

Invited review

Functional genomics studies on the innate immunity of disease vectors

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Abstract The increasing availability of genome sequences and the development of high-throughput techniques for gene expression profiling and functional characterization are transforming the study of innate immunity and other areas of insect biology. Already, functional genomic approaches have enabled a quantum advance in the characterization of mosquito immune responses to malaria parasite infection, and similar high-throughput functional genomic studies of other vector–pathogen interactions can be expected in the near future. The application of microarray-based and other expression analyses provide genome-wide transcriptional profiles that can be used to identify insect immune system components that are differentially regulated upon exposure to various classes of pathogens, including many important etiologic agents of human and animal diseases. The role of infection-responsive or other candidate immune genes identified through comparative genomic approaches can then be functionally characterized, either *in vivo*, for instance in adult mosquitoes, or *in vitro* using cell lines. In most insect vectors of human pathogens, germ-line transgenesis is still technically difficult and maintenance of multiple transgenic lines logistically demanding. Consequently, transient RNA interference (RNAi)-mediated gene-silencing has rapidly become the method of choice for functional characterization of candidate innate immune genes. The powerful combination of transcriptional profiling in conjunction with assays using RNAi to determine gene function, and identify regulatory pathways, together with downstream cell biological approaches to determine protein localization and interactions, will continue to provide novel insights into the role of insect innate immunity in a variety of vector–pathogen interactions. Here we review advances in functional genomics studies of innate immunity in the insect disease vectors, over the past decade, with a particular focus on the *Anopheles* mosquito and its responses to malaria infection.

Key words *Anopheles*, genomics, innate immunity, insect, mosquito, malaria, microarray, parasite, *Plasmodium*, RNA interference, transcriptomics, transgenesis, vector

Introduction

The study of the innate immune system of insect disease vectors has undergone a profound revolution within the last decade, like many other areas of biological research

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(Hill *et al.*, 2005). This revolution has been facilitated by the work in model non-vector insects (primarily the fruitfly *Drosophila melanogaster*), the increasing public availability of genome sequences of insect disease vectors, and the development of high-throughput technologies for gene and protein expression profiling and for transient reverse genetic characterization of gene function. Together, these new resources comprise the tools for functional genomics: the high-throughput identification and characterization of genes of biological interest, through genome-wide expression screens and functional assays.

In the field of mosquito-malaria interactions, the publication and continuing annotation of the genome sequence of *Anopheles gambiae* (Holt *et al.*, 2002; Kalume *et al.*, 2005; Kriventseva *et al.*, 2005; Li *et al.*, 2006; Ribeiro *et al.*, 2004; Sharakhova *et al.*, 2007), the major vector throughout sub-Saharan Africa of the human malaria parasite *Plasmodium falciparum*, heralded a new era in research on mosquito innate immunity with unprecedented opportunities for investigating the determinants of mosquito vector competence (Christophides *et al.*, 2002; Dimopoulos, 2003). Similar genome sequencing and/or associated expressed sequence tag (EST) projects are now currently near completion, in progress and/or pending for a number of other medically important invertebrate vector species of human disease, including: the mosquitoes *Aedes aegypti* (<http://msc.tigr.org/aedes/aedes.shtml>) (Severson *et al.*, 2004) and *Culex pipiens quinquefasciatus* (http://msc.tigr.org/c_pipiens/index.shtml); the sandflies *Lutzomyia longipalpis* (http://www.sanger.ac.uk/Projects/L_longipalpis/) and *Phlebotomus papatasi* (http://genome.wustl.edu/genome_group_index.cgi); the tsetse fly *Glossina morsitans morsitans* (http://www.sanger.ac.uk/Projects/G_morsitans/) (Aksoy *et al.*, 2005); the kissing bug *Rhodnius prolixus* (http://genome.wustl.edu/genome_group_index.cgi); the louse *Pediculus humanus* (Pittendrigh *et al.*, 2006); and the tick *Ixodes scapularis* (<http://www.entm.purdue.edu/IGP/>) (Hill & Wikel, 2005). The genome sequence and expression data generated by these projects are made available on-line as a public resource to be exploited by the research community (e.g. <http://www.vectorbase.org>) (Lawson *et al.*, 2007).

