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Insect immunity and its implication in mosquito–malaria interactions

George Dimopoulos

Centre for Molecular Microbiology and Infection,
Department of Biological Sciences, Imperial College of
Science, Technology and Medicine, SW7 2AZ London, UK

Summary

Insects' resistance to infectious agents is essential for their own survival and also for the health of the plant, animal and human populations with which they closely interact. Several of the major human diseases are spread by insects and are rapidly expanding as a result of the development of insecticide resistance in vectors and drug resistance in parasites. A vector insects' permissiveness to a pathogen, and hence the spread of the disease, will largely depend on the compatibility of the molecular interactions between the two species and the capability of the insect immune system to recognize and kill the pathogen. The innate immune system comprises a variety of components and mechanisms that can discriminate between different microorganisms and mount specific responses to control pathogenic infections. An impressive body of knowledge on the insects' innate immunity has been generated from studies in the model organism *Drosophila*. These studies are now guiding the exploration of the immune system in the vector mosquito of human malaria, *Anopheles*, and its implication in the elimination of parasites. *Anopheles* immune responses have been linked to parasite losses and some refractory mosquitoes can kill all parasites through specific defence mechanisms. The recently sequenced *Drosophila* and *Anopheles* genomes provide a detailed and comparative view on their immune gene repertoires that in combination with post-genomic analyses is used to further dissect the complex mechanisms of *Plasmodium* killing in the mosquito.

Introduction

Insects are exposed to a variety of infectious microbes in their habitats throughout their life cycle and some species feed on animal or human blood infected with parasites and viruses. In order to cope with the risk of infection, from the frequent and diverse microbial exposure, insects have developed several structural barriers and a multifaceted innate immune system comprising a variety of synergistic defence mechanisms (Fig. 1). The first line defence against microbes is represented by the structural barriers which includes the hardened outer exoskeleton, the peritrophic matrix of the midgut and the chitinous linings of the trachea. The midgut epithelium, apart from being an immune competent organ, is also serving as a structural barrier for microbes and parasites. The exoskeleton protects the insect organs and haemolymph from direct exposure to microbes of the environment and upon breakage it is rapidly sealed by coagulation and melanization reactions (Söderhäll and Cerenius, 1998; Theopold *et al.*, 2002). The peritrophic matrix is a chitinous sack that facilitates digestion and also protects the midgut epithelium from direct contact with the meal and a large proportion of the microbial midgut flora which can be boosted up to a 16 000-fold after a blood meal in some haematophagous insects (Demaio *et al.*, 1996; Shao *et al.*, 2001). Malaria parasites have developed a specific mechanism, utilizing a chitinase, to traverse the peritrophic matrix before invasion of the mosquito midgut epithelium (Shahabuddin *et al.*, 1993). Pathogens that have made it through the insects' structural barriers will encounter its innate immune system that is less complex than the adaptive immune system of vertebrates due to the absence of antibodies and B-cell memory. It is however, rapidly activated after challenge and has a certain degree of specificity to different microbial classes. It comprises a variety of effector mechanisms that ultimately can control infection. Insect innate immune responses involve both cellular and humoral defence mechanisms that are triggered by PRRs (pattern recognition receptors) capable of specific binding to PAMPs (pathogen associated molecular patterns) (Medzhitov and Janeway Jr., 2002). These PRRs can mediate microbial killing directly, through phagocyto-

Received 13 September, 2002; revised 4 November, 2002; accepted 4 November, 2002. For correspondence. E-mail g.dimopoulos@ic.ac.uk; Tel. (+44) 0207 5943205; Fax (+44) 0207 5943069.

