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Microreview

Drosophila innate immunity and response to fungal infections

Anastasia Levitin[1] and Malcolm Whiteway[2,3]*
[1]*Department of Biology, Stanford University, Stanford, CA 94305-5020, USA.*
[2]*Genetics Group, Biotechnology Research Institute, National Research Council of Canada, 6100 Royalmount Ave., Montreal, Quebec, Canada H4P 2R2.*
[3]*Department of Biology, McGill University, Montreal, Quebec, Canada H3A 1B1.*

Summary

The fruit fly *Drosophila melanogaster* is an important model for the analysis of the interaction between host immune systems and fungal pathogens. Recent experiments have extended our understanding of the Toll-based signalling pathway critical to response to fungal infections, and identified new elements involved in cellular and humoral-based defences. The fly immune system shows remarkable sophistication in its ability to discriminate among pathogens, and the powerful genetics available to researchers studying the adult fly response, and the ability to manipulate cultured phagocytic cell lines with RNAi, are allowing researchers to dissect the molecular details of the process.

Introduction

The interactions between insects and fungi are complex and fascinating, and include such intriguing phenomena as the cultivation of fungi as food sources by ants (Martin, 1970) and the existence of yeast-like endosymbionts living within cells of specialized structures of some beetles and scale insects (Noda and Kodama, 1996). Several fungal species are successful pathogens of insects; these include generalists such as *Beauveria bassiana* and *Metarhizium anisopliae*, and specialists such as *Furia ithacensis* infecting snipe flies (Kramer, 1981) and *Entomophaga grylli* infecting grasshoppers (Ramoska *et al*., 1988). The identification and use of specific fungal pathogens for biological control of insects that are economic or health concerns of humans has become an increasingly important endeavour.

In addition to investigations on fungal pathogens of insects as biocontrol agents, the research community has used the relationships between fungal pathogens and insects to probe basic biological questions. In particular, the interaction between the fruit fly *Drosophila melanogaster* and bacterial or fungal pathogens has been extensively studied on a molecular level, and these investigations have led to fundamental insights into the insect and subsequently the mammalian immune system. There have been a number of excellent recent reviews that cover the use of *Drosophila* in studies on insect–microbial interactions (Hetru *et al*., 2003; Naitza and Ligoxygakis, 2004; Mylonakis and Aballay, 2005; Cherry and Silverman, 2006; Fuchs and Mylonakis, 2006); here we will focus on recent developments in this field with respect to fungal pathogenesis.

Important insight into insect immunity arose through the identification of *Drosophila*-encoded peptides such as drosomycin (Fehlbaum *et al*., 1994) and metchnikowin (Levashina *et al*., 1995). Some of these insect-produced molecules had clear similarity to plant-derived defence molecules, and were subsequently shown to have antipathogen activity in their own right. The observation that these peptides were induced in response to particular pathogens directed the identification of the signalling pathways leading to the defence-molecule induction (Lemaitre *et al*., 1995). These studies, in turn, led first to the realization that components of the fly dorsal-ventral patterning system were critical for the fungal-induced expression of the host defence molecules (Lemaitre *et al*., 1996), and then to the fact that Toll-like receptors were key components of the pathogen recognition systems of both insects and mammals (Akira *et al*., 2006). In mammals the immune response consists of both innate and acquired components; these act in synergy to defend the organism against infection. The fruit fly, on the other hand, lacks the classic acquired immune system, and is thus inherently a useful model to study innate immune responses in the absence of antibody-based acquired immunity. This insect innate immune system is composed

Received 26 November, 2007; revised 24 December, 2007; accepted 2 January, 2008. *For correspondence. E-mail Malcolm.whiteway@cnrc-nrc.gc.ca; Tel. (+1) 514 496 6146; Fax (+1) 514 496 6213.

