Infec 15(69):544–551

Wheat LJ (2003) Rapid diagnosis of invasive aspergillosis by antigen detection. Transpl Infect Dis 5 (4):158–166

Wheat LJ, Walsh TJ (2008) Diagnosis of invasive aspergillosis by galactomannan antigenemia detection using an enzyme immunoassay. Eur J Clin Microbiol Infect Dis 27(4):245–251

White PL, Mengoli C et al (2011) Evaluation of *Aspergillus* PCR protocols for testing serum specimens. J Clin Microbiol 49(11):3842–3848

White PL, Perry MD et al (2010) Critical stages of extracting DNA from *Aspergillus* fumigatus in whole-blood specimens. J Clin Microbiol 48(10):3753–3755

Wiederhold NP (2009) Paradoxical echinocandin activity: a limited in vitro phenomenon? Med Mycol 47 (Suppl 1):S369–S375

Wieser A, Schneider L et al (2012) MALDI-TOF MS in microbiological diagnostics-identification of microorganisms and beyond (mini review). Appl Microbiol Biotechnol 93(3):965–974

Willinger B (2006) Laboratory diagnosis and therapy of invasive fungal infections. Curr Drug Targets 7 (4):513–522

Willinger B, Hillowoth C et al (2001) Performance of Candida ID, a new chromogenic medium for presumptive identification of *Candida* species, in comparison to CHROMagar *Candida*. J Clin Microbiol 39(10):3793–3795

Willinger B, Manafi M (1999) Evaluation of CHROMagar *Candida* for rapid screening of clinical specimens for *Candida* species. Mycoses 42(1–2):61–65

Wilson LS, Reyes CM et al (2002) The direct cost and incidence of systemic fungal infections. Value Health 5(1):26–34

Winkelstein JA, Marino MC et al (2000) Chronic granulomatous disease. Report on a national registry of 368 patients. Med (Baltimore) 79(3):155–169

Yeo SF, Wong B (2002) Current status of nonculture methods for diagnosis of invasive fungal infections. Clin Microbiol Rev 15(3):465–484

Treatment of Fungal Infections

14 Therapeutic Strategies in Fungal Infections

Maria J.G.T. Vehreschild[1], Kerstin Wahlers[1], Oliver A. Cornely[2]

CONTENTS

I. Definition of Antifungal Treatment Strategies

In the care of patients at risk for invasive aspergillosis (IA), candidiasis (IC), or other invasive fungal diseases (IFD), clinicians may consider different treatment strategies. The choice for or against a particular strategy should take risk for development of IFD, timing of treatment initiation, and diagnostic aspects into account. Four terms are commonly used in the literature to facilitate classification and communication on the subject: prophylactic, empiric, pre-emptive, and targeted treatment (Fig. 14.1). In the **prophylactic treatment** situation, an antifungal agent is administered to patients at risk for IFD, but in the absence of any attributable signs and symptoms. Depending on the local incidence of IFD, this may require treatment of a considerable number of patients who may never develop IFD. **Empiric treatment** is commonly defined as antifungal treatment in patients at risk of IFD and with established clinical signs and symptoms. In clinical practice, persistent fever under broad-spectrum antibiotics is the most common trigger for initiation of empiric antifungal treatment, particularly in the neutropenic patient. If results of a diagnostic workup (e.g., chest CT scans, blood cultures, or galactomannan testing) have yielded results suspicious of IFD, the treatment situation is commonly termed **pre-emptive**. A **targeted treatment** strategy may be applied if diagnostic criteria allow for definite pathogen identification, i.e. culture from a physiologically sterile site or histopathological evidence of IFD.

In clinical practice, these situations may often overlap, making an exact classification difficult. However, the implied categorization of the probability of IFD can be of considerable help in the evaluation and choice of antifungal treatment for particular patients and entire patient populations.

II. Prophylaxis

IFD have a profoundly negative impact on patient survival (Gudlaugsson et al. 2003). If treatment is delayed, mortality increases even further (Morrell et al. 2005; Garey et al. 2006). This is a severe clinical problem in the context

[1]Department I of Internal Medicine, Hospital of the University of Cologne, Kerpener Strasse 62, Köln 50937, Germany
[2]Department I of Internal Medicine, Hospital of the University of Cologne, Kerpener Strasse 62, Köln 50937, Germany; e-mil: oliver.corely@ctuc.e

Human Fungal Pathogens, 2nd Edition
The Mycota XII
O. Kurzai (Ed.)

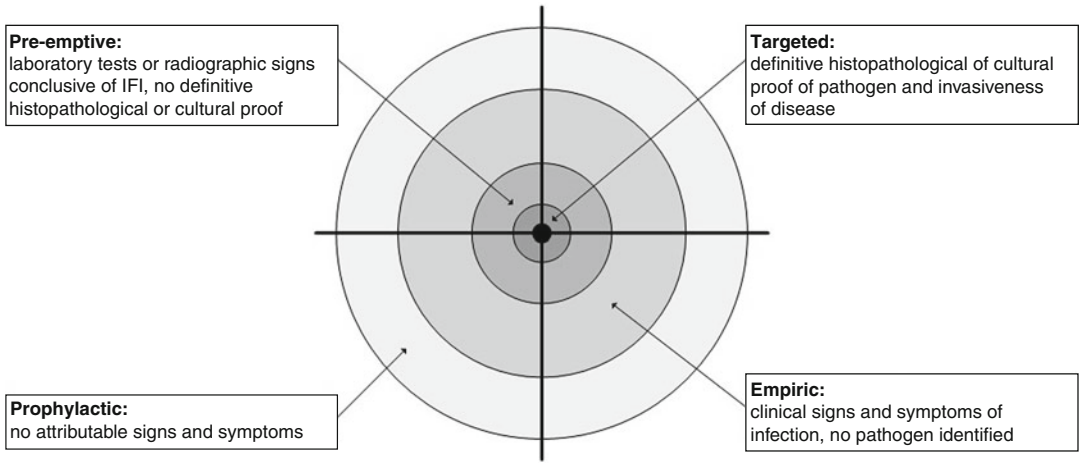


Fig. 14.1. Antifungal treatment strategies for patients at high risk for fungal infection [Taken from Ruping et al. (2008)]

of unreliable diagnostic tools, such that the concept of prophylaxis becomes attractive.

Prophylactic strategies follow the a priori risk assessment of a patient, or – more likely – a patient group because prophylaxis should be an institutional policy rather than a case-by-case decision.

Classical immunosuppressed patient groups, i.e., long-lasting neutropenia and allogeneic hematopoietic stem cell transplant (HSCT) recipients, need a different approach to patients in intensive care or undergoing abdominal surgery.

A. Neutropenia and Hematopoietic Stem Cell Transplantation

Neutropenic patients and hematopoietic stem cell recipients have been treated prophylactically in about 100 clinical trials (Cornely et al. 2003). Some of these trials showed convincing benefits. In the mid-1990s, fluconazole was shown to improve survival in stem cell transplantation (Slavin et al. 1995; Marr et al. 2000). It almost instantly became standard in many transplant protocols, although lacking activity against filamentous fungi, specifically aspergillosis. The logical next step were randomized comparisons of fluconazole versus itraconazole. In stem cell transplantation, itraconazole improved protection from aspergillosis, but did not lead to a further increase in survival rates. Approximately one third of the patients receiving itraconazole had gastrointestinal adverse effects and were finally unable to tolerate this prophylaxis (Marr et al. 2004b). Micafungin proved to be as effective as fluconazole in the hematopoietic transplant setting (van Burik et al. 2004). Posaconazole was evaluated in a randomized clinical trial in patients immunosuppressed for graft-versus-host disease. Numerically more fungal infections were diagnosed in patients receiving the comparator drug fluconazole, although this difference did not reach statistical significance (Ullmann et al. 2007). In neutropenic patients undergoing chemotherapy for remission induction in acute myelogenous leukemia, posaconazole reduced the incidence rate of invasive fungal infection to a mere 2 %, whereas the comparators fluconazole and itraconazole resulted in a rate of 8 %. **This difference had a profound impact on 100-day survival** (Cornely et al. 2007b). The patient numbers needed to treat to prevent a fungal infection and to prevent a death until day 100 were 16 and 14, respectively, which has been considered low (Cornely and Ullmann 2008). Posaconazole became the standard approach for the above populations.

Other trials compared voriconazole and fluconazole, and voriconazole and itraconazole. Although no benefit of voriconazole over these comparators was demonstrated, voriconazole was non-inferior and better tolerable than itraconazole (Wingard et al. 2010; Marks et al. 2011). Table 14.1 summarizes our recommendations on the use of antifungal prophylaxis in the neutropenic and/or transplanted patient.

B. Intensive Care

In contrast to the patient populations mentioned above, the number of clinical trials in intensive care unit (ICU) populations is very limited. One likely reason is the heterogeneity of patient groups. Today, the patients groups appropriate for prophylaxis have not been defined.

Many trials have aimed to identify risk factors that might enable physicians to predict occurrence of IC; however, none of these risk scores has made its way into clinical practice, so far (Michalopoulos et al. 2003; Ostrosky-Zeichner et al. 2007; Dupont et al. 2003; Shorr et al. 2009; Paphitou et al. 2005). The most promising approach evaluated fluconazole in a placebo-controlled clinical trial in a population of patients who had undergone recent abdominal surgery and who had recurrent gastrointestinal perforations or anastomotic leakages. Intraabdominal candidiasis occurred significantly less often with fluconazole prophylaxis (Eggimann et al. 1999). The study was well designed, and the population was successfully predicted to be at high risk. However, the study was too small to warrant a strong recommendation (Cornely et al. 2012). A larger trial evaluated the comparison in a population of surgical patients with an expected ICU stay of $\geq$3 days. Time to fungal infection was significantly longer under fluconazole prophylaxis (Pelz et al. 2001). From today's perspective, the study used outdated definitions of invasive fungal infection and was conducted in a setting of unusually high *Candida* infection incidence rates. This might have prevented the study from leading to widespread use of fluconazole in this indication (Pelz et al. 2001).

Meta-analyses found fluconazole at 400 mg/day to be superior to placebo in preventing invasive fungal infection in critically ill surgical patients, but could not identify a patient population appropriate for prophylaxis (Cruciani et al. 2005; Shorr et al. 2005; Playford et al. 2006a, b; Vardakas et al. 2006).

In contrast to trials in the hematological settings above, none of the clinical trials in ICU settings have led to reduced mortality.

III. Empiric Treatment

Most studies on empiric antifungal treatment have been performed in the setting of persistent febrile neutropenia. In this setting, amphotericin B deoxycholate (D-AmB) used to be considered the gold standard until it was challenged by three major randomized trials. All three trials used similar composite endpoints, including survival, defervescence during neutropenia, successful treatment of baseline IFD, prevention of breakthrough infections, and absence of early discontinuation due to side effects.

Initially, **D-AmB and liposomal amphotericin B** (L-AmB) were compared in a randomized, double blind, multicenter study. There was no difference in treatment success and survival rates between groups, but breakthrough infections and nephrotoxicity were observed less often in the L-AmB group. Thereafter, L-AmB was established as the new standard treatment in the setting of persistent febrile neutropenia (Walsh et al. 1999).

Voriconazole was compared to this new gold standard in a subsequent open-label, randomized, multicenter trial. Although breakthrough IFD and nephrotoxic events were significantly lower in the voriconazole arm, non-inferiority criteria for voriconazole were not met. In addition, voriconazole was associated with more episodes of transient visual changes and hallucinations (Walsh et al. 2002). Voriconazole was thus not established as an antifungal of choice in the empiric setting.

Finally, **caspofungin and L-AmB** were compared in a double-blind, multinational non-inferiority trial including 1,095 patients with hematological cancer. Overall success rates were similar in both treatment groups, and

Table 14.1 Prophylaxis and treatment of IFD in neutropenic patients

Strategy	Underlying condition	Risk factor	Antifungal	Dose	Reference
Prophylaxis	Acute leukemia or myelodysplastic syndrome	Induction therapy	Posaconazole	200 mg tid po	Cornely et al. (2007b)
	Allogeneic SCT	Neutropenia and immunosuppression	Posaconazole	200 mg tid po	Cornely et al. (2007b); Ullmann et al. (2007)
			Micafungin[a]	50 mg qd iv	van Burik et al. (2004)
Empiric treatment	Total duration of neutropenia >10 days	Persistent fever for >72–96 h	Caspofungin	70 mg loading dose and 50 mg qd iv	Walsh et al. (2004)
			Liposomal amphotericin B	3 mg/kg qd iv	Walsh et al. (1999)
Pre-emptive treatment	Total duration of neutropenia >10 days or prior allogeneic SCT	Laboratory or radiographic results conclusive of IFD	Unknown		NA
Targeted treatment[b]	Any	Any	Voriconazole[c]	6 mg/kg bid loading dose, followed by 4 mg/kg bid iv	Herbrecht et al. (2002)
			Liposomal amphotericin B	3 mg/kg qd iv	Cornely et al. (2007a)

Adapted after Ruping et al. (2008)

SCT stem cell transplantation, *NA* not available, *qd* once daily, *bid* twice a day, *tid* three times a day, *po* by mouth, *iv* intravenously

[a] If oral medication not possible

[b] For proven or probable invasive aspergillosis

[c] If patient is receiving azole prophylaxis, liposomal amphotericin B should be preferred

caspofungin fulfilled statistical criteria for non-inferiority. Furthermore, fewer premature treatment discontinuations due to adverse events were recorded in the caspofungin group (Abzug and Walsh 2004).

Table 14.1 summarizes the outcome of these trials with respect to current empiric treatment recommendations.

The current basis of evidence does not favor empiric antifungal therapy in non-neutropenic ICU patients with blood cultures negative for *Candida* spp. (Zilberberg et al. 2010). Some authors would classify patients with blood cultures positive for *Candida* spp., but pending information on species and susceptibility, as potential recipients of empiric treatment (Cruciani and Serpelloni 2008). However, we prefer to discuss these patients in the context of targeted treatment.

IV. Targeted and Pre-emptive Treatment

A. Invasive Aspergillosis

Three major groups are at high risk for contracting IA: patients with hematologic cancer, recipients of solid organ or stem cell transplants, and a heterogeneous group of otherwise immunosuppressed patients, e.g. by long-term corticosteroid administration, acquired immunodeficiency syndrome without antiretroviral therapy, chronic obstructive pulmonary disease (COPD), or liver failure. In patients outside these risk groups, occurrence of IA is unlikely and suggestive clinical signs and symptoms should be scrutinized to avoid initiation of unwarranted antifungal treatment. Even in patients at risk, a suspected diagnosis of IA should be consolidated by a thorough diagnostic workup. Nevertheless, many patients remain without proof of IFD such that treatment is initiated in a pre-emptive as opposed to a targeted treatment setting. This is also reflected in the design of clinical studies assessing the treatment of IA. Usually, they allow for the inclusion of patients in both pre-emptive and targeted settings. In this section, relevant results from these studies will be presented and discussed.