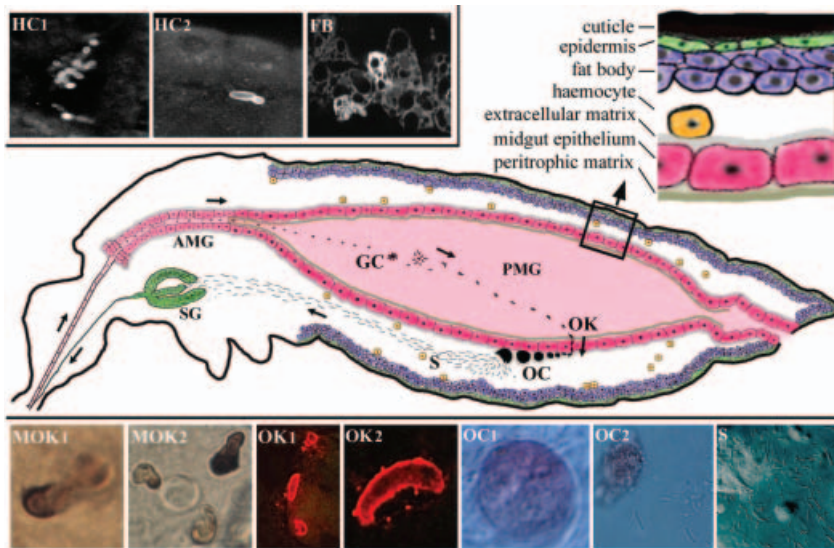


Fig. 1. Middle panel: Organs and cell types involved in *Plasmodium* interaction and immune response, and the parasites life cycle. Gametocytes (GC) enter the posterior midgut (PMG) through the anterior midgut (AMG), fertilize and develop into an ookinete (OK) that traverses the peritrophic matrix and midgut epithelium to form an oocyst (OC) under the basal lamina. After maturation, sporozoites (S) translocate from the oocyst (OC) to the salivary glands (SG). Drawings of organs and cells are not proportional. Upper left panel: Haemocytes stained with a FBN-specific antibody are aggregated and attached to the fat body (HC1) or on midgut cells (HC2). Only a subset of the highly vacuolated fat body cells (FB) express high levels of FBN. Lower panel: Melanized ookinetes in the midgut epithelium (MOK1, MOK2). Melanization is stronger on the apical end that is facing the haemolymph. Ookinetes (OK1, OK2) can be visualized in the midgut epithelium when stained with an ookinete surface protein-specific antibody. Infact mature oocyst on the basal side of the midgut epithelium (OC1). Sporozoites escaping from a ruptured mature oocyst (OC2). Sporozoites escaping from a damaged infected salivary gland (S2, provided by Dr. R. Sinden).

sis, or indirectly by the triggering of serine protease cascades that in turn can activate defence reactions such as melanotic encapsulation or initiate intracellular immune signalling pathways which regulate the transcription of antimicrobial peptide genes and other effector genes (Hoffmann *et al.*, 1996; Hoffmann and Reichhart, 2002). *Anopheles* mosquitoes are strongly activating their immune system when the parasites are invading the epithelial tissues and subsequently when they migrate through their open circulatory system. These immune responses peak at the stages when the largest parasite losses occur and have been linked to parasite elimination (discussed below; Dimopoulos *et al.*, 1997; 1998; Richman *et al.*, 1997; Luckhart *et al.*, 1998; Lowenberger *et al.*, 1999). A genetically selected refractory mosquito strain, L 3-5, can completely block *Plasmodium* development through a melanotic encapsulation mechanism that appears to involve components of the innate immune system (Collins *et al.*, 1986). A detailed understanding of the mechanisms involved in *Plasmodium* killing and the determination of mosquito permissiveness to malaria infection can ultimately be used for the development of novel malaria control strategies (Collins, 1994). The existence of a high density genetic map, several immune competent cell lines, the complete genome sequence, EST collections, expression profiling tools and transgenic tools, renders *A. gambiae* an important model organism for the study of the insect innate immune system and its interactions with a protozoan parasite (Zheng *et al.*, 1997; Müller *et al.*, 1999; Dimopoulos *et al.*, 2000; 2002; Grossman *et al.*, 2001; Blandin *et al.*, 2002; Holt *et al.*,

2002). Using the *D. melanogaster* model as a reference, this review will mainly focus on aspects of the innate immune system that are relevant to *Anopheles-Plasmodium* interactions.

Recognition of non-self

Infections with different microorganisms will selectively activate different effector genes and defence reactions. The molecular basis of discrimination between different microbial classes and the activation of elicitor specific immune responses is attributed to the binding specificity of PRRs to PAMPs such as LPS (lipopolysaccharide) and PGLC (peptidoglycan) (Medzhitov *et al.*, 2002). Two protein families, GGBP (Gram-negative bacteria-binding protein) and PGRP (peptidoglycan recognition protein), have been shown to activate intracellular immune signalling pathways in *D. melanogaster* upon interaction with PAMPs. GGBP contain a region with high similarity to β -1,3 glucan binding domains of bacterial glucanases, and was initially isolated from *Bombyx mori* as a Gram-negative bacteria-binding protein (Lee *et al.*, 1996). One GGBP gene from the mosquito *A. gambiae* has been molecularly characterized and shown to be strongly upregulated upon malaria infection (Dimopoulos *et al.*, 1997; 1998). One of the *D. melanogaster* GGBPs exists in both soluble and membrane bound forms and can activate NF κ B-dependent immune gene transcription upon LPS challenge (Kim *et al.*, 2000). A GGBP homologue has also been linked to the activation of melanization reactions in a moth (Lee *et al.*, 2000).