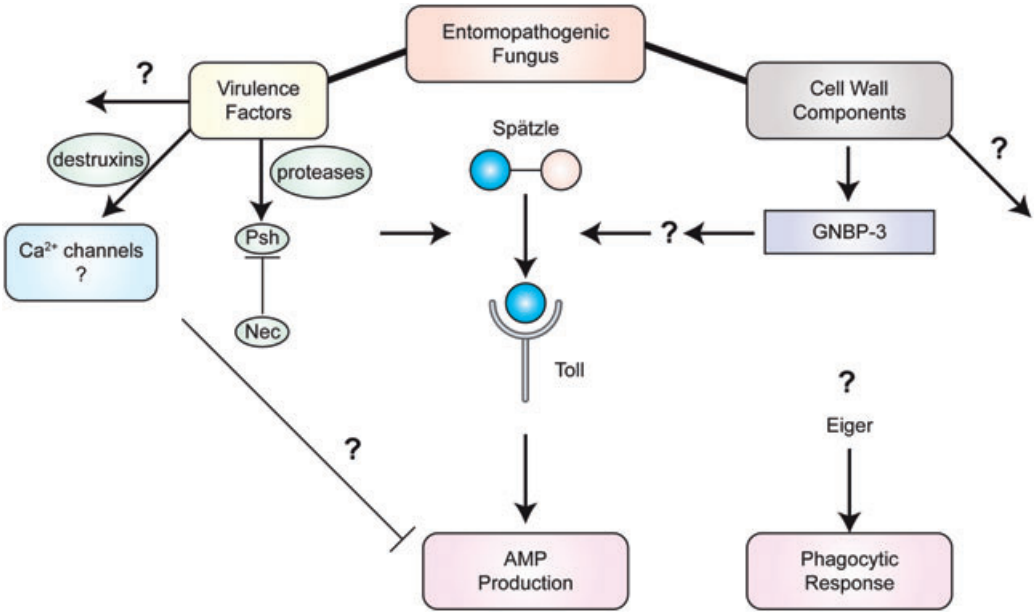

Fig. 1. *Drosophila* immune response to fungal pathogens. Two major components of fly immunity are the production of antimicrobial peptides (AMPs) and the activation of a phagocytic response. The tumour necrosis factor homologue Eiger is implicated in the activation of the phagocytic response, while the Toll receptor plays a major role in activation of AMP production in response to fungal pathogens. The Toll pathway is activated by interaction with the product of proteolytic cleavage of the ligand spätzle; this can occur in response to the recognition of fungal wall components through the pattern recognition receptor GNBP-3, or through detection of protease virulence factors through activation of the Persephone gene product. Other virulence factors such as cyclic peptides of the destruxin family serve to inhibit AMP production.

of both humoral and cellular constituents, and is sophisticated enough to be able to distinguish among different classes of pathogens; in particular fungi and Gram-positive bacteria are dealt with differently from Gram-negative bacteria. The humoral components are concerned with biosynthesis of elements such as various antimicrobial peptides (AMPs) (Meister *et al*., 1997), whereas cellular reactions involve blood cells or haemocytes. These two responses act in concert (Elrod-Erickson *et al*., 2000) with phagocytosis of pathogens ultimately serving as an important part of the defence mechanism.

Fly-based analysis

Advances in our understanding of *Drosophila* response to fungi have been made using both natural fungal pathogens, as well as artificial infections using fungi that are normally human pathogens. Both *B. bassiana* and *M. anisopliae*, two generalist fungal pathogens, have been used to probe the immune response of *Drosophila*. Although the basic pattern of response to fungal pathogens involved the Toll receptor and the induction of the AMP drosomycin, many significant details of the upstream signalling pathway have been uncovered by recent studies in the fly (Fig. 1). Infection studies using *B. bassiana* suggested that the Persephone protease was critical to the activation of the Toll receptor in response to fungal infection. Persephone (*psh*) (Ligoxygakis *et al*., 2002) was itself initially identified as a suppressor of the constitutive melanization and early death exhibited by *Drosophila* mutants of the Necrotic (*nec*) gene; *nec* mutants have a constitutively activated Toll pathway due to loss of a *nec*-encoded serine protease inhibitor or serpin (Levashina *et al*., 1999). These results implied that the *nec* and *psh* gene products played active roles in the Toll-mediated response to fungal pathogens, but did not identify the specific pathogen recognition machinery involved although such specificity was expected as the innate immunity networks were able to induce directed responses to different classes of pathogens.

