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Mosquito immune defenses against *Plasmodium* infection

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Abstract

The causative agent of malaria, *Plasmodium*, has to undergo complex developmental transitions and survive attacks from the mosquito's innate immune system to achieve transmission from one host to another through the vector. Here we discuss recent findings on the role of the mosquito's innate immune signaling pathways in preventing infection by the *Plasmodium* parasite, the identification and mechanistic description of novel anti-parasite molecules, the role that natural bacteria harbored in the mosquito midgut might play in this immune defense, and the crucial parasite and vector molecules that mediate midgut infection.

I. Introduction

Within the mosquito vector, malaria parasites must go through a series of complex developmental transitions before transmission to a human host occurs. After being ingested by a mosquito, male and female gametocytes fuse within the midgut lumen and will over a period of approximately 18 hours develop into a motile ookinete that will migrate to the midgut epithelium and invades a single epithelial cell. The ookinete must travel to the basal lamina before the infected cell is extruded from the epithelial layer. Once it arrives at the basal lamina, the parasite differentiates into an oocyst and then further develops over a period of about 10 days into thousands of sporozoites that are released into the mosquito hemolymph. Sporozoites migrate to and invade the salivary glands and can be transmitted when the mosquito takes another blood meal. A major bottleneck for *Plasmodium* development takes place during the ookinete invasion of the midgut epithelium. The majority of the parasite loss can be attributed to lumenal and epithelial immune responses mounted by the mosquito.

Insect (and especially dipteran) innate immunity has generally been resolved using the fruit fly *Drosophila melanogaster* as a model and bacteria or fungi as the challenging microbe. For a more thorough coverage of *Drosophila* immunity, the reader is referred to a review by Lemaitre and Hoffmann [1]. Cellular and humoral factors are major players in the response to microbial challenge, especially within the hemolymph (blood) of the insect. Hemocytes, the insect blood cells, are constantly circulating and can either engulf (by phagocytosis) or surround (encapsulation) a foreign invader as a defense mechanism. Humoral responses to pathogens involve melanization and antimicrobial effector molecules. During melanization, a serine protease cascade activates pro-phenoloxidases that, through a second catalytic

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cascade, generate the melanin and free radicals that are involved in killing microbes. Production of antimicrobial effector molecules are regulated by intracellular immune signaling pathways that are activated by pattern recognition receptors (PRRs) upon interaction with pathogen associated molecular patterns (PAMPs).

The intracellular immune signaling pathways have been extensively studied in *Drosophila*, with most information having been obtained by injection of bacteria or fungus directly into the fly hemolymph. Toll pathway activation occurs through pathogen detection by soluble peptidoglycan recognition proteins (PGRPs) that stimulate a serine protease cascade, culminating in the proteolytic activation of the extracellular ligand, Spätzle. Activation of a second pathway, the immune deficiency (IMD) pathway, occurs when a pathogen is detected by a membrane-bound class of PGRPs. From either pathway, extracellular signals initiate a series of intracellular reactions that lead to an increased expression of select immune-related genes, including antimicrobial peptides (Figure 1).

Here we describe recent findings concerning the role of immune signaling pathways in preventing infection of the mosquito vector by the malaria parasite, the identification and mechanistic description of novel anti-parasite molecules, the role that natural bacteria harbored in the mosquito midgut might play in this overall immune response, and the crucial parasite and vector molecules that mediate midgut infection. The role of pattern recognition receptors in activating anti-*Plasmodium* defense will be discussed in the different sections.

II. Immune signaling pathways and *Plasmodium* infection

Immune signaling pathways, which direct insect immune responses to a variety of pathogens, have recently been shown to regulate anti-*Plasmodium* immunity in mosquitoes. The three major immune signaling pathways (Toll, IMD, and Jak/Stat) that were originally described in *Drosophila* or mammals have been identified through orthology in *Anopheles gambiae* [2]. A schematic representation of the Toll and IMD pathways is provided in Figure 1.

The Toll Pathway

The classical Toll pathway is activated upon infection with Gram-positive (G+) bacteria and fungi. It has also been implicated in the defense against viruses in fruit flies [3] and mosquitoes [4] and against the rodent malaria parasite *P. berghei* in *Anopheles* mosquitoes [5] (see below). PAMP recognition by Toll pathway PRRs is well documented, but the underlying mechanism is currently unresolved.

While *Drosophila* has two different transcription factors that separate the expression of Toll-mediated immune and developmental gene expression (Dif and Dorsal, respectively), *Anopheles* mosquitoes appear to express only an ortholog of Dorsal, the NF-kappaB-like REL1 (originally described as Gambif1) [2], [6], [7], [8], [9], [10]. Cactus, identified in *Drosophila* as a negative regulator of the pathway, sequesters REL1 to the cell cytoplasm [11], [12], [13], [14]. Directed degradation of cactus frees REL1 for nuclear translocation and subsequent transcription initiation at kappaB sites located upstream of canonical Toll pathway effector genes [6], [15], [16].