Peptidoglycan recognition proteins have been isolated from both insects and vertebrates, they share a highly conserved 160 residue PGR-region and comprise both secreted, transmembrane and cytoplasmic forms (Werner *et al.*, 2000). As many as 13 members of the PGRP gene family have been identified in the *D. melanogaster* genome and seven in the *A. gambiae* genome (Christophides *et al.*, 2002). A predicted *D. melanogaster* transmembrane PGRP (PGRP-LC) specifically activates the Imd pathway upon stimulation with Gram-negative bacteria whereas another soluble member, PGRP-SA, activate the Toll pathway upon stimulation with Gram-positive bacteria but not fungi (Michel *et al.*, 2001; Choe *et al.*, 2002; Gottar *et al.*, 2002; Ramet *et al.*, 2002). The mosquitoes' PGRP genes can produce several isoforms, through alternative splicing, with potential different ligand binding specificities. Interestingly, some of these isoforms are differentially regulated upon immune challenge, suggesting splice selection by immune signals (Christophides *et al.*, 2002). The *D. melanogaster* PGRP-LC gene, which can activate the Imd signalling pathway and mediate phagocytosis, has also been shown to produce several isoforms (Choe *et al.*, 2002; Gottar *et al.*, 2002; Ramet *et al.*, 2002). Several *A. gambiae* PGRP genes are strongly upregulated by both bacteria and malaria infection (Dimopoulos *et al.*, 2000; 2002; Christophides *et al.*, 2002). In moths, a PGRP has also been implicated in the activation of melanization reactions (Ochiai and Ashida, 1999). The binding specificities of the PGRP family members is not restricted to peptidoglycan and, together, they are most likely providing a broad recognition repertoire for a variety of microorganisms and are thereby playing a key role in the insects' immune surveillance system.

Another PRR family comprises proteins with a globular domain similar to the C-terminus of the fibrinogen gamma chain. The snails' (*Biomphalaria glabrata*) fibrinogen-related domain proteins, FREPs, are induced by infection with a digenic trematode *Echinostoma paraensei* and can precipitate parasite-derived molecules (Adema *et al.*, 1997). The Tachylectins5 from the horseshoe crab *Tachypleus tridentatus* are present in the haemolymph, can agglutinate bacteria and enhance the activity of big defensin. These horseshoe crab FREPs are now considered as the primary lectins involved in microbial recognition. Similarly to the vertebrate mannose binding lectins and Ficolins, Tachylectins can associate to form hexameric and octameric bouquet-like arrangements that is believed to increase their pathogen recognition specificity (Gokudan *et al.*, 1999; Kairies *et al.*, 2001). Five FREP genes have been cloned from *A. gambiae* and another 52 members have been identified from the genome sequence. Two of the characterized mosquito FREP genes are highly expressed in haemocytes and fat body and are, together with several other members, upregu-

lated by bacteria and malaria challenge (Dimopoulos *et al.*, 2000; 2002; unpublished data; Christophides *et al.*, 2002; Zdobnov *et al.*, 2002). Other proteins with potential pattern recognition function are represented by the TEPs (thioester containing proteins; discussed below), lectins, chitin binding domain proteins and scavenger receptor domain proteins (Dimopoulos *et al.*, 1996; 1997; 1998; Levashina *et al.*, 1999; Ramet *et al.*, 2001).

Transduction of immune response signals

Serine protease cascades

Pattern recognition receptors that have bound to PAMPs are believed to activate proteolytic cascades, involving serine proteases and serpins. These cascades will transduce and amplify the immune response signal to activate intracellular signalling pathways that control antimicrobial peptide gene expression, or effector systems such as melanization reactions. Most serine proteases of the invertebrate innate immune system have a mosaic structure consisting of a carboxy-terminal serine protease domain and a variety of other domains in their amino-terminal region. The clip-domain is the most frequently found amino-terminal domain and consists of a cysteine knot. Clip-domains are believed to be implicated in interactions with other proteins and have a similar structure to big defensin (Jiang and Kanost, 2000; Gorman and Paskewitz, 2001). The *D. melanogaster* genome contain about 200 serine proteases of which 35 carry clip-domains (Rubin *et al.*, 2000; Christophides *et al.*, 2002). The activity of serine protease cascades is tightly regulated by serpins. A clip-domain serine protease, persephone and a serine protease inhibitor, Spn43Ac, have been linked to the activation of the Toll pathway in *D. melanogaster* (Levashina *et al.*, 1999; Ligoxygakis *et al.*, 2002). Both serine proteases and serpins that are involved in the control of melanization reactions have been identified in moths (Jiang and Kanost, 1997; Jiang *et al.*, 1998; Satoh *et al.*, 1999; Park *et al.*, 2000) and some of the *A. gambiae* clip-domain serine proteases, AgSP14D1, AgSP14D2 and AgSP14A, have sequence characteristics of PPO (prophenoloxidase)-activating enzymes (Dimopoulos *et al.*, 2001).