Recently, identification of a pattern recognition receptor for fungal pathogens has added intriguing layers of complexity in the fungal pathogen response pathway (Fig. 1). GNBP-3, a member of a class of β-glucan recognition proteins that includes Gram-negative binding protein-1 (GNBP-1), was shown to act as a recognition factor for fungal surface components (Gottar *et al*., 2006). Because the related GNPB-1 served as a component of the Toll pathway-inducing recognition element for Gram-negative bacteria (Gobert *et al*., 2003), the connection of GNBP-3 to Toll pathway activation in response to fungal pathogens had a logical molecular symmetry. When mutant flies defective in GNBP-3 were challenged with fungal cell wall

components like β1-3 glucans or with heat-killed *Candida albicans* cells or extracts from *Aspergillus nidulans* cells, they were unable to properly induce drosomycin expression (Gottar *et al*., 2006). Surprisingly, infections with *B. bassiana* were not particularly lethal in *gnbp-3*-defective flies, although the mutant flies were highly sensitive to infections from live *C. albicans*, and Toll pathway-defective flies were quite susceptible to *B. bassiana* infection. However, loss of both Persephone and GNBP-3 function created flies that were lethally sensitive to entomopathogenic fungal infection (Gottar *et al*., 2006); this overlap in GNPB-3 and *psh* appears to arise because *psh* is implicated in responding to the direct influence of fungal virulence factors generated by entomopathogenic fungi, while GNBP-3 acts to activate the Toll response in response to opportunistic fungal infections and cell surface markers.

Investigations using flies defective in the *eiger* gene, the *D. melanogaster* tumour necrosis factor homologue, suggest this gene also plays a role in pathogen recognition (Schneider *et al*., 2007). In *eiger* mutant flies, extracellular pathogens such as *B. bassiana* and *Staphylococcus aureus* were more lethal, while there was no heightened sensitivity to intracellular pathogens such as *Salmonella typhimurium*. This suggests that in addition to pattern recognition systems that classify pathogens on the basis of cell surface components, the fly innate immune system can differentiate pathogens on the basis of the interaction of the pathogen with the haemocyte system. In addition, fungal products, such as the peptide destruxin A produced by *M. anisopliae*, appear to have the ability to suppress humoral responses in flies, and this suppression can lead to non-pathogenic organisms such as *Escherichia coli*, becoming pathogenic (Pal *et al*., 2007). Thus overall the relationship between the host immune system and the fungal pathogen is multifaceted, and much work remains to be done to fully establish the links between natural fungal pathogens and the fly response.

Several lines of evidence show that the Toll pathway also serves to defend flies against artificially induced infections with fungal pathogens that are normally limited to mammalian hosts. Because these pathogens have not evolved to deal with the insect cuticle, it is necessary to infect *Drosophila* by injecting the fungi into the fly by pricking with a pathogen-coated needle. Initial infections with *Aspergillus fumigatus* (Lemaitre *et al*., 1996) and subsequently with *C. albicans* (Alarco *et al*., 2004) and *Cryptococcus neoformans* (Apidianakis *et al*., 2004) established that human pathogens could be lethally injected into *Drosophila* adults, and that the lethality of these infections was influenced by the Toll pathway. This ability to infect the genetically tractable fly with human pathogens has led to efforts to expand the use of the *Drosophila* model to investigate antifungal drugs. Mutations that affect the virulence of the human fungal pathogen *C. albicans* can reduce virulence in a *Drosophila* infection model (Alarco *et al*., 2004; Chamilos *et al*., 2006), suggesting that mechanisms of virulence may be related in mammals and insects. In addition, *Aspergillus* infections of *Drosophila* Toll mutants were influenced by the virulence state of the pathogen (Lionakis *et al*., 2005). It was possible to reduce the severity of *Aspergillus* infections with voriconazole treatment (Lionakis *et al*., 2005), and to treat *C. albicans* infections with fluconazole added to the fly food, although infection from the naturally resistant *Candida krusei* was not affected by the drug treatment (Chamilos *et al*., 2006). This opens up the possibility of using the fly model in screens for new antifungal drugs, or in tests of function of new candidate compounds (Tournu *et al*., 2005).