A milestone randomized, open-label clinical trial focused on immunocompromised patients undergoing allogeneic HSCT, autologous HSCT, or chemotherapy for acute leukemia. As a minimum diagnostic requirement for inclusion into the study and randomization to intravenous treatment with voriconazole or amphotericin B deoxycholate (D-AmB), patients needed to present with an infiltration considered typical of IA (halo or air-crescent sign). Presentation of further microbiological proof of IFD was encouraged, but not compulsory. Based on these criteria, 144 patients in the voriconazole arm and 133 patients in the D-AmB arm were included into the final efficacy analysis. Treatment success had been defined as complete or partial clinical and radiological response and was observed in 53 % and 32 % of patients in the voriconazole and D-AmB groups, respectively. At week 12, survival rates of 71 % and 58 %, respectively, were reported. Based on these findings, the primary endpoint, defined as the non-inferiority of voriconazole as compared with D-AmB at week 12, could be fulfilled. Furthermore, significantly fewer severe drug-related adverse events were observed in the voriconazole arm. This trial established voriconazole as the treatment of choice for IFD in the pre-emptive and targeted treatment setting (Herbrecht et al. 2002).

A consecutive multinational, double-blind trial compared the clinical efficacy of liposomal amphotericin B (L-AmB) 3 mg/kg qd iv (once daily, intravenously) or 10 mg/kg qd iv for 14 days, followed by 3 mg/kg qd iv. Concerning patient eligibility, the same risk factors and diagnostic criteria as in the previously described trial were applied. However, for patients with radiological criteria, only, confirmatory microbiology or pathology results needed to be obtained within 4 days of randomization ("up-grading") to allow for continuation of study treatment. The primary endpoint was defined as favorable outcome, i.e. complete or partial response at the end of study treatment. Secondary endpoints included survival up to 12 weeks and the safety profiles of the treatment regimens.

Favorable response rates of 50 % and 46 % were reported from the 3 and 10 mg/kg group,

respectively, revealing no significant difference. However, significantly higher rates of nephrotoxicity and hypokalemia were observed in the high-dose L-AmB group (Cornely et al. 2007a).

Based on these findings, L-AmB at a dosage of 3 mg/kg qd is commonly recommended as an alternative to voriconazole in the setting of targeted or pre-emptive treatment. Although preference in patients without prior azole therapy and lack of contraindications may be given to voriconazole as first-line treatment, L-AmB may be preferred in patients with prior azole exposition, e.g. those receiving posaconazole prophylaxis.

For both studies, analyses of outcome by presence of diagnostic criteria (radiology, microbiology, and pathology) revealed superior response rates for patients included into the studies on the basis of a halo or air-crescent sign, only (Cornely et al. 2007a; Herbrecht et al. 2002; Greene et al. 2007). In these patients, therapy of IA was probably initiated earlier than in patients who had undergone a thorough workup and presented with further diagnostic criteria at randomization. These findings underline the necessity of early treatment initiation. However, this interpretation should not discourage physicians from completion of further diagnostic procedures, even after initiation of antifungal treatment, because alternative diagnoses might otherwise be missed.

The role of echinocandins as first-line treatment agents for IA has not been satisfactorily assessed. A small number of uncontrolled studies evaluated the role of caspofungin and micafungin in neutropenic cancer patients and/or those undergoing allogeneic SCT. From these studies, success rates between 33 % and 44 % have been reported (Herbrecht et al. 2010; Viscoli et al. 2009; Denning et al. 2006). The large number of severely ill patients enrolled into these studies might be partly responsible for these comparatively unfavorable response rates. Of note, in a phase II dosage escalation study using caspofungin 70, 100, 150 and 200 mg as first-line treatment for IA, escalation was associated with an increase of response to treatment (Cornely et al. 2011). However, further studies will be necessary to consolidate this hypothesis.

Concerning the efficacy of combination antifungal therapy as first-line treatment, there are no results available from properly randomized, controlled clinical trials. However, observational studies have provided some evidence for the use of voriconazole plus caspofungin (Marr et al. 2004a) or L-AmB plus caspofungin as second-line treatment (Raad et al. 2008; Maertens et al. 2006; Caillot et al. 2007).

Based on the presented evidence, a treatment approach for patients at risk of IA has been compiled (Fig. 14.2). Available antifungal agents are given in Table 14.1.

B. Invasive Candidiasis

Although the only reliable diagnosis of IC is currently based on positive results from blood cultures, the true diagnostic yield of this method remains unknown, as no other gold standard has been established so far. This unsatisfactory situation has led to the development of various **surrogate markers and risk scores**, intended for early identification of patients at risk of IC. Similar attempts have been made to identify patients who might profit from early initiation of antifungal prophylaxis for IC (see Sect. II); however, these scoring systems usually do not include any surrogate markers. In the following section, an overview on the most relevant concepts will be given (Table 14.3).

In the 1990s, Pittet et al. identified the high relevance of colonization with *Candida* spp. prior to the development of candidemia on the basis of a prospective cohort study. To identify patients at risk, the *Candida* colonization index (CI) and the corrected *Candida* colonization index (CCI) were developed (Table 14.3) (Pittet et al. 1994). This work established colonization as a crucial risk factor to be incorporated in most successive scoring systems. For example, in a prospective study including surgical ICU patients, the CCI with a threshold of 0.4 was used to identify patients eligible for pre-emptive therapy with fluconazole. In this setting, the incidence of proven IC acquired in the ICU decreased from 2.2 % to 0 % ($P < .001$). This intervention did not lead to a detectable emergence of *Candida* strains resistant to fluconazole (Piarroux et al. 2004).

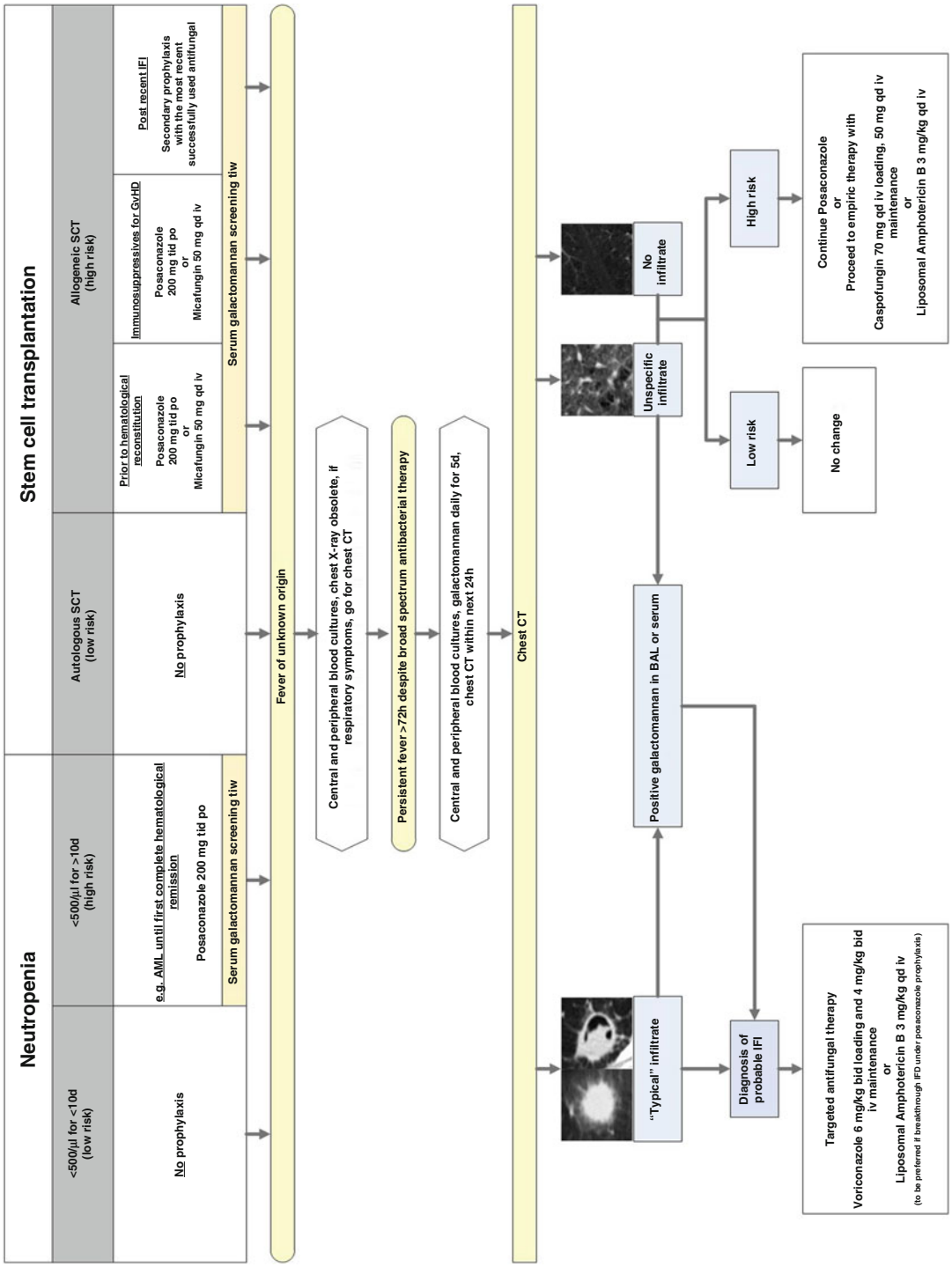


Fig. 14.2. Antifungal prophylaxis and treatment in the neutropenic patient [Adapted after Ruping et al. (2008)]

Table 14.2 Treatment of invasive candidiasis in ICU and neutropenic patients

Indication	Antifungal agent	Dosage	Reference
Non-neutropenic patients	Anidulafungin	200 mg qd loading dose and 100 mg qd maintenance iv	Reboli et al. (2007)
	Micafungin	100 mg qd iv	Kuse et al. (2007); Pappas et al. (2007)
	Caspofungin	70 mg qd loading dose, 50 mg qd maintenance iv	Mora-Duarte et al. (2002); Pappas et al. (2007)
Neutropenic patients	Micafungin	100 mg qd iv	Pappas et al. (2007); Kuse et al. (2007); van Burik et al. (2004)
	Caspofungin	70 mg qd loading dose and 50 mg qd maintenance iv	Mora-Duarte et al. (2002); Abzug and Walsh (2004); Pappas et al. (2007)
Second-line neutropenic and non-neutropenic patients	L-AmB	3 mg/kg qd iv	Kuse et al. (2007)
	Voriconazole	6 mg/kg bid loading dose, followed by 4 mg/kg bid iv	Kullberg et al. (2005)

Adapted after Ruping et al. (2008)
qd once daily, *bid* twice a day, *iv* intravenously

Two further analyses in medical and surgical ICU patients, however, failed to identify an association between previous colonization with *Candida* spp. and patient outcome (Charles et al. 2005; Troughton et al. 2010). It was hypothesized that the identification of further independent risk factors for IC, besides colonization, might result in an improved predictive model.

Consequently, Leon et al. identified four independent risk factors for development of IC in a population of 1,669 non-neutropenic ICU patients. The statistical weight of each of these risk factors was used to create the *Candida* score. Patients with at least 2.5 points were found to be at a 7.75-fold risk of IC and therefore eligible for pre-emptive treatment (Leon et al. 2006). In a follow-up study by the same authors, the optimum cutoff was adjusted to 3 points (Leon et al. 2009). In a more recent study, the authors presented the combination of a β-D-glucan value of >259 pg/ml and a positive *Candida albicans* germ tube antibody test as a reliable predictor of IC (Leon et al. 2012). Table 14.3 provides an overview on studies analyzing scoring systems for pre-emptive treatment.

D-AmB used to be considered the gold standard in the treatment of candidemia until a series of clinical trials assessing the clinical efficacy of less toxic antifungals was initiated in the 1990s with a multicenter, randomized trial comparing the clinical efficacy of D-AmB and fluconazole in the treatment of IC in immunocompetent, non-neutropenic patients. With corresponding response rates of 79 ($n=81$) and 70 % ($n=72$), respectively, there was no significant difference in treatment outcome ($P=0.22$). However, treatment with fluconazole was accompanied by less side effects than with D-AmB (Rex et al. 1994).

Because the proportion of fluconazole-resistant *Candida* non-*albicans* strains (e.g. *Candida krusei* or *Candida glabrata*) has been continuously growing, other antifungals with a broader spectrum than fluconazole have quickly become the focus of further studies. A multicenter, randomized, non-inferiority study compared intravenous voriconazole to D-AmB followed by oral or intravenous fluconazole in non-neutropenic patients with candidemia. The primary endpoint, defined as sustained clinical and mycological response,

Table 14.3 *Candida* spp. pre-emptive treatment scores

Reference	Study type	Inclusion criteria	Number of patients (*n*)	Best rule	Sensitivity (specifity) (%)	PPV (NPV) (%)
Pittet et al. (1994)	Prospective cohort	Colonization with *Candida* spp., defined as detection in three or more samples taken from the same or different body sites on at least two consecutive screening days.	650 screened, 29 included	CI[a] CCI[b]	100 (69) 100 (100)	66 (100) 100 (100)
Piarroux et al. (2004)	Prospective cohort Retrospective cohort	Surgical ICU ≥5 days	Prospective cohort: 899 screened, 413 included Retrospective cohort: 906 screened, 455 included	CCI[b] ≥0.4	NA	NA
Charles et al. (2005)	Prospective cohort	Medical ICU ≥7 days,	593 screened, 92 included	CI[a] ≥0.5	NA	NA
Leon et al. (2006)	Prospective cohort	Medical and/or surgical ICU ≥7 days	1,765 screened, 1,669 included	CS[c] ≥2.5	81 (74)	NA
Leon et al. (2009)	Prospective cohort	Medical and/or surgical ICU ≥7 days	1,205 screened, 1,107 included	CS[c] ≥3	78 (66)	NA
Troughton et al. (2010)	Retrospective cohort	Surgical ICU	3,500 screened, 974 included	*Candida* spp. colonization at two or more non-sterile sites and signs of unexplained sepsis	NA	NA
Leon et al. (2012)	Prospective cohort	Medical and/or surgical ICU ≥7 days, severe abdominal condition	338 screened, 176 included	BDG >259 pg/ml and positive CAGTA	90 (55)	42 (94)

Adapted after Ruping et al. (2008)

PPV positive predictive value, *NPV* negative predictive value, *CI Candida* colonization index, *CCI* corrected *Candida* colonization index, *CS Candida* score, *NA* not available or applicable, *ICU* intensive care unit, *BDG*, β-D-glucan, *CAGTA Candida albicans* germ tube antibody

[a]CI = number of non-blood distinct body sites (dbs) colonized by *Candida* spp/total number of dbs cultured

[b]CCI = CI x number of dbs showing heavy growth/total number of dbs growing *Candida* spp.