Intracellular signalling pathways

The intracellular signalling pathways are transducing the signal for immune response from the serine protease cascades to the transcriptional machinery for the production of immune gene mRNAs. Two major immune signalling pathways have been identified and studied in *D. melanogaster*. The Toll pathway is activated by fungal and Gram-positive bacteria elicitors and the Imd pathway is activated by Gram-negative bacteria elicitors (Hoffmann and

Reichhart 1996; 2002). Toll itself is a transmembrane protein with an extracellular domain containing leucine-rich repeats and cysteine knots. The cytoplasmic domain of Toll is part of a multimeric protein kinase complex together with MyD88, Tube and Pelle. Activation of Toll by the cleaved Spaetzle ligand will lead to phosphorylation of Cactus and release of the Rel family transcription factors DIF and Dorsal. These transcription factors will induce immune genes after entry into the nucleus (Tauszig *et al.*, 2000; Shen and Manley 2002; Tauszig-Delamasure *et al.*, 2002). Another eight Toll-related genes are expressed in *D. melanogaster* and they are mainly implicated in developmental processes (Tauszig *et al.*, 2000). The *A. gambiae* orthologue of Dorsal, Gambif, is translocated to the nucleus upon bacteria infection but not malaria infection (Barillas-Mury *et al.*, 1996). Imd is a protein containing a death domain and it also shares similarity to the mammalian tumour necrosis factor- α (TNF- α) receptor interacting protein (RIP). Activation of Imd results in the endoproteolytic cleavage of the transcription factor Relish through a cascade that involves a caspase-8 homologue, DREDD and a mitogen-activated protein 3 (MAP3) kinase. The released Rel homology domain can translocate into the nucleus and then activate gene transcription of effector genes (Leulier *et al.*, 2000; Stoven *et al.*, 2000; Georgel *et al.*, 2001; Vidal *et al.*, 2001). The STAT pathway has also been linked to immune regulation in *A. gambiae* (Barillas-Mury *et al.*, 1999).

Killing mechanisms

Antimicrobial peptides

The majority of insect antimicrobial peptides are composed of 20–40 amino acids and are rapidly produced by the fat body and epithelial tissues upon infection. Their activity is in most cases specific for different classes of pathogens and the killing mechanism is believed to rely on the disintegration of the bacterial membrane or the interference with membrane assembly or bacterial proteins (Otvos, 2000). The *D. melanogaster* Toll pathway specifically control transcription of the antifungal peptide Drosomycin, whereas the Imd pathway is activating transcription of the anti-Gram-negative bacteria Dipterocins and Drosocins. Cecropins, Attacins and Defensins appear to be controlled by both pathways (Hoffmann *et al.*, 1996). As many as 24 immune inducible peptides, with antimicrobial activity and regulatory dependence on the Toll and Imd pathways, have been identified in the *D. melanogaster* haemolymph through a powerful mass spectrometry approach (Uttenweiler-Joseph *et al.*, 1998). The *A. gambiae* genome harbours four defensin and four cecropin genes (Christophides *et al.*, 2002). Members from both families are highly induced by malaria infection

(Richman *et al.*, 1997; Dimopoulos *et al.*, 1997; 1998; Vizioli *et al.*, 2000). The mosquito-specific Gambicin has been shown to possess both antimicrobial and anti-Plasmodial activity. (Dimopoulos *et al.*, 2000; Vizioli *et al.*, 2001; Christophides *et al.*, 2002).

Melanotic encapsulation

Two types of melanotic encapsulation mechanisms have been described in insects. Cellular encapsulation is mediated by haemocytes that surround and attach to the invading microorganism to form a haemocytic capsule that subsequently becomes melanized by PPOs (Gotz, 1986). Humoral encapsulation is the formation of a melanized proteinaceous capsule around the invading microorganism without the participation of haemocytes. Upon humoral encapsulation, PPOs are activated by the phenoloxidase activating system that comprises pattern recognition receptors linked to serine protease cascades (Söderhäll *et al.*, 1998). In mosquitoes, the proteolytically activated PPO together with Dopachrome conversion enzyme and DOPA decarboxylase is believed to mediate conversion of tyrosine to melanine through a series of oxidation reactions (Beerntsen *et al.*, 2000). *Anopheles gambiae* has nine PPO genes of which six have been molecularly characterized and shown to be highly expressed after the blood meal when *Plasmodium* encapsulation occurs. Immune challenge of mosquitoes does not lead to the activation of PPO gene transcription and their activity is therefore likely to be regulated at the post-transcriptional level (Müller *et al.*, 1999).

Phagocytosis

Phagocytosis involves killing of microorganisms through engulfment and subsequent degradation by haemocytes. It is mediated by pattern recognition receptors that can bind to the particle and trigger intracellular cascades leading to its internalization through an actin-dependent mechanism (Aderem and Underhill, 1999). In *D. melanogaster*, Croquemort is implicated in phagocytosis of apoptotic cells (Franc *et al.*, 1996). In *A. gambiae*, a complement-like thioester containing protein aTEP-1 has been shown to be implicated in phagocytosis of *E. coli* by an immune competent cell line. This phagocytosis appeared to involve binding of aTEP-1 to the bacterial surface through a thioester bond, similarly to the human complement factor C3 (Levashina *et al.*, 2001). A PGRP from *D. melanogaster* has also been implicated in phagocytosis of Gram-negative bacteria by haemocytes (Ramet *et al.*, 2002).

