Role of mini-host models in the study of medically important fungi

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Mini-host models have emerged as simple experimental systems to study the pathogenesis and host innate immune responses in fungal invaders and also to test drug efficacy against these organisms. A growing number of medically important fungi, including *Aspergillus* spp, *Candida* spp, *Cryptococcus* spp, and species in the class Zygomycetes, have been shown to infect and kill invertebrates such as roundworms, fruit flies, and wax moths. These studies have shown that several genes implicated in the virulence of fungi in mammalian models also have a similarly important pathogenic role in mini-host organisms. These mini-host models provide a unique opportunity of simultaneously exploring the molecular mechanisms of fungal pathogenicity and candidate agents with antifungal activity. Furthermore, the fact that some of these mini-hosts have well-defined genetics and conserved innate immunity offers the advantage of a comprehensive analysis of the molecular aspects of host immune response. We examine the relevance, advantages, and pitfalls of experimental systems of fungal infections in various mini-hosts and compare them with what is known in experimental systems in mammalian animal models.

Introduction

In recent years, opportunistic fungi have emerged as leading causes of morbidity and mortality in immunocompromised individuals.[1,2] The epidemiology of invasive fungal infections has evolved over the past two decades because of the widespread use of antifungal agents, the increasing use of medical devices, and the growing spectrum of immunosuppressive agents.[2,3] Yeasts remain the most common fungal pathogens in various groups of immunocompromised patients.[1,2,4] *Candida* spp—commensal fungi of mucosal surfaces—are the main pathogenic yeasts in human beings, causing systemic life-threatening infections in a wide range of debilitated patients.[4] Also, *Cryptococcus neoformans* is a common cause of severe opportunistic fungal infections in patients with AIDS, as well as in other groups of immunosuppressed patients—eg, solid organ transplant recipients.[5] Importantly, the extensive use of azole antifungals over the past decade has led to the emergence of non-*albicans* *Candida* species less susceptible to antifungals, such as *Candida glabrata* and *Candida krusei*.[4,6]

Meanwhile, ubiquitous airborne saprophytic moulds have become the main fungal pathogens in severely immunocompromised patients since the early 1990s.[1–3] *Aspergillus* spp are by far the most common of these moulds, and mortality rates for invasive aspergillosis exceed 90% in haematopoietic stem cell transplant recipients.[7] Even more concerning, however, is that infections caused by multidrug-resistant non-*fumigatus* *Aspergillus* species[8] and other difficult-to-treat opportunistic moulds, such as those of the class Zygomycetes, are increasingly reported in several cancer centres.[9,10] The increase in the frequency and spectrum of invasive fungal infections in immunocompromised patients, together with the increasing rates of resistance in fungi despite the expansion of the antifungal armamentarium, underscores the need to expand our knowledge of the pathogenesis of opportunistic fungal infections and develop novel therapeutic approaches.

Researchers have made substantial strides in the field of fungal pathogenesis over the past decade, but much remains to be learned. The rarity of invasive fungal infections in immunocompetent individuals provides strong evidence that normal host immune mechanisms mediate effective resistance to this class of pathogens.[11,12] However, opportunistic fungi use diverse, sophisticated virulence strategies to take advantage of the underlying host immunodeficiency.[11–13] For example, switching from budding yeast to a filamentous (hyphal) form is a pivotal virulence mechanism that allows *Candida* spp to invade host tissues and escape phagocytic destruction.[14,15] Similarly, *Aspergillus* spp produce potent hydrolytic enzymes and toxins with immunomodulating properties that facilitate the development of invasive infections,[16] whereas the pathogenicity of *C neoformans* has been largely attributed to its unique mucopolysaccharide capsule and the production of melanin.[17] Notably, Zygomycetes elaborate alternate virulence mechanisms, including the secretion of siderophores, which scavenge iron and facilitate fungal growth in the settings of iron overload and deferoxamine-based treatment.[18] The versatility of the pathogenetic mechanisms of fungi indicates the importance of understanding the nature of host–pathogen interactions in robust experimental systems.

Over the past decade, invertebrate mini-host models with well-characterised genetics and simple immunity have been effectively used to explore several aspects of both fungal pathogenicity and host immune response.[5,19–23] Several factors sparked the development of these models. First, the traditional animal models remain logistical barriers to large-scale studies. Second, the realisation that innate immune mechanisms are evolutionarily conserved between invertebrates and mammals and that several common virulence factors are involved in fungal pathogenesis in phylogenetically disparate hosts—eg, fruit flies, nematodes, and mammals—further expanded the field.[24–28] Third, invertebrate organisms have been

increasingly used as in-vivo assays for antifungal drug efficacy studies because of their low cost and simplicity.[29,30] We outline recent advances in the study of medically important fungi in mini-host models and discuss future implications and challenges in the use of these elegant pathosystems. We have attempted to put this new knowledge into a conceptual framework of what has been achieved in studies with mammalian systems.

Emergence of alternative models of fungal pathogens

Ideally, an animal model of fungal pathogenesis should closely mimic the pathophysiology of the corresponding infection in human beings, including colonisation and invasion at a specific route of entry, and also appropriately reproduce host immune defences (panel 1). Additionally, the tempo of experimental infection should be sufficiently protracted to effectively account for virulence attributes of the fungal pathogen.

Mammalian animal models are invaluable tools for the elucidation of the molecular and cellular pathogenesis of fungal infections.[31,32] Apart from the ethical dilemmas associated with experimentation in human beings, the main advantage of modelling infections in animals is that both the host and its environment can be precisely controlled, allowing for comprehensive analysis of host–pathogen interactions. Thus, small mammals, including, rats, mice, and rabbits, are the gold standard for pathogenesis studies because of their relative anatomic and immunological similarity to human beings.

However, several problems remain with the use of these pathosystems. Although mammalian models are amenable to reverse genetics through the generation of knockout mutants, identification of genes by large-scale forward genetic screening is challenging.[33] Furthermore, the use of large numbers of mammals is difficult for logistical, economical, and ethical reasons. This is an especially timely issue, since the genome sequences of medically important fungi such as aspergillus,[34] candida,[35] and cryptococcus[36] have been completed. This surge in genetic information has created an increasing need for simple innovative ways to screen for virulence mechanisms and assess the contribution of individual genes to fungal pathogenesis.

Pioneering studies over the past decade have shown that a variety of pathogenic fungi can infect and cause fatal disease in a variety of simple non-vertebrate hosts, such as the fruit fly *Drosophila melanogaster*,[19,20,37,38] the roundworm *Caenorhabditis elegans*,[21] the greater wax moth *Galleria mellonella*,[30] and the free-living soil amoeba *Dictyostelium discoideum*.[23] Also, research has shown that important aspects of innate immunity have been evolutionarily conserved across phylogeny.[27,28,39] Hence, comparative genomic studies show that a high proportion of human protein homologues, especially those involved in pathogen recognition, signal transduction, and the innate immune responses, exist within the various invertebrates, such as *D melanogaster* (60%) and *C elegans* (55%). Thus, because of their simple immunity and because both the host and pathogen are amenable to genetic analysis and high-throughput screening in each of these pathosystems, the use of invertebrate models has increased in studies of microbial virulence and host immunity.[33,40–43]

Common innate immune mechanisms in invertebrates and vertebrates

Although they do not exhibit adaptive immunity, invertebrates are capable of mounting efficient responses against an array of pathogens in the natural environments where they spend their lives. Their self-defence system is simple and consists of epithelial responses, a primitive phagocytic response, and the release of natural defensins through stimulation of innate immunity (figure 1). Because epithelial surfaces are where potentially invading pathogens come into contact with these hosts, physical barriers such as chitin-rich rigid membranes and a low pH constitute the first line of defence and prevent direct contact and colonisation by invaders.[24–26,45] When these physical barriers are breached, the introduction of microbes within the insect body induces a strong, highly coordinated immune response that has both cellular and humoral components and many similarities with mammalian immune responses against pathogens.