Equally important to recent advances in the field of mosquito-malaria interactions has been the concurrent development of a suite of novel tools enabling high-throughput transcriptomic studies and analyses of gene function in mosquitoes. Two of the most important tools that have been developed are microarray-based platforms for genome-wide expression profiling (Chen *et al.*, 2004; Dimopoulos *et al.*, 2002; Dong *et al.*, 2006a; Kriventseva *et al.*, 2005; Marinotti *et al.*, 2006; Marinotti *et al.*, 2005; Vlachou *et al.*, 2005) and transient RNA interference (RNAi)-mediated gene silencing (Blandin *et al.*, 2002; Boisson *et al.*, 2006; Brown & Catteruccia, 2006; Hoa *et al.*, 2003; Levashina *et al.*, 2001). Identification of candidate immune genes involved in mosquito anti-*Plasmodium* defense through comparative genomics and/or transcriptional profiling, followed by functional characterization using RNAi, have rapidly become the paradigmatic approach for functional genomic analysis of vector innate immunity.

Here, we critically review the recent progress in the functional genomic characterization of the disease vector

innate immune system, with especial attention to its role in the mosquito as a determinant of *Plasmodium* infection.

The innate immune system of insect disease vectors: a brief overview

In broad terms, the innate immune system of insect disease vectors comprises three functional categories of genes involved in: (i) pathogen recognition; (ii) signaling pathways mediating signal amplification, modulation and transduction; and (iii) effector mechanisms mediating pathogen clearance from the host. Once pathogens are recognized by specific binding of insect pattern recognition receptors to pathogen-associated molecular patterns, that are unique to particular classes of microbial organisms, a variety of defense reactions can be activated (Medzhitov & Janeway, 2002). This immune response activation can either occur directly as in the case of phagocytosis and melanization, or indirectly through the intracellular immune signaling pathways that initiate transcriptional activation of appropriate antimicrobial peptides and other immune effector genes (for detailed reviews of the members and functions of these three functional categories see Christophides *et al.*, 2004; Dimopoulos, 2003; Osta *et al.*, 2004b). Comparative phylogenomic analyses using the much-studied model organism *D. melanogaster* have enabled the *in silico* characterization of the immune system repertoire of the malaria vector *An. gambiae* (Christophides *et al.*, 2002). These comparative bioinformatic studies have resulted in a catalog of putative mosquito immune genes, although not necessarily exhaustive, which provide the basis for further systematic exploration of the mosquito innate immune system and its role in malarial and other microbial infections. Of the approximately 14 000 genes currently recognized in the *An. gambiae* genome, 282 (approximately 2.0% of the total) have been identified as belonging to gene families or functional classes related to innate immunity (Christophides *et al.*, 2002, 2004). Comparisons between the genomes of *D. melanogaster* and *An. gambiae* show that components of immune signaling pathways exhibit the highest degree of conservation, mostly being found as 1:1 orthologs, while pattern recognition receptors and genes encoding effector molecules such as antimicrobial peptides exhibit the greatest divergence and diversity between these two dipteran species (Christophides *et al.*, 2002). The evolutionary conservation of the components of the immune signaling pathways across diverse insect phyla, as well as mammalian taxa, suggests that these signaling pathways perform fundamental and functionally similar roles in pathogen defense in divergent organisms (Cherry & Silverman, 2006;

Hoffmann *et al.*, 1999; Hoffmann & Reichhart, 2002; Tanji & Ip, 2005). Therefore, even in the absence of evidence for differential expression in response to microbial challenge, the conservation of immune signaling pathways implies *a priori* that these pathways are also key regulators of insect disease vector responses to pathogen infection. In accord with this theoretical prediction, an increasing number of studies are revealing a role for these evolutionarily conserved immune signaling pathways in mediating insect vector responses to pathogen infection (Bian *et al.*, 2005; Cheon *et al.*, 2006; Dillon *et al.*, 2006; Frolet *et al.*, 2006; Hu & Aksoy, 2006; Lehane *et al.*, 2003; Luna *et al.*, 2006; Meister *et al.*, 2005; Shin *et al.*, 2002, 2003, 2005, 2006).

As the genome sequences of other insect disease vectors become available, comparative phylogenomic analyses will continue to play an important initial role in the preliminary identification of the components of different insect innate immune systems. Furthermore, this will also allow significantly expanded and detailed phylogenomic comparisons of invertebrate innate immune systems, which will provide insight into the evolutionary dynamics driving conservation and diversity of the innate immune system across different insect phyla, inhabiting diverse and distinct ecological niches each associated with unique microbial exposures (Christophides *et al.*, 2002).