Spatial organization of insect immunity: immune competent organs and cell types

Different compartments, organs and cell types of the

insect are performing specialized immune functions and are together protecting against infectious microorganisms (Fig. 1).

Fat body

The fat body is a liver analogue in insects and is the principal storage organ and the major site of metabolism. It is also the major producer of immune proteins that it supplies into the haemolymph. It usually consists of a several cell layers thick sheet that is located underneath the epidermis in the abdominal and thoracic compartments (Fig. 1). The visceral fat body that is found in the posterior part of the insect is mainly involved in protein production, whereas the peripheral fat body in the thoracic part is the major storage site for glycogen (Paskewitz and Christensen, 1996; van Heudsen, 1997).

Epithelia

Epithelial tissues can protect against infection both as structural barriers, as in the case of the midgut, and through their immune competence that enables them to produce defence components. The epidermis, gut, salivary glands, genitals and malpighian tubules produce specific sets of immune components that are likely to be involved in specialized defence reactions (Dimopoulos *et al.*, 1997, 1998; Ferrandon *et al.*, 1998; Tzou *et al.*, 2000; Onfelt *et al.*, 2001). The regulation of immune responses in epithelial tissues is different from that of the fat body. For instance, only the Imd pathway has been implicated in transcriptional regulation of antimicrobial peptide genes in the *D. melanogaster* epidermis (Ferrandon *et al.*, 1998; Tzou *et al.*, 2000). The midgut is the most important epithelial barrier to infection as it surrounds the microbe rich lumen and is furthermore the entry point for many parasites and viruses that are transmitted by insects. Invasion of the midgut epithelium by *Plasmodium* ookinetes results in upregulation of several immune genes (Dimopoulos *et al.*, 1997; 1998; Luckhart *et al.*, 1998). The salivary gland is another major immune organ capable of producing an array of different immune peptides that may ensure sterility of the ingested food and the host wound upon blood feeding. Similarly to the midgut, the *A. gambiae* salivary glands activate immune gene transcription upon invasion by sporozoite stage Plasmodia (Dimopoulos *et al.*, 1998). Within a tissue, different cell types appear to carry out specialized immune functions (Fig. 1). Only the mosquitoes' anterior midgut expresses high levels of Defensin, and in the salivary glands, GNBP and Defensin are mainly expressed in the proximal lateral lobes (Dimopoulos *et al.*, 1997; 1998; Richman *et al.*, 1997).

Haemolymph and haemocytes

The haemolymph which is surrounding the different organs of the insect contain large quantities of immune components as well as haemocytes that can circulate freely or be attached to different organs (Fig. 1). Haemocytes produce immune components and are also capable of phagocytosing and encapsulating microorganisms. Four types of haemocytes with specialized functions have been described in *D. melanogaster*, including secretory cells, plasmatocytes, lamellocytes and crystal cells. The plasmatocytes are involved in phagocytosis. The lamellocytes are implicated in cellular encapsulation of parasites whereas the crystal cells are important for melanization (Rizki and Rizki, 1984; Shresta and Gateff, 1982; Lanot *et al.*, 2000). The JAK/STAT pathway has been shown to regulate lamellocyte differentiation and the Toll pathway has been linked to the proliferation of haemocytes (Qiu *et al.*, 1998; Zeidler *et al.*, 2000). Several *A. gambiae* immune competent cell line have been established and used extensively to study defence reactions that are likely to be carried out by haemocytes. The haemocyte-like characteristics of these cell lines includes highly inducible expression of immune genes, phagocytic activity and capacity to produce components of melanization reactions (Dimopoulos *et al.*, 1999; 2000; Müller *et al.*, 1999; Levashina *et al.*, 2001).

Comparative genomic analyses of the innate immune system

The genome sequences of *D. melanogaster* and *A. gambiae* have provided an unprecedented insight on their immune gene repertoires and allowed a genome-wide comparison of immune gene families between two dipteran species that diverged approximately 250 million years ago (Christophides *et al.*, 2002; Holt *et al.*, 2002; Zdobnov *et al.*, 2002). The two insects differ substantially with respect to their ecological niches and lifestyles. Whereas *D. melanogaster* feeds on yeast and sugar and lays its eggs in semidry locations, the female *A. gambiae* is feeding on blood and lays its eggs in water ponds where the offspring will develop until the adult stages. The vertebrate blood exposes the mosquito to viruses and parasites that can migrate from the midgut and infect other tissues. These adaptations have resulted in a quite different microbial exposure of the two species, including malaria infection, which in turn have led to a significant divergence of their innate immune systems. An analysis of 242 immunity-related genes from *A. gambiae* and their 185 *D. melanogaster* homologues have only revealed a relatively small number of 1:1 orthologues whereas homologous genes are highly abundant because of species-specific gene family expansions. Many orthologues