Epithelial immune responses

In *Drosophila* spp, antimicrobial peptide-encoding genes, which are normally expressed in the fat body (an equivalent of the mammalian liver), are also constitutively expressed in respiratory, digestive tract, and reproductive system epithelia that are in contact with the external

For more information on **protein homologues** see http://www.ncbi.nlm.nih.gov/sutils/taxik2.cgi

Panel 1: **Preferable characteristics of animal models of pathogenic fungi**

- Simulate the pathophysiology of infection in human beings
  - Route of challenge (colonisation, local infection, disseminated infection)
  - Type of immunosuppression (steroids, neutropenia, genetically engineered animals)
  - Tempo of infection sufficiently protracted as in human beings
- Reproducibility
  - Inoculum standardisation
  - Reliable methods of measurement of fungal burden (colony forming units, PCR)
  - Assessment of other surrogate markers (eg, galactomannan antigen, DNA, chitin)
- Reasonable cost
- Ethically acceptable
- Simplicity
- Amenable to genetic studies
  - Reverse and forward genetics
  - Microarray analysis
- Assessment of virulence factors
- Assessment of therapeutic agents
  - Pharmacodynamic and pharmacokinetic studies
  - Multiple routes of administration of agents
  - Mass screening of agents

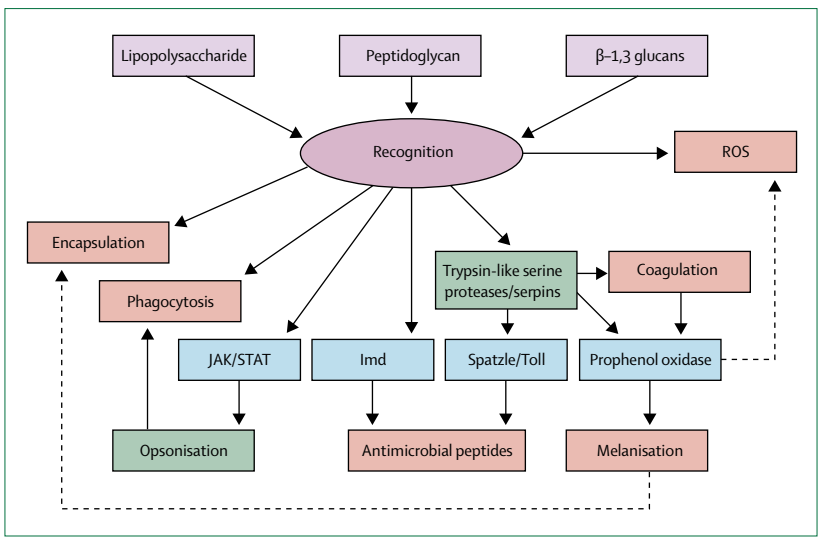

Figure 1: **Overview of innate immune response of insects**
Detection of pathogenic organisms by pattern recognition receptors results in specific activation of cellular and humoral immune responses. In addition to the main role of the Toll and Imd pathways in orchestrating innate immunity against Gram-positive bacteria/fungi and Gram-negative microorganisms, respectively, melanisation cascades, generation of reactive oxygen species (ROS), and activation of other immune-related pathways, such as the JAK/STAT pathway, are essential for effective defence against the wide range of pathogens that insects combat in their natural environments. Adapted from Tzou and colleagues.[41]

environment.[44] However, by contrast with the systemic immune responses mediated by the fat body where the Toll pathway modulates the immune responses against Gram-positive bacteria and fungi, epithelial immune responses in *Drosophila* spp seem to be controlled by another pathway, the immune deficiency (Imd) pathway.[44] Furthermore, there is evidence that genes involved in oxidative stress or detoxification of reactive oxygen species, such as oxidases and cytochromes, are crucial aspects of epithelial defences.[46,47] Studies of *Drosophila* spp mutants defective in genes that control either the homoeostasis of redox balance[46] or the generation of reactive oxygen species[47] indicate that these mutants were exquisitely susceptible to infection by a wide array of microbes, including the non-pathogenic yeast *Saccharomyces cerevisiae*.

In *C elegans*, most ingested pathogens are unable to invade intestinal cells, implying that *C elegans* epithelial cells possess powerful defence responses against microbial and fungal invaders.[42,43,48] For example, *Drechmeria coniospora*, a nematophagous fungus that infects roundworms by pentatrating the cuticle, is unable to infect *C elegans* by the oral route because it is sufficiently eliminated by the grinder of the roundworm, an apparatus that is normally used to process food.[49] Additionally, *C elegans* has been shown to be able to mount inducible epithelial and systemic immune responses through activation of evolutionarily conserved pathways that closely resemble the innate immune response of insects and mammals.[42,43,48] Despite the lack of a Toll-like signalling cascade with subsequent transcriptional activation of nuclear factor (NF) κB homologues, roundworms have at least three signalling pathways involved in antimicrobial and antifungal defences: the P38-like mitogen-activated protein kinase (PMK)-1 pathway, the transforming growth factor (TGF)-β pathway, and the insulin-like growth factor-1 pathway. Activation of these pathways after exposure to pathogens results in a coordinated response, with the production of antimicrobial peptides and lysozymes, the induction of programmed cell death, and possibly other immune defence responses.[42,43,48]

Cellular responses

In terms of cellular responses, professional macrophages—mainly plasmatocytes—that engulf incoming bacteria through phagocytosis operate in *Drosophila* spp.[50] Nonetheless, cellular immune responses of insects are better characterised in *G mellonella*, in which at least six types of haemocytes (prohaemocytes, coagulocytes, spherulocytes, oenocytoids, plasmacytes, and granulocytes) participate in specialised processes including phagocytosis, nodulation, and melanisation.[45,51] Of these cells, plasmatocytes and granulocytes are the predominant phagocytic cell types. Infection of *G mellonella* larvae with non-pathogenic fungi (eg, *S cerevisiae*) results in high haemocyte concentrations in the haemolymph, whereas pathogenic fungi (eg, *C albicans*) induce a substantial reduction in the number of haemocytes, suggesting that measurement of the concentrations of haemocytes could serve as a marker of pathogenicity of fungal invaders in the galleria model.[52]