Transcriptional profiling of insect innate immune responses to pathogen infection

Evaluation of pathogen-induced transcriptional changes in insect disease vectors has been critical for identifying the role of both known and novel contributors to anti-microbial responses, as well as providing global overviews of the pathogenic processes occurring during infection. Gene transcription is intimately related to gene function, and can therefore be used to study functional responses of an organism to different stimuli. This is especially true for investigating insect responses to infection, where transcriptional induction/repression is a major mechanism regulating innate immunity. Accordingly, microarray-based high throughput gene expression analyses of insect vector responses to pathogen infection have been particularly fruitful, and are largely responsible for the major advances in our understanding of the *Anopheles* defense against *Plasmodium* (Dimopoulos *et al.*, 2002). One prerequisite for such analyses is the availability of gene sequence information that derive from either ESTs and/or genome sequence data (Abraham *et al.*, 2004; Christophides *et al.*, 2002; Dana *et al.*, 2006; Dimopoulos *et al.*, 2000; Holt *et al.*, 2002; Kriventseva *et al.*, 2005; Li *et al.*, 2006; Srinivasan *et al.*, 2004). The early availability of such resources for the

malaria vector *An. gambiae* has allowed for particularly detailed transcriptomic analyses of its innate immune system, and its regulation during bacterial, malarial and viral infections (Aguilar *et al.*, 2005b; Bonnet *et al.*, 2001; Christophides *et al.*, 2002; Dimopoulos *et al.*, 2002; Dong *et al.*, 2006a; Sim *et al.*, 2005; Tahar *et al.*, 2002; Vlachou *et al.*, 2005; Xu *et al.*, 2005), as well as characterization of the gene expression changes in uninfected mosquitoes during development and hematophagy (Dana *et al.*, 2005; Marinotti *et al.*, 2005, 2006; Warr *et al.*, 2007). Similar microarray-based studies have also been conducted for the yellow fever mosquito *Ae. aegypti* (Xi & Dimopoulos, 2007, unpublished) (Chen *et al.*, 2004; Sanders *et al.*, 2003, 2005), and can be expected in the near future for those insect disease vectors for which genome and/or EST projects are currently ongoing (Dillon *et al.*, 2006; Hao *et al.*, 2001; Lehane *et al.*, 2003; Ramalho-Ortigao *et al.*, 2001; Ursic-Bedoya & Lowenberger, 2007). A number of studies are also beginning to characterize tissue-specific patterns of gene transcription, especially for those tissues with which pathogens interact during infection of the insect vector. For example, Warr *et al.* (2007) characterized the spatial variation in gene expression throughout different regions of the *An. gambiae* midgut. In particular these authors found that the cardia was enriched for a variety of immune genes, including the antimicrobial peptides *Cec2*, *Cec3*, *Defl* and gambicin. Several studies have begun to characterize gene and protein expression in hemocytes and the hemolymph (Baton, Robertson, Warr, Strand, Dimopoulos, 2007, unpublished data) (Bartholomay *et al.*, 2004; Castillo *et al.*, 2006; Li *et al.*, 2006; Paskewitz & Shi, 2005), which are believed to be a major source of immune effector molecules mediating anti-*Plasmodium* responses (Abraham *et al.*, 2005; Blandin *et al.*, 2004; Castillo *et al.*, 2006; Frolet *et al.*, 2006; Volz *et al.*, 2005).

Transcriptional profiling of Anopheles responses to Plasmodium infection

Early studies of mosquito immune gene expression during malaria infection, characterized both local and systemic induction of immune gene expression, for a limited number of genes in different mosquito tissues (Dimopoulos *et al.*, 1997, 1998; Richman *et al.*, 1997). Subsequent studies using whole genome microarrays have provided characterization of changes in immune gene expression in whole mosquitoes and hemocyte-like cell lines, following various bacterial and malarial challenges, as well as other stresses (Christophides *et al.*, 2002; Dimopoulos *et al.*, 2002; Kumar *et al.*, 2003). These microarray analyses identified innate immunity as one of the major functional