[c]CS = 1 point indicates total parenteral nutrition, surgery, or multifocal *Candida* species colonization; CS = 2 points indicates clinically severe sepsis

12 weeks after end of treatment, was reached by 41 % of patients in each group. In the voriconazole group, fewer serious adverse events, especially those classified as renal toxicity were observed (Kullberg et al. 2005).

Similarly favorable results were expected from the newly developed echinocandins. Caspofungin was compared to D-AmB in the treatment of IC in a mixed population of non-neutropenic ($n=200$) and neutropenic patients ($n=24$). With 73.4 % and 61.7 %, respectively, response rates between caspofugin and D-AmB did not differ significantly. Patients in the D-AmB group, however, experienced more nephrotoxic effects and hypokalemia (Mora-Duarte et al. 2002). In a double-blind, randomized, multinational non-inferiority trial, first-line treatment with micafungin and L-AmB was compared in non-neutropenic ($n=469$) and neutropenic ($n=62$) patients. Non-inferiority criteria were met, and micafungin was associated with fewer treatment-related adverse events than L-AmB (Kuse et al. 2007). Another randomized, double-blind trial compared micafungin 100 mg qd ($n=191$) to micafungin 150 mg qd ($n=199$) and the standard dosage of caspofungin ($n=188$) in patients with invasive candidiasis. There was no significant difference in the response rate between the three treatment arms. Therefore, dosages of micafungin 100 mg qd and 150 mg qd were regarded as non-inferior to a standard dosage of caspofungin (Pappas et al. 2007).

Finally, a randomized, double-blind, multicenter trial comparing anidulafungin with fluconazole for the treatment of IC was conducted in a predominantly non-neutropenic population. Significantly higher response rates (75.6 % versus 60.2 %) and better survival (74 % versus 69 %) were documented for patients treated with anidulafungin ($P=0.01$) (Reboli et al. 2007). Results from clinical trials on the treatment of IC are summarized in Table 14.2.

C. Emerging Fungi

As the range of emerging fungi is diverse, only the clinically most important ones will be covered in this section, including Mucorales, *Fusarium* spp., *Scedosporium* spp., and rare yeasts. There is very limited data from controlled randomized clinical trials available for any of the emerging fungi. Available data remains mostly restricted to microbiological studies, case series, and animal studies.

For all patients suffering from infections with emerging fungi, consideration of antifungal and surgical treatment are of primary interest; however, reduction of immunosuppression or time and duration of neutropenia should also be included into any treatment strategy.

Because no data on optimum therapy duration is available for these infections, decisions should be made on an individual basis. In general, complete resolution of clinical and radiological signs and symptoms is recommended before termination of antifungal therapy. Furthermore, antifungal treatment should be continued in the sense of secondary antifungal prophylaxis if subsequent periods of immunosuppression are to be expected.

For initial treatment of mucormycosis, most authors advocate L-AmB at a dosage of at least 5 mg/kg. In addition, the possibility of a surgical resection should be assessed, because response to antifungal treatment is often limited (Rüping et al. 2009; Skiada et al. 2011). Posaconazole at 200 mg po qid (by mouth, four times a day) or 400 mg po bid (by mouth, twice a day) is a valuable option as salvage therapy or if a switch to oral treatment is desired after a satisfactory response to L-AmB (Rüping et al. 2009; van Burik et al. 2006; Greenberg et al. 2006; Skiada et al. 2011). Combination therapy of L-AmB with an echinocandin has shown promising results in murine models of disseminated mucormycosis (Ibrahim et al. 2008; Spellberg et al. 2005), but clinical data remains limited to retrospective cases series (Reed et al. 2008).

One of the few randomized controlled trials conducted in patients with emerging fungal infections assessed the efficacy of deferasirox as an add-on to L-AmB in 20 patients with proven or probable mucormycosis. Unfortunately, death was more frequent in the deferasirox than in the placebo arm at 30 days (45 % versus 11 %, $P=0.1$) and 90 days (82 % versus 22 %, $P=0.01$) (Spellberg et al. 2012).

Influencing the intensity and duration of neutropenia in immunocompromised patients may also impact on patient outcome. Case reports have shown favorable outcomes for patients receiving granulocyte transfusions or granulocyte colony-stimulating factor (G-CSF). Even in non-neutropenic patients with refractory infection, administration of other recombinant growth factors, i.e., granulocyte-macrophage colony-stimulating factor (GM-CSF) or interferon (IFN)-γ is supposed to augment antifungal host responses (Abzug and Walsh 2004; Kullberg and Anaissie 1998; Grigull et al. 2006; Slavin et al. 2002; Gonzalez et al. 1997; Ma et al. 2001; Mastroianni 2004).

Few antifungals seem useful as first-line treatment agents for invasive fusarosis. AmB formulations have long been considered the standard of care for these infections; however, in vitro susceptibility results are not uniformly favorable for all species (Tortorano et al. 2008). Although the echonocandins and older azoles (i.e., fluconazole and itraconazole) display no or minimal activity against *Fusarium* spp., at least some potency has been reported for voriconazole and posaconazole (Gonzalez et al. 2005; Lewis et al. 2005; Paphitou et al. 2002). Furthermore, these new generation azoles have been shown to be effective in animal models of invasive fusariosis (Graybill et al. 2003; Lozano-Chiu et al. 1999; Ruiz-Cendoya et al. 2009). As for L-AmB, susceptibility by species may vary substantially for posaconazole and voriconazole, e.g., *Fusarium verticilloides* displays significantly lower posaconazole minimum inhibitory concentrations (MICs) than other *Fusarium* spp. (Tortorano et al. 2008).

In clinical practice, initial treatment with a combination of L-AmB and a new generation azole may be a safe and effective strategy to avoid problems arising from unexpected variations in susceptibility. Once identification to the species level and resistance testing have been completed, treatment may be reduced to one of the two antifungals.

The duration of neutropenia has been shown to be significantly associated with patient outcome (Nucci et al. 2003), and favorable results were reported from a retrospective study on the efficacy of granulocyte transfusions for the treatment of invasive fusariosis in patients with hematologic malignancies (Boutati and Anaissie 1997). However, granulocyte infusions should be initiated soon after the diagnosis of infection has been established and may only be used to gain time for patients who are soon expected to recover from neutropenia by themselves (Dignani et al. 1997).

Within the genus *Scedosporium*, two hyphomycetes of medical importance have been identified, *Scedosporium apiospermum* and *Scedosporium prolificans*. In the literature, *S. apiospermum* is often referred to by use of its teleomorph (sexual state), *Pseudallescheria boydii*. Infections with *Scedosporium* spp. have been observed in immunocompetent and immunocompromised patients and are particularly difficult to treat if caused by *S. prolificans*, which is largely refractory to antifungal treatment (Cortez et al. 2008; Guarro et al. 2006; Rodriguez-Tudela et al. 2009). Nevertheless, based on a large case series, targeted treatment with voriconazole has been established as first-line treatment for both *S. apiospermum* and *S. prolificans* (Troke et al. 2008). However, antifungal drug susceptibilities of individual isolates are difficult to predict. Therefore, susceptibility testing of clinical isolates remains essential for targeted treatment (Lackner et al. 2012).

In addition to antifungal treatment, improved response rates have been reported for patients undergoing surgical resection of infected tissues (Rodriguez-Tudela et al. 2009). In vitro studies further suggest synergistic activity between neutrophils and certain antifungal agents, i.e., amphotericin B lipid complex and triazoles (Gil-Lamaignere et al. 2002a, b). Because there are no case series on granulocyte transfusions available in the literature, the practical value of these findings remains uncertain. Similarly, in animal models of scedosporiosis, modest protective effects were observed after addition of G-CSF or GM-CSF, but clinical data is currently lacking (Ortoneda et al. 2002, 2004).

The treatment of infections with rare yeasts (e.g., *Trichosporon* spp., *Geotrichum* spp., *Rhodotorula* spp., or *Saccharomyces* spp.) depends largely on the individual susceptibility

Table 14.4 In vitro susceptibility patterns of most frequent emerging yeasts

	Antifungal agent								
Yeast	Isavuconazole	Posaconazole	Voriconazole	Itraconazole	Fluconazole	Flucytosin	Amphotericin B	Echinocandins	References
Trichosporon spp.	S	S	S	SDD/R	R	R	I	R	Thompson et al. (2009); Guinea et al. (2010a); Pfaller et al. (2007); Ruan et al. (2009)
Geotrichum spp.	S	S	S	SDD	S/SDD/R	I	S	R	Thompson et al. (2009); D'Antonio et al. (1996); Girmenia et al. (2003); Pfaller et al. (2007)
Rhodotorula spp.	S/SDD	S/SDD/R	S/SDD/R	SDD/R	R	S	S	R	Thompson et al. (2009); De Almeida et al. (2008); Zaas et al. (2003); Pfaller et al. (2007)
Sachcharomyces spp.	S	S/SDD	S	R	SDD/R	S	S	S	Thompson et al. (2009); Munoz et al. (2005); Guinea et al. (2010a)

S susceptible, *SDD* susceptible dose dependent, *R* resistant, *I* intermediate

pattern of each isolated species. Although some case series have been published, they do not contain any analyses relating in vitro susceptibilities with actual response to treatment (Ruan et al. 2009; Thompson et al. 2010; Guinea et al. 2010b; Girmenia et al. 2003; Zaas et al. 2003). Table 14.4 displays a summary of susceptibility patterns found for the most common emerging yeasts identified in the literature. As for all emerging fungi, susceptibility testing should always be attempted to ensure adequate targeted treatment.

V. Conclusion

In the treatment of patients at risk for invasive aspergillosis (IA), candidiasis (IC), or an emerging invasive fungal disease (IFD), the risk for development of IFD, timing of treatment initiation, and diagnostic aspects should be taken into account. Although physicians should strive for a thorough diagnostic workup, its completion should not delay timely initiation of antifungal treatment, which has a major impact on morbidity and mortality rates. To this end, early treatment strategies, i.e. prophylactic, empiric, and pre-emptive, have been assessed for different risk groups. The results are presented in this chapter and summarized in Tables 14.1 and 14.2, as well as in Fig. 14.2. In the case of a proven IFD, these strategies should be abandoned in favor of an appropriate targeted therapy (Tables 14.1, 14.2 and 14.4).

References

Abzug MJ, Walsh TJ (2004) Interferon-gamma and colony-stimulating factors as adjuvant therapy for refractory fungal infections in children. Pediatr Infect Dis J 23:769–773

Boutati EI, Anaissie EJ (1997) Fusarium, a significant emerging pathogen in patients with hematologic malignancy: ten years' experience at a cancer center and implications for management. Blood 90:999–1008

Caillot D, Thiebaut A, Herbrecht R, de Botton S, Pigneux A, Bernard F, Larche J, Monchecourt F, Alfandari S, Mahi L (2007) Liposomal amphotericin B in combination with caspofungin for invasive aspergillosis in patients with hematologic malignancies: a randomized pilot study (combistrat trial). Cancer 110:2740–2746

Charles PE, Dalle F, Aube H, Doise JM, Quenot JP, Aho LS, Chavanet P, Blettery B (2005) *Candida* spp. colonization significance in critically ill medical patients: a prospective study. Intensive Care Med 31:393–400

Cornely OA, Ullmann AJ (2008) Numbers needed to treat with posaconazole prophylaxis to prevent invasive fungal infection and death. Clin Infect Dis 46:1626–1627; author reply 1627–1628

Cornely OA, Ullmann AJ, Karthaus M (2003) Evidence-based assessment of primary antifungal prophylaxis in patients with hematologic malignancies. Blood 101:3365–3372

Cornely OA, Maertens J, Bresnik M, Ebrahimi R, Ullmann AJ, Bouza E, Heussel CP, Lortholary O, Rieger C, Boehme A, Aoun M, Horst HA, Thiebaut A, Ruhnke M, Reichert D, Vianelli N, Krause SW, Olavarria E, Herbrecht R, AmBiLoad Trial Study (2007a) Liposomal amphotericin B as initial therapy for invasive mold infection: a randomized trial comparing a high-loading dose regimen with standard dosing (AmBiLoad trial). Clin Infect Dis Official Publ Infect Dis Soc Am 44:1289–1297

Cornely OA, Maertens J, Winston DJ, Perfect J, Ullmann AJ, Walsh TJ, Helfgott D, Holowiecki J, Stockelberg D, Goh YT, Petrini M, Hardalo C, Suresh R, Angulo-Gonzalez D (2007b) Posaconazole vs. fluconazole or itraconazole prophylaxis in patients with neutropenia. N Engl J Med 356:348–359

Cornely OA, Vehreschild JJ, Vehreschild MJ, Wurthwein G, Arenz D, Schwartz S, Heussel CP, Silling G, Mahne M, Franklin J, Harnischmacher U, Wilkens A, Farowski F, Karthaus M, Lehrnbecher T, Ullmann AJ, Hallek M, Groll AH (2011) Phase II dose escalation study of caspofungin for invasive Aspergillosis. Antimicrob Agents Chemother 55:5798–5803

Cornely OA, Bassetti M, Calandra T, Garbino J, Kullberg BJ, Lortholary O, Meersseman W, Akova M, Arendrup MC, Arikan-Akdagli S, Bille J, Castagnola E, Cuenca-Estrella M, Donnelly JP, Groll AH, Herbrecht R, Hope WW, Jensen HE, Lass-Flörl C, Petrikkos G, Richardson MD, Roilides E, Verweij PE, Viscoli C, Ullmann AJ, f. t. E. F. I. S. (EFISG) (2012) ESCMID* guideline for the diagnosis and management of *Candida* diseases 2012: non-neutropenic adult patients. CMI 18:19–37

Cortez KJ, Roilides E, Quiroz-Telles F, Meletiadis J, Antachopoulos C, Knudsen T, Buchanan W, Milanovich J, Sutton DA, Fothergill A, Rinaldi MG, Shea YR, Zaoutis T, Kottilil S, Walsh TJ (2008) Infections caused by *Scedosporium* spp. Clin Microbiol Rev 21:157–197