Phagocytosis is a hallmark of the cellular immune response and exhibits considerable similarity across phylogeny from simple monocellular eukaryotic organisms (eg, *Acanthamoeba castellanii*) to mammals.[28,39,45] Hence, opsonisation and recognition by specific receptors mediate the initial stages of phagocytosis in both invertebrates and mammals. For example, in *Drosophila* spp, peptidoglycan recognition proteins (PGRPs) such as PGRP-LC and Croquemort (a human CD36 homologue) participate in the recognition and phagocytosis of Gram-negative bacteria,[53,54] whereas the recently identified transmembrane receptor Eater has been shown to bind both bacterial (eg, *S aureus* and *Escherichia coli*) and fungal (*Candida silvata*) cells.[55] In many insects (eg, fruit flies, mosquitoes), thioester-containing proteins exhibit a complement-like activity and facilitate phagocytocis of invading pathogens.[56] Recently, a RNAi library of *Drosophila melanogaster* S2 embryonic phagocytic cells comprising 7216 fly genes was used to study phagocytosis of *C albicans*.[57] The investigators identified a novel protein, macroglobulin complement related (Mcr), which is a member of the a2-macroglobulin/complement family and is selectively induced after exposure of drosophila phagocytic cells to *C albicans*. Mcr is able to bind specifically on the surface of yeast cells and subsequently enhance phagocytosis.[57] Similarly, *G mellonella* apolipophorin III, a novel insect

apolipoprotein homologous to mammalian apolipoprotein E, has been shown to bind to fungal conidia and β-1,3-glucan acting as a pattern recognition receptor.[58] Additionally, apolipophorin III was shown to promote cellular encapsulation of entomopathogenic fungi and induction of antifungal peptides exhibiting broad-spectrum activity against fungal invaders.[58] Other types of receptors with mammalian counterparts, including the *N*-acetylglucosamine-specific lectins, also have a crucial role in the recognition of invading pathogens by invertebrates.[45,51]

The mechanisms of killing of phagocytic cells in insects seem to be analogous to those in mammals. Thus, insect phagocytic cells are capable of generating an oxidative burst of oxygen radical intermediates in a way analogous to that in mammals.[59] For example, recent immunoblotting studies in *G mellonella* haemocytes with antibodies specific against human neutrophil phox proteins revealed the conservation of human protein homologues involved in the generation of reactive oxygen species, such as gp91phox, p67phox, p47phox, and the GTP binding protein rac2.[59] Additionally, inhibition of nitric oxide synthase has been shown to result in increased susceptibility to infection in drosophila larvae.[60] Furthermore, numerous antimicrobial peptides contained within human neutrophil granules, such as lysozyme, lipases, metalloproteases (like the mammalian gelatinases or collagenases), and nucleases, are also produced by the phagocytic haemocytes of most insects in response to infection.[45,51]

However, unique cellular responses such as encapsulation and melanisation mediated by unique effector cells could have a role in insect immunity against larger pathogens such as parasites.[24–26,45] Phagocytic cells exhibit clear differentiation of pathogen specificity and killing mechanisms among invertebrates, probably a result of their unique environmental lifestyles. For example, in *C elegans*, macrophage-like cells called coelomocytes have not been shown to be involved in phagocytosis of pathogenic organisms.[42,43,48]

Humoral responses

Invading pathogens induce potent humoral responses in a variety of hosts. Notably, more than 800 such antimicrobial peptides with activity against a wide range of bacterial and fungal pathogens have been isolated from unicellular and multicellular eukaryotic organisms, from plants to mammals.[45] In insects, cuticle breakage leads to rapid activation of proteolytic cascades that provoke coagulation and melanisation in an attempt to restrict the spread of infection. Nevertheless, the hallmark of humoral immune response against pathogenic micro-organisms in invertebrates is the induction of a battery of antimicrobial peptides, which are secreted by the fat body into the haemolymph.[24–26,44]

Despite the broad spectrum of antimicrobial peptides, specificity exists upon their induction against various microbial pathogens across a variety of hosts. For example, in drosophila, fungi and Gram-positive bacteria mainly induce drosomycin and metchnikowin via the Toll pathway[19] (analogous to mammalian Toll/interleukin-1 receptor signalling), whereas Gram-negative microbes induce diptericin, attacin, and cecropin through the Imd pathway (analogous to the mammalian tumour necrosis factor [TNF] signalling).[19,20,24–26] Of interest, *C elegans* infection with the nematophagous fungus *D coniospora* was recently shown to lead to activation of the novel Toll-interleukin 1 receptor (TIR1), with subsequent induction of an antifungal peptide (NLP31) belonging to the family of neuropeptide-like proteins.[61] Importantly, NLP31 exhibited activity against *D coniospora*, *Neurospora crassa,* and *Aspergillus fumigatus* in vitro, whereas RNAi silencing of TIR1 in *C elegans* was associated with increased susceptibility to infection by *D coniospora*. Furthermore, RNAi studies in *C elegans* showed that TIR1 functions upstream of the evolutionarily conserved PMK1 pathway.[62]

In *G mellonella*, gallerimycin, a cysteine-rich defensin-like peptide similar to drosomycin, was recently identified.[63] Recombinant gallerimycin possessed substantial activity against the entomopathogenic fungus *Metarhizium anisopliae*, but not against bacteria and yeasts. Importantly, injection of larvae of *G mellonella* with a non-lethal dose of *C albicans*, *S cerevisiae*, or glucan has been shown to protect against subsequent infection with a lethal dose of *C albicans* via induction of a battery of novel immune-related molecules, including an inducible metalloproteinase inhibitor, a transferrin-like protein, and several antifungal peptides.[64] Furthermore, proteomic analysis of *G mellonella* haemolymph after infection with the pathogenic yeast *C albicans*, compared with the non-pathogenic model yeast *S cerevisiae*, led to identification of several other candidate immune inducible antifungal molecules.[64]

The specificity of innate immunity is conferred through pattern recognition receptors (PRRs).[53] PRRs are soluble or transmembrane proteins common to various mammals and insects, which recognise essential molecules present exclusively in microbes, such as lipopolysaccharide, lipoteichoic acids, and peptidoglycan—the so-called pathogen-associated molecular patterns (PAMPs). Recent studies in drosophila indicated that PGRPs and Gram-negative bacteria-binding proteins (GBNP) comprise the main PRR.[53] Recently, mutation analysis in drosophila implicated that GNBP3 is a candidate PRR for fungal pathogens.[53] Other types of soluble PRRs, such as ficolins and C-type lectins that recognise microbial carbohydrates (eg, D-mannose, *N*-acetylglucosamine, lipoteichoic acids), are also present in both invertebrates and mammals and are induced after bacterial immune challenge.[28,45] This class of PRRs seems to have an additional role in the activation of melanisation cascades.[45,51]

Evolutionarily conserved innate immunity pathways in insects

The Toll pathway

In both insects and mammals, interaction of invading pathogens with a specific PRR leads to activation of intracellular phosphorylation cascades with subsequent upregulation of antimicrobial peptide-encoding genes through the translocation of NFκB-like transcriptional factors to the nucleus (figure 2). In drosophila, PGRP-SA and GNBP1 activate the serpin Persephone through an as yet unidentified mechanism.[25] Persephone is implicated in the cleavage of another serpin called Spatzle, which physically interacts with and activates the transmembrane receptor Toll.[25] Another serpin inhibitor, Necrotic, seems to be a negative regulator of Spatzle activation.[25] Besides drosophila, similar serine protease cascades and Toll-like receptors (TLRs) have been identified in mosquitoes, silkworms, and mammals and have been shown to be activated by interaction with PAMPs of microbial pathogens.[39,44,53]

Upon activation, the Toll receptor recruits the adaptor proteins MyD88 and Tube and the threonine-serine kinase Pelle, which are homologues of the human proteins MyD88, Mal (functional equivalent), and IRAK, respectively.[25,27,28] This proteolytic cascade ultimately leads to degradation of an inhibitor of κB (IκB) known as Cactus, and nuclear translocation of the NFκB-like transcriptional factors Dorsal and Dif, which induce the expression of antimicrobial peptide-related genes. However, there are important differences in the aspects of innate immunity across various hosts. Hence, of the ten drosophila Toll homologues, only Toll has a role in immune defence, whereas most of the ten mammalian TLRs have important roles in innate immune response, which seems to be pathogen specific.[25,27,28] Additionally, the Toll pathway does not seem to be functionally conserved in *C elegans*, since homologues of the drosophila Toll, dTraf, Pelle, and Cactus proteins (tol-1, trf-1, pik-1, and ikb-1, respectively) do not seem to be involved in immune defence.[61]