Using RNA interference (RNAi)-mediated gene silencing of *cactus* gene expression, Frolet and colleagues (2006) have shown that Toll pathway activation significantly decreases *P. berghei* parasite burden, while REL1 depletion increases infection levels in mosquito midguts. Importantly, they demonstrated that removal of a negative regulator could activate an immune response without pathogen challenge. They hypothesized that pre-formed effector molecules are able to immediately attack an invading pathogen and that early

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transcriptional activation is a means of replenishing molecules used in the directed immune response [5]. However, Toll pathway-mediated control of *Plasmodium* parasite infection may not be universal. Using multiple parasite-mosquito species combinations, Garver and coworkers (2009) showed for the first time that *P. berghei* infection of *A. gambiae, A. stephensi*, and *A. albimanus* is controlled through Toll pathway activation, while *P. falciparum* infection of the same mosquito species is independent of *cactus* depletion. Expression of numerous genes from diverse functional groups was regulated by *cactus* depletion, likely as a result of Toll pathway involvement in processes other than immunity (such as development); this widespread effect may contribute to the significant reduction in fitness seen in *cactus*-silenced mosquitoes [17].

In an *Aedes aegypti-P. gallinaceum* infection model, *cactus* silencing has been shown to protect mosquitoes from parasite infection via a mechanism that requires the expression of a second transcription factor, RUNT related transcription factor 4 (RUNX4) [18]. In this model, parasite melanization was mediated by prophenoloxidase gene expression, which was controlled cooperatively by REL1 and RUNX4. This dual-factor mechanism, or potentially another transcription factor such as REL2 (see below), might be used in mosquitoes to separate Toll pathway-mediated expression of different gene sets from disparate functional groups.

The IMD pathway

A second major immune signaling pathway, the IMD pathway, is likened to the tumor necrosis factor (TNF) signaling pathway in mammals [19], [20]. Microbe detection by PGRPs initiates intracellular signaling through the adaptor IMD protein and various caspase-like proteins and kinases, leading to a functional split in the pathway [21], [22], [23], [24], [25], [26], [27], [28]. One branch is similar to the c-Jun/JNK pathway of mammals and uses JNK to activate the transcription factor AP-1, while the other branch, an NF-kappaB activating branch, culminates in the processing of the transcription factor REL2 (Relish in *Drosophila*) [2], [29], [30], [31], [32], [33], [34], [35], [36], [37], [38].

In the absence of immune stimulation, REL2 exists in two splice variants: a short form (REL2-S) lacking the inhibitory ankyrin domain that is constitutively active and responsible for basal immune gene expression, and a full-length form (REL2-F) that is inactive until immune stimulation occurs [29], [39]. IMD pathway activation stimulates cleavage of the carboxy-terminal end of REL2-F, exposing the nuclear localization signal for nuclear translocation and subsequent transcription initiation [30], [40]. Because REL2-S has no unique features when compared to REL2-F, dsRNA-mediated depletion of the short form alone is not possible. However, Meister *et al.* (2005) have suggested that REL2-F is essential for anti-*Plasmodium* activity because targeted depletion of the full-length form only or of both REL2 forms results in similar infection phenotypes in a *P. berghei* model. Their data also suggest that processing of REL2-F, carried out and regulated by components of the IMD pathway, is important for anti-*Plasmodium* gene transcription [29].

Depletion of *caspar*, a Fas-associating factor homolog that inhibits Relish activation in *Drosophila* [41] and is a putative IMD pathway inhibitor in *Anopheles*, produces a *P. falciparum*-refractory phenotype in *A. gambiae* females, indicating that the IMD pathway (at least downstream of caspar-mediated inhibition) controls transcription of genes involved in parasite elimination [17]. *Caspar* silencing is effective in limiting *P. falciparum* infection of three anopheline species but is less effective against *P. berghei*, indicating that IMD pathway-mediated control of parasite infection is mosquito species-independent and parasite species-dependent. It is interesting that the IMD and Toll pathways are both mosquito species-independent but limit infection for distinct parasite species. Depletion of caspar does

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not confer noticeable fitness effects in the laboratory environment, a finding that reflects the strictly immune-responsive nature of IMD [17].

The IMD pathway of *A. gambiae* may be more complex than has been reported for *Drosophila*. Preliminary data suggest that immune responses directed by components of the IMD pathway may be IMD protein-independent, and REL2 isoforms are regulated and utilized in a multi-faceted manner [29], [39], [42], [43].

The Jak/Stat Pathway

The third major immune signaling pathway, the Jak/Stat pathway, is named for the kinases (Jak) and transcription factors (STAT) that control its activation. Research on this pathway has intensified in *Drosophila*, where Jak/Stat has been shown to play an important role in the immune response against pathogenic bacterial infection in the gut [44], [45]. The pathway has also been associated with antiviral immunity in *Drosophila* [46] and *Ae. aegypti* [47] but until recently had been less extensively explored in the context of *Anopheles-Plasmodium* interactions.