Cruciani M, Serpelloni G (2008) Management of *Candida* infections in the adult intensive care unit. Expert Opin Pharmacother 9:175–191

Cruciani M, de Lalla F, Mengoli C (2005) Prophylaxis of *Candida* infections in adult trauma and surgical

intensive care patients: a systematic review and meta-analysis. Intensive Care Med 31:1479–1487

D'Antonio D, Mazzoni A, Iacone A, Violante B, Capuani MA, Schioppa F, Romano F (1996) Emergence of fluconazole-resistant strains of *Blastoschizomyces capitatus* causing nosocomial infections in cancer patients. J Clin Microbiol 34:753–755

De Almeida GM, Costa SF, Melhem M, Motta AL, Szeszs MW, Miyashita F, Pierrotti LC, Rossi F, Burattini MN (2008) Rhodotorula spp. isolated from blood cultures: clinical and microbiological aspects. Med Mycol 46:547–556

Denning DW, Marr KA, Lau WM, Facklam DP, Ratanatharathorn V, Becker C, Ullmann AJ, Seibel NL, Flynn PM, van Burik JA, Buell DN, Patterson TF (2006) Micafungin (FK463), alone or in combination with other systemic antifungal agents, for the treatment of acute invasive aspergillosis. J Infect 53:337–349

Dignani MC, Anaissie EJ, Hester JP, O'Brien S, Vartivarian SE, Rex JH, Kantarjian H, Jendiroba DB, Lichtiger B, Andersson BS, Freireich EJ (1997) Treatment of neutropenia-related fungal infections with granulocyte colony-stimulating factor-elicited white blood cell transfusions: a pilot study. Leukemia 11:1621–1630

Dupont H, Bourichon A, Paugam-Burtz C, Mantz J, Desmonts JM (2003) Can yeast isolation in peritoneal fluid be predicted in intensive care unit patients with peritonitis? Crit Care Med 31:752–757

Eggimann P, Francioli P, Bille J, Schneider R, Wu MM, Chapuis G, Chiolero R, Pannatier A, Schilling J, Geroulanos S, Glauser MP, Calandra T (1999) Fluconazole prophylaxis prevents intra-abdominal candidiasis in high-risk surgical patients. Crit Care Med 27:1066–1072

Garey KW, Rege M, Pai MP, Mingo DE, Suda KJ, Turpin RS, Bearden DT (2006) Time to initiation of fluconazole therapy impacts mortality in patients with candidemia: a multi-institutional study. Clin Infect Dis 43:25–31

Gil-Lamaignere C, Roilides E, Maloukou A, Georgopoulou I, Petrikkos G, Walsh TJ (2002a) Amphotericin B lipid complex exerts additive antifungal activity in combination with polymorphonuclear leucocytes against *Scedosporium prolificans* and *Scedosporium apiospermum*. J Antimicrob Chemother 50:1027–1030

Gil-Lamaignere C, Roilides E, Mosquera J, Maloukou A, Walsh TJ (2002b) Antifungal triazoles and polymorphonuclear leukocytes synergize to cause increased hyphal damage to *Scedosporium prolificans* and *Scedosporium apiospermum*. Antimicrob Agents Chemother 46:2234–2237

Girmenia C, Pizzarelli G, D'Antonio D, Cristini F, Martino P (2003) In vitro susceptibility testing of *Geotrichum capitatum*: comparison of the E-test, disk diffusion, and Sensititre colorimetric methods with the NCCLS M27-A2 broth microdilution reference method. Antimicrob Agents Chemother 47:3985–3988

Gonzalez CE, Couriel DR, Walsh TJ (1997) Disseminated zygomycosis in a neutropenic patient: successful treatment with amphotericin B lipid complex and granulocyte colony-stimulating factor. Clin Infect Dis Official Publ Infect Dis Soc Am 24:192–196

Gonzalez GM, Fothergill AW, Sutton DA, Rinaldi MG, Loebenberg D (2005) In vitro activities of new and established triazoles against opportunistic filamentous and dimorphic fungi. Med Mycol 43:281–284

Graybill JR, Najvar LK, Gonzalez GM, Hernandez S, Bocanegra R (2003) Improving the mouse model for studying the efficacy of voriconazole. J Antimicrob Chemother 51:1373–1376

Greenberg RN, Mullane K, van Burik JA, Raad I, Abzug MJ, Anstead G, Herbrecht R, Langston A, Marr KA, Schiller G, Schuster M, Wingard JR, Gonzalez CE, Revankar SG, Corcoran G, Kryscio RJ, Hare R (2006) Posaconazole as salvage therapy for zygomycosis. Antimicrob Agents Chemother 50:126–133

Greene RE, Schlamm HT, Oestmann JW, Stark P, Durand C, Lortholary O, Wingard JR, Herbrecht R, Ribaud P, Patterson TF, Troke PF, Denning DW, Bennett JE, de Pauw BE, Rubin RH (2007) Imaging findings in acute invasive pulmonary aspergillosis: clinical significance of the halo sign. Clin Infect Dis 44:373–379

Grigull L, Beilken A, Schmid H, Kirschner P, Sykora KW, Linderkamp C, Donnerstag F, Goudeva L, Heuft HG, Welte K (2006) Secondary prophylaxis of invasive fungal infections with combination antifungal therapy and G-CSF-mobilized granulocyte transfusions in three children with hematological malignancies. Support Care Cancer 14:783–786

Guarro J, Kantarcioglu AS, Horre R, Rodriguez-Tudela JL, Cuenca Estrella M, Berenguer J, de Hoog GS (2006) *Scedosporium apiospermum*: changing clinical spectrum of a therapy-refractory opportunist. Med Mycol 44:295–327

Gudlaugsson O, Gillespie S, Lee K, Vande Berg J, Hu J, Messer S, Herwaldt L, Pfaller M, Diekema D (2003) Attributable mortality of nosocomial candidemia, revisited. Clin Infect Dis 37:1172–1177

Guinea J, Recio S, Escribano P, Pelaez T, Gama B, Bouza E (2010a) In vitro antifungal activities of isavuconazole and comparators against rare yeast pathogens. Antimicrob Agents Chemother 54:4012–4015

Guinea J, Recio S, Escribano P, Torres-Narbona M, Pelaez T, Sanchez-Carrillo C, Rodriguez-Creixems M, Bouza E (2010b) Rapid antifungal susceptibility determination for yeast isolates by use of Etest

performed directly on blood samples from patients with fungemia. J Clin Microbiol 48:2205–2212

Herbrecht R, Denning DW, Patterson TF, Bennett JE, Greene RE, Oestmann JW, Kern WV, Marr KA, Ribaud P, Lortholary O, Sylvester R, Rubin RH, Wingard JR, Stark P, Durand C, Caillot D, Thiel E, Chandrasekar PH, Hodges MR, Schlamm HT, Troke PF, de Pauw B, Invasive Fungal Infections Group of the European Organisation for Research and Treatment of Cancer and the Global Aspergillus Study Group (2002) Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. N Engl J Med 347:408–415

Herbrecht R, Maertens J, Baila L, Aoun M, Heinz W, Martino R, Schwartz S, Ullmann AJ, Meert L, Paesmans M, Marchetti O, Akan H, Ameye L, Shivaprakash M, Viscoli C (2010) Caspofungin first-line therapy for invasive aspergillosis in allogeneic hematopoietic stem cell transplant patients: an European Organisation for Research and Treatment of Cancer study. Bone Marrow Transplant 45:1227–1233

Ibrahim AS, Gebremariam T, Fu Y, Edwards JE Jr, Spellberg B (2008) Combination echinocandin-polyene treatment of murine mucormycosis. Antimicrob Agents Chemother 52:1556–1558

Kullberg BJ, Anaissie EJ (1998) Cytokines as therapy for opportunistic fungal infections. Res Immunol 149:478–488, discussion 515

Kullberg BJ, Sobel JD, Ruhnke M, Pappas PG, Viscoli C, Rex JH, Cleary JD, Rubinstein E, Church LW, Brown JM, Schlamm HT, Oborska IT, Hilton F, Hodges MR (2005) Voriconazole versus a regimen of amphotericin B followed by fluconazole for candidaemia in non-neutropenic patients: a randomised non-inferiority trial. Lancet 366: 1435–1442

Kuse ER, Chetchotisakd P, da Cunha CA, Ruhnke M, Barrios C, Raghunadharao D, Sekhon JS, Freire A, Ramasubramanian V, Demeyer I, Nucci M, Leelarasamee A, Jacobs F, Decruyenaere J, Pittet D, Ullmann AJ, Ostrosky-Zeichner L, Lortholary O, Koblinger S, Diekmann-Berndt H, Cornely OA, G. Micafungin Invasive Candidiasis Working (2007) Micafungin versus liposomal amphotericin B for candidaemia and invasive candidosis: a phase III randomised double-blind trial. Lancet 369:1519–1527

Lackner M, de Hoog GS, Verweij PE, Najafzadeh MJ, Curfs-Breuker I, Klaassen CH, Meis JF (2012) Species-specific antifungal susceptibility patterns of *Scedosporium* and *Pseudallescheria* species. Antimicrob Agents Chemother 56:2635–2642

Leon C, Ruiz-Santana S, Saavedra P, Almirante B, Nolla-Salas J, Alvarez-Lerma F, Garnacho-Montero J, Leon MA (2006) A bedside scoring system ("Candida score") for early antifungal treatment in non-neutropenic critically ill patients with Candida colonization. Crit Care Med 34:730–737

Leon C, Ruiz-Santana S, Saavedra P, Galvan B, Blanco A, Castro C, Balasini C, Utande-Vazquez A, Gonzalez de Molina FJ, Blasco-Navalproto MA, Lopez MJ, Charles PE, Martin E, Hernandez-Viera MA, G. Cava Study (2009) Usefulness of the "Candida score" for discriminating between Candida colonization and invasive candidiasis in non-neutropenic critically ill patients: a prospective multicenter study. Crit Care Med 37:1624–1633

Leon C, Ruiz-Santana S, Saavedra P, Castro C, Ubeda A, Loza A, Martin-Mazuelos E, Blanco A, Jerez V, Ballus J, Alvarez-Rocha L, Utande-Vazquez A, Farinas O (2012) Value of beta-D-glucan and *Candida albicans* germ tube antibody for discriminating between *Candida* colonization and invasive candidiasis in patients with severe abdominal conditions. Intensive Care Med 38:1315–1325

Lewis RE, Wiederhold NP, Klepser ME (2005) In vitro pharmacodynamics of amphotericin B, itraconazole, and voriconazole against *Aspergillus*, *Fusarium*, and *Scedosporium* spp. Antimicrob Agents Chemother 49:945–951

Lozano-Chiu M, Arikan S, Paetznick VL, Anaissie EJ, Loebenberg D, Rex JH (1999) Treatment of murine fusariosis with SCH 56592. Antimicrob Agents Chemother 43:589–591

Ma B, Seymour JF, Januszewicz H, Slavin MA (2001) Cure of pulmonary *Rhizomucor pusillus* infection in a patient with hairy-cell leukemia: role of liposomal amphotericin B and GM-CSF. Leuk Lymphoma 42:1393–1399

Maertens J, Glasmacher A, Herbrecht R, Thiebaut A, Cordonnier C, Segal BH, Killar J, Taylor A, Kartsonis N, Patterson TF, Aoun M, Caillot D, Sable C, G. Caspofungin Combination Therapy Study (2006) Multicenter, noncomparative study of caspofungin in combination with other antifungals as salvage therapy in adults with invasive aspergillosis. Cancer 107:2888–2897

Marks DI, Pagliuca A, Kibbler CC, Glasmacher A, Heussel CP, Kantecki M, Miller PJ, Ribaud P, Schlamm HT, Solano C, Cook G (2011) Voriconazole versus itraconazole for antifungal prophylaxis following allogeneic haematopoietic stem-cell transplantation. Br J Haematol 155:318–327

Marr KA, Seidel K, Slavin MA, Bowden RA, Schoch HG, Flowers ME, Corey L, Boeckh M (2000) Prolonged fluconazole prophylaxis is associated with persistent protection against candidiasis-related death in allogeneic marrow transplant recipients: long-term follow-up of a randomized, placebo-controlled trial. Blood 96:2055–2061

Marr KA, Boeckh M, Carter RA, Kim HW, Corey L (2004a) Combination antifungal therapy for invasive aspergillosis. Clin Infect Dis Official Publ Infect Dis Soc Am 39:797–802

Marr KA, Crippa F, Leisenring W, Hoyle M, Boeckh M, Balajee SA, Nichols WG, Musher B, Corey L (2004b) Itraconazole versus fluconazole for prevention of fungal infections in patients receiving allogeneic stem cell transplants. Blood 103: 1527–1533

Mastroianni A (2004) Paranasal sinus mucormycosis in an immunocompetent host: efficacy and safety of combination therapy with liposomal amphotericin B and adjuvant rHuGM-CSF. Infez Med 12: 278–283

Michalopoulos AS, Geroulanos S, Mentzelopoulos SD (2003) Determinants of candidemia and candidemia-related death in cardiothoracic ICU patients. Chest 124:2244–2255

Mora-Duarte J, Betts R, Rotstein C, Colombo AL, Thompson-Moya L, Smietana J, Lupinacci R, Sable C, Kartsonis N, Perfect J (2002) Comparison of caspofungin and amphotericin B for invasive candidiasis. N Engl J Med 347:2020–2029

Morrell M, Fraser VJ, Kollef MH (2005) Delaying the empiric treatment of candida bloodstream infection until positive blood culture results are obtained: a potential risk factor for hospital mortality. Antimicrob Agents Chemother 49: 3640–3645

Munoz P, Bouza E, Cuenca-Estrella M, Eiros JM, Perez MJ, Sanchez-Somolinos M, Rincon C, Hortal J, Pelaez T (2005) *Saccharomyces cerevisiae* fungemia: an emerging infectious disease. Clin Infect Dis Official Publ Infect Dis Soc Am 40: 1625–1634

Nucci M, Anaissie EJ, Queiroz-Telles F, Martins CA, Trabasso P, Solza C, Mangini C, Simoes BP, Colombo AL, Vaz J, Levy CE, Costa S, Moreira VA, Oliveira JS, Paraguay N, Duboc G, Voltarelli JC, Maiolino A, Pasquini R, Souza CA (2003) Outcome predictors of 84 patients with hematologic malignancies and *Fusarium* infection. Cancer 98:315–319

Ortoneda M, Capilla J, Pujol I, Pastor FJ, Mayayo E, Fernandez-Ballart J, Guarro J (2002) Liposomal amphotericin B and granulocyte colony-stimulating factor therapy in a murine model of invasive infection by *Scedosporium prolificans*. J Antimicrob Chemother 49:525–529