appear to either have been lost or forced to diversify. Interestingly, the largest number of orthologues and smallest diversification between the two species is found in gene families that are involved in the highly conserved signal transduction pathways of the innate immune system and developmental processes. Mosquito orthologues have for instance been identified for all Toll pathway components. In contrast, the largest family expansions and diversifications have occurred within gene families implicated in pathogen recognition, serine protease cascades and effector systems. Diversification of the protein repertoire for some gene families is also increased through alternative splicing of transcripts, as in the case of the PGRP genes (discussed above). The TEP families (discussed above) are represented by six and 15 members in *D. melanogaster* and *A. gambiae*, respectively. Only one 1:1 orthologous pair has been identified and most of the other members have resulted from species-specific expansions which may reflect adaptations to different microbial exposure (Christophides *et al.*, 2002). One of the most remarkable examples of a massive mosquito-specific gene family expansion is represented by the FREP family (described in earlier sections). Whereas only 13 FREP homologues have been identified in the *D. melanogaster* genome, the *A. gambiae* has as many as 58 different members (Zdobnov *et al.*, 2002). The reason for this massive expansion of the *A. gambiae* family is probably linked to haematophagy and exposure to *Plasmodium*. The bacteria and blood cell binding nature of FREPs may be important in controlling the midgut bacteria flora, which is highly boosted upon blood feeding, or prevent blood coagulation through competitive inhibition (Demaio *et al.*, 1996; Gokudan *et al.*, 1999). The malaria infection responsive nature of some mosquito members suggest them being a part of the antimalarial defence system (Christophides *et al.*, 2002; Dimopoulos *et al.*, 2000; 2002; unpublished data). The *A. gambiae* antimicrobial peptide, Gambicin, has also been identified in the *Aedes aegypti* mosquito but does not exist in the *D. melanogaster* genome (Dimopoulos *et al.*, 2000; Vizioli *et al.*, 2001; Christophides *et al.*, 2002). Gambicin may have evolved specifically to cope with the mosquitos' microbial flora or the malaria parasite.

The mosquitos' innate immune responses and *Plasmodium* killing

The lifecycle of *Plasmodium* in the mosquito is complex and involve several developmental transformations and spatial translocations through and between epithelial tissues (Fig. 1). The parasite suffers large losses during its sporogonic development. A small proportion of ingested gametocytes will develop into ookinetes and of these only a fraction will reach the oocysts stage. At the

later stages of infection, more than 80% of the haemocoel sporozoites will fail to relocate into the salivary glands and are instead rapidly cleared from the haemolymph through unknown mechanisms. The magnitude of these losses can differ greatly between infections with different parasite and mosquito species combinations and their molecular basis is unknown (Beier, 1998; Ghosh *et al.*, 2000; Dimopoulos *et al.*, 2002b). Parasite elimination in mosquitoes have been linked to the innate immune system and may be crucial for the successful transmission of malaria (discussed below; Luckhart *et al.*, 1998; Lowenberger *et al.*, 1999). High infection levels may seriously affect the mosquitos' fitness whereas a too low infection level may prevent transmission of the parasite. The lowest possible infection level that can permit transmission appears to be most favourable for both the parasite and the vector. In fact, several studies have indicated increased mosquito mortality upon high malaria infection levels that are more likely to occur in infections between unnatural mosquito-parasite species as opposed to low infection levels that are characteristic for natural mosquito-parasite combinations (Ferguson and Read, 2002). A finely tuned equilibrium between the mosquitos' immune system and the parasites immune evasive capability is likely to contribute towards the establishment of a low but transmissible infection level. Incompatibility of the biochemical environment in the mosquito, the receptor-ligand interactions involved in epithelial invasion and the temporal kinetics of parasite development and mosquito processes, such as blood digestion, are also likely to constitute important determinants of mosquito permissiveness to malaria infection and thus important regulators of transmission.