Two STAT transcription factors (STAT-A and STAT-B) have been identified in *A. gambiae*, while only one STAT is present in *Ae. aegypti* and *Drosophila*. STAT-B apparently regulates the transcription of STAT-A, the ancestral transcription factor and predominant form expressed in adult mosquitoes. Translocation of STAT-A into the nucleus leads to up-regulation of anti-*Plasmodium* effector molecule expression. Recently, the pathway was shown to mediate the killing of *P. falciparum* and *P. berghei* parasites at a later infection stage, after midgut invasion. Depletion of STAT-A increased *P. berghei* oocyst intensity, while activation of the pathway (through depletion of the negative regulator SOCS) decreased infection levels [48].

Pathway divergence from Drosophila

Recent evidence suggests that the single pathogen-single pathway paradigm of immune activation in *Drosophila* may not be as clear-cut in mosquitoes. PGRP-LC, a receptor for IMD pathway activation, has been found to be essential for mosquito survival following Gram-negative (G-) and G+ bacterial challenge [42]. Also, the IMD pathway provides early protection from G+ bacterial infections and long-lasting protection against G- bacteria [43], in contrast to the pathogen specificity of the Toll (G+ bacteria and fungi) and IMD (G- bacteria) pathways that has been noted in *Drosophila*. Although *Drosophila* is a good model system for forward genetic screens and has been used in previous studies to identify anti-*Plasmodium* effectors, the data obtained correlate with mosquito immunity, especially with regard to *Plasmodium* infection, on a case-by-case basis [49].

Other pathways have been implicated in the defense against *Plasmodium* infection of the mosquito, including a kinase-kinase signaling cascade (MAPK-ERK) that is apparently regulated by mosquito ingestion of a human cytokine [50]. However, the specifics of how different microbes elicit immune responses, how the pathways discriminate among different methods of activation, how the alternative activation methods influence downstream gene transcription and microbe destruction, and why such branching is beneficial to the mosquito are all unclear at this point. When and where in the mosquito body these pathways are activated and on which parasite stage the response is targeted are areas of interest that are only beginning to be elucidated.

III. Effectors of the anti-*Plasmodium* response

The ultimate result of immune signaling pathway activation is an up-regulation of specific gene expression that is PAMP- and pathway-dependent. These immune effector genes form

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an important line of defense for the mosquito against invading pathogens. Microarray and RNAi-based studies have shown that effector molecules from diverse gene families play important roles in the killing of *Plasmodium* ookinetes and/or oocysts. The mechanisms employed by anti-parasite molecules are just beginning to be unraveled as new protein-protein interactions are discovered.

Leucine rich-repeat (LRR) domain-containing proteins

LRR domain-containing proteins play a key role in mediating anti-*Plasmodium* immunity in mosquitoes. This protein family encodes secreted, membrane-bound, and cytoplasmic proteins with numerous leucine-rich repeats (LRRs); these proteins are up-regulated in *A. gambiae* following infection with *Plasmodium* [2], [51], [52]. In this gene family, LRIM1 is a potent *P. berghei* antagonist, while LRRD19 (also known as APL1) and LRRD7 are involved in the defense against both human and rodent *Plasmodium* parasites [52], [53], [54].

Manual re-annotation has revealed that the LRRD19 (or APL1) locus encodes three distinct genes (APL1A, -B, and -C) that have arisen from recent duplications. Of the three, APL1A and -B have been proposed to have no effect on *P. berghei* infection while APL1C is suggested as the sole *P. berghei* antagonist [55]. LRIM1 and LRRD19 interact and play an important role in parasite melanization and killing during early *P. berghei* infection [53], [56], [57]. In *A. gambiae*, LRIM1 and APL1C form a disulfide-linked, high-molecular-weight complex that is secreted into the hemolymph. The heterodimeric complex interacts with the complement C3-like protein TEP1 and may be necessary to promote cleavage of TEP1 into an active form. The reactive TEP1 subsequently localizes to the surface of midgut-invading *P. berghei*, targeting the parasite for destruction. LRIM1 and APL1C are required for hemolymph circulation and binding of TEP1 to the parasite surface [43], [58]. These results reveal a role for LRR proteins as complement control factors that may function as part of a complement-like system in killing *Plasmodium* parasites.

LRIM1 does not interfere with natural *P. falciparum* infection but does control melanization in an anopheline species that is naturally refractory to *P. berghei* [56]. APL1A has however been suggested to be involved in *P. falciparum* resistance, both in laboratory colonies and field-collected mosquitoes [56], [54], [55], [59]. Other proteins such as fibrinogen immunolectin 9 (FBN9) [60] (see below) and/or other LRR proteins (such as LRRD7) may provide alternate mechanisms for TEP1-mediated parasite killing of human *Plasmodium* species.

More than 20 LRIM1 -related proteins have been identified in the genomes of vector mosquito species, but no orthologs have been found in other organisms, suggesting a mosquito-specific immune role for these genes [43]. APL1C and LRIM1 appear to be regulated by the Toll pathway [5], [58], [55]; however, the IMD pathway plays an essential role in mounting the anti-*P. falciparum* immune response through FBN9, LRRD7, and TEP1 in diverse anopheline mosquito species [17]. Expression of TEP1 from hemocytes circulating in the mosquito hemolymph may also be regulated by the Jak-Stat and Toll pathways, suggesting a multipartite regulation of this potent anti-*Plasmodium* effector [5], [48], [58].