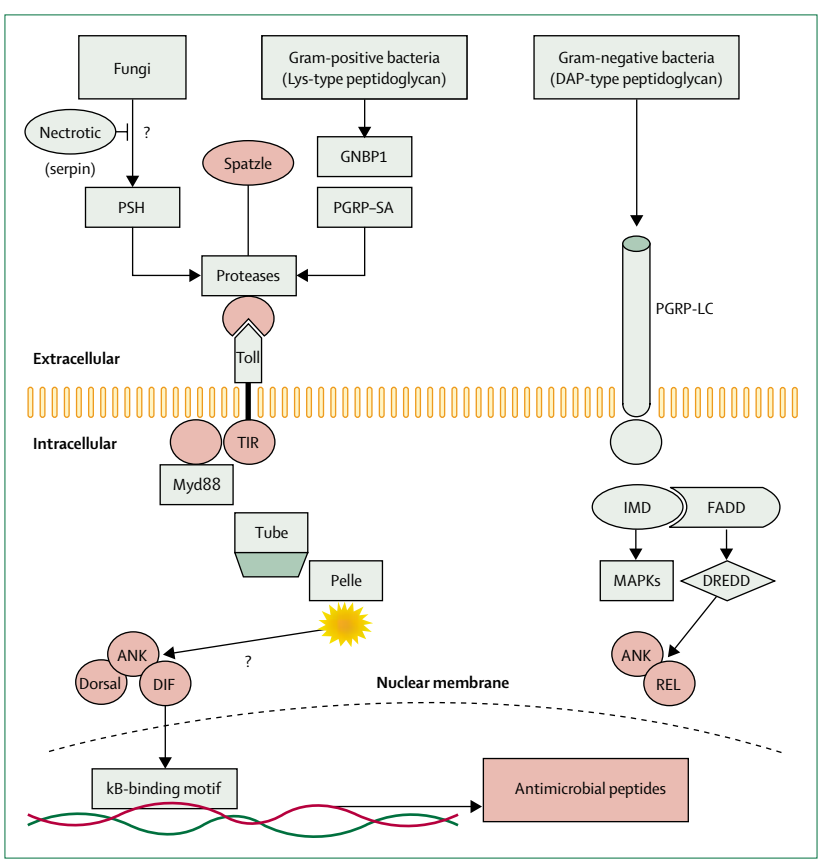

Figure 2: **Evolutionarily conserved innate immunity pathways**
The Toll and Imd pathways regulate the genes that encode antimicrobial peptides in drosophila and most insects. The Toll pathway is activated mainly by fungi and Gram-positive bacteria, whereas the Imd pathway is activated mainly by Gram-negative bacteria. ANK=ankyrin-repeat domain; DIF=dorsal-related immunity factor; FADD=FAS-associated death domain; MyD88=myeloid differentiation primary-response protein 88; PSH=Persephone; TIR domain=Toll/interleukin-1 receptor domain.

The Imd pathway

The Imd pathway in drosophila is analogous to the TNF signalling pathway in human beings and seems to be especially important for immune responses against Gram-negative bacteria.[25–28] Similar to the TNF pathway, various proteins containing death domains—which have homologue counterparts in mammals—are recruited after interaction between PGRP-LC/PGRP-LE and the Imd receptor (figure 2). The final stage of the Imd cascade in drosophila involves the phosphorylation of Relish, a member of the Rel family of transcriptional regulators. Importantly, two well-defined cascades mediate phosphorylation of Relish: a pathway that includes the caspase DREDD and a mitogen-activated protein kinase pathway (figure 2).[25]

Other evolutionarily conserved immunity pathways in invertebrates

The level of complexity of the immune defences in insects is higher than initially perceived, and crosstalk exists between the Imd and Toll pathways after exposure to both Gram-negative and Gram-positive microbes, such as *Staphylococcus aureus* and *Enterococcus faecalis*.[65–67] In addition to both the Toll and Imd signalling cascades, other pathways associated with developmental or stress resistance processes are induced in response to infections in both invertebrates and mammals.[26,28,45] For example, expression profile analyses in drosophila indicated that several genes induced by microbial challenge are regulated by independent pathways such as the Jun N-terminal kinase and the JAK/STAT pathways.[65–67] The latter pathway seems to branch out from the Imd pathway and is activated in response to Gram-negative bacterial challenge, regulating expression of the complement-like thioester-containing proteins.[68]

Besides its pivotal role in orchestrating the immune responses in *C elegans*, the phylogenetically conserved

	Fruit fly (*D melanogaster*)	Roundworm (*C elegans*)	Wax moth (*G mellonella*)	Silkworm (*B mori*)	Soil amoeba (*D discoideum*)
Genetic tractability	++	++	–	+	++
Sequenced genome	Completed	Completed	–	Pending	Completed
Adaptive immunity	–	–	–	–	–
Survival at mammalian physiologic temperature (37°C)	–	–	+	+	–
Need for simple laboratory resources	+	+	++	++	++
Phagocytic cell studies	+	–	++	+	+
Suitable for antifungal studies	+	±	++	++	–
Correlation of virulence factors in mammalian models					
Candida spp	++	NA	±	NA	NA
Cryptococcus neoformans	+	++	++	NA	+
Aspergillus spp	±	NA	+	NA	NA
Potential for high-throughput screening	++	++	±	+	++

++=excellent; +=very good; ±=good; –=absent; NA=not available.

Table 1: **Comparison of different mini-host models of fungal pathogens**

PMK-1 P38 mitogen-activated protein kinase is also involved in mediating resistance to osmotic stress.[69] Specifically, that there is considerable crosstalk between different MAPK pathways that regulate the immune responses and stress responses in *C elegans* has been shown recently.[69] Furthermore, another evolutionarily conserved important protective response against invading pathogens in *C elegans,* which is modulated upstream by PMK1, is the induction of programmed cell death.[70] Although the physiological mechanisms implicated in protection of the roundworm by apoptosis after intestinal infection are obscure, apoptosis is also induced as a general defence mechanism to stress in *C elegans*.

Evidence from expression profiling analyses in fruit flies and roundworms further suggests that the stress response, innate immunity, and life span are under the control of interrelated pathways such as the insulin-like and transforming growth factor-β receptor pathways.[70–72] Importantly, the *C elegans* long-lived mutants daf-2 and age-1, which are defective in genes of the insulin-like pathway, possess substantial resistance to oxidative stress and effectively combat infection by various pathogenic organisms.[72,73] Furthermore, male *C elegans* differ in susceptibility to fungal infection compared with hermaphrodite animals.[74] Specifically, male roundworms possess increased resistance to killing by *C neoformans*, and overexpression of the male sex-determination pathway in hermaphrodite animals resulted in increased resistance to infection and increased survival, mediated by activation of the stress response transcriptional factor daf-16. These observations imply that in *C elegans* longevity, immunity, and stress responses are regulated by common pathways.

Moreover, the TGFβ pathway seems to be involved in immune responses of some invertebrates (eg, *C elegans*) by inducing the expression of antimicrobial peptides such as lipases, lysozyme, and caenopores upon bacterial challenge.[48] Finally, members of the transferrin family of glycoproteins that are involved in iron homeostasis and have a pivotal role in the acute phase of immune response in human beings also exist in insects.[45,51] Overall, resistance to pathogens across a range of hosts seems to develop through the interplay between immune defence, apoptosis, and general stress mechanisms.