Cell-based analysis

An alternative to working with the whole organism is to scale down to a smaller model. There are a number of *Drosophila* cell lines derived from mixed embryonic tissues including the most common Schneider 2 (alternative names S2, SL2 and L2) and Kc cells. Recently, cell lines from specific tissues, larval central nervous system and imaginal discs have become available as well. Gorr *et al*. (2004) studied the *Drosophila* hypoxia-inducible factor (HIF), the key regulator of survival and adaptation during oxygen deprivation. In this work S2 cells were used to study the ability of flies to sustain oxygen deprivation as opposed to the highly oxygen-dependent organs and tissues of mammals. As these cells can function in low oxygen, an environment preferred by many pathogens, they represent a good tool to study host–pathogen interactions. Although the cell lines exhibit similar properties, they are not identical in their responses to various treatments and conditions (Cherbas and Cherbas, 2000). For example, when S2 and KC cell lines were compared, only the former exhibited scavenger receptor-mediated endocytosis, an activity observed in mammalian macrophages (Abrams *et al*., 1992).

The Schneider 2 cells are frequently used as a tool to study the *Drosophila* defence response (Echalier, 1997). In *Drosophila*, 95% of blood cells are a specific type of haemocyte, termed the plasmatocyte, which fulfil the functions of mammalian neutrophils and macrophages (Tepass *et al*., 1994). The S2 cells are *Drosophila* embryonic haemocytes (Schneider, 1972) that can phagocytose invading microbes and cell debris (Ramet *et al*., 2001; 2002). These cells have been established as a model to study host–pathogen interactions primarily due to the ability to genetically manipulate these cells with RNAi; various *Drosophila* plasmatocytes such as S2, KC, BG2-C6 and Shi are sensitive to double-stranded RNAi

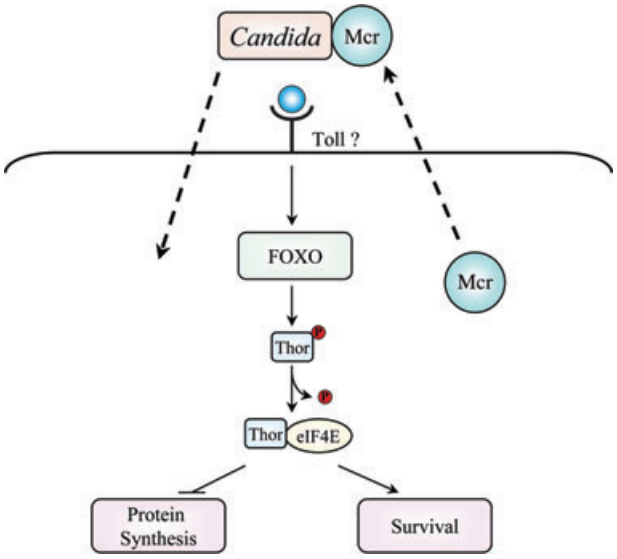

Fig. 2. *Drosophila* S2 cells response to *Candida albicans*. *Drosophila* Mcr protein is required for *Candida* recognition and promotes subsequent phagocytosis of the pathogen. The engulfment of *Candida* by S2 cells triggers expression of Thor gene, regulated by a transcriptional activator, FOXO. Thor plays a role in host survival during *Candida* infections in *Drosophila* flies by interacting with the member of translation-initiation machinery, eIF4E.

and have been successfully used to study pathogenesis of various microbes. For example, a systematic functional genomic screen was used to pinpoint the genes involved in the uptake and growth of *Mycobacterium fortuitum* (Philips *et al*., 2005), and researchers have used S2 cells in genome-wide RNAi screens for factors required by the host during infections of the cytosolic pathogen *Listeria monocytogenes* as well as *M. fortuitum*, a vacuolar pathogen (Agaisse *et al*., 2005).