The fibrinogen-related proteins (FREPs)

FREPs share a fibrinogen-like domain (FBG) that is evolutionarily conserved from invertebrates to mammals [61], [62], [63]. FREP gene families have been identified in mosquitoes and flies, with a significant expansion in the *A. gambiae* genome (58 genes) when compared to *A. aegypti* (37 genes) and *D. melanogaster* (14 genes) [60], [63], [64],

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[65]. A strong correlation between phylogeny, chromosomal location, and the expression pattern of FREP genes was identified in *A. gambiae*, implying conserved functions among the duplicated family members that apparently arose through tandem duplication and shuffling of FBG domains [60].

Transcriptomic and functional analyses have shown that FREP genes are involved in the mosquito's immune response to bacteria and *Plasmodium* parasites. RNAi-mediated gene silencing assays have implicated FBN8, FBN9, and FBN39 in the anti-*Plasmodium* defense, where FBN39 specifically protects the mosquito against *P. falciparum* [2], [51], [52], [66]. Functional studies of 38 members of this gene family in *A. gambiae* have revealed that FREP proteins have complementary and synergistic functions that are mediated by inter- and intra-molecular associations. Interestingly, *in vitro* bacterial binding assays show that FBN9 forms homodimers (and possibly heterodimers with other FREP proteins) that bind to bacterial surfaces with different affinities [60]. FREPs may use a mechanism similar to LRIM1/APL1C to form multimers as a means of increasing the mosquito's PRR repertoire and mediating anti-pathogen responses. However, the molecular basis for this mechanism remains to be elucidated.

C-type lectins (CTLs)

The C-type lectin (CTL) family is one of the largest and most diverse animal lectin families. CTLs bind carbohydrates in a Ca^{2+}-dependent manner through the C-terminal carbohydrate recognition domain. These binding events mediate processes such as cell adhesion, cell/cell interactions, glycoprotein turnover, and pathogen recognition. In vertebrates, the collectins (collagenous lectins) serve as acute-phase proteins that mediate opsonization, clearance of microbial agents, and complement activation through the lectin pathway [67], [68], [69].

Twenty-three genes encoding C-type lectin domains have been identified in the *A. gambiae* genome. CTL4 and CTLMA2 have been identified as agonists of the rodent *Plasmodium* parasite, and silencing of either of these genes induces massive melanization of *P. berghei* ookinetes in the basal labyrinth of the midgut epithelium, blocking their development at the pre-oocyst stage [53]. However, CTLs do not appear to be involved in the defense against human *Plasmodium* parasites. A more recent examination has shown that CTL4 and CTLMA2 also significantly contribute to the mosquito's defense against G-, but not G+ bacteria. Like LRIM1/APLC, CTL4 and CTLMA2 each exist in the mosquito hemolymph as a disulfide-linked hetero-dimeric complex, a similarity that partially explains their similar roles in antibacterial defense and the melanization response to *P. berghei* [70]. However, whether a complement-like killing mechanism of action is utilized remains to be elucidated at the molecular level.

Other effector molecules

Other molecules have been implicated in killing *Plasmodium* parasites. Nitric oxide synthase (NOS), which is induced by the Jak-Stat or TGF-β1/MEK-ERK pathway, has potent anti-*Plasmodium* activity in the mosquito midgut and may be a late-stage line of defense against *Plasmodium* [48], [50]. NOS and antimicrobial proteins, such as gambicin and cecropin, were among the first anti-*Plasmodium* factors to be identified [71], [72]. Gambicin and a novel putative short secreted peptide, IRSP5, are more specific for defense against the rodent parasite *P. berghei* [52]. Among the putative pattern recognition receptors (PRRs) of *A. gambiae, s*plice variants of the *A. gambiae* Down syndrome cell adhesion molecule gene (*AgDscam*) have been shown to protect mosquitoes against challenge with either *P. berghei* or *P. falciparum* [73] (Dong *et al.*, unpublished data). An MD2-like receptor, AgMDL1, shows specificity in regulating resistance only to *P. falciparum* [52]. Among the 138 predicted immunoglobulin superfamily proteins of *A. gambiae*, IRID4 and

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IRID6 have been shown to be involved in limiting *P. falciparum* infection [74]. G- bacteria-binding proteins (GNBPs) are functionally diverse components of the *A. gambiae* innate immune system, within which GNBP4 acts as a key factor in the anti-*Plasmodium* responses [76], [75]. Figure 2 shows anti-*Plasmodium* effector molecules involved in parasite killing and at what stage of parasite development the killing may occur.