Ortoneda M, Capilla J, Pastor FJ, Serena C, Guarro J (2004) Interaction of granulocyte colony-stimulating factor and high doses of liposomal amphotericin B in the treatment of systemic murine scedosporiosis. Diagn Microbiol Infect Dis 50:247–251

Ostrosky-Zeichner L, Sable C, Sobel J, Alexander BD, Donowitz G, Kan V, Kauffman CA, Kett D, Larsen RA, Morrison V, Nucci M, Pappas PG, Bradley ME, Major S, Zimmer L, Wallace D, Dismukes WE, Rex JH (2007) Multicenter retrospective development and validation of a clinical prediction rule for nosocomial invasive candidiasis in the intensive care setting. Eur J Clin Microbiol Infect Dis 26:271–276

Paphitou NI, Ostrosky-Zeichner L, Paetznick VL, Rodriguez JR, Chen E, Rex JH (2002) In vitro activities of investigational triazoles against *Fusarium* species: effects of inoculum size and incubation time on broth microdilution susceptibility test results. Antimicrob Agents Chemother 46:3298–3300

Paphitou NI, Ostrosky-Zeichner L, Rex JH (2005) Rules for identifying patients at increased risk for candidal infections in the surgical intensive care unit: approach to developing practical criteria for systematic use in antifungal prophylaxis trials. Med Mycol 43:235–243

Pappas PG, Rotstein CM, Betts RF, Nucci M, Talwar D, De Waele JJ, Vazquez JA, Dupont BF, Horn DL, Ostrosky-Zeichner L, Reboli AC, Suh B, Digumarti R, Wu C, Kovanda LL, Arnold LJ, Buell DN (2007) Micafungin versus caspofungin for treatment of candidemia and other forms of invasive candidiasis. Clin Infect Dis Official Publ Infect Dis Soc Am 45:883–893

Pelz RK, Hendrix CW, Swoboda SM, Diener-West M, Merz WG, Hammond J, Lipsett PA (2001) Double-blind placebo-controlled trial of fluconazole to prevent candidal infections in critically ill surgical patients. Ann Surg 233:542–548

Pfaller MA, Diekema DJ, Gibbs DL, Newell VA, Meis JF, Gould IM, Fu W, Colombo AL, Rodriguez-Noriega E, S. Global Antifungal Surveillance (2007) Results from the ARTEMIS DISK Global Antifungal Surveillance study, 1997 to 2005: an 8.5-year analysis of susceptibilities of *Candida* species and other yeast species to fluconazole and voriconazole determined by CLSI standardized disk diffusion testing. J Clin Microbiol 45:1735–1745

Piarroux R, Grenouillet F, Balvay P, Tran V, Blasco G, Millon L, Boillot A (2004) Assessment of preemptive treatment to prevent severe candidiasis in critically ill surgical patients. Crit Care Med 32:2443–2449

Pittet D, Monod M, Suter PM, Frenk E, Auckenthaler R (1994) Candida colonization and subsequent infections in critically ill surgical patients. Ann Surg 220:751–758

Playford EG, Webster AC, Sorrell TC, Craig JC (2006a) Antifungal agents for preventing fungal infections in non-neutropenic critically ill and surgical patients: systematic review and meta-analysis of randomized clinical trials. J Antimicrob Chemother 57:628–638

Playford EG, Webster AC, Sorrell TC, Craig JC (2006b) Antifungal agents for preventing fungal infections in non-neutropenic critically ill patients. Cochrane Database Syst Rev (Online):CD004920

Raad II, Hanna HA, Boktour M, Jiang Y, Torres HA, Afif C, Kontoyiannis DP, Hachem RY (2008) Novel antifungal agents as salvage therapy for invasive

aspergillosis in patients with hematologic malignancies: posaconazole compared with high-dose lipid formulations of amphotericin B alone or in combination with caspofungin. Leukemia 22:496–503

Reboli AC, Rotstein C, Pappas PG, Chapman SW, Kett DH, Kumar D, Betts R, Wible M, Goldstein BP, Schranz J, Krause DS, Walsh TJ (2007) Anidulafungin versus fluconazole for invasive candidiasis. N Engl J Med 356:2472–2482

Reed C, Bryant R, Ibrahim AS, Edwards J Jr, Filler SG, Goldberg R, Spellberg B (2008) Combination polyene-caspofungin treatment of rhino-orbital-cerebral mucormycosis. Clin Infect Dis Official Publ Infect Dis Soc Am 47:364–371

Rex JH, Bennett JE, Sugar AM, Pappas PG, van der Horst CM, Edwards JE, Washburn RG, Scheld WM, Karchmer AW, Dine AP et al (1994) A randomized trial comparing fluconazole with amphotericin B for the treatment of candidemia in patients without neutropenia. Candidemia Study Group and the National Institute. N Engl J Med 331:1325–1330

Rodriguez-Tudela JL, Berenguer J, Guarro J, Kantarcioglu AS, Horre R, de Hoog GS, Cuenca-Estrella M (2009) Epidemiology and outcome of *Scedosporium prolificans* infection, a review of 162 cases. Med Mycol 47:359–370

Ruan SY, Chien JY, Hsueh PR (2009) Invasive trichosporonosis caused by *Trichosporon asahii* and other unusual *Trichosporon* species at a medical center in Taiwan. Clin Infect Dis Official Publ Infect Dis Soc Am 49:e11–e17

Ruiz-Cendoya M, Marine M, Rodriguez MM, Guarro J (2009) Interactions between triazoles and amphotericin B in treatment of disseminated murine infection by *Fusarium oxysporum*. Antimicrob Agents Chemother 53:1705–1708

Ruping MJ, Vehreschild JJ, Cornely OA (2008) Patients at high risk of invasive fungal infections: when and how to treat. Drugs 68:1941–1962

Rüping MJGT, Heinz W, Kindo A, Rickerts V, Lass-Flörl C, Beisel C, Herbrecht R, Roth Y, Silling G, Ullmann AJ, Borchert K, Egerer G, Maertens J, Maschmeyer G, Simon A, Wattad M, Fischer G, Vehreschild JJ, Cornely OA (2009) Forty-one recent cases of invasive zygomycosis from a global clinical registry. J Antimicrob Chemother 65:296–302

Shorr AF, Chung K, Jackson WL, Waterman PE, Kollef MH (2005) Fluconazole prophylaxis in critically ill surgical patients: a meta-analysis. Crit Care Med 33:1928–1935, quiz 1936

Shorr AF, Tabak YP, Johannes RS, Sun X, Spalding J, Kollef MH (2009) Candidemia on presentation to the hospital: development and validation of a risk score. Crit Care 13:R156

Skiada A, Pagano L, Groll A, Zimmerli S, Dupont B, Lagrou K, Lass-Flörl C, Bouza E, Klimko N, Gaustad P, Richardson M, Hamal P, Akova M, Meis JF, Rodriguez-Tudela JL, Roilides E, Mitrousia-Ziouva A, Petrikkos G (2011) Zygomycosis in Europe: analysis of 230 cases accrued by the registry of the European Confederation of Medical Mycology (ECMM) Working Group on Zygomycosis between 2005 and 2007. Clin Microbiol Infect 17:1859–1867

Slavin MA, Osborne B, Adams R, Levenstein MJ, Schoch HG, Feldman AR, Meyers JD, Bowden RA (1995) Efficacy and safety of fluconazole prophylaxis for fungal infections after marrow transplantation–a prospective, randomized, double-blind study. J Infect Dis 171:1545–1552

Slavin MA, Kannan K, Buchanan MR, Sasadeusz J, Roberts AW (2002) Successful allogeneic stem cell transplant after invasive pulmonary zygomycosis. Leuk Lymphoma 43:437–439

Spellberg B, Fu Y, Edwards JE Jr, Ibrahim AS (2005) Combination therapy with amphotericin B lipid complex and caspofungin acetate of disseminated zygomycosis in diabetic ketoacidotic mice. Antimicrob Agents Chemother 49:830–832

Spellberg B, Ibrahim AS, Chin-Hong PV, Kontoyiannis DP, Morris MI, Perfect JR, Fredricks D, Brass EP (2012) The Deferasirox-AmBisome Therapy for Mucormycosis (DEFEAT Mucor) study: a randomized, double-blinded, placebo-controlled trial. J Antimicrob Chemother 67:715–722

Thompson GR 3rd, Wiederhold NP, Sutton DA, Fothergill A, Patterson TF (2009) In vitro activity of isavuconazole against *Trichosporon, Rhodotorula, Geotrichum, Saccharomyces* and *Pichia* species. J Antimicrob Chemother 64:79–83

Thompson SD, Sudman M, Ramos PS, Marion MC, Ryan M, Tsoras M, Weiler T, Wagner M, Keddache M, Haas JP, Mueller C, Prahalad S, Bohnsack J, Wise CA, Punaro M, Zhang D, Rose CD, Comeau ME, Divers J, Glass DN, Langefeld CD (2010) The susceptibility loci juvenile idiopathic arthritis shares with other autoimmune diseases extend to PTPN2, COG6, and ANGPT1. Arthritis Rheum 62:3265–3276

Tortorano AM, Prigitano A, Dho G, Esposto MC, Gianni C, Grancini A, Ossi C, Viviani MA (2008) Species distribution and in vitro antifungal susceptibility patterns of 75 clinical isolates of *Fusarium* spp. from northern Italy. Antimicrob Agents Chemother 52:2683–2685

Troke P, Aguirrebengoa K, Arteaga C, Ellis D, Heath CH, Lutsar I, Rovira M, Nguyen Q, Slavin M, Chen SC (2008) Treatment of scedosporiosis with voriconazole: clinical experience with 107 patients. Antimicrob Agents Chemother 52:1743–1750

Troughton JA, Browne G, McAuley DF, Walker MJ, Patterson CC, McMullan R (2010) Prior colonisation with *Candida* species fails to guide empirical therapy for candidaemia in critically ill adults. J Infect 61:403–409

Ullmann AJ, Lipton JH, Vesole DH, Chandrasekar P, Langston A, Tarantolo SR, Greinix H, Morais de Azevedo W, Reddy V, Boparai N, Pedicone L, Patino H, Durrant S (2007) Posaconazole or fluconazole for prophylaxis in severe graft-versus-host disease. N Engl J Med 356:335–347

van Burik JA, Ratanatharathorn V, Stepan DE, Miller CB, Lipton JH, Vesole DH, Bunin N, Wall DA, Hiemenz JW, Satoi Y, Lee JM, Walsh TJ (2004) Micafungin versus fluconazole for prophylaxis against invasive fungal infections during neutropenia in patients undergoing hematopoietic stem cell transplantation. Clin Infect Dis Official Publ Infect Dis Soc Am 39:1407–1416

van Burik JA, Hare RS, Solomon HF, Corrado ML, Kontoyiannis DP (2006) Posaconazole is effective as salvage therapy in zygomycosis: a retrospective summary of 91 cases. Clin Infect Dis Official Publ Infect Dis Soc Am 42:e61–e65

Vardakas KZ, Samonis G, Michalopoulos A, Soteriades ES, Falagas ME (2006) Antifungal prophylaxis with azoles in high-risk, surgical intensive care unit patients: a meta-analysis of randomized, placebo-controlled trials. Crit Care Med 34:1216–1224

Viscoli C, Herbrecht R, Akan H, Baila L, Sonet A, Gallamini A, Giagounidis A, Marchetti O, Martino R, Meert L, Paesmans M, Ameye L, Shivaprakash M, Ullmann AJ, Maertens J, Infectious Disease Group of the EORTC (2009) An EORTC phase II study of caspofungin as first-line therapy of invasive aspergillosis in haematological patients. J Chemother 64:1274–1281

Walsh TJ, Finberg RW, Arndt C, Hiemenz J, Schwartz C, Bodensteiner D, Pappas P, Seibel N, Greenberg RN, Dummer S, Schuster M, Holcenberg JS (1999) Liposomal amphotericin B for empirical therapy in patients with persistent fever and neutropenia. National Institute of Allergy and Infectious Diseases Mycoses Study Group. N Engl J Med 340:764–771

Walsh TJ, Pappas P, Winston DJ, Lazarus HM, Petersen F, Raffalli J, Yanovich S, Stiff P, Greenberg R, Donowitz G, Schuster M, Reboli A, Wingard J, Arndt C, Reinhardt J, Hadley S, Finberg R, Laverdiere M, Perfect J, Garber G, Fioritoni G, Anaissie E, Lee J (2002) Voriconazole compared with liposomal amphotericin B for empirical antifungal therapy in patients with neutropenia and persistent fever. N Engl J Med 346:225–234

Walsh TJ, Teppler H, Donowitz GR, Maertens JA, Baden LR, Dmoszynska A, Cornely OA, Bourque MR, Lupinacci RJ, Sable CA, dePauw BE (2004) Caspofungin versus liposomal amphotericin B for empirical antifungal therapy in patients with persistent fever and neutropenia. N Engl J Med 351:1391–1402

Wingard JR, Carter SL, Walsh TJ, Kurtzberg J, Small TN, Baden LR, Gersten ID, Mendizabal AM, Leather HL, Confer DL, Maziarz RT, Stadtmauer EA, Bolanos-Meade J, Brown J, Dipersio JF, Boeckh M, Marr KA (2010) Randomized, double-blind trial of fluconazole versus voriconazole for prevention of invasive fungal infection after allogeneic hematopoietic cell transplantation. Blood 116:5111–5118

Zaas AK, Boyce M, Schell W, Lodge BA, Miller JL, Perfect JR (2003) Risk of fungemia due to *Rhodotorula* and antifungal susceptibility testing of *Rhodotorula* isolates. J Clin Microbiol 41:5233–5235

Zilberberg MD, Kollef MH, Arnold H, Labelle A, Micek ST, Kothari S, Shorr AF (2010) Inappropriate empiric antifungal therapy for candidemia in the ICU and hospital resource utilization: a retrospective cohort study. BMC Infect Dis 10:150

15 Antifungal Pharmacokinetics

Fedja Farowski[1], Oliver A. Cornely[2]

CONTENTS

I. Introduction

The introduction of new antifungal drugs has led to a considerable improvement in the therapeutic options against invasive fungal diseases (IFD). However, despite new targeted antifungal therapies, the attributable mortality of IFD is still high at 5–71% for invasive *Candida* infections (candidiasis) and 60–90% for invasive aspergillosis (IA) (Lin et al. 2001; Falagas et al. 2006; Wisplinghoff et al. 2004). **Unreliable pharmacokinetics, with vast inter- and intra-individual variances in the plasma concentrations, may be one cause for treatment failures**. Hence, there is an increasing demand for therapeutic drug monitoring during antifungal therapies. Understanding the pharmacokinetics and pharmacodynamics of a compound measured is crucial for the interpretation of, e.g., serum concentrations. Knowledge about the tissue distribution of antifungal drugs is often very limited. In some animal models, the ratio of the area under the concentration–time curve (AUC) to minimal inhibitory concentration (MIC) (AUC:MIC ratio), as well as the ratio of peak concentration (C_{max}) to MIC (C_{max}:MIC ratio), and the ratio of C_{max} to the minimum effective concentration (MEC) (C_{max}:MEC ratio) have been suggested as predictors for outcome. However, it is not fully understood which antifungal plasma concentrations are required to effectively treat infections caused by pathogens with a certain MIC in humans (Pound et al. 2010).