gene classes exhibiting differential expression during pathogen infection/challenge. More recently, an increasing number of studies have characterized tissue-specific transcriptional profiles, especially mosquito midgut gene expression during the period of ookinete invasion and subsequent oocyst formation (Abraham *et al.*, 2004; Dana *et al.*, 2006; Dong *et al.*, 2006a; Srinivasan *et al.*, 2004; Vlachou *et al.*, 2005; Xu *et al.*, 2005). These studies have identified a number of immune and other genes that are differentially regulated during ookinete invasion, both within the midgut itself and throughout other tissues of the mosquito (Dong *et al.*, 2006a; Vlachou *et al.*, 2005). For example, Dong *et al.* (2006a) compared transcriptional responses of *An. gambiae* to infection with either the rodent malaria *P. berghei* or the human malaria *P. falciparum*, and thereby identified substantial qualitative and quantitative differences in mosquito gene expression following midgut infection with the ookinete stages of these two different malaria parasite species. Furthermore, while *P. berghei* triggers differential regulation of more than twice as many genes as *P. falciparum* (8.1% vs. 3.4% of the transcriptome), the latter malaria species induces a greater number of putative immune genes, with limited overlap between the transcription profiles for the two malaria parasite species (Dong *et al.*, 2006a). These studies highlight the importance of parasite variation in determining the outcome of malaria infection in the mosquito vector. Such studies also demonstrated the differential expression of genes associated with oxidative stress, apoptosis and/or cytoskeletal reorganization (Abraham *et al.*, 2004; Vlachou *et al.*, 2005; Xu *et al.*, 2005), consistent with the pathology and remodeling of the midgut epithelium accompanying ookinete invasion.

In contrast, relatively few studies have investigated salivary gland-specific immune gene expression following sporozoite invasion (Dimopoulos *et al.*, 1998). Rosinski-Chupin *et al.* (2007) recently reported a serial analysis of gene expression (SAGE) analysis of the salivary gland transcriptome during malaria parasite infection. These authors identified 37 immune genes expressed within the salivary glands, including up-regulation of *Cec2*, *Def1*, *GNBP* and *SRPN6* during the period of sporozoite invasion. These genes also exhibited varying spatial and temporal patterns of transcriptional expression in different regions of the salivary glands. Rosinski-Chupin *et al.* (2007) did not observe differential expression of genes associated with oxidative stress, apoptosis or cytoskeletal reorganization; consistent with previous observations that sporozoite invasion of the salivary glands is not accompanied by the host cell pathology occurring during ookinete invasion of the midgut epithelium.

Transcriptional profiling of mosquito responses to arboviral infection

Arboviruses constitute an important class of arthropod-borne infectious diseases, including dengue and yellow fever, but the molecular responses of insect vectors to infection with these agents of human disease have, as yet, not been widely studied. However, this situation will surely change within the next few years, with the imminent publication of the genome of *Ae. aegypti*, a major vector of both dengue and yellow fever. Several microarray analyses have been undertaken describing mosquito responses to alphavirus infection (Sanders *et al.*, 2005; Sim *et al.*, 2005). Sim *et al.* (2005) identified 18 genes differentially regulated in *An. gambiae* following o'nyong-nyong virus infection, while Sanders *et al.* (2005) reported the transcriptional responses of *Ae. aegypti* to Sindbis virus infection. The authors of the latter study found that components of both the Toll and JNK pathways were differentially regulated upon Sindbis virus infection, suggesting a role for these pathways in antiviral as well as antibacterial, antifungal and antimalarial immune responses (Sanders *et al.*, 2005).

Transcriptional profiling of other insect disease vector responses to microbial infection

Relatively few studies have described the transcriptional responses of other disease vectors to pathogen infection (Hao *et al.*, 2001; Rudenko *et al.*, 2005; Ursic-Bedoya & Lowenberger, 2007). Hao *et al.* (2001) reported differential expression of antimicrobial peptide genes in tsetse flies following bacterial and trypanosome challenge, while Ursic-Bedoya and Lowenberger (2007) recently identified a number of immune-related transcripts induced in *R. prolixus* following challenge with the causative agent of Chagas disease, *Trypanosoma cruzi*. Several large-scale EST studies have been undertaken for *G. m. morsitans* and *L. longipalpis*, and the ticks *I. scapularis* and *Rhipicephalus appendiculatus* (Attardo *et al.*, 2006; Dillon *et al.*, 2006; Lehane *et al.*, 2003; Nene *et al.*, 2004; Ribeiro *et al.*, 2006; Rudenko *et al.*, 2005). Such EST studies can provide a basis for the development of cDNA microarray platforms for subsequent profiling of insect vector transcriptional responses following pathogen infection.