IV. The influence of bacteria on the anti-*Plasmodium* response

When immune signaling pathways and anti-*Plasmodium* effectors are examined, a common theme emerges: The immune responses are active against both bacterial and plasmodial challenge. Therefore, it is not surprising that immune responses mounted against endogenous and exogenous bacteria are capable of modulating the infection by *Plasmodium* parasites, especially in the gut.

The process of blood feeding causes an extreme bloom in mosquito gut microbiota, presumably through an increase in available nutrients. Twenty-four hours after a blood meal, bacterial densities can reach 10^7 colony-forming units per milliliter and return to pre-blood meal concentrations in 3-5 days [77]. This dramatic increase in bacterial content loosely coincides with the invasion of the midgut by the parasite, and an immune response against bacteria at that time would be mounted when the parasite is most vulnerable.

There is considerable overlap between the anti-bacterial and anti-*Plasmodium* immune responses, with many molecules having activity against more than one type of microbe. Effector proteins including TEP1, FBN9, FBN39, LRRD7, and CTL4 have been identified through a microarray analysis of *P. falciparum* and *P. berghei*-infected mosquitoes and found to control resistance to bacterial and plasmodial challenge [52]. CTL4 and CTLMA2, first identified on the basis of their *P. berghei* melanization properties, are involved in anti-G- bacterial responses. Depletion of either molecule results in increased pathogenicity and decreased clearance of G- bacteria but has no effect on G+ bacteria [70]. GNBPs have been shown to regulate immune gene expression through the Toll or IMD pathway, and certain members are able to mediate *Plasmodium* oocyst intensities in *A. gambiae* [75].

Strong evidence for anti-bacterial/anti-*Plasmodium* immune synergy has recently been presented by Dong et al. (2009). Co-feeding live or heat-inactivated bacteria with parasites has been found to decrease the prevalence of *P. falciparum* oocysts after 8 days of incubation [78]. Experiments in which the bacterial populations in the midgut were lowered (through ingestion of antibiotic-containing sugar) have shown that mosquitoes without detectable bacteria in the midgut are more susceptible to *P. falciparum* infection [78], [79]. Differences in parasite infection have been observed when select immune genes are silenced in antibiotic-treated and untreated mosquitoes, allowing the identification of the natural gut microbiota as a major influence on *Plasmodium* development [78]. Basal immune gene expression is activated by gut bacteria and is responsible for controlling their proliferation, possibly acting through an IMD-mediated mechanism [42], [78]. This possibility is in line with the results of *Drosophila* studies suggesting that the gut epithelium-associated antibacterial immune responses that control proliferation of commensal microflora are dependent exclusively on the IMD pathway, while systemic responses are governed by both the IMD and Toll pathways [1].

Orthologs of *Drosophila* proteins that control commensal gut bacteria may play a role in early antiplasmodial immunity. The regulatory transcription factor Caudal inhibits IMD-dependent AMP over-expression in the absence of pathogenic challenge [80]. Removal of Caudal from Rel2 binding sites may be necessary for the expression of the IMD pathway components that are effective in killing *Plasmodium* in *Anopheles* midguts. The expression and activity of dual oxidase (DUOX), a membrane-associated protein involved in the

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regulation of reactive oxygen species (ROS), is dependent on the gut bacterial load [81]. PGRP-LC plays an important role in the DUOX-mediated response, but Relish (Rel2) is not involved in the regulation of ROS generation [82]. ROS are important for antibacterial and anti-*P. berghei* immunity in *A. gambiae* [83], suggesting that DUOX could play a role in parasite killing by controlling the production of free radicals.

If bacteria present in the mosquito midgut are capable of stimulating an immune response sufficient to interfere with *Plasmodium* development, then why are natural mosquito populations susceptible to parasite infection at all? Bacteria are not isolated from every field-collected mosquito, but this is most likely an artifact of the isolation processes that are culture-dependent and rely on the presence of bacteria capable of growth in the culture medium used. Also, the species composition of the midgut-associated bacteria may play a role: G- bacteria more robustly interfere with *Plasmodium* parasite infection than do G+ bacteria, and species-specific differences in G- inhibition are apparent [84], [85] (Cirimotich and Dimopoulos, unpublished results). Laboratory and field strains of G- bacteria have potent inhibitory effects on *Plasmodium* infection, significantly inhibiting parasite development when bacteria and parasite are introduced in the same blood meal [42], [77], [84], [85] (Cirimotich and Dimopoulos, unpublished results). However, no data have been collected on the co-prevalence of specific bacteria and *Plasmodium* infection in wild-caught mosquitoes. It would be interesting to look at the gut microbiome of mosquitoes from malaria-endemic and non-endemic areas to identify possible associations between bacteria species and natural parasite refractoriness. Increasing the prevalence of inhibitory bacteria, possibly through artificially baited sugar solutions, may create mosquito populations that are naturally refractory to parasite infection.