II. Pharmacokinetic Profiles of Common Antifungal Drugs

Currently, there are three major classes of systemic antifungal drugs, i.e., the polyenes, the echinocandins, and the azoles, in use for the treatment of IFD. Pharmacokinetic properties of selected antifungal drugs are presented in Table 15.1.

A. Polyenes

Amphotericin B (AmB) and its various formulations, deoxycholate AmB (D-AmB), AmB colloidal dispersion (ABCD), AmB lipid complex (ABLC), and liposomal AmB (L-AmB), belong to the class of polyene antifungals. **For many years, AmB was the "gold standard" and has proven to be a reliable antifungal agent, but a toxic compound.** However, details regarding its

[1]Clinic I for Internal Medizin, Clinical Infectiology, University Hospital Cologne, Cologne 50924, Germany; e-mail: Fedja.Farowski@ctuc.de
[2]Department I of Internal Medicine, Hospital of the University of Cologne, Kerpener Strasse 62, Köln 50937, Germany

Human Fungal Pathogens, 2nd Edition
The Mycota XII
O. Kurzai (Ed.)

Table 15.1 Pharmacokinetic parameters of selected antifungal drugs

Pharmacokinetic Parameter	D-AmB	L-AmB	Anid	Cas	Mica	Flu	Itr	Pos	Vor
Oral bioavailability (%)	< 5	< 5	2-7	< 10	< 10	95	50	8-47	96
$t_{½}$ (h)	50	100-153	24-26	9-11	11-17	31	24	25-31	6
Protein binding (%)	> 95	> 95	> 99	96-97	99.8	10	99.8	99	58
Metabolism	Hepatic	Unknown	None	Hepatic	Hepatic	Hepatic	Hepatic	Hepatic	Hepatic
Elimination	Feces	Unknown	Feces	Urine	Feces	Urine	Hepatic	Feces	Renal
Food effect	n.a.	n.a.	n.a.	n.a.	n.a.	No effect	Solid oral: increased absorption Liquid oral: decreased absorption	Increased absorption (with high fat food)	Decreased absorption
Acid effect	n.a.	n.a.	n.a.	n.a.	n.a.	No effect	Solid oral: increased absorption Liquid oral: No effect	Increased absorption	No effect
Dose adjustment in renal insufficiency	None	None	None	None	None	Decrease Dose	Caution: CrCl < 30 mL/min (IV formul.)	None	Caution: CrCl < 50 mL/min (IV formul.)
Dose adjustment in hepatic insufficiency	None	None	None	Moderate insufficiency: Decrease Dose	None	None	None	None	Moderate cirrhosis: Decrease Dose

Pharmacokinetic parameters of selected antifungal drugs, i.e. deoxycholate Amphotericin B (D-AmB), liposomal Amphotericin B (L-AmB), anidulafungin (Anid), caspofungin (CAS), micafungin (Mica), fluconazole (Flu), intraconazole (Itr), posaconazole (Pos), and voriconazole (Vor).

D-AmB deoxycholate Amphotericin B, *L-AmB* liposomal Amphotericin B, *Anid* anidulafungin, *CAS* caspofungin, *Mica* micafungin, *Flu* fluconazole, *Itr* intraconazole, *Pos* posaconazole *Vor* voriconazole, *n.a.* not applicable, *IV formul.* intravenous formulation containing sulfobutylether-β-cyclodextrin

pharmacokinetics, especially the impact of either renal or hepatic organ dysfunction on its pharmacokinetics, are not available (Lepak and Andes 2011). Thus, whereas **the dose-limiting toxicity is predominantly nephrotoxicity, dose reductions are unlikely to produce sufficient infection site concentrations** (Lepak and Andes 2011). Among the lipid formulations there are marked differences in kinetic profiles (Groll et al. 2000a, 2006; Lewis et al. 2007). Lewis et al. compared the pharmacokinetics of AmB lung accumulation and fungal clearance in ABLC- and L-AmB-treated mice; both formulations were effective in reducing the fungal burden at a dose of $\geq$ 5 mg/kg/day. Though ABLC produced a more rapid antifungal clearance than L-AmB at 5 mg/kg/day, there was no difference at a higher dose of 10 mg/kg/day (Groll et al. 2006). Within the cerebrospinal fluid (CSF), the concentrations of all four polyenes were remarkable similar. L-AmB achieved 6–10 times higher brain tissue concentrations than all other AmB formulations (Groll et al. 2000a). In another study, the distributions of D-AmB, ABCD, ABLC, and L-AmB were investigated. At a dose of 5 mg/kg/day **all AmB lipid formulations achieved at least fourfold higher concentrations in the bone marrow than conventional AmB** at a dose of 1 mg/kg/day. In contrast, all AmB formulations accumulated relatively poorly within fat tissue, with L-AmB achieving the highest absolute concentration (Groll et al. 2000b).

B. Echinocandins

Echinocandins are also only available for intravenous administration. **They all share a low oral bioavailability, high protein binding, and extensive distribution into most tissues including liver, spleen, lungs, and kidneys** (Eschenauer et al. 2007; Dowell et al. 2007; Hebert et al. 2005; Goicoechea et al. 2004; Carver 2004; Pfizer 2006; Merck 2001; Astellas 2005). **However,** due to their high protein binding and large molecular weights, **they only exhibit low penetration into urine, CSF, brain, and ocular fluid** (Vazquez and Sobel 2006; Deresinski and Stevens 2003; Chandrasekar and Sobel 2006; Okugawa et al. 2007). The

pharmacokinetics of all three echinocandins are linear, but whereas caspofungin and micafungin are mainly degraded in the liver by hydrolysis and N-acetylation (Eschenauer et al. 2007), anidulafungin undergoes chemical degradation within the plasma and not within the liver (Pfizer 2006). The mainly inactive metabolites of the echinocandins are then slowly excreted over many days, primarily via the bile. Echinocandins are usually administered once daily and have half-lives ranging from 9 to 26 h, i.e., 9–11 h for caspofungin, 11–17 h for micafungin, and 24–26 h for anidulafungin (Astellas 2005; Merck 2001; Pfizer 2006).

C. Triazoles

Among the currently used triazole antifungals, fluconazole is the only drug that is soluble in water without the need for a solvent. **Fluconazole** is very well absorbed from the gastrointestinal tract and it exhibits very low protein binding and good distribution in all kinds of tissues, including ocular and central nervous system (CNS) sites (Brammer et al. 1990; O'Day et al. 1990; Savani et al. 1987; Mian et al. 1998; Arndt et al. 1988; Foulds et al. 1988). In the CSF, the fluconazole concentrations reach ~70% of the serum concentration. The pharmacokinetics of fluconazole are linear over dosages ranging from 50 to 800 mg/day (McLachlan and Tett 1996; Anaissie et al. 1995). Most of the drug (~80%) is excreted unchanged via the urine; hence, the hepatic CYP2C9 enzyme only plays a minor role in its metabolism. Fluconazole dosage reduction is recommended in cases of marked renal dysfunction (Pfizer 1990).

Voriconazole is available as an oral and as a sulfobutylether-β-cyclodextrin intravenous formulation (Pfizer 2002). Despite its structural similarity to fluconazole it is only poorly soluble in water. The oral bioavailability of voriconazole as tablet or solution is 96%; however, its absorption may be decreased by 22% by the concomitant intake of food. Voriconazole disseminates into most infection sites, including the CNS and eye (Lutsar et al. 2003; Hariprasad et al. 2004). It is metabolized in the liver, mainly by CYP2C19, CYP3A4, and to a lesser extent by CYP2C9; hence, the genotype status for CYP2C19 and/or co-administration of drugs that modulate CYP2C19 or CYP3A4 activities may affect voriconazole plasma levels (Hyland et al. 2003).

The effect of CYP2C19 polymorphism on the pharmacokinetics of voriconazole was studied for healthy CYP2C19 homozygous extensive metabolizers (EMs), heterozygous extensive metabolizers (HEMs), and poor metabolizers (PMs) (Lee et al. 2012). The steady state trough concentrations and AUCs were five and three times higher for PMs than for EMs, respectively. Thus, the CYP2C19 polymorphism is a major covariate and accountable for some of the wide pharmacokinetic variability of voriconazole (Lee et al. 2012). But still, the total variability of voriconazole plasma concentrations is only partly accounted for by sex, age, and CYP2C19 genotype (FDA 2001).

Although voriconazole is usually well tolerated (Walsh et al. 2002; Ally et al. 2001), high concentrations may be associated with visual changes and hepatotoxicity, which mainly manifests by elevation of AST/ALT (Tan et al. 2006). Congruently, various studies have shown that therapeutic drug monitoring of trough concentrations is important as a guide to therapy (Smith et al. 2006; Pascual et al. 2008; Denning et al. 2002). Because the sulfobutylether-β-cyclodextrin, which is used as solvent for the intravenous formulation, may accumulate in patients with impaired renal function extended use of this formulation should be avoided in patients with moderate to severe renal dysfunction, defined as a creatinine clearance (CrCl) below 50 ml/min or a creatinine concentration above 2.5 mg/dl (FDA 2001).

Itraconazole is another member of the triazole antifungal class. Like voriconazole, it is poorly soluble in water at physiological pH, hence its liquid formulations, i.e., the liquid oral (LO) and intravenous formulations, contain cyclodextrin as a solubilizing agent (Janssen 1999). Similar to the cyclodextrin-containing parenteral formulation of voriconazole, the parenteral formulations of itraconazole are indicated in patients with severe renal impairment (defined as CrCl below 30 ml/min) only if the benefit outweighs the risk (Janssen 1999). In patients with mild (defined as CrCl

50–80 ml/min) and moderate (defined as CrCl 30–49 ml/min) renal impairment, parenteral formulations should be used with caution, while monitoring serum creatinine concentrations (Janssen 1999). The pharmacokinetic parameters of the drug are not markedly affected by renal or hepatic dysfunction. Capsules containing itraconazole (i.e., solid oral formulations) do not require any solvent; however, its absorption is influenced by the gastric acidity and significantly varies with the intake of food or acidic beverages (Barone et al. 1993; Jaruratanasirikul and Kleepkaew 1997). The absorption of the LO formulation is not affected by the gastric acidity and, in contrast to the solid oral formulation, is best absorbed without a meal (Janssen 1999; Johnson et al. 2003; Barone et al. 1998; Van de Velde et al. 1996). One study compared the relative bioavailability of the solid oral and LO formulation in adult cystic fibrosis patients; relative bioavailability for itraconazole capsules was 82% compared with the solution (Hennig et al. 2007). Nausea and diarrhea is more common with the LO formulation, possibly due to the osmotic effects of the cyclodextrin. The distribution of itraconazole into most infection sites, except the CNS and eye, is good (Janssen 1999; Savani et al. 1987). The pharmacokinetics of itraconazole have been described using nonlinear (Hardin et al. 1988) and linear concentration–time profile models (Hardin et al. 1988; Hennig et al. 2006, 2007; Koks et al. 2003). In healthy volunteers, the terminal half-life was 24 h, and the steady state was reached after 13–14 days (Hardin et al. 1988). Itraconazole is mainly metabolized within the liver and/or the intestine, facilitating the CYP3A4 pathway (Poirier and Cheymol 1998). The first step consists of the formation of hydroxy-itraconazole (OH-itraconazole), which, unlike metabolites of other azoles, exhibits similar antifungal activity to the native drug (Odds and Bossche 2000). Later on, this metabolite is converted to the inactive keto-itraconazole and *N*-dealkyl-itraconazole (ND-itraconazole). Both active compounds (i.e., itraconazole and OH-itraconazole) usually circulate at comparable concentrations, whereas the concentrations of keto-itraconazole and ND-itraconazole are significantly lower (Templeton et al. 2008). Itraconazole is predicted to partially saturate CYP3A4, but its metabolites, and ND-itraconazole in particular, also contribute to inhibition of CYP3A4-mediated metabolism (Templeton et al. 2008). Because the half-life of ND-itraconazole appears to be longer it might be responsible for the clinically observed persistent inhibition of CYP3A4-mediated clearance of immunosuppressants co-administered with itraconazole (Templeton et al. 2008; Banerjee et al. 2001; Kwan et al. 1987).

Posaconazole is currently only available as an oral solution (Schering 2006), but solid oral and intravenous formulations are in development (Merck 2010; Merck 2013). Posaconazole displays linear pharmacokinetics between 50 and 800 mg, with saturation of absorption occurring above 800 mg/day (Courtney et al. 2003). The terminal half-life ranges from 25 to 31 h, supporting once- or twice-daily dosing (Courtney et al. 2003). However, divided daily doses (every 12 or 6 h) significantly increases the posaconazole exposure in healthy adults under fasting conditions (Ezzet et al. 2005), while administration with food also increases the bioavailability (Courtney et al. 2004). The relative oral bioavailability of posaconazole significantly depends on the prandial state. When posaconazole is administered with a high-fat or non-fat meal, its mean AUC (0, 72 h) and maximum concentrations (C_{max}) are increased fourfold and 2.6- to 3.0-fold, respectively, compared with administration in the fasting state (Courtney et al. 2004). Still, the pharmacokinetics of posaconazole are not very predictable and subject to huge inter-individual variability (Smith et al. 2009). The elimination of posaconazole is increased in patients with diarrhea (Kohl et al. 2010). However, despite the relatively frequent occurrence of low posaconazole serum concentrations, no cut-off for its prophylactic efficacy has yet been established. Therefore, it is currently unclear whether therapeutic drug monitoring (TDM) may increase its clinical efficacy (Kohl et al. 2010; Hope et al. 2008a). Posaconazole is mainly metabolized by hepatic glucuronidation, but not by the CYP450 system enzymes; however,

it is a moderate inhibitor of CYP3A4 (Wexler et al. 2004). The drug is primarily excreted via the bile and the urine as unchanged drug or inactive metabolites.