Functional analysis of innate immunity genes of insect disease vectors

Once candidate insect innate immune genes have been identified through comparative genomics and/or transcrip-

tional profiling, the next task is to functionally characterize the role (if any) of those candidate genes in antipathogen defense. Unlike the fruitfly *D. melanogaster*, tools for large-scale, high-throughput insertional mutagenesis screens are not available for any insect disease vectors. Consequently, transient gene-silencing using RNAi has rapidly become the method of choice for characterizing gene function in insect disease vectors, because of its high efficiency and ease of performance in comparison to transgenic approaches in these organisms. One limitation of RNAi is the inability to directly over-express specific genes. (It should be noted that indirect gene over-expression is sometimes possible using RNAi, for example silencing of the negative regulator of the Toll/REL1 pathway, *Cactus*, results in endogenous over-expression of other immune-related factors [Cheon *et al.*, 2006; Frolet *et al.*, 2006]). In this context, the recent description in mosquitoes of tissue-specific over-expression of episomally maintained genes using “whole body transfection” is especially encouraging (Isoe *et al.*, 2007). Another potential problem with RNAi is the occurrence of so-called “off-target” effects, where non-target genes sharing homology with the intended target of gene silencing are concurrently silenced (Kulkarni *et al.*, 2006; Ma *et al.*, 2006). However, a recent study of CLIP domain serine proteases in *An. gambiae* reported minimal silencing of related but non-target genes, suggesting that RNAi specificity is typically robust (Volz *et al.*, 2006). Although genome-wide RNAi screens are not yet feasible in insect disease vectors, moderately high-throughput RNAi screens are being performed (Osta *et al.*, 2004a; Volz *et al.*, 2006). A notable example was the identification of *in vivo* regulators of bacterial phagocytosis in *An. gambiae* undertaken by Moita *et al.* (2005).

Tools for screens of gene function in mosquitoes

Although tools have been developed for stable germ-line transformation of mosquitoes (Catteruccia *et al.*, 2000; Grossman *et al.*, 2001; Jasinskiene *et al.*, 1998; Lycett *et al.*, 2004), the inherent technical and logistical difficulties of transgenesis and mutagenesis in anopheline mosquitoes have led investigators to rely on RNAi for rapid characterization of gene function (Blandin *et al.*, 2002; Boisson *et al.*, 2006; Brown & Catteruccia, 2006; Hoa *et al.*, 2003). Only one study has so far used transgenesis to investigate innate immunity in *An. gambiae*, demonstrating a role for *Cec1* in regulating rodent malaria infection (Kim *et al.*, 2004). In *Ae. aegypti*, transgenesis has been more exploited for investigation of mosquito innate immunity, especially for the Imd and Toll immune signaling pathways (Bian *et al.*, 2005; Cheon *et al.*, 2006; Shin *et al.*, 2003). The relative

ease and high efficiency of RNAi-mediated gene-silencing has greatly facilitated identification and functional characterization of mosquito innate immunity genes. The typical experimental strategy involves microinjection of gene-specific dsRNA into the thorax of adult mosquitoes, followed several days later by challenge of the injected adult mosquitoes with bacterial or malarial or other pathogens, and subsequent examination of the mosquitoes to score the phenotype resulting from dsRNA injection (i.e. mosquito survival rate, prevalence/intensity of microbial infection, melanization of foreign substrates, *in vivo* quantification of hemocyte phagocytosis etc) (Blandin *et al.*, 2002; Li & Paskewitz, 2006; Moita *et al.*, 2005; Paskewitz *et al.*, 2006; Warr *et al.*, 2006). RNAi in *An. gambiae* has even been used to show that the RNAi pathway itself acts as an antiviral mechanism against o'nyong-nyong virus infection (Keene *et al.*, 2004). As well as *in vivo* studies in adult mosquitoes, RNAi is also increasingly used in mosquito-derived immune competent cell lines, especially as a means of defining the pathways regulating immune gene expression (Fallon & Sun, 2001; Levashina *et al.*, 2001; Luna *et al.*, 2006; Meister *et al.*, 2005; Muller *et al.*, 1999; Shin *et al.*, 2005). A further emerging functional genomic strategy to identify the components regulated within the same/different immune pathways, is to simultaneously perform double or triple RNAi-mediated gene knock-downs (e.g. Moita *et al.*, 2005; Volz *et al.*, 2006) or to perform expression profiling of cell lines or individual insects, which have been subjected to RNAi (Frolet *et al.*, 2006; Meister *et al.*, 2005).