S2 cells have recently been used as a model to study cell-mediated innate immunity of *Drosophila* against fungal pathogens such as *C. albicans*, as it was shown that S2 cells are capable of engulfing *Candida* and its close relative *Saccharomyces cerevisiae* (Stroschein-Stevenson *et al*., 2006; Levitin *et al*., 2007). Stroschein-Stevenson *et al*. have specifically investigated phagocytosis of *C. albicans* through an RNAi-based screen to identify genes involved in engulfment of *Candida* by *Drosophila* S2 cells. They found 184 genes representing a variety of functions to be important for *Candida* phagocytosis. The study further concentrated on one of the findings, involving the Macroglobulin complement-related (Mcr) gene product (Stroschein-Stevenson *et al*., 2006). The *Mcr* gene is closely related to a family of four *Drosophila* thioester proteins (Tep). Mcr was found to be secreted by S2 cells and to be preferentially and tightly bound to *C. albicans*, which promoted subsequent *Candida* phagocytosis (Fig. 2). The study illustrated the specificity of different members of this conserved group of Tep genes for different pathogens including Gram-negative *E. coli* and Gram-positive *S. aureus*.

Another aspect of *C. albicans* engulfment by *Drosophila* S2 cells was recently investigated through a microarray analysis that identified a number of genes differentially expressed as a result of *Candida* internalization by S2 cells. *Candida* infection was shown to trigger a production of Thor (Levitin *et al*., 2007), a translational regulator previously shown to be involved in starvation and oxidative stress resistance in *Drosophila* (Tettweiler *et al*., 2005), as well as to resistance to bacterial infection (Bernal and Kimbrell, 2000). Using the live *Drosophila* model, Thor was found to be involved in fly survival in response to *Candida* infection, suggesting a significant component of the fruit fly's cell-based immunity may involve regulation of translation (Fig. 2) (Levitin *et al*., 2007). This validation of the results derived from *Drosophila* macrophage-like cells by using the whole fly helps to confirm S2 cells as a useful model to study *Drosophila–Candida* interactions (Levitin *et al*., 2007).

Conclusions

Therefore, both derived cell lines and the fruit fly itself have proven to be impressive tools for the investigation of insect–fungi relationships. These studies have illuminated key components of the innate immune system that apply even to mammals, and promise to provide useful approaches for investigations into antifungal drugs. The recent application of transcriptional profiling and of RNAi to insect cell lines interacting with fungal pathogens has added powerful new tools to these studies, and should provide both further fundamental insights and new practical approaches to questions of fungal pathogen function and treatment. In the future these technologies will allow researchers to probe deeply into the interactions between the insect host and fungal pathogens; likely some of these interactions will prove specific to the insect case, while others will highlight general functions. A major need is a greater molecular understanding of the processes controlling aspects of innate immunity, such as phagocytosis, melanization and clotting, that are not yet as advanced as those that control production of antimicrobial proteins. Further use of RNAi will provide greater information about the engulfment process in phagocytic cell lines, while identification of cell lines specialized in other immune processes would provide novel tools, and exploiting the multiple *Drosophila* genome sequences with standardized infection assays and comparative genomics should provide a powerful screening approach. However, the development of assays for immune functions and the identification of mutants affected in these processes, the approach exploited brilliantly in the dissection of the AMP

production process, is perhaps the most powerful strategy to gain insight into these functions in the whole organism. Ultimately we need to understand how the multiple processes are coordinated to provide such an impressive defence against fungal pathogens, and for this we will have to make good use of all the advantages of the fly as an experimental organism.

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