Current research efforts are devoted to teasing apart the tripartite interactions among bacteria, the parasite, and vector immunity. It is possible that the pathway-directed immune responses against parasites are elicited by the post-blood meal bacterial bloom, with an indirect effect on parasite survival. A second stimulus could be the introduction of bacteria into the hemolymph during the mechanical disruption of the midgut that occurs when the parasite invades the epithelial layer. However, the parasites themselves might be immunogenic, or a combination of anti-bacterial and anti-plasmodial mechanisms could be occurring. If mosquitoes indeed mount a *Plasmodium*-specific response, what are the parasite surface moieties that are detected by the mosquito's immune system, and which mosquito receptors are detecting them? Arrighi *et al.* (2008) have recently shown that the *A. gambiae* immune response can be stimulated through the introduction of parasite-derived glycosylphosphatidylinositols (GPI). Immune gene expression controlled by both the IMD and Jak-Stat pathways was increased soon after artificial bloodfeed; however, a more drastic increase in immune gene expression was observed when the mosquitoes were fed a GPI from *Toxoplasma gondii*, a response that potentially represents a less-conserved response to the unnatural pathogen [86]. In *A. stephensi* mosquitoes, NOS expression is induced by feeding with *P. falciparum*-derived GPI, and a second parasite-derived molecule, *P. falciparum* hemozoin, has been shown to trigger an immune response that may be mediated by the IMD pathway [87], [88]. The identification of the PRRs responsible for recognition of the parasite molecules will be crucial to determining the existence of a true *Plasmodium*-directed mosquito immune response.

V. *Plasmodium*–mosquito midgut interactions

In addition to the immune-mediated killing of *Plasmodium* parasites, physical barriers also present an obstacle to productive infection of the mosquito. Motile ookinetes in the midgut lumen must traverse the peritrophic matrix and invade the midgut epithelial cells. The ookinetes migrate through the epithelial cell to the intracellular space between the epithelial

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surface and basal lamina, where they form oocysts. Eventual maturation leads to rupture and release of sporozoites into the mosquito hemocoel. Crossing the peritrophic matrix and midgut epithelium are obligatory steps in the eventual transmission of the parasite by the mosquito vector.

Interactions with the peritrophic matrix

The peritrophic matrix (PM) is a thick chitin-containing layer that coats the lumenal side of the midgut epithelium after blood ingestion. The PM poses a major physical barrier to ookinete invasion because of its 1- to 20-μm thickness [89] and because the time of its maturation (24 hours after a blood meal) roughly coincides with the time of ookinete invasion of the midgut epithelium [90]. Although an artificially thickened PM in *Ae. aegypti* reduces *P. gallinaceum* oocyst formation [91], PM disruption by silencing chitin synthase results in a markedly lower oocyst count in the same vector-parasite system [92]. One possible explanation for these results is that the PM slows the diffusion of hydrolytic enzymes that may be detrimental to the parasite. However, the interaction of the parasite with the PM might be necessary for *Plasmodium* maturation. The *A. gambiae* PM proteome has recently been characterized by mass spectrometry [93]. A better understanding of the composition and structure of the PM may lead to modifications that can make it a complete barrier to *Plasmodium* infection.

Interactions with the midgut epithelium

Invasion of the midgut epithelium occurs once the ookinete has bound to an unknown receptor on the lumenal surface. The *P. berghei* membrane-attack ookinete protein (MAOP) is essential for midgut invasion and most likely acts by creating pores in target membranes. MAOP-disrupted ookinetes attach to the epithelium but are unable to enter the cytoplasm [94]. Other *P. berghei* ookinete surface proteins that are essential for invasion include the micronemal proteins circumsporozite and TRAP related protein (CTRP) and secreted ookinete adhesive protein (SOAP). Although the precise role of these molecules in midgut invasion has not been determined, CTRP (a member of the TRAP family of proteins, which are involved in motility and invasion in the Apicomplexa) plays a role in ookinete locomotion, while SOAP binds to laminin and may be required for adhesion to the basal lamina [95], [96].

Invasion may also require enzymatic processing of bound ligands for subsequent detachment and entry into the host cell. *P. berghei* Rhomboid 1 (PbROM1), a member of the rhomboid family of serine proteases, is able to cleave TRAP family members within their transmembrane domains [97]. PbROM1 gene disruptants are impaired in the ookinete-to-oocyst transition [98], indicating that proteolytic processing of invasion proteins may be required for efficient entry into midgut epithelial cells. Depletion of the *Anopheles* homologue of Croquemort, a *Drosophila* scavenger receptor expressed in the midgut in response to blood feeding, results in the inhibition of oocyst formation, suggesting an as-yet unknown role for this molecule in *Plasmodium*-midgut interactions [99].

Once inside the cell cytoplasm, the ookinete must quickly travel to the basal lamina before the cell undergoes apoptosis and is extruded from the epithelium; this process is known as the time-bomb model of ookinete invasion [90]. A key protein in this process is the cell-traversal protein for ookinetes and sporozoites (CelTOS). CelTOS-disrupted ookinetes are able to invade the midgut epithelium but fail to traverse the cell, indicating that the protein is needed for migration through the cytoplasm. The authors speculate that CelTOS may anchor the ookinete to molecules in the cytoplasm [100].