Common to all systemic triazole antifungals is that they are inhibitors of cytochrome P450 (CYP) isozymes, such as CYP3A4, CYP2C9, and CYP2C19, to varying degrees. Whereas fluconazole and voriconazole inhibit CYP2C9, CYP2C19, and CYP3A4; itraconazole and posaconazole only inhibit CYP3A4. Some triazoles are substrates (fluconazole, itraconazole, and posaconazole) and/or inhibitors (itraconazole) of drug transporters such as the multidrug resistance-1 gene product, P-glycoprotein (Nivoix et al. 2008). A detailed review of this important topic has been published by Nivoix et al. (2008).

III. Pharmacodynamics of Common Antifungal Drugs

Different indices are commonly used to measure or describe the relationship between the pharmacokinetic parameters of a drug and the MIC of a pathogen. Various studies have been undertaken to address the question of which index best describes the activity of a certain antifungal drug against different pathogens in order to predict treatment outcomes. For AmB it was shown that the C_{max}:MIC is closely linked to treatment efficacy, with drug concentrations exceeding the MIC two- to tenfold (Andes et al. 2001; Lewis et al. 2005; Wiederhold et al. 2006).

In a neutropenic mouse model of disseminated candidiasis, AmB efficacy correlated with the percentage of time above MIC (%t>MIC), AUC:MIC, and C_{max}:MIC (Andes et al. 2001). The best predictive index to determine the treatment outcome was C_{max}:MIC, whereas the AUC:MIC and %t>MIC were only slightly less predictive (Andes et al. 2001). For filamentous fungi, a species-specific concentration-dependent activity of AmB (Lewis et al. 2005) and a close correlation between increased survival and C_{max}:MIC ratio was shown (Wiederhold et al. 2006). For the echinocandins, similar results regarding their activity against *Candida* spp. have been shown, with a maximum efficacy at free-drug AUC:MIC ratios close to 10 (Ernst et al. 1999, 2002a; Andes et al. 2008a, b, 2003a; Andes and Safdar 2005; Gumbo et al. 2006, 2007; Groll et al. 2001; Hope et al. 2008b; Clancy et al. 2006; Walsh et al. 1991). In a murine model of invasive pulmonary aspergillosis, a C_{max}:MEC ratio for caspofungin appeared to be the parameter most closely associated with the reduction of pulmonary fungal burden (Wiederhold et al. 2004). However, increasing drug concentration of echinocandins above the organism's MIC may result in a paradoxical increase of in vitro fungal growth (Pound et al. 2010). For the triazole antifungals, exposure–response relationships are well studied, with concentrations one to two times above the MIC showing optimal time–dependent (t>MIC) antifungal activity (Turnidge et al. 1994; Ernst et al. 2000, 2002b; Andes and van Ogtrop 1999; Andes et al. 2003b, c, 2004, 2006; Ernst et al. 1998; Warn et al. 2009; Louie et al. 1998). A study on the pharmacodynamics of fluconazole in patients with candidemia showed that AUC:MIC ratios below 11.5 were associated with an increased mortality (Baddley et al. 2008). For multiple *Candida* isolates, efficacy was observed with free-drug 24 h AUC:MIC ratios near 25 (Lepak and Andes 2011). The in vivo efficacy of posaconazole against four clinical *Aspergillus fumigatus* isolates, with posaconazole MICs ranging from 0.03 to 16 mg/l, was assessed in a non-neutropenic murine model of disseminated aspergillosis. Near-maximum and 50% survival were reached at AUC:MIC ratios of nearly 1,000 (corresponding to a free-drug AUC:MIC ratio of 10) and 321.3, respectively (Mavridou et al. 2010). Similar results were observed using a murine inhalational model of invasive pulmonary aspergillosis, with a dose-dependent decline in serum galactomannan concentrations. AUC:MIC ratios of 89.88, 166.90, 308.51, and 440.91 resulted in 20%, 50%, 80%, and 90% of the maximum antifungal effect, respectively (Howard et al. 2011).

Transparency Declarations

FF received travel grants from Astellas and Schering-Plough. OAC is supported by the German Federal Ministry of Research and Education (BMBF grant 01KN1106) and has received research grants from, is an advisor to, or received lecture honoraria from 3 M, Actelion, Astellas, Basilea, Bayer, Biocryst, Celgene, Cubist, F2G, Genzyme, Gilead, GSK, Merck/Schering, Miltenyi, Optimer, Pfizer, Quintiles, Sanofi Pasteur, Viropharma.

References

Ally R, Schurmann D, Kreisel W, Carosi G, Aguirrebengoa K, Dupont B, Hodges M, Troke P, Romero AJ (2001) A randomized, double-blind, double-dummy, multicenter trial of voriconazole and fluconazole in the treatment of esophageal candidiasis in immunocompromised patients. Clin Infect Dis 33:1447–1454

Anaissie EJ, Kontoyiannis DP, Huls C, Vartivarian SE, Karl C, Prince RA, Bosso J, Bodey GP (1995) Safety, plasma concentrations, and efficacy of high-dose fluconazole in invasive mold infections. J Infect Dis 172:599–602

Andes D, Safdar N (2005) Efficacy of micafungin for the treatment of candidemia. Eur J Clin Microbiol Infect Dis 24:662–664

Andes D, van Ogtrop M (1999) Characterization and quantitation of the pharmacodynamics of fluconazole in a neutropenic murine disseminated candidiasis infection model. Antimicrob Agents Chemother 43:2116–2120

Andes D, Stamsted T, Conklin R (2001) Pharmacodynamics of amphotericin B in a neutropenic-mouse disseminated-candidiasis model. Antimicrob Agents Chemother 45:922–926

Andes D, Marchillo K, Lowther J, Bryskier A, Stamstad T, Conklin R (2003a) In vivo pharmacodynamics of HMR 3270, a glucan synthase inhibitor, in a murine candidiasis model. Antimicrob Agents Chemother 47:1187–1192

Andes D, Marchillo K, Stamstad T, Conklin R (2003b) In vivo pharmacodynamics of a new triazole, ravuconazole, in a murine candidiasis model. Antimicrob Agents Chemother 47:1193–1199

Andes D, Marchillo K, Stamstad T, Conklin R (2003c) In vivo pharmacokinetics and pharmacodynamics of a new triazole, voriconazole, in a murine candidiasis model. Antimicrob Agents Chemother 47:3165–3169

Andes D, Marchillo K, Conklin R, Krishna G, Ezzet F, Cacciapuoti A, Loebenberg D (2004) Pharmacodynamics of a new triazole, posaconazole, in a murine model of disseminated candidiasis. Antimicrob Agents Chemother 48:137–142

Andes D, Forrest A, Lepak A, Nett J, Marchillo K, Lincoln L (2006) Impact of antimicrobial dosing regimen on evolution of drug resistance in vivo: fluconazole and *Candida albicans*. Antimicrob Agents Chemother 50:2374–2383

Andes D, Diekema DJ, Pfaller MA, Prince RA, Marchillo K, Ashbeck J, Hou J (2008a) In vivo pharmacodynamic characterization of anidulafungin in a neutropenic murine candidiasis model. Antimicrob Agents Chemother 52:539–550

Andes DR, Diekema DJ, Pfaller MA, Marchillo K, Bohrmueller J (2008b) In vivo pharmacodynamic target investigation for micafungin against *Candida albicans* and *C. glabrata* in a neutropenic murine candidiasis model. Antimicrob Agents Chemother 52:3497–3503

Arndt CA, Walsh TJ, McCully CL, Balis FM, Pizzo PA, Poplack DG (1988) Fluconazole penetration into cerebrospinal fluid: implications for treating fungal infections of the central nervous system. J Infect Dis 157:178–180

Astellas (2005) Mycamine. U.S. Food and Drug Administration. http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm?fuseaction=Search.Set_Current_Drug&ApplNo=021506&DrugName=MYCAMINE&ActiveIngred=MICAFUNGIN%20SODIUM&SponsorApplicant=ASTELLAS&ProductMktStatus=1&goto=Search.DrugDetails. Accessed 9 July 2013

Baddley JW, Patel M, Bhavnani SM, Moser SA, Andes DR (2008) Association of fluconazole pharmacodynamics with mortality in patients with candidemia. Antimicrob Agents Chemother 52:3022–3028

Banerjee R, Leaver N, Lyster H, Banner NR (2001) Coadministration of itraconazole and tacrolimus after thoracic organ transplantation. Transplant Proc 33:1600–1602

Barone JA, Koh JG, Bierman RH, Colaizzi JL, Swanson KA, Gaffar MC, Moskovitz BL, Mechlinski W, Van de Velde V (1993) Food interaction and steady-state pharmacokinetics of itraconazole capsules in healthy male volunteers. Antimicrob Agents Chemother 37:778–784

Barone JA, Moskovitz BL, Guarnieri J, Hassell AE, Colaizzi JL, Bierman RH, Jessen L (1998) Food interaction and steady-state pharmacokinetics of itraconazole oral solution in healthy volunteers. Pharmacotherapy 18:295–301

Brammer KW, Farrow PR, Faulkner JK (1990) Pharmacokinetics and tissue penetration of fluconazole in humans. Rev Infect Dis 12(Suppl 3):S318–S326

Carver PL (2004) Micafungin. Ann Pharmacother 38:1707–1721

Chandrasekar PH, Sobel JD (2006) Micafungin: a new echinocandin. Clin Infect Dis 42:1171–1178

Clancy CJ, Huang H, Cheng S, Derendorf H, Nguyen MH (2006) Characterizing the effects of caspofungin on *Candida albicans, Candida para-*

psilosis, and *Candida glabrata* isolates by simultaneous time-kill and postantifungal-effect experiments. Antimicrob Agents Chemother 50:2569–2572

Courtney R, Pai S, Laughlin M, Lim J, Batra V (2003) Pharmacokinetics, safety, and tolerability of oral posaconazole administered in single and multiple doses in healthy adults. Antimicrob Agents Chemother 47:2788–2795

Courtney R, Wexler D, Radwanski E, Lim J, Laughlin M (2004) Effect of food on the relative bioavailability of two oral formulations of posaconazole in healthy adults. Br J Clin Pharmacol 57:218–222

Denning DW, Ribaud P, Milpied N, Caillot D, Herbrecht R, Thiel E, Haas A, Ruhnke M, Lode H (2002) Efficacy and safety of voriconazole in the treatment of acute invasive aspergillosis. Clin Infect Dis 34:563–571

Deresinski SC, Stevens DA (2003) Caspofungin. Clin Infect Dis 36:1445–1457

Dowell JA, Stogniew M, Krause D, Damle B (2007) Anidulafungin does not require dosage adjustment in subjects with varying degrees of hepatic or renal impairment. J Clin Pharmacol 47:461–470

Ernst EJ, Klepser ME, Pfaller MA (1998) In vitro interaction of fluconazole and amphotericin B administered sequentially against *Candida albicans*: effect of concentration and exposure time. Diagn Microbiol Infect Dis 32:205–210

Ernst EJ, Klepser ME, Ernst ME, Messer SA, Pfaller MA (1999) In vitro pharmacodynamic properties of MK-0991 determined by time-kill methods. Diagn Microbiol Infect Dis 33:75–80

Ernst EJ, Klepser ME, Pfaller MA (2000) Postantifungal effects of echinocandin, azole, and polyene antifungal agents against *Candida albicans* and *Cryptococcus neoformans*. Antimicrob Agents Chemother 44:1108–1111

Ernst EJ, Roling EE, Petzold CR, Keele DJ, Klepser ME (2002a) In vitro activity of micafungin (FK-463) against *Candida* spp.: microdilution, time-kill, and postantifungal-effect studies. Antimicrob Agents Chemother 46:3846–3853

Ernst EJ, Yodoi K, Roling EE, Klepser ME (2002b) Rates and extents of antifungal activities of amphotericin B, flucytosine, fluconazole, and voriconazole against *Candida lusitaniae* determined by microdilution, Etest, and time-kill methods. Antimicrob Agents Chemother 46:578–581

Eschenauer G, Depestel DD, Carver PL (2007) Comparison of echinocandin antifungals. Ther Clin Risk Manag 3:71–97

Ezzet F, Wexler D, Courtney R, Krishna G, Lim J, Laughlin M (2005) Oral bioavailability of posaconazole in fasted healthy subjects: comparison between three regimens and basis for clinical dosage recommendations. Clin Pharmacokinet 44:211–220

Falagas ME, Apostolou KE, Pappas VD (2006) Attributable mortality of candidemia: a systematic review of matched cohort and case-control studies. Eur J Clin Microbiol Infect Dis 25:419–425

FDA (2001) Briefing document for voriconazole (oral and intravenous formulations). Antiviral Drugs Advisory Committee, U.S. Food and Drug Administration, Silver Spring. Available at http://www.fda.gov/ohrms/dockets/ac/01/briefing/3792b2_01_Pfizer.pdf. Last accessed 9 July 2013

Foulds G, Brennan DR, Wajszczuk C, Catanzaro A, Garg DC, Knopf W, Rinaldi M, Weidler DJ (1988) Fluconazole penetration into cerebrospinal fluid in humans. J Clin Pharmacol 28:363–366

Goicoechea M, Fierer J, Johns S (2004) Treatment of candidal cholangitis with caspofungin therapy in a patient with a liver transplant: documentation of biliary excretion of caspofungin. Clin Infect Dis 38:1040–1041

Groll AH, Giri N, Petraitis V, Petraitiene R, Candelario M, Bacher JS, Piscitelli SC, Walsh TJ (2000a) Comparative efficacy and distribution of lipid formulations of amphotericin B in experimental *Candida albicans* infection of the central nervous system. J Infect Dis 182:274–282

Groll AH, Mickiene D, Piscitelli SC, Walsh TJ (2000b) Distribution of lipid formulations of amphotericin B into bone marrow and fat tissue in rabbits. Antimicrob Agents Chemother 44:408–410

Groll AH, Mickiene D, Petraitis V, Petraitiene R, Ibrahim KH, Piscitelli SC, Bekersky I, Walsh TJ (2001) Compartmental pharmacokinetics and tissue distribution of the antifungal echinocandin lipopeptide micafungin (FK463) in rabbits. Antimicrob Agents Chemother 45:3322–3327

Groll AH, Lyman CA, Petraitis V, Petraitiene R, Armstrong D, Mickiene D, Alfaro RM, Schaufele RL, Sein T, Bacher J, Walsh TJ (2006) Compartmentalized intrapulmonary pharmacokinetics of amphotericin B and its lipid formulations. Antimicrob Agents Chemother 50:3418–3423