Invertebrate models of fungal pathogenesis

G mellonella

Larvae of Lepidoptera insects such as the greater wax moth *G mellonella* and the silkworm *Bombyx mori* have been successfully used as models of fungal pathogenesis because of their relatively large size (about 2 cm and 5 cm long, respectively), which allows for the injection of standardised fungal inocula and studies of drug pharmacodynamics.[30,75,76] Furthermore, in-vivo studies of phagocytic cell function are feasible with these invertebrates (table 1).[52,59] Importantly, although their optimum temperature of growth and maintenance is 29°C, *G mellonella* and *B mori* larvae are able to survive at the mammalian physiological temperature (37°C), which could allow for the expression of certain temperature-regulated virulence factors of fungal pathogens in these models.[30] Nevertheless, the effect of increased temperatures on the *G mellonella* immune response has not been studied in detail. In fact, some have shown that *G mellonella* exhibits increased susceptibility to pathogenic fungi at mammalian physiological temperatures.[30] By contrast, in all other invertebrate models, infection experiments are typically done at optimum rearing temperatures, ranging from 22°C to 30°C.[33] The major disadvantage of Lepidoptera insects is the absence of methods for genetic analysis and lack of genome sequencing. However, the *B mori* genome sequencing project is near completion, and the number of genetic tools and techniques for lepidopteran genetic analysis are quickly accumulating.

For more information on **lepidopteran genetic analyses** see http://www.ab.a.u-tokyo.ac.jp/lep-genome

C elegans

The transparent roundworm *C elegans* is much smaller (about 1 mm long) than all other mini-host models.[33] Nevertheless, *C elegans* has a sequenced genome and fully developed genetic tools. Additionally, its hermaphroditic lifestyle and short lifespan (2–3 weeks) facilitate genetic studies with this organism. *C elegans* is typically maintained in Petri dishes that contain a lawn of its standard laboratory food source, *E coli* OP50.[42,43] Pathogenicity studies are typically done by feeding worms in lawns of the bacterial or fungal pathogens of interest.[21,42,43] So far, only the nematophagous fungus *Drechmeria coniospora* has been shown to infect *C elegans* through invasion of the cuticle.[49] Survival is typically assessed under a dissecting microscope with worms considered dead if there is no response after stimulation with a wire pick. Importantly, worms need to be transferred to a fresh plate every 48 h after infection with pathogens that allow progeny production (eg, *C neoformans*), to separate the originally infected worms from their growing progenies. Of interest, worms infected with less virulent *C neoformans* mutants produce substantially more progeny than worms infected with wild-type *C neoformans* strains.[77] Thus, instead of counting survival, high throughput screening for *C neoformans* mutants with attenuated virulence can be done on the basis of counting differences in the number of progenies of *C elegans*. The simplicity of experimental infection in *C elegans* allows for individual screening of thousands of candidate mutants of a pathogenic fungus for attenuated virulence through feeding. Several studies have illustrated that the roundworm is a rewarding in-vivo assay for high-throughput screening of virulence genes[78,79] and candidate antimicrobial compounds.[80]

Thus far, *C elegans* has proven to be a valuable model for studying the virulence of *Cryptococcus* spp, but studies of this model with other medically important pathogenic fungi—eg, *Aspergillus* spp and *Candida* spp—are scarce. Furthermore, *C elegans* is rather selective with regard to the growth medium and pathogenic organisms, which could further complicate analysis of results. For example, poor growth of *C neoformans* in complete media substantially reduces its pathogenicity in *C elegans*.[30,42] The age of this worm also has a role in its susceptibility to pathogens, although the generation of synchronous populations obviates this problem.[72]

D melanogaster

The fruit fly *D melanogaster* (about 3 mm long) is larger than the roundworm but substantially smaller than caterpillars.[33] The pathogen of interest is normally injected into the dorsal thorax of fruit flies; however, other more physiological methods of infection (eg, rolling, feeding) are also used.[40,41] Female flies are typically used in infection experiments because of their larger size and relative resistance to injection injury compared with male flies. Because wild-type drosophila is resistant to most pathogenic fungi, mutants deficient in various components of the Toll cascade are used to model infections. In most cases, crossing different loss-of-function alleles is required to generate homozygous Toll-mutant flies.[19,29] The genetic tractability[40] and well-characterised immune system[24,25] of drosophila is a major advantage. Hence, drosophila is amenable to both forward and reverse genetics, and large collections of drosophila mutants and transgenic cell lines are commercially available. Also, the drosophila genome sequence was one of the first to be completed and is probably one of the most fully annotated eukaryotic genomes found in a database. As a result, double-stranded RNA has been synthesised for all the drosophila genes. Application of RNA interference technology in the drosophila S2 phagocytic cell line enabled a functional genome-wide analysis of host–pathogen interaction at the cellular level.[57,81–83]

Thermotolerance is a universal virulence trait across pathogenic fungi.[11,12,15,17,18] Hence infection and maintenance of drosophila as well as most invertebrate hosts at a lower temperature might be an important limitation. For example, a recent study found that a gene that regulates the expression of the nucleolar protein CgrA has a pivotal role in aspergillus thermotolerance and that a *ΔCgrA* mutant displayed attenuated virulence in mice.[84] However, the *ΔCgrA* mutant was fully virulent in Toll-mutant flies infected and maintained at 25°C, showing that certain aspects of fungal virulence in mammals might not be appropriately modelled in mini-hosts.

Methods of infection

Few fungi that naturally infect invertebrates have been identified.[40,41,49] Therefore, most fungal pathogens are directly introduced into the body cavity by pricking of the insect cuticle with a sharp needle or microinjecting a precise dose of fungal cells into the insect body cavity.[41] However, this procedure bypasses natural routes of infection, including colonisation and initial interaction of the pathogen with specific epithelial receptors, and induces a wide spectrum of antimicrobial expression, which might be caused by physical injury in some cases.[41]

A more physiological method of infection that is closer to natural infection is typically achieved by feeding insects in a lawn of yeast or moulds or rolling insects over a fresh carpet of fungal spores.[40,42,49,51] In the latter case, establishment of infection takes place by penetration of the insect exoskeleton by the fungal pathogen. Furthermore, this method results in a more protracted model of infection compared with injection and thus might allow for more reliable assessment of certain attributes of fungal pathogenicity. For example, we recently reported that the *alb1 A fumigatus* mutant that was hypovirulent in mice exhibited attenuated virulence in Toll-deficient flies when introduced with the rolling and ingestion methods but not injection.[29] However, standardisation of the infecting inocula is difficult with natural infection methods such as ingestion.

For more information on **the genetics of *C elegans*** see http://www.wormbase.org/

For more information on **drosophila mutants** see http://flybase.net/

For more information on **the annotated drosophila genome** see http://flybase.net/annot/

For more information on **drosophila double-stranded RNA** see http://www.flyrnai.org

Screening of candidate antifungal compounds in invertebrate models

The value of a new animal model is largely dependent on its capability to assess the efficacy of therapeutic agents. In drosophila, the easiest way to expose a large number of flies to antifungal compounds is by mixing drugs with fly food, which is typically achieved by dissolving them into a yeast-sucrose medium.[29,85] However, screening of antifungal compounds with decreased solubility in aqueous solvents makes delivery through the oral route challenging. Furthermore, precise estimation of the amount of the drug ingested with this method is difficult. A simple method to overcome this problem, especially when testing novel compounds, is to verify drug ingestion by adding dye into the fly food.[29] Overall, feeding is regarded as the method of choice when long-term treatment is required.