Functional characterization of Anopheles genes implicated in anti-Plasmodium immune responses

Since the first report of RNAi in *An. gambiae* (Blandin *et al.*, 2002), a large number of mosquito immune and other genes have been screened for their role in *Plasmodium* infection of the mosquito vector (Abraham *et al.*, 2005; Blandin *et al.*, 2004; Cheon *et al.*, 2006; Cohuet *et al.*, 2006; Dong *et al.*, 2006a, 2006b; Frolet *et al.*, 2006; Meister *et al.*, 2005; Michel *et al.*, 2005, 2006; Osta *et al.*, 2004a; Shiao *et al.*, 2006; Vlachou *et al.*, 2005; Volz *et al.*, 2005, 2006). The first anti-*Plasmodium* factors identified through RNAi-mediated gene silencing were the pattern recognition receptors: Thioester-containing protein 1 (TEP1), a complement-like protein (Blandin *et al.*, 2004); the leucine-rich repeat domain-containing protein, LRIM1; and two C-type lectins, CTL4 and CTLMA2 (Osta *et al.*, 2004a). While silencing of either *TEP1* or *LRIM1* resulted in increased levels of *P. berghei* oocyst infection, knock-down of the two *CTLs* resulted in increased parasite melanization (Blandin *et al.*, 2004; Osta *et al.*, 2004a). Subse-

quent studies using field isolates of *P. falciparum* and a local *An. gambiae* mosquito strain found that CTL4, CTLMA2 and LRIM1 had no effect on either levels of oocyst infection or parasite melanization (Cohuet *et al.*, 2006). The effect of TEP1 in vector-parasite field populations has yet to be reported, although silencing of *TEP1* does result in increased levels of laboratory *P. falciparum* infection in *An. gambiae* (Dong *et al.*, 2006a). The exact roles of TEP1 and LRIM1 in parasite killing and melanization have been unclear (Blandin *et al.*, 2004; Osta *et al.*, 2004a), but recent studies suggest that parasite lysis and melanization are functionally distinct events, albeit both mediated by TEP1 and LRIM1 (Shiao *et al.*, 2006; Warr *et al.*, 2006). More recently, other pattern recognition receptors mediating susceptibility of *An. gambiae* to *P. berghei* and/or *P. falciparum* infection have been identified (Dong *et al.*, 2006a, 2006b; Riehle *et al.*, 2006). These include: AgDscam (*An. gambiae* Down syndrome cell adhesion molecule), a protein encoded by a 101-exon gene potentially capable of producing over 31 000 pathogen-specific variants through alternative splicing (Dong *et al.*, 2006b); three members of the fibrinogen domain-containing (FBN) family (FBN8, FBN9 and FBN39); an MD2-like receptor, AgMDL1, specific for immune defense against *P. falciparum* (Li, Xi, and Dimopoulos, 2007, unpublished data) (Dong *et al.*, 2006a); and the leucine-rich repeat domain-containing proteins APL1/LRRD19 and APL2/LRRD7 (Dong *et al.*, 2006a; Riehle *et al.*, 2006). Several immunoglobulin-domain encoding genes, implicated in *An. gambiae* innate immunity, have also been identified in the *An. gambiae* genome, including IRID6 (Infection Responsive with Immunoglobulin Domain 6) (Garver & Dimopoulos, 2007, unpublished data). IRID6 contains multiple immunoglobulin domains as well as two kinase domains. Preliminary studies show that IRID6 is up-regulated following infection with either *P. berghei* or *P. falciparum*. Further, when silenced by RNAi, *P. falciparum* oocyst infection increases greater than two-fold (Garver & Dimopoulos, 2007, unpublished data).

Other studies investigated the role of serine proteases and their serpin inhibitors in innate immune responses to *Plasmodium*, especially in regulating melanization (Abraham *et al.*, 2005; Dong *et al.*, 2006a; Michel *et al.*, 2005, 2006; Paskewitz *et al.*, 2006; Volz *et al.*, 2005, 2006). These studies have shown that the regulation of ookinete lysis and melanization are complex processes involving the interaction between multiple serine proteases/serpin partners, some of which promote and some of which prevent innate immune reactions (Volz *et al.*, 2006). Furthermore, RNAi screens have revealed that the function of these serine proteases and their serpin partners is context-dependent: In different genetic backgrounds, silenc-

ing of certain CLIPA and CLIPB family serine proteases, and the serpin SRPN6, have either differing or opposite effects on parasite infection (Abraham *et al.*, 2005; Volz *et al.*, 2005, 2006). Furthermore, the effect of RNAi-mediated silencing of the serpin *SRPN2* in the same *An. gambiae* strain differs for *P. berghei* and *P. falciparum* (Michel *et al.*, 2006). Similar to the studies of TEP1 and LRIM1, investigations of CLIP serine proteases and serpins support the belief that parasite killing and melanization are functionally separate events, which are mediated by common effector molecules.