Gumbo T, Drusano GL, Liu W, Ma L, Deziel MR, Drusano MF, Louie A (2006) Anidulafungin pharmacokinetics and microbial response in neutropenic mice with disseminated candidiasis. Antimicrob Agents Chemother 50:3695–3700

Gumbo T, Drusano GL, Liu W, Kulawy RW, Fregeau C, Hsu V, Louie A (2007) Once-weekly micafungin therapy is as effective as daily therapy for disseminated candidiasis in mice with persistent neutropenia. Antimicrob Agents Chemother 51:968–974

Hardin TC, Graybill JR, Fetchick R, Woestenborghs R, Rinaldi MG, Kuhn JG (1988) Pharmacokinetics of itraconazole following oral administration to normal volunteers. Antimicrob Agents Chemother 32:1310–1313

Hariprasad SM, Mieler WF, Holz ER, Gao H, Kim JE, Chi J, Prince RA (2004) Determination of vitreous, aqueous, and plasma concentration of orally administered voriconazole in humans. Arch Ophthalmol 122:42–47

Hebert MF, Smith HE, Marbury TC, Swan SK, Smith WB, Townsend RW, Buell D, Keirns J, Bekersky I (2005) Pharmacokinetics of micafungin in healthy volunteers, volunteers with moderate liver disease, and volunteers with renal dysfunction. J Clin Pharmacol 45:1145–1152

Hennig S, Wainwright CE, Bell SC, Miller H, Friberg LE, Charles BG (2006) Population pharmacokinetics of itraconazole and its active metabolite hydroxy-itraconazole in paediatric cystic fibrosis and bone marrow transplant patients. Clin Pharmacokinet 45:1099–1114

Hennig S, Waterhouse TH, Bell SC, France M, Wainwright CE, Miller H, Charles BG, Duffull SB (2007) A d-optimal designed population pharmacokinetic study of oral itraconazole in adult cystic fibrosis patients. Br J Clin Pharmacol 63:438–450

Hope WW, Billaud EM, Lestner J, Denning DW (2008a) Therapeutic drug monitoring for triazoles. Curr Opin Infect Dis 21:580–586

Hope WW, Mickiene D, Petraitis V, Petraitiene R, Kelaher AM, Hughes JE, Cotton MP, Bacher J, Keirns JJ, Buell D, Heresi G, Benjamin DK Jr, Groll AH, Drusano GL, Walsh TJ (2008b) The pharmacokinetics and pharmacodynamics of micafungin in experimental hematogenous *Candida meningoencephalitis*: implications for echinocandin therapy in neonates. J Infect Dis 197:163–171

Howard SJ, Lestner JM, Sharp A, Gregson L, Goodwin J, Slater J, Majithiya JB, Warn PA, Hope WW (2011) Pharmacokinetics and pharmacodynamics of posaconazole for invasive pulmonary aspergillosis: clinical implications for antifungal therapy. J Infect Dis 203:1324–1332

Hyland R, Jones BC, Smith DA (2003) Identification of the cytochrome P450 enzymes involved in the N-oxidation of voriconazole. Drug Metab Dispos 31:540–547

Janssen (1999) Sporanox. U.S. Food and Drug Administration. http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm?fuseaction=Search.Set_Current_Drug&ApplNo=020966&DrugName=SPORANOX&ActiveIngred=ITRACONAZOLE&SponsorApplicant=JANSSEN%20PHARMS&ProductMktStatus=3&goto=Search.Label_ApprovalHistory. Accessed 9 July 2013

Jaruratanasirikul S, Kleepkaew A (1997) Influence of an acidic beverage (Coca-Cola) on the absorption of itraconazole. Eur J Clin Pharmacol 52:235–237

Johnson MD, Hamilton CD, Drew RH, Sanders LL, Pennick GJ, Perfect JR (2003) A randomized comparative study to determine the effect of omeprazole on the peak serum concentration of itraconazole oral solution. J Antimicrob Chemother 51:453–457

Kohl V, Muller C, Cornely OA, Abduljalil K, Fuhr U, Vehreschild JJ, Scheid C, Hallek M, Ruping MJ (2010) Factors influencing pharmacokinetics of prophylactic posaconazole in patients undergoing allogeneic stem cell transplantation. Antimicrob Agents Chemother 54:207–212

Koks CH, Huitema AD, Kroon ED, Chuenyam T, Sparidans RW, Lange JM, Beijnen JH (2003) Population pharmacokinetics of itraconazole in Thai HIV-1-infected persons. Ther Drug Monit 25:229–233

Kwan JT, Foxall PJ, Davidson DG, Bending MR, Eisinger AJ (1987) Interaction of cyclosporin and itraconazole. Lancet 2:282

Lee S, Kim BH, Nam WS, Yoon SH, Cho JY, Shin SG, Jang IJ, Yu KS (2012) Effect of CYP2C19 polymorphism on the pharmacokinetics of voriconazole after single and multiple doses in healthy volunteers. J Clin Pharmacol 52(2):195–203

Lepak AJ, Andes DR (2011) Antifungal PK/PD considerations in fungal pulmonary infections. Semin Respir Crit Care Med 32:783–794

Lewis RE, Wiederhold NP, Klepser ME (2005) In vitro pharmacodynamics of amphotericin B, itraconazole, and voriconazole against *Aspergillus*, *Fusarium*, and *Scedosporium* spp. Antimicrob Agents Chemother 49:945–951

Lewis RE, Liao G, Hou J, Chamilos G, Prince RA, Kontoyiannis DP (2007) Comparative analysis of amphotericin B lipid complex and liposomal amphotericin B kinetics of lung accumulation and fungal clearance in a murine model of acute invasive pulmonary aspergillosis. Antimicrob Agents Chemother 51:1253–1258

Lin SJ, Schranz J, Teutsch SM (2001) Aspergillosis case-fatality rate: systematic review of the literature. Clin Infect Dis 32:358–366

Louie A, Drusano GL, Banerjee P, Liu QF, Liu W, Kaw P, Shayegani M, Taber H, Miller MH (1998) Pharmacodynamics of fluconazole in a murine model of systemic candidiasis. Antimicrob Agents Chemother 42:1105–1109

Lutsar I, Roffey S, Troke P (2003) Voriconazole concentrations in the cerebrospinal fluid and brain tissue of guinea pigs and immunocompromised patients. Clin Infect Dis 37:728–732

Mavridou E, Bruggemann RJ, Melchers WJ, Mouton JW, Verweij PE (2010) Efficacy of posaconazole against three clinical *Aspergillus fumigatus* isolates with mutations in the cyp51A gene. Antimicrob Agents Chemother 54:860–865

McLachlan AJ, Tett SE (1996) Pharmacokinetics of fluconazole in people with HIV infection: a population analysis. Br J Clin Pharmacol 41:291–298

Merck (2001) Cancidas. U.S. Food and Drug Administration. http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm?fuseaction=Search.Set_Current_Drug&ApplNo=021227&DrugName=CANCIDAS&ActiveIngred=CASPOFUNGIN%20ACETATE&SponsorApplicant=MERCK&ProductMktStatus=1&goto=Search.DrugDetails. Accessed 9 July 2013

Merck (2010) Pharmacokinetics, safety, and tolerability of intravenous posaconazole solution followed by oral posaconazole suspension in subjects at high risk for invasive fungal infections (P05520 AM3). Clinicaltrials.gov identifier NCT01075984. Available at http://clinicaltrials.gov/ct2/show/NCT01075984. Last accessed 9 July 2013

Merck (2013) Pharmacokinetics and safety of posaconazole tablet in participants at high risk for invasive fungal infections (MK-5592-065 AM1). Clinicaltrials.gov identifier NCT01777763. Available at http://www.clinicaltrials.gov/ct2/show/NCT01777763. Last accessed 9 July 2013

Mian UK, Mayers M, Garg Y, Liu QF, Newcomer G, Madu C, Liu W, Louie A, Miller MH (1998) Comparison of fluconazole pharmacokinetics in serum, aqueous humor, vitreous humor, and cerebrospinal fluid following a single dose and at steady state. J Ocul Pharmacol Ther 14:459–471

Nivoix Y, Leveque D, Herbrecht R, Koffel JC, Beretz L, Ubeaud-Sequier G (2008) The enzymatic basis of drug-drug interactions with systemic triazole antifungals. Clin Pharmacokinet 47:779–792

O'Day DM, Foulds G, Williams TE, Robinson RD, Allen RH, Head WS (1990) Ocular uptake of fluconazole following oral administration. Arch Ophthalmol 108:1006–1008

Odds FC, Bossche HV (2000) Antifungal activity of itraconazole compared with hydroxy-itraconazole in vitro. J Antimicrob Chemother 45:371–373

Okugawa S, Ota Y, Tatsuno K, Tsukada K, Kishino S, Koike K (2007) A case of invasive central nervous system aspergillosis treated with micafungin with monitoring of micafungin concentrations in the cerebrospinal fluid. Scand J Infect Dis 39:344–346

Pascual A, Calandra T, Bolay S, Buclin T, Bille J, Marchetti O (2008) Voriconazole therapeutic drug monitoring in patients with invasive mycoses improves efficacy and safety outcomes. Clin Infect Dis 46:201–211

Pfizer (1990) Diflucan. U.S. Food and Drug Administration. http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm?fuseaction=Search.Set_Current_Drug&ApplNo=019949&DrugName=DIFLUCAN&ActiveIngred=FLUCONAZOLE&SponsorApplicant=PFIZER&ProductMktStatus=1&goto=Search.DrugDetails. Accessed 9 July 2013

Pfizer (2002) Vfend. U.S. Food and Drug Administration. http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm?fuseaction=Search.Set_Current_Drug&ApplNo=021267&DrugName=VFEND&ActiveIngred=VORICONAZOLE&SponsorApplicant=PFIZER&ProductMktStatus=1&goto=Search.DrugDetails. Accessed 9 July 2013

Pfizer (2006) Eraxis. U.S. Food and Drug Administration. http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm?fuseaction=Search.Set_Current_Drug&ApplNo=021948&DrugName=ERAXIS&ActiveIngred=ANIDULAFUNGIN&SponsorApplicant=PFIZER&ProductMktStatus=1&goto=Search.Label_ApprovalHistory. Accessed 9 July 2013

Poirier JM, Cheymol G (1998) Optimisation of itraconazole therapy using target drug concentrations. Clin Pharmacokinet 35:461–473

Pound MW, Townsend ML, Drew RH (2010) Echinocandin pharmacodynamics: review and clinical implications. J Antimicrob Chemother 65:1108–1118

Savani DV, Perfect JR, Cobo LM, Durack DT (1987) Penetration of new azole compounds into the eye and efficacy in experimental *Candida endophthalmitis*. Antimicrob Agents Chemother 31:6–10

Schering, (2006) Noxafil. U.S. Food and Drug Administration

Smith J, Safdar N, Knasinski V, Simmons W, Bhavnani SM, Ambrose PG, Andes D (2006) Voriconazole therapeutic drug monitoring. Antimicrob Agents Chemother 50:1570–1572

Smith WJ, Drew RH, Perfect JR (2009) Posaconazole's impact on prophylaxis and treatment of invasive fungal infections: an update. Expert Rev Anti Infect Ther 7:165–181

Tan K, Brayshaw N, Tomaszewski K, Troke P, Wood N (2006) Investigation of the potential relationships between plasma voriconazole concentrations and visual adverse events or liver function test abnormalities. J Clin Pharmacol 46:235–243

Templeton IE, Thummel KE, Kharasch ED, Kunze KL, Hoffer C, Nelson WL, Isoherranen N (2008) Contribution of itraconazole metabolites to inhibition of CYP3A4 in vivo. Clin Pharmacol Ther 83:77–85

Turnidge JD, Gudmundsson S, Vogelman B, Craig WA (1994) The postantibiotic effect of antifungal agents against common pathogenic yeasts. J Antimicrob Chemother 34:83–92

Van de Velde VJ, Van Peer AP, Heykants JJ, Woestenborghs RJ, Van Rooy P, De Beule KL, Cauwenbergh GF (1996) Effect of food on the pharmacokinetics of a new hydroxypropyl-beta-cyclodextrin formulation of itraconazole. Pharmacotherapy 16:424–428

Vazquez JA, Sobel JD (2006) Anidulafungin: a novel echinocandin. Clin Infect Dis 43:215–222

Walsh TJ, Lee JW, Kelly P, Bacher J, Lecciones J, Thomas V, Lyman C, Coleman D, Gordee R, Pizzo PA (1991) Antifungal effects of the nonlinear pharmacokinetics of cilofungin, a 1,3-beta-glucan synthetase inhibitor, during continuous and intermittent intravenous infusions in treatment of experimental disseminated candidiasis. Antimicrob Agents Chemother 35:1321–1328

Walsh TJ, Pappas P, Winston DJ, Lazarus HM, Petersen F, Raffalli J, Yanovich S, Stiff P, Greenberg R, Donowitz G, Schuster M, Reboli A, Wingard J, Arndt C, Reinhardt J, Hadley S, Finberg R, Laverdiere M, Perfect J, Garber G, Fioritoni G, Anaissie E, Lee J (2002) Voriconazole compared with liposomal amphotericin B for empirical antifungal therapy in patients with neutropenia and persistent fever. N Engl J Med 346:225–234

Warn PA, Sharp A, Parmar A, Majithiya J, Denning DW, Hope WW (2009) Pharmacokinetics and pharmacodynamics of a novel triazole, isavuconazole: mathematical modeling, importance of tissue concentrations, and impact of immune status on antifungal effect. Antimicrob Agents Chemother 53:3453–3461

Wexler D, Courtney R, Richards W, Banfield C, Lim J, Laughlin M (2004) Effect of posaconazole on cytochrome P450 enzymes: a randomized, open-label, two-way crossover study. Eur J Pharm Sci 21: 645–653

Wiederhold NP, Kontoyiannis DP, Chi J, Prince RA, Tam VH, Lewis RE (2004) Pharmacodynamics of caspofungin in a murine model of invasive pulmonary aspergillosis: evidence of concentration-dependent activity. J Infect Dis 190:1464–1471

Wiederhold NP, Tam VH, Chi J, Prince RA, Kontoyiannis DP, Lewis RE (2006) Pharmacodynamic activity of amphotericin B deoxycholate is associated with peak plasma concentrations in a neutropenic murine model of invasive pulmonary aspergillosis. Antimicrob Agents Chemother 50:469–473

Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB (2004) Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin Infect Dis 39:309–317

Biosystematic Index

Human Fungal Pathogens, 2nd Edition
The Mycota XII
O. Kurzai (Ed.)

Subject Index

Human Fungal Pathogens, 2nd Edition
The Mycota XII
O. Kurzai (Ed.)