Mosquito refractoriness to Plasmodium infection

Several *Anopheles-Plasmodium* combinations are incompatible because of highly efficient parasite killing by mosquito refractory mechanisms. The best characterized refractory mosquitoes belong to the genetically selected L3-5 *A. gambiae* strain which melanotically encapsulates late ookinetes and early oocysts as they reach the basal side of the midgut epithelium between 20 and 40 h after infection (Fig. 1). This humoral encapsulation mechanism has a certain degree of specificity and key components of the melanization reaction appear to originate from the haemolymph (Collins *et al.*, 1986; Gorman *et al.*, 1998; Paskewitz *et al.*, 1998; Dimopoulos *et al.*, 2001). Melanized Plasmodia are rarely seen in field collected *A. gambiae* mosquitoes (Schwartz and Koella, 2002). Three quantitative trait loci (QTL) are controlling the refractory trait (Zheng *et al.*, 1997) and a 528 kb section of the major QTL, *Pen1*, chromosomal region has been sequenced and revealed 48 genes that are currently being analysed for potential implication in the refractory mech-

anism (Thomasova *et al.*, 2002). Factors such as pattern recognition receptor molecules or components of serine protease cascades represent promising candidates for the regulation of melanotic encapsulation. Microarray gene expression studies on the refractory L 3–5 mosquitoes have indicated major differences from the susceptible mosquitoes in expression signatures involving immunity and oxidoreductive genes. The potential implication of the innate immune system and/or the mosquitoes' oxidative status in the determinants of the refractory phenotype is currently investigated (C. Barillas-Mury, R. Cantera, G. Christophides, G. Dimopoulos, F. C. Kafatos, unpublished data).

Other refractory mosquito species and strains that kill Plasmodia with different mechanisms exist. A genetically selected strain of the oriental vector *Anopheles dirus* is almost totally refractory to *Plasmodium yoelii nigeriensis* infection whereas it will permit normal development of *P. falciparum* and *Plasmodium vivax*. In this strain, *Plasmodium yoelii* oocyst development is arrested within 12 h and then followed by melanization of dead early oocysts. The melanization reaction does not seem mediate the killing itself but occurs as a subsequent event (Somboon *et al.*, 1999). In a genetically selected *A. gambiae* strain, all *Plasmodium gallinaceum* ookinetes die in the midgut epithelium through a lytic mechanism. The dying ookinetes are first vacuolated and will then appear as degraded and broken within the midgut cell cytoplasm. Genetic crossing experiments suggest that this lytic killing mechanism is controlled by a single dominant locus (Vernick *et al.*, 1995). The Mexican malaria vector *Anopheles albimanus* is highly refractory to the *P. vivax* CS VK247 variant. The development of this parasite is compromised at three different stages in the midgut: the first portion of parasites is believed to be destroyed by mosquito digestive enzymes in the ectoperitrophic space close to the internal midgut surface; a second portion disintegrates within the midgut epithelium and; a third portion is arrested during early oocyst development on the basal side of the midgut epithelium. (Gonzalez-Ceron *et al.*, 2001). Although the implication of the mosquitoes' immune system in these refractory mechanisms is strongly suggested, their molecular basis remains unknown. The same mechanisms that are responsible for total refractoriness are also most likely operating at a lower level in susceptible mosquitoes where they reduce parasites at the crucial transition stages within and between epithelia. For instance, melanized Plasmodia have been documented in natural mosquito populations (Schwartz and Koella, 2002). Other killing mechanisms, such as lysis, are more difficult to document because of the lack of a visible phenotype. Hence, genetically selected refractory strains are important for the study of mechanisms mediating *Plasmodium* killing in natural susceptible mosquitoes.

Anopheles gambiae immune responses

Upon malaria infection, the mosquito is mounting robust local and systemic immune responses that correlate temporally and spatially with the parasite's development. During midgut invasion by the ookinete, several immune genes have been shown to be upregulated in both the midgut epithelium and in the fat body. The activation of immune responses in the fat body at this stage, when the parasites are still located within the midgut epithelium, strongly suggest the existence of immune signalling cascades between the different tissues. At the later stages of infection, when sporozoites translocate from the oocysts to the salivary glands, immune responses have been documented in the salivary gland and in the fat body. Genes implicated in these immune responses encode putative pattern recognition receptors, serine proteases, signalling pathway components, antimicrobial peptides and nitricoxide synthase (Dimopoulos *et al.*, 1997; 1998; 2002; Richman *et al.*, 1997; Luckhart *et al.*, 1998; Oduol *et al.*, 2000). A significant degree of overlap between responses to bacteria and malaria challenge has been demonstrated by gene expression analysis of 2300 genes on a microarray. The spectrum of genes that are regulated by malaria infection is significantly smaller than that of the bacteria infection induced genes and do not overlap with sterile injury induced genes. In this assay, the malaria infection responsive immune genes included a GGBP, a PGRP, a FREP, a TEP, a serine protease, a phagocytic component and a leucine rich repeat protein gene that share homology with Toll receptors (Dimopoulos *et al.*, 2002). The documented immune responses to malaria infection may partly result from the injury that is caused by parasite invasion of epithelial tissues as well as microbial components of the midgut that may be present at the invasion site. However, the lack of overlap between sterile injury responsive and malaria infection responsive gene expression signatures, and the strong activation of immune genes in antibiotic-treated malaria-infected mosquitoes strongly suggest the existence of a *Plasmodium* recognition-specific mechanism of immune induction (Richman *et al.*, 1997; Dimopoulos *et al.*, 2002). A comprehensive gene expression study using a whole genome microarray, representing the entire *A. gambiae* transcriptome, will provide a much more detailed view on the regulation of malaria infection responses and the implicated components. The significance of the documented immune responses in the elimination of parasites is strongly supported by the lower prevalence of malaria infection in mosquitoes that have been preimmune challenged with bacteria, the implication of the immune responsive nitricoxide synthase in *Plasmodium* killing and the correlation of immune