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Cellular components also play a role in controlling ookinete invasion and oocyst formation. Decreasing actin polymerization increases *P. berghei* oocyst numbers, while depleting lipid transport activity reduces *P. berghei* oocyst formation [101], [102]. Although the role of lipid transport has not been fully determined, there appears to be a requirement for the host's cellular pathways in the parasite life cycle. There is apparent conservation involved here, since increasing actin polymerization or decreasing lipid transport components has similar effects on oocyst counts in *P. falciparum* field isolates [103].

There are other important interactions between the *Plasmodium* parasite and mosquito that must occur before transmission to a vertebrate host. Among the most important is parasite invasion of the salivary glands and movement into the salivary ducts. For further information on these interactions, a recent review by Ghosh and Jacobs-Lorena (2009) is recommended [104].

VI. Conclusions

The mosquito's immune response is paramount in limiting pathogen infection and, in the case of *Plasmodium* parasites, transmission. Using anti-pathogen molecules that are expressed as a result of immune signaling pathway activation, the mosquito is able to combat this infection process. These effector molecules are members of diverse protein families that in some instances appear to be mosquito-specific and may have evolved as anti-*Plasmodium* and anti-bacterial factors. Indeed, bacteria harbored in the mosquito midgut may play a significant role in modulating *Plasmodium* transmission through the stimulation of an immune response.

Recent efforts in the areas of vector biology and vector-borne diseases have focused on blocking infection of the insect as a means of disease control. A better understanding of the interactions between the parasite and the mosquito vector is crucial to achieving this goal. For instance, boosting the anti-*Plasmodium* immune response by temporally expressing Rel2 or a specific effector molecule in transgenic mosquitoes could create mosquito populations refractory to parasite infection. The introduction into and stable transmission of inhibitory bacteria in natural mosquito populations might also be a means of controlling *Plasmodium* transmission that does not require genetic modification or release of mosquitoes. Exploiting the anti-*Plasmodium* immune response of the mosquito is yet another potential mechanism for combating *Plasmodium* infections worldwide.

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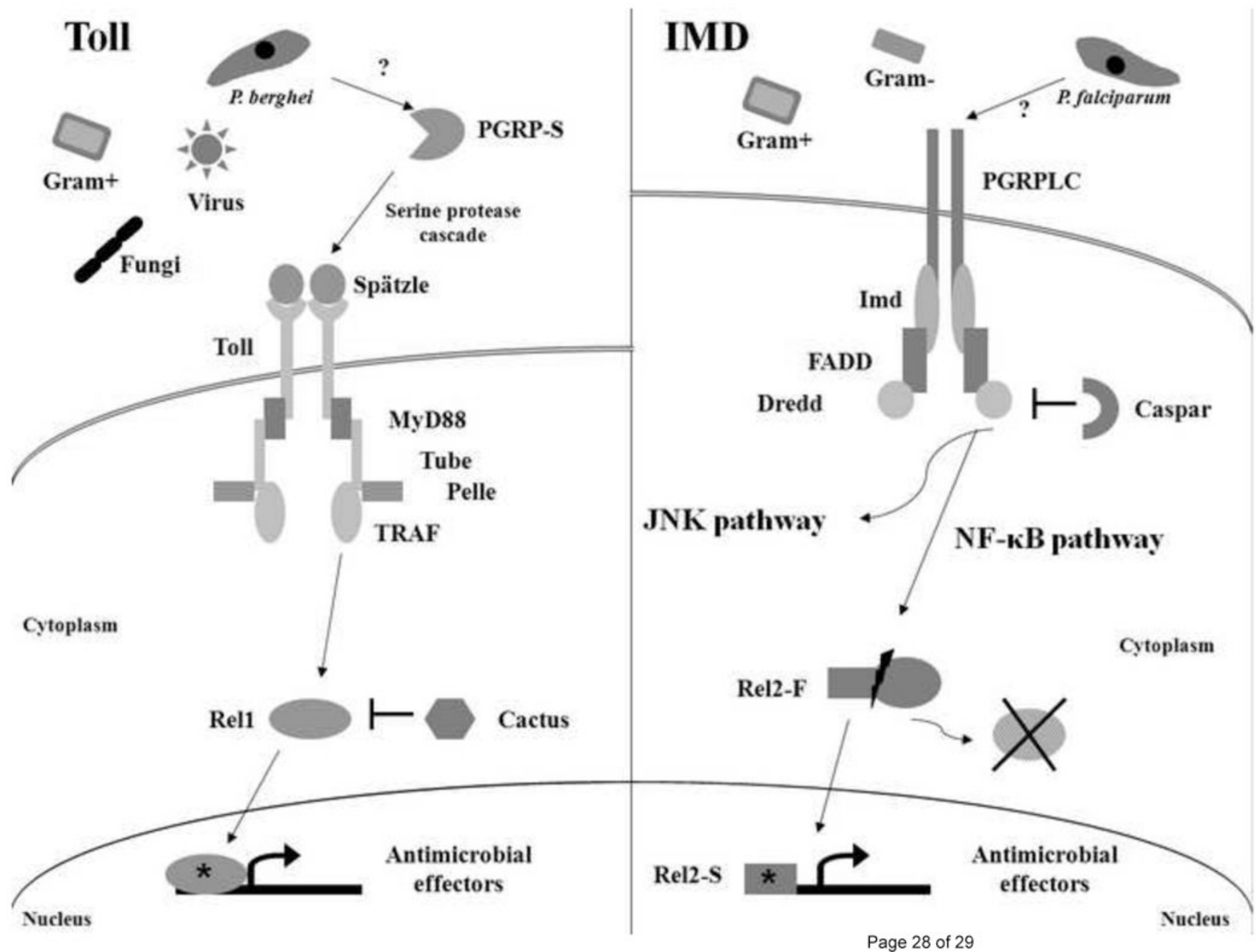