Studies using cell lines and adult mosquitoes show that the Toll/REL1 and Imd/REL2 intracellular immune signaling pathways regulate expression of a variety of antimicrobial peptides and pattern recognition receptors, including cecropins 1 and 3 (Cec1/Cec3), defensin 1 (Def 1), gambicin, TEP1 and LRIM1 (Frolet *et al.*, 2006; Luna *et al.*, 2006; Meister *et al.*, 2005). In aedine mosquitoes, the Toll/REL1 pathway has been shown to function in antifungal immune responses (Shin *et al.*, 2005, 2006) while the Imd/REL2 pathway in anopheline mosquitoes is involved in defense against both Gram-positive and Gram-negative bacteria (Meister *et al.*, 2005). The role of the Toll/REL1 and Imd/REL2 pathways in anti-*Plasmodium* defenses are currently unclear, as contradictory findings have been reported. Meister *et al.* (2005) showed that RNAi-mediated silencing of *Rel2* increased levels of oocyst infection in *An. gambiae* of the rodent malaria parasite *Plasmodium berghei*. In contrast, Frolet *et al.* (2006) found that only double *Rel1/Rel2* knock-down, as opposed to single knock-downs of either *Rel1* or *Rel2*, resulted in increased levels of rodent malaria infection. Additionally, RNAi-mediated knock-down of *Cactus*, a negative regulator of the Toll/REL1 pathway, has been shown to decrease both rodent and avian malaria oocyst infection (Cheon *et al.*, 2006; Frolet *et al.*, 2006). It will be important to determine the extent to which the findings of these studies, using unnatural model laboratory systems, apply to human malaria parasites in natural vector-parasite combinations. As well as the Imd and Toll immune signaling pathways, there is evidence implicating other pathways in mosquito immune responses to *Plasmodium* infection, although the roles of these pathways in vector-pathogen interactions are currently less well understood (Lieber & Luckhart, 2004; Lim *et al.*, 2005; Luckhart *et al.*, 2003; Luckhart & Riehle, 2007; Vodovotz *et al.*, 2004).

RNAi-mediated gene silencing has facilitated great progress in the characterization of the pattern recognition receptors and immune signaling pathways regulating anti-*Plasmodium* immune responses. In contrast, the exact effector molecules mediating parasite killing remain elusive. Although there is evidence that antimicrobial peptides

have some activity against *Plasmodium*, in general, antimicrobial peptides appear not to be major effectors of mosquito innate immune responses against malaria parasites (Baton & Dimopoulos, 2007, unpublished) (Blandin *et al.*, 2002). For example, knockdown of *gambicin* increases levels of *P. berghei* oocyst infection, but has no effect on *P. falciparum* in *An. gambiae* (Dong *et al.*, 2006a). However, several immune-responsive putative short secreted peptides (IRSP1 and IRSP5) with anti-*Plasmodium* effects may be novel antimicrobial peptides (Dong *et al.*, 2006a).

Intriguingly, functional genomic analyses employing RNAi also implicate several genes involved in lipid metabolism and transport in innate immune responses to *Plasmodium*. Such factors include apolipoprotein D, the glycoprotein precursor of apolipoprotein I and II (RFABG), and lipophorin and its receptor (Cheon *et al.*, 2006; Dong *et al.*, 2006a; Vlachou *et al.*, 2005). The significance of these findings has yet to be determined.