responses and parasite losses (Dimopoulos *et al.*, 1997; 1998, 2000; 2002; Richman *et al.*, 1997; Luckhart *et al.*, 1998; Lowenberger *et al.*, 1999). The immune responsive mosquito-specific antimicrobial peptide Gambicin has been shown to possess lethal activity against ookinete stage Plasmodia (Vizioli *et al.*, 2001). Anti-plasmodial activity against the oocyst and sporozoite stages of *Plasmodium* has also been shown *in vitro* for defensins from other insects (Shahabuddin *et al.*, 1998).

Other insect–parasite models

Whereas most of our knowledge on the insect innate immune system and its interactions with parasites has derived from studies in *D. melanogaster* and *A. gambiae*, respectively, studies of other insect–pathogen models are revealing novel and complementary aspects on insects' immune system and resistance to human parasites.

For example, ookinete stage Plasmodia that have been injected into the *D. melanogaster* hemocoel will develop oocysts and produce infectious sporozoites that are rapidly cleared from the haemolymph. Parasite development was not compromised in mutants with constitutively active Toll receptors, nor was transcription of antimicrobial peptide genes induced in infected flies. Killing of sporozoites in the haemolymph appeared to involve haemocytes and other unknown components (Schneider and Shahabuddin, 2000). Drosophila immune responses and killing mechanisms of Plasmodia are likely to differ significantly from those taking place in the co-adapted mosquito vector. However, the fly with its powerful genetic and transgenic tools may provide a useful model to identify, study and engineer anti-Plasmodial proteins.

Studies in tsetse (*Glossina* spp.) vectors of African sleeping sickness and nagana have shown upregulation of several antimicrobial peptides upon infection with *Trypanosoma*. Interestingly, the tsetse's immune surveillance system appears to be capable of discriminating between parasites and bacteria, and between different parasite life stages. The implication of tsetse immune responses in parasite killing is suggested by the significantly lower *Trypanosoma* infection levels in flies that have been preimmune challenged with bacteria (Hao *et al.*, 2001; Boulanger *et al.*, 2002). A recently implemented gene discovery project have identified over 60 tsetse fly genes with potential implication in its immune system and will permit a more detailed dissection of tsetse responses to *Trypanosoma* infection (M. J. Lehane, personal communication).

Conclusions and discussion

The past decade has experienced a revolution in our knowledge on the insect innate immune system and

novel aspects are continuously added. For instance, the apparent connections between immune cascades and the apoptotic machinery may prove to play a key role in the mosquitoes' interactions with the malaria parasite (Christophides *et al.*, 2002; Hoffmann and Reichhart, 2002). *Plasmodium* infection has been suggested to induce apoptosis of both invaded mosquito midgut cells and follicular epithelial cells (Han *et al.*, 2000; Hopwood *et al.*, 2001).

The available *A. gambiae* genome sequence in combination with high throughput gene expression analysis and transgenic technologies will allow a more comprehensive dissection of the mosquitoes' responses to infection. These studies can be expanded and include different refractory mosquito strains, that are easily selected in the laboratory (H. Hurd, personal communication), and mosquitoes from the field that may have diverged significantly from the currently studied lab strains.

The accumulated knowledge on vector–parasite interactions will ultimately allow the development of disease control strategies based on transgenic refractory insects and other transmission blocking approaches where host antibodies can block the parasites' lifecycle in the mosquito (Collins, 1994; Stowers and Carter, 2001; Ito *et al.*, 2002). The mosquitoes' innate immune system could be utilized in various ways for the development of malaria control strategies based on genetically engineered mosquitoes or the spread of resistance genes in mosquito populations with mobile elements. In one scenario the mosquito could have a boosted anti-Plasmodial immune-surveillance system that would recognize the parasite as non-self more efficiently and/or stronger activate *Plasmodium* killing mechanisms. In another scenario, the engineered mosquito could express a blood meal inducible immune protein that would kill ookinetes in the midgut epithelium. The implementation of such control strategies will, in addition to a comprehensive dissection of the mosquitoes' immune system, also require consideration of the transgenic mosquitoes' fitness, the possible selection of resistant parasites to the killing mechanism and a detailed knowledge of the field mosquito populations that frequently are composed of several sympatric, but reproductively isolated, sibling species (Coetzee *et al.*, 2000; Alpey *et al.*, 2002; Enserink, 2002).

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