Figure 1. Toll and IMD immune signaling pathways involved in anti-*Plasmodium* defense
Following recognition of a microbe, or unknown *Plasmodium* ligand, by soluble PGRP molecules, the Toll pathway is stimulated by binding of the ligand Spätzle with the Toll transmembrane receptor. This triggers a series of molecular events that culminate in the activation (*) and translocation of Rel1 into the nucleus, up-regulating transcription of immune genes that are responsible for microbial killing. The IMD pathway is stimulated when the transmembrane PGRPLC receptor binds peptidoglycan or an unknown *Plasmodium* ligand that leads to the cleavage of Rel2-F and translocation of active Rel2-S (*) into the nucleus. A different set of anti-*Plasmodium* genes are up-regulated when the IMD pathway is stimulated. Branching of the IMD pathway is indicated, but the JNK pathway has not been extensively characterized in *Anopheles* mosquitoes.

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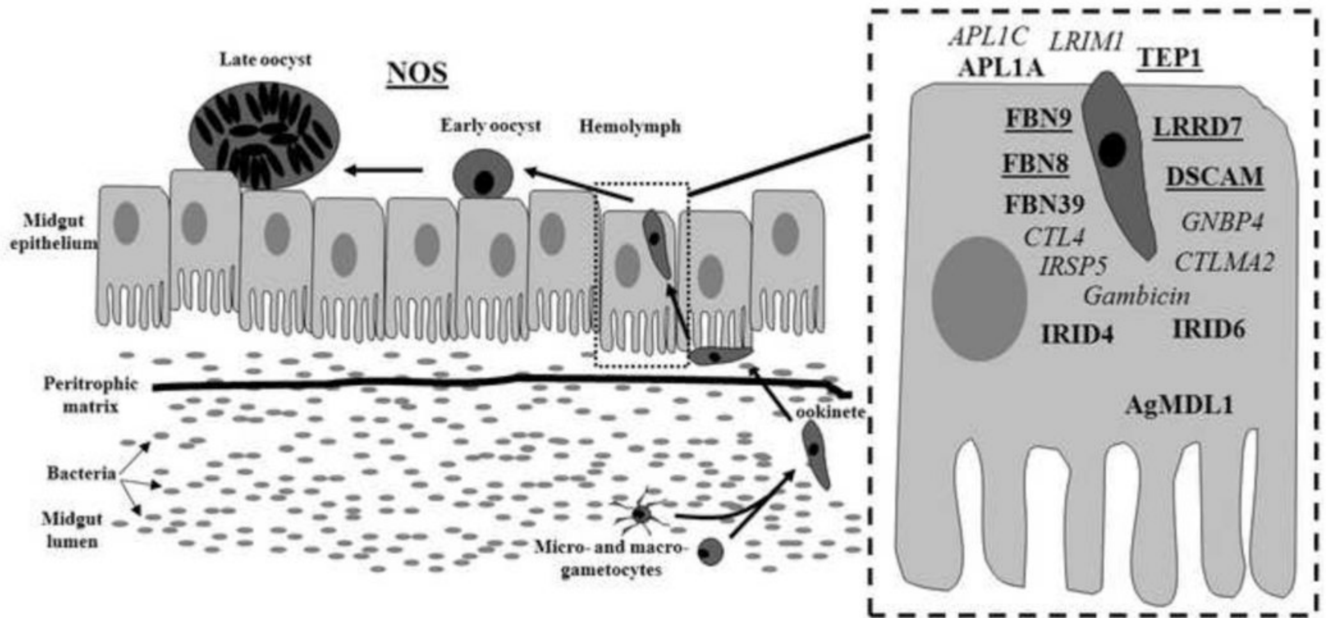

Figure 2. Parasite development and anti-*Plasmodium* effectors in the mosquito

The *Plasmodium* parasite develops into the motile ookinete stage and will invade the midgut epithelium at approximately 18 hours after ingestion of an infected blood meal. At this stage, a number of effector molecules target the parasite for destruction (inset). NOS targets the parasite at a later developmental stage- the early/late oocyst transition. Effector molecules in **underlined bold** are involved in killing human and rodent *Plasmodium* species, those in ***bold italics*** have been shown to be effective against *P. berghei* only, and those in **bold** target *P. falciparum*.