Despite these limitations, our group has recently shown that drosophila is a reliable model for testing orally absorbed antifungals with anti-aspergillus activity.[29] Specifically, Toll-mutant flies fed in vials containing voriconazole and infected with *A fumigatus* had substantially better survival rates and lower fungal burdens than did control (untreated) Toll-mutant flies infected and maintained in regular vials without drugs. Additionally, we found that the fungal burden was substantially decreased as assessed with quantitative real-time PCR and histopathology in voriconazole-treated flies when compared with control (untreated) flies. Furthermore, the combination of voriconazole and terbinafine, two drugs that block sequential steps in the ergosterol pathway and show synergy in vitro against aspergillus, was synergistic in the drosophila model of invasive aspergillosis.[29]

Similarly, ingestion of fluconazole mixed into fly food resulted in substantially improved survival rates and lower fungal burdens in Toll-mutant flies injected with fluconazole-susceptible *Candida albicans* compared with control flies.[86] Importantly, fluconazole was not active in flies injected with a fluconazole-resistant *Candida krusei* strain. These findings imply that drosophila is also a promising model for studying the correlation between in-vitro susceptibility testing and in-vivo efficacy of antifungal compounds for infections caused by candida strains that have different minimum inhibitory concentrations.[86]

For most experimental invertebrate models, the preferable method of administration for precisely studying the pharmacology of agents is injection. However, although injection is relatively easy to do for large insects such as caterpillars,[30,87] this approach is time-consuming and requires technical sophistication and specialised equipment for fruit flies.[85]

Mylonakis and colleagues[30] recently developed an elegant model of cryptococcosis in the wax moth *G mellonella* and tested the efficacy of amphotericin B deoxycholate, fluconazole, and flucytosine, the preferred antifungal agents for *Cryptococcus* spp infections in human beings. They administered all the antifungal agents parenterally by injection of galleria larvae with pharmacological doses equivalent to those used for the treatment of cryptococcosis in human beings. In agreement with the results of clinical studies, the authors found that the combination of amphotericin B deoxycholate and flucytosine resulted in better survival rates and lower fungal burdens than did all the other antifungal agent combinations in the *G mellonella* moths. However, the investigators did not attempt to measure antifungal drug levels in galleria larvae.

Hamamoto and colleagues[76] recently reported that injection of either amphotericin B deoxycholate or fluconazole offered substanial protection in silkworms infected with clinical isolates of *C albicans* and *Candida tropicalis* that are susceptible to both antifungals in vitro. Of interest, these authors did pharmacodynamic studies and found good correlation between the dose of each antifungal that protected 50% of silkworms infected with candida and the dose of the corresponding antifungal in a mouse model of candidiasis.

An important limitation of the studies described above is that drug levels were not measured, nor were pharmacokinetic analyses included. Although high-performance liquid chromatography analysis[85] and bioassay methods are feasible in invertebrates,[29] such studies are more cumbersome, imprecise, and technically challenging in these models compared with mammals. Additionally, injection of multiple doses of antifungals for long periods, a requirement for pharmacokinetic studies, is difficult to accomplish in invertebrates since it results in increased mortality associated with repeat injury. Furthermore, little is known regarding the metabolism and elimination pathways of drugs and the potential for drug–drug interactions in mini-host models. However, despite these limitations, invertebrates are attractive models for the mass screening of candidate antifungal compounds that will require subsequent validation in mammalian systems. Such approaches have been successfully used in drosophila to select life-extending compounds[86] and in *C elegans* to screen for novel anthelmintic microbial molecules.[87]

Fungal infections in invertebrate animal models

Aspergillus spp

Only a few entomopathogenic fungi are able to infect fruit flies in nature, via penetration of the fly exoskeleton.[20,41] Even with experimental introduction of fungal pathogens directly into the fly haemolymph, wild-type flies are still capable of effectively combating infection.[19,20] For example, injection of 10^4 conidia of *A fumigatus* in wild-type *D melanogaster* resulted in a survival rate of almost 100%.[19] In the mid-1990s, Lemaitre and colleagues[19,20] were the first to show that *A fumigatus* was able to infect and kill flies carrying mutations in various aspects of the Toll pathway.[19,20] The usefulness of the fly model in studying virulence mechanisms in *A fumigatus* is suggested by the

fact that the *alb1 A fumigatus* mutant, which lacks the ability to produce melanin and exhibits attenuated virulence in a mouse model of invasive aspergillosis,[88] also displayed a hypovirulent phenotype in Toll-mutant flies infected by ingestion or rolling.[29] Nevertheless, similar to recent findings with the *ΔCgrA* mutant, putative virulent factors of *A fumigatus* with a role in thermotolerance might not be effectively encountered in drosophila or other invertebrate models, because infection in these mini-hosts takes place at temperatures much lower (25°C) than the mammalian physiological temperature.[84]

Virulence studies of *A fumigatus* have been done thus far only in *G mellonella*.[89,90] Of interest, *G mellonella*, like other invertebrates, is enormously resistant to infection by *A fumigatus*. Importantly, the stage of conidial germination of *A fumigatus* has recently been shown to have a substantial effect on virulence of *A fumigatus* in *G mellonella* larvae because of the associated differences in the rate of phagocytosis. Specifically, whereas resting conidia of *A fumigatus* were avirulent in larvae of *G mellonella* even when injected in high inocula (up to 10^7 conidia per insect), swollen conidia (greater than 3 μm in size) or germinating conidia were highly virulent and were associated with substantially reduced rates of phagocytosis by haemocytes.[90]

Furthermore, the same group of investigators reported that *G mellonella* was extremely susceptible to injection of conidia of the *A fumigatus* strain ATCC 26933.[89] This *A fumigatus* isolate was shown to produce gliotoxin, an aspergillus metabolite that exhibits immunosuppressive and apoptotic activity against immune effector cells in vitro,[13,16] implying a role for this toxin as a virulent factor in the galleria model of invasive aspergillosis.[89] However, comparative studies of gliotoxin gene-deletion *A fumigatus* mutants with isogenic controls are needed to provide definitive answers regarding the role of gliotoxin in aspergillus virulence. Recently, an *A fumigatus* mutant deleted in calcineurin A (*CnaA*), the catalytic subunit of the calcineurin pathway, exhibited substantial defects in conidial cell wall structure and lateral filamentation, and was shown to be hypovirulent both in the mouse model of aspergillosis and in *G mellonella*.[91] Similarly, another *A fumigatus* mutant (*ΔPes1*), deleted for a non-ribosomal peptide synthetase with a potential role in tolerance against oxidative stress, was found to be hypovirulent in *G mellonella*.[92]

Candida spp

Alarco and colleagues[38] found that the Toll pathway is strongly induced and has a central role in the immunity of drosophila against *Candida* spp, much the same as the important role of TLRs against invasive candidiasis in human beings. The investigators tested a group of *C albicans* mutants defective in filamentation (*cdc35* and *cla4*) as well as a mutant with a deletion in three aspartyl-protease genes (*sap4*, *sap5*, and *sap6*) in *Tl*-mutant flies and reported that all the mutants displayed attenuated virulence much the same as that seen in mammalian models of candidiasis (table 2). We studied in *Tl*-mutant flies the pathogenicity of the *efg1/efg1*, *cph1/cph1*, and *efg1/efg1 cph1/cph1 C albicans* mutants, which have well-characterised defects in filamentous growth and attenuated virulence in a mouse model of systemic candidiasis.[86,93,97] We found that, much the same as has been shown in the mouse model of candidiasis,[93] the *efg1/efg1* and *cph1/cph1* mutants were hypovirulent, whereas the double mutant *efg1/efg1 cph1/cph1* was almost avirulent in *Tl*-mutant flies.[86] We also used an alternative strategy for virulence studies in drosophila, using the conditional *C albicans* strain *tet-NRG1* in which filamentation is under the control of a tetracycline-promoter system.[95] Unlike conventional gene disruption, the tetracycline-promoter system allows for exogenous modulation of gene expression both in vivo and in vitro, even for essential genes.[98] We found that exogenous modulation of *NRG1* expression is achievable in the drosophila model by feeding flies with doxycycline at various concentrations.[86] Additionally, the virulence of the *tet-NRG1* strain under different doxycycline concentrations in *Tl*-mutant flies was much the same as that seen in the mouse model of candidiasis.[95]