RNA interference functional screens in other insect disease vectors

Novel RNAi protocols have recently been developed for several other invertebrate vectors of medically and/or veterinary important diseases, including: *G. m. morsitans* (Hu & Aksoy, 2006), *R. prolixus* (Araujo *et al.*, 2006), and *Amblyomma americanum* and *I. scapularis* (Karim *et al.*, 2004; Narasimhan *et al.*, 2004). However, these tools for reverse genetic analysis have generally yet to be utilized for the functional characterization of innate immunity in these organisms, although Hu & Aksoy (2006) used RNAi to characterize the roles of Relish and attacin in tsetse fly infection with trypanosomes. These authors recently identified the *G. m. morsitans* ortholog of the immune transcription factor Relish, and demonstrated its regulation of induction of the antimicrobial peptides attacin and cecropin in the fat body and cardia following microbial challenge. Hu & Aksoy (2006) further showed that RNAi-mediated silencing of either attacin or Relish resulted in significant increases in both the prevalence and intensity of the two etiologic agents of African sleeping sickness, *Trypanosoma brucei brucei* and *T. b. rhodesiense* (Hu & Aksoy, 2006). Given the evolutionary conservation of the Imd and Toll immune signaling pathways, and their roles in regulating insect and mammalian innate immune responses, it seems highly likely that roles for either or both these pathways will be identified in a variety of other vector–pathogen interactions.

Several investigations have used RNAi to characterize the role of various tick salivary gland proteins in the transmission of the etiologic agent of Lyme disease, *Bor-*

relia burgdorferi, and the *Rickettsia*-like pathogen *Anaplasma phagocytophilum* (Pal *et al.*, 2004; Pedra *et al.*, 2006; Ramamoorthi *et al.*, 2005; Sukumaran *et al.*, 2006), but no studies have yet been published reporting the functional characterization of tick innate immunity genes and their role in determining vector competence.

Future perspectives and research directions

Recent technological developments have provided invaluable tools enabling high-throughput functional genomic analysis of the role of *Anopheles* innate immunity in anti-*Plasmodium* defense. However, our view of mosquito immune responses toward malaria parasites is still far from complete, but several key issues have emerged with relevance to future studies and general applicability to other vector–pathogen interactions. First, it has become apparent that innate immune genes may have different functions in different genetic backgrounds, between different strains of the same of insect disease vector species, and between different but relatively closely related insect disease vector species (Abraham *et al.*, 2005; Volz *et al.*, 2005, 2006). Second, different strains and species of the same pathogen are likely to elicit different, and exhibit differing susceptibilities to, the innate immune responses of insect disease vectors (Cohuet *et al.*, 2006; Dong *et al.*, 2006a; Michel *et al.*, 2006; Shiao *et al.*, 2006; Tahar *et al.*, 2002). These two observations emphasize the importance of specificity in vector–pathogen interactions, and raise issues regarding the applicability of findings from laboratory models to the vector–pathogen systems occurring in the field (Aguilar *et al.*, 2005a; Cohuet *et al.*, 2006). Another important issue for consideration in future functional genomic studies of vector–pathogen interactions will be to dissect the contribution to differential gene expression of different immune elicitors associated with hematophagous transmission of vector-borne pathogens. Previous studies indicate that insect vector immune responses are elicited not just by the infective stages of the relevant pathogen, but also by a variety of other factors such as other microbial flora of the insect vector (Baton & Dimopoulos, 2007, unpublished) (Azambuja *et al.*, 2005) and the infection status of the blood of the vertebrate host (Bonnet *et al.*, 2001; Dong *et al.*, 2006a; Lim *et al.*, 2005; Luckhart *et al.*, 2003). Generally, functional genomic studies so far conducted on vector–pathogen interactions have not controlled for these potentially confounding effects. Lastly, as the elegant study by Riehle *et al.* (2006) recently demonstrated, there is tremendous potential to integrate functional genomics approaches with classical genetic approaches to the mutual empowerment of both techniques (Jansen & Nap, 2001). In particular,

the prospect of using microarray-based techniques for high-throughput genotyping of polymorphic loci, in both vector and pathogen genomes, promises to provide a powerful means of performing association and linkage studies both in the laboratory and in the field (Turner *et al.*, 2005). As genetic analyses continue to identify the genes encoding components of the innate immune system mediating antipathogen defense, the remaining future and non-trivial challenge will be to characterize the interactions of the protein products of those genes with one-another, and with the pathogen itself.

In summary, the approach of functional genomics provides powerful new tools for rapid high-throughput characterization of the innate immune system of insect disease vectors. Progress of similar magnitude to that recently seen for mosquito–malaria interactions is expected to occur for other insect disease vectors within the near future, through ongoing and future developments of the research infrastructure required for functional genomics.

Acknowledgments

We thank other members of the Dimopoulos Group for fruitful discussions on the reviewed topics, and for sharing unpublished data. Work produced by the Dimopoulos Group has been supported by the Ellison Medical Foundation, the Johns Hopkins Malaria Research Institute, NIH/NIAID 1R01AI061576-01A1, the NSF and the WHO/TDR.

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Accepted May 28, 2007