Investigators have tested various *Candida* spp and mutants in a *G mellonella* model of invasive candidiasis. Unlike drosophila, wild-type galleria larvae were susceptible to infection with injection of 10^6 yeast cells of various *Candida* spp.[75,96,99] Notably, *C albicans* was the most virulent *Candida* species, whereas *C krusei* and *C glabrata* isolates, which have reduced virulence in mice compared with *C albicans*, were essentially avirulent in galleria larvae.[75,99] Interestingly, the pathophysiology of candida infection seems to be different in *G mellonella*, where budding of yeast cells are predominantly observed in infected tissue, by contrast with both *D melanogaster* and mammalian models of invasive candidiasis, where filamentatious hyphae predominate.[86] Not surprisingly, candida mutants defective in filamentation, such as *efg1/efg1* and *cph1/cph1*, retain their virulence in *G mellonella*.[75] Nonetheless, another hypovirulent *C albicans* mutant, which is deleted in the myosin-I gene and is defective in filamentation, was found to be completely avirulent in *G mellonella* larvae;[96] notably, yeast cells of myosin-I-defective mutants were rapidly removed from larvae haemolymph, possibly because of increased susceptibility to lysozyme and other effectors of humoral immune response.[96] Overall, *G mellonella* is emerging as a useful model for the study of effector cell activity against candida in vivo because there are considerable similarities in the mechanisms of phagocytosis and oxidative killing by immune effector cells in *G mellonella* and mammals.[59]

C neoformans

By contrast with other medically important fungi, *C neoformans* is an encapsulated fungus that has the ability

Mutant strain	Relevant genotype/ mutation	Mini-host model tested	Phenotype	Comments
C albicans				
CA LJ3	*Δcacla4/Δcacla4*	*D melanogaster, G mellonella*	Defective in filamentation	Avirulent in mice, hypovirulent in invertebrates[38,75]
CDH10	*Δhst7/Δhst7*	*G mellonella*	Defective in filamentation	Virulent in mice and *G mellonella*[75]
CDH22	*Δcst20/Δcst20*	*G mellonella*	Defective in filamentation	Virulent in mice and *G mellonella*[75]
CDH107	*Δcaras1/Δcaras1*	*G mellonella*	Defective in filamentation	Hypovirulent in mice, virulent in *G mellonella*[75]
CP29-1-7	*Δcpp1/Δcpp1*	*G mellonella*	Defective in filamentation	Hypovirulent in mice, virulent in *G mellonella*[75]
CK43B-16	*Δcek1/Δcek1*	*G mellonella*	Defective in filamentation	Hypovirulent in mice, virulent in *G mellonella*[75]
CR 216	*Δcacdc35Δ*	*D melanogaster, G mellonella*	Defective in filamentation	Avirulent in mice, near avirulent in invertebrates[38,75]
HLC 69	*Δefg1/Δefg1, Δcph1/Δcph1*	*D melanogaster, G mellonella*	Defective in filamentation	Avirulent in mice, almost avirulent in *D melanogaster*, virulent in *G mellonella*[93,94]
JKC 19	*Δcph1/Δcph1*	*D melanogaster, G mellonella*	Defective in filamentation	Hypovirulent in mice and *D melanogaster*, virulent in *G mellonella*[93,94]
HLC 67	*Δefg1/Δefg1*	*D melanogaster, G mellonella*	Defective in filamentation	Hypovirulent in mice and invertebrates[93,94]
DSY 459	*Δsap6 /sap6, Δsap4/Δsap4, Δsap5/Δsap5*	*D melanogaster*	Defective in production of aspartyl-protease genes	Hypovirulent in mice and *D melanogaster*[38]
SSY50-B	*tet-NRG1*	*D melanogaster*	Tetracycline-regulatable expression of *NRG1*, results in blocking of filamentation	Avirulent in mice and almost avirulent in *D melanogaster* (in the absence of tetracycline)[95]
COU 42	*Δ/ ΔG myo5*	*G mellonella*	myosin-I-defective; defective in filamentation	Avirulent in mice and *G mellonella*[96]
***Cryptococcus* spp**				
H99 gpa1	*Δgpa1*	*G mellonella, C elegans*	*GPA1* encodes a G-protein alpha subunit, involved in capsule formation and melanin production	Avirulent in mice, hypovirulent in invertebrates[21,30]
H99 pka1	*Δpka1*	*D melanogaster, G mellonella, C elegans*	*PKA1* encodes the major cAMP-dependent protein kinase catalytic subunit, involved in capsule formation and melanin production	Avirulent in mice, hypovirulent in invertebrates[21,30,37]
H99 ras1	*Δras1*	*D melanogaster, G mellonella, C elegans*	*RAS1* regulates the ras-signalling cascade in mammals, involved in capsule formation and melanin production	Avirulent in mammals and *G mellonella*, hypovirulent in *C elegans* and *D melanogaster*[21,30,37]
H99 pkr1	*Δpkr1*	*D melanogaster, G mellonella, C elegans*	*PKR1* encodes the PKA regulatory subunit, *pkr1* mutant overproduces capsule	Hypervirulent in mice and invertebrates[21,30,37]
H99 M001	*Δade2*	*G mellonella, C elegans*	Adenine auxotroph	Hypovirulent in mice and invertebrates[21,30]
H99 cap 59	*Δcap59*	*D melanogaster, G mellonella, C elegans, D discoideum*	*CAP59* is essential for capsule formation, acapsular mutant	Avirulent in mammals and *G mellonella*, almost fully virulent in *D melanogaster* and *C elegans*[21,30,37]
ATCC #208819 923-tu4)	*MATα cnlac1*	*G mellonella, C elegans, A castellanii*	Defective in melanin production, laccase negative	Hypovirulent in mice, *C elegans*, and *G mellonella*; virulent in amoeba[21,22,30,37]
H99 Cap 67	*Δcap67*	*A castellanii*	Acapsular mutant of 3501 strain	Avirulent in mammals and amoeba[22]
H99 Plb1	*ΔPlb1*	*A castellanii*	H99 phospholipase-deficient mutant	Hypovirulent in mammals and amoeba[22]
KN99α *kin1*	*Δkin1*	*C elegans*	KIN1 encodes for a serine/threonine protein kinase, kin1 mutants display increased binding to macrophages	Hypovirulent in mice and *C elegans*[79]
KN99α *rom2*	*Δrom2*	*C elegans*	*S cerevisiae* homologous gene required for cell integrity under heat and osmolar stress	Avirulent in mice; hypovirulent in *C elegans*[77]
JEC20 (ATCC 96910); JEC21 (ATCC 96909)	*MATα (JEC21); MATa (JEC20)*	*G mellonella*	Isogenic strains with opposite mating types	MATα (JEC21) is more virulent in mammals and *G mellonella*[30]
A fumigatus				
H237	*cgrA*	*D melanogaster*	*CGRA* encodes a nucleolar protein implicated in thermotolerance	Hypovirulent in mice, virulent in *D melanogaster*[84]
B-5233/ RGD12-8	*Alb1*	*D melanogaster*	Defective in melanin production	Hypovirulent in mice and *D melanogaster* (feeding assay)[29]
	ΔcnaA	*G mellonella*	*CnaA* encodes for the catalytic subunit in the calcineurin pathway; Δ*cnaA* exhibits decrease in filamentation	Almost avirulent in mice and *G mellonella*[91]
	Δpes1	*G mellonella*	*Pes1*, is a nonribosomal peptide synthetase encoding for secondary metabolites; Δ*pes1* exhibits increased sensitivity to oxidative stress	Reduced virulence in *G mellonella* (no data in mammalian models)[92]

ATCC=American type culture collection; cAMP=cyclic AMP; PKA=protein kinase A.

Table 2: **Overview of virulence studies of pathogenic fungi in mini-host models**

to infect a wide range of hosts by exploiting conserved virulence traits.[17] In fact, it has been increasingly recognised that the pathogenetic mechanisms of *C neoformans* might have evolved as a result of selection pressure imposed by environmental predators such as amoeba. Pioneering studies of the free-living amoeba *A castellanii*[17] and the roundworm *C elegans*[21] indicated that *C neoformans* is able to infect and kill these mini-host organisms by use of similar pathogenetic strategies. For example, *C neoformans* infection of *A castellanii* closely resembles *C neoformans* infection of human macrophages.[17] Furthermore, infection with mutants of *C neoformans* that are acapsular or defective in melanin production (laccase negative) result in attenuated killing of *A castellanii*.[17] Investigators recently used the soil amoeba *D discoideum* as a successful genetically amenable model to dissect *C neoformans* pathogenesis.[23] Also, serial passages of *C neoformans* through *D discoideum* substantially increased the virulence of *C neoformans* in mice because of an increase in capsular size and melanisation.[23]

In *C elegans*, Mylonakis and colleagues[21,77] tested a series of *C neoformans* mutants defective in capsule production, melanisation, or cell integrity and osmolar stress (table 2). These investigators also tested these mutants in drosophila[37] and galleria[30] and found comparable results. Additionally, considerable similarities exist in the role of the mating locus in the pathogenesis of *C neoformans* in *G mellonella* and mammals. Specifically, the MATα strain JEC21 was shown to be more virulent compared with its isogenic strain MATa strain JEC20 (differing in the mating locus).[30] Furthermore, it has been shown that the *MF*α1 gene, which regulates the production of α mating type, is induced during the proliferative stage of infection of *C neoformans* in the central nervous system of mammals and in *G mellonella* tissues.[30] Notably, by contrast with galleria, wild-type drosophila was remarkably resistant to killing by injection of *C neoformans*. However, oral infection by *C neoformans* resulted in fly death that was shown to be independent of Toll-pathway or Imd-pathway mechanisms.[37] Another interesting observation was that ingestion of heat-killed conidia resulted in death in drosophila and *C elegans* but not in galleria larvae. Subtle differences were shown to exist between the models used. The importance of a variety of virulence factors seems to depend on the host background and method of infection.

Emerging fungal pathogens

Zygomycetes have recently emerged as an important cause of serious angioinvasive infections in immunocompromised individuals.[8,9] Few animal models of Zygomycetes exist, and the immunopathogenesis of zygomycosis is largely unknown.[18] However, sequencing of the genome of *Rhizopus oryzae*, the predominant Zygomycetes species in human beings, is ongoing, and genetic tools have become available to dissect the molecular mechanisms of Zygomycetes pathogenicity. Although Zygomycetes species have not been reported to be entomopathogenic, we recently found that injection of different Zygomycetes species in wild-type *D melanogaster* results in a hyperacute and disseminated infection with a high mortality rate within 48 hours.[94] Increased iron supply through exogenous administration and increased iron availability through administration of deferoxamine increased the virulence of Zygomycetes infection in flies. Hence, Zygomycetes species seem to have developed common virulence strategies to invade evolutionarily disparate organisms such as drosophila and human beings. The simplicity and genetic tractability of mini-host models might facilitate our understanding of molecular mechanisms that mediate the pathogenicity of Zygomycetes.

Of interest, *Paecilomyces lilacinus*, an emerging hyalohyphomycetes in severely immunocompromised individuals,[100] has been shown to be able to kill *C elegans* through toxin production. Specifically, culture filtrates of *P lilacinus* isolates exhibiting increased protease and chitinase activity were able to rapidly kill roundworms, within 24 hours of exposure, implying a toxin-mediated mechanism of death.[101]

Conclusions

In recent years, mini-host models have been increasingly appreciated as important tools to dissect the molecular mechanisms of fungal pathogenesis and host innate immunity. A variety of genetically amenable invertebrate hosts are expected to become key components of genomic strategies to scan the entire genomes of medically important fungi for pathogenicity-related genes. Also, these elegant pathosystems could be adapted as high-throughput assays to screen for new types of antifungal compounds that target specific virulence attributes of pathogenic fungi. However, important questions concerning the future role of mini-host models continue to evolve (panel 2). Despite the

***Panel 2*: Future questions regarding the use of invertebrates to study the immunopathogenesis of medically important fungi**

- What is the pathophysiology of mini-host death caused by opportunistic fungal pathogens?
- Which particular types or classes of fungi are better modelled in each mini-host model?
- Which fungal virulence factors are equally important for both mammalian and mini-host pathosystems?
- What are the immune mechanisms that mediate resistance to fungi in invertebrate epithelia?
- What are the essential molecular structures of fungi that mediate recognition by the Toll pathway?
- Is recognition of different classes of fungi induced by the same PAMPs?
- Are there pathogenic fungi other than *C neoformans* that kill *C elegans*?

For more information on ***Rhizopus oryzae*** see http://www.broad.mit.edu/annotation/fungi/rhizopus_oryzae/

Search strategy and selection criteria

We searched the Medline database via PubMed (from 1966 to September, 2006) and abstracts from major infectious diseases meetings held from 1992 to 2005 for published English language reports pertaining to invertebrate models of medically important fungi. Articles describing the immunopathogenesis of yeast and mould infections in invertebrate (mini-host models) were reviewed. We identified articles by cross-referencing the terms "invertebrates", "mini host models", and "insects" to specific medically important fungi such as *Aspergillus* spp, *Candida* spp, *Cryptococcus* spp, and the *Zygomycetes* spp. Studies were selected on the basis of importance of data (as determined by citation frequency by subsequent publications in the specialty) and originality. Additionally, review papers written by leaders in the fields of mycology and invertebrate host immunity were included in our search.

considerable similarities in innate immune mechanisms, invertebrate models are not directly comparable to mammals. Thus, one could reasonably speculate that some of the virulence attributes of pathogenic fungi that affect mammals might not be important in invertebrate mini-host models. Each of the existing mini-host models has advantages and disadvantages, which highlight the need for the use of many models to understand the mechanisms of fungal pathogenicity.

Conflicts of interest

DPK has received research support and honoraria from Merck & Co Inc, Fujisawa Inc, Enzon Pharmaceuticals, and Schering-Plough. REL has received research support from Merck & Co Inc, Fujisawa Inc, Pfizer Inc, and Schering-Plough. All other authors have no conflicts of interest to